Cadherin-7 enhances Sonic Hedgehog signaling by preventing Gli3 repressor formation during neural tube patterning

Rie Kawano, Kunimasa Ohta and Giuseppe Lupo

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Final acceptance: 9 November 2017

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSOB-17-0025.R0 (Original submission)

Review form: Reviewer 1

Recommendation
A very good paper making an important contribution to the field

Are each of the following suitable for general readers?

a) Title
   Yes

b) Summary
   Yes

c) Introduction
   Yes
Is the length of the paper justified?  
Yes

Should the paper be seen by a specialist statistical reviewer?  
No

Is it clear how to make all supporting data available?  
Yes

Is the supplementary material necessary; and if so is it adequate and clear?  
Yes

Do you have any ethical concerns with this paper?  
No

Comments to the Author  
The Sonic Hedgehog (SHH) signalling pathway plays a central role in ventral patterning of the spinal cord. Previous work from the Redies lab had shown that the cell adhesion molecule Cadherin 7 (Cdh7) is expressed in a distinctive lateral domain in the embryonic spinal cord of chick embryos, and that the dorsal border of this domain is set by the expression of Pax7, a class I transcription factor that is repressed by SHH. In the manuscript reviewed here, Kawano et al. describe a set of compelling in vivo and in vitro experiments that conclusively demonstrate that Pax7 is not only a readout, but surprisingly also an effector of SHH signalling that appears to function as a highly specific co-receptor of this ligand.

The experiments are well thought-out and overall the data are convincing. There are a few minor points that require clarification. Provided that the authors address these I strongly support the publication of this interesting, original piece of work in Open Biology.

It would be good to see the Cdh7-siRNA construct used a bit more extensively to provide additional evidence that endogenous Cdh7 is required in SHH-mediated DV patterning. For example, rather than just knocking down co-transfected Cdh7 in Fig. 3B, is there a difference in reporter activity detectable between control and Cdh7-siRNA electroporated explants? Can the knockdown be confirmed at the protein level, using Cdh7 antibody that appears to work well in Western blots? And are the effects on Sufu localisation (Fig. 5AA-AK) neutralised by Cdh7-siRNA? Can the effects on GliR formation in Fig. 6 be reversed by Cdh7-siRNA?

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Review form: Reviewer 2

Recommendation  
A paper that is of insufficient interest or importance, or which contains errors in experimental method, analysis or logic
Comments to the Author

The study by Rie Kawano and cols, entitled “Cadherin-7 enhances Sonic Hedgehog signaling by preventing Gli3 repressor formation during neural tube patterning”, proposes an interaction between the Shh-signaling pathway and Cadherin7 to regulate dorsal ventral patterning of the spinal cord. Although the hypothesis is of relevance for the field, in many cases data summarized in the present format of the manuscript do not fully support authors conclusions. Moreover, the manuscript is, in general, too long and poorly organized. Images are of poor quality and, in most of the cases, lack quantitative data.

Authors take advantage of two model system; chick in ovo electroporation and transiently transfected NIH3T3 cells, for manipulating both Shh-signaling components and Cadh7 expression. Experiments performed in the NT are far more relevant that those in the exogenous system, and should be shown in the main figures (in particular S11).

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Shh protein in the developing NT has been addressed by many laboratories by means of many different in vivo assays to show that diffusion of the Shh-ligand is ventrally restricted. On the contrary, both SUFU and Gli3 are expressed within the Cadh7 expression domain in the NT. Again in this case these analyses should be performed in equivalent systems and should be shown together.

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Specific Points.
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Figure3: Next authors test for a functional Shh/Cadh7 interaction by luciferase assays performed in chick intermediate NT explants that are in vitro treated with various doses of recombinant N-Shh. Authors should show whether explants respond to Shh addition as expected (statistical significance of increased Luc-activity) 4nM increase statistical should be shown in the main figure (not is supplementary).

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Figure 7 shows a model of Cdh7-dependent specification of the Pax7+/Pax7- NT boundary via regulation of Gli3R production and Pax7 expression. As previously published Wnt activity directly regulates Gli3 expression in the dorsal spinal cord, which should be indicated in the model.

Li et al (Development. 2009 Dec;136(23):4055-63)

Decision letter (RSOB-17-0025)

13-Mar-2017

Dear Dr Kawano,

We are writing to inform you that your manuscript RSOB-17-0025 entitled "Cadherin-7 enhances Sonic Hedgehog signaling by preventing Gli3 repressor formation during neural tube patterning" has, in its current form, been rejected for publication in Open Biology.

The referees have recommended that major revisions are necessary but that the manuscript has potential; hence, we would like to actively encourage you to revise the manuscript accordingly, and resubmit. Nevertheless, please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript and will re-enter the review process. Every attempt will be made to use the original referees, but this cannot be guaranteed. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. Please upload a ‘response to referees’ document including details of how you have responded to the comments, and the adjustments you have made.

To upload a resubmitted manuscript, log into http://mc.manuscriptcentral.com/rsob and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

Sincerely,

The Open Biology Team
mailto: openbiology@royalsociety.org

Reviewer(s)’ Comments to Author(s):

Referee: 1

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Li et al (Development. 2009 Dec;136(23):4055-63)

Author's Response to Decision Letter for (RSOB-170025)

See Appendix A

RSOB-17-0025.R1 (Revision)

Review form: Reviewer 1

Recommendation
A very good paper making an important contribution to the field

Are each of the following suitable for general readers?

  a) Title
     Yes

  b) Summary
     Yes

  c) Introduction
     Yes

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Is it clear how to make all supporting data available?
Yes

Is the supplementary material necessary; and if so is it adequate and clear?
Yes

Do you have any ethical concerns with this paper?
No
Comments to the Author
The efforts that you have made to address the issues raised by both referees are commendable. I am confident that your paper will be of great interest beyond the field of dorsoventral neural patterning. I therefore strongly support the publication of this study in Open Biology.

Review form: Reviewer 2

Recommendation
A very good paper making an important contribution to the field

Are each of the following suitable for general readers?

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   Yes

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   Yes

c) Introduction
   Yes

Is the length of the paper justified?
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The experimental design is appropriate and in general support well the results, although part of the mechanism is inferred from in vitro experiments using cell lines, which leaves a doubt of whether all the described interactions indeed occur in the neuroepithelium. The different electroporation experiments in ovo however fit well with the authors’ interpretations, making the manuscript worth of publication.

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Decision letter (RSOB-17-0225.R1)

17-Oct-2017

Dear Dr Kawano,

We are pleased to inform you that your manuscript RSOB-17-0225 entitled "Cadherin-7 enhances Sonic Hedgehog signaling by preventing Gli3 repressor formation during neural tube patterning" has been accepted by the Editor for publication in Open Biology. The reviewer(s) have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, we invite you to respond to the reviewer(s)' comments and revise your manuscript.

Please submit the revised version of your manuscript within 14 days. If you do not think you will be able to meet this date please let us know immediately and we can extend this deadline for you.

To revise your manuscript, log into https://mc.manuscriptcentral.com/rsob and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

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When submitting your revised manuscript, you will be able to respond to the comments made by the referee(s) and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referee(s).

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2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. Please note that PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file from the main text and meet our ESM criteria (see http://royalsocietypublishing.org/instructions-
Authors#question5). All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rsob.2016[last 4 digits of e.g. 10.1098/rsob.20160049].

4) A media summary: a short non-technical summary (up to 100 words) of the key findings/importance of your manuscript. Please try to write in simple English, avoid jargon, explain the importance of the topic, outline the main implications and describe why this topic is newsworthy.

Images
We require suitable relevant images to appear alongside published articles. Do you have an image we could use? Images should have a resolution of at least 300 dpi, if possible.

Data-Sharing
It is a condition of publication that data supporting your paper are made available. Data should be made available either in the electronic supplementary material or through an appropriate repository. Details of how to access data should be included in your paper. Please see http://royalsocietypublishing.org/site/authors/policy.xhtml#question6 for more details.

Data accessibility section
To ensure archived data are available to readers, authors should include a ‘data accessibility’ section immediately after the acknowledgements section. This should list the database and accession number for all data from the article that has been made publicly available, for instance:

- DNA sequences: Genbank accessions F234391-F234402
- Phylogenetic data: TreeBASE accession number S9123
- Final DNA sequence assembly uploaded as online supplemental material
- Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311

Once again, thank you for submitting your manuscript to Open Biology, we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,

The Open Biology Team
mailto:openbiology@royalsociety.org

Reviewer(s)' Comments to Author:

Referee: 1

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Response to Reviewers:
We were pleased to hear that the editor considers our work to be of sufficient interest to allow a resubmission. We have now addressed the constructive concerns raised by the Reviewers and have significantly extended our data with many new experiments and analyses. Amongst the changes, we have performed several experimental controls requested by the Reviewers, we have considerably improved image presentation and provide much more extensive quantitation of results. Our detailed comments to both Reviewers are documented below.

Referee: 1

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We would like to thank the Reviewer for providing constructive comments that have helped to improve the manuscript. We hope that we have addressed the Reviewer's concerns in the resubmitted manuscript and in this rebuttal letter, as described below.

It would be good to see the Cdh7-siRNA construct used a bit more extensively to provide additional evidence that endogenous Cdh7 is required in SHH-mediated DV patterning. For example, rather than just knocking down co-transfected Cdh7 in Fig. 3B, is there a difference in reporter activity detectable between control and Cdh7-siRNA electroporated explants?

Although Cdh7 expression is induced in explants treated with 2 nM N-Shh (Fig. 1AD, AE), no significant reporter activation is detected in the explants in the absence of exogenous Cdh7 (Fig. 3A), indicating that the endogenous levels of Cdh7 expression are not sufficient to promote reporter expression in this in vitro assay. Higher doses of N-Shh (4 nM) can increase reporter activity in the absence of exogenous Cdh7 (Fig. 3A), but the endogenous expression of Cdh7 is repressed at these doses of N-Shh (Fig. 1AD, AE), indicating that high doses...
of N-Shh promote Shh signalling independently of Cdh7.

In conclusion, the explant reporter assay used for Fig. 3 is not suitable for studying the requirement of endogenous Cdh7 in the regulation of Shh signalling.

Can the knockdown be confirmed at the protein level, using Cdh7 antibody that appears to work well in Western blots?

Western blot and immunocytochemistry data of Cdh7 knockdown were already reported in the Supplementary Materials and we have now moved them to Fig. 2N and O of the revised manuscript for increased visibility.

And are the effects on Sufu localisation (Fig. 5AA-AK) neutralised by Cdh7-siRNA? Can the effects on GliR formation in Fig. 6 be reversed by Cdh7-siRNA?

We have performed these additional controls requested by the Reviewer and the results are shown in Figure S13 in the Supplementary Materials of the revised manuscript. The effects of Cdh7 on both Sufu localisation (Fig. 6K) and GliR repressor formation (Fig. 6AJ) were largely prevented when cells were co-transfected with Cdh7-siRNA (Fig. S13E, M).

I find it difficult to see the supposed difference between Cdh7 and Ncdh localisation in Fig. 5H,I,K,L. It is possible that something got lost in the PDF conversion, but to me Ncdh looks clustered into spots in a manner that is quite similar to Cdh7 in Fig. 5I (compared to Fig. 5H), whereas the overall Ncdh signals seems much weaker in Fig. 5L. Please clarify what exactly the reader is meant to see here—a higher magnification might be useful.

We have repeated these experiments and, as requested by the Reviewer, provide new images in Figure S8 in the Supplementary Materials of the revised manuscript. These new experiments confirm the lack of endogenous Ncdh aggregation in cells showing strong aggregation of Cdh7 (Fig. S8D, E, G, H). It should be noted that, even in the presence of higher Ncdh expression levels due to transfection of exogenous Ncdh plasmids, no aggregation of Ncdh comparable to that of Cdh7 was detectable (Fig. 5B, F).

Minor point: In the Introduction, the authors state that “this DV pattern results from gradients of dorsally-enriched Bone Morphogenetic Proteins (BMPs) and Wnts, and ventrally enriched Sonic Hedgehog (Shh).” To my knowledge the evidence for a graded function of BMPs and Wnts is somewhat less convincing than for Shh.

We have modified this sentence according to the Reviewer’s suggestion.

Referee: 2

Comments to the Author(s)
The study by Rie Kawano and cols, entitled “Cadherin-7 enhances Sonic
Hedgehog signaling by preventing Gli3 repressor formation during neural tube patterning proposes an interaction between the Shh-signaling pathway and Cadherin7 to regulate dorsal ventral patterning of the spinal cord. Although the hypothesis is of relevance for the field, in many cases data summarized in the present format of the manuscript do not fully support authors conclusions. Moreover, the manuscript is, in general, too long and poorly organized. Images are of poor quality and, in most of the cases, lack quantitative data.

Authors take advantage of two model system; chick in ovo electroporation and transiently transfected NIH3T3 cells, for manipulating both Shh-signaling components and Cadh7 expression. Experiments performed in the NT are far more relevant that those in the exogenous system, and should be shown in the main figures (in particular S11).

We are grateful to the Reviewer for the detailed and critical review of our manuscript. We agree with the Reviewer that the quantitative analyses in our work needed to be strengthened and that data presentation in the manuscript was not sufficiently clear. We hope that both issues have been addressed in this resubmitted manuscript, where we have included several new experimental controls as requested by both Reviewers.

To propose a genetic interaction between Cadh7 and the Shh-signaling pathway, authors show that over-expression of Shh (Figure 1) and the Shh- signaling activator SmoM2 (Supplementary Fig S11) alters Cadh7 distribution in the NT. On one case (Shh) induces a dorsal shift on Cadh7 expression (down-regulation?), on the other case (SmoM2) authors claim an ectopic expression (over-expression?). Authors need to reconcile these apparently contradictory findings.

We have performed additional experiments and quantifications to address the Reviewer's concerns. New data reported in Fig.1R,S of the revised manuscript show that the overexpression of Shh in a broad domain spanning the intermediate and the dorsal spinal cord results in the repression of the endogenous Cdh7 domain in the intermediate region and ectopic Cdh7 expression in the dorsal region. Data about SmoM2 electroporation have now been moved to Fig. 1X-AA, and a quantification of SmoM2-expressing and Cdh7-positive cells following SmoM2 overexpression is now provided in Fig. 1AA. These results show that SmoM2-expressing cells falling within the endogenous Cdh7 domain in the intermediate spinal cord are mostly Cdh7-negative (indicating Cdh7 downregulation), whereas those falling within the dorsal region are mostly Cdh7-positive (indicating Cdh7 upregulation). Altogether, the two datasets show that both Shh and SmoM2 overexpression cause similar effects: downregulation of Cdh7 in the intermediate spinal cord and ectopic expression in the dorsal spinal cord. The likely explanation for these results is that Shh or SmoM2 electroporation causes higher level of Shh signalling in the intermediate than in the dorsal spinal cord due to additive effects with endogenous signalling in the intermediate region, and to the fact that SmoM2 can upregulate Shh in the intermediate, but not in the dorsal region (Fig. 1AB). These observations suggest a dose-dependent regulation of Cdh7
expression by Shh signalling. To further confirm the dose-dependent effects of Shh signalling on Cdh7 expression, we have treated spinal cord explants with different doses of the Smo agonist SAG and found that, similar to treatments with N-Shh, Cdh7 was upregulated by moderate doses of SAG (0.5 µM) and repressed at higher doses. These new results are now shown in Fig. 1AF of the revised manuscript.

Moreover, in neither case, authors show quantitative data supporting either observation, which is an absolute requirement. In addition, these analyses should be performed at equivalent developmental stages, and at equivalent hours post electroporation, and should be shown together.

As requested by the reviewer, we now provide quantitative data for these experiments in Fig. 1AA and Fig. 1AE.

All electroporation experiments were performed by electroporating embryos at HH st.10. For the majority of the experiments, electroporated embryos or explants were then incubated until st.15-17, when they were harvested for endpoint analysis (slight variations in the stage of the embryos at the time of harvesting depended on slight fluctuations in the rate of growth of different embryo batches). Embryos were harvested at a later stage (st. 23) only for the experiments of Shh overexpression shown in Fig. 1L-W. Since Shh protein is diffusible and can act at a considerable distance from the electroporation site, these experiments were analysed at stage 23 to allow for further growth of the neural tube and better resolution of the effect of exogenous Shh on the intermediate and the dorsal regions of the spinal cord.

As requested by the Reviewer, the results of Shh and SmoM2 overexpression are now shown side-by-side in Fig. 1, along with the results of treatments of spinal cord explants with different doses of N-Shh or SAG.

To propose a direct protein-protein interaction between Cdh7 and components of the Shh-signalling pathway, authors analyze the direct binding of Cdh7 with components of the Shh-signaling pathway; Shh itself (Figure 2), SUFU and the transcription factor Gli3FL (Figure 4). These experiments are performed in exogenous systems (L cells for Shh and NIH3T3 for SUFU and Gli3FL). It is difficult to reconcile the relevance of a direct binding of Shh/Cdh7 in the NT, since these two proteins are not co-expressed in this tissue. Cdh7 expression is restricted to the intermediate NT, as shown previously, and as it is also shown in this study. The distribution of Shh protein in the developing NT has been addressed by many laboratories by means of many different in vivo assays to show that diffusion of the Shh-ligand is ventrally restricted. On the contrary, both SUFU and Gli3 are expressed within the Cdh7 expression domain in the NT. Again in this case these analyses should be performed in equivalent systems and should be shown together.

As requested by the Reviewer, we have repeated the co-IP assays of N-Shh with Cdh7, Ncdh and Cdh20 in NIH3T3 cells, so that all the pull-down assays have now been performed with the same cell type (NIH3T3 cells). As requested by the Reviewer, these data are now all shown in Fig. 4 of the revised manuscript.
Data reporting the binding affinity of Cdh7 for N-Shh (Fig. 4E, F, Fig. S6, Fig. S7) and the mapping of the Cdh7 domains involved in the interaction with N-Shh (Fig. 4G, H) are still provided based on COS-7 cells. This is due to the fact that the lower transfection efficiency of NIH3T3 cells in comparison with COS-7 cells make them less suitable for this kind of experiments.

To propose a mechanism of Cadh7-mediated regulation of Shh activity, authors address whether these direct interactions between Cadh7 and Shh-signaling components might regulate Cadh7 sub-cellular distribution. However, as shown in Figures 4, 5 and supplementary Figure S11, these analyses are inconclusive, since for the most part lack quantitative data (except for Fig5, panelA,B), and in addition they lack membrane markers. These are important points, since authors propose a functional mechanism based on the role played by Cadh7 in controlling the sub-cellular distribution of Shh-signaling components, and hence the activity of the pathway.

Again in this case these analyses should be performed in equivalent systems, preferably in the NT, and should be shown together.

As requested by the Reviewer, we have grouped together within the same figure the main results of the experiments investigating the interaction between Cdh7 and Shh pathway components (Fig. 4), the effects of Shh signalling on Cdh7 localization (Fig. 5) and the effects of Cdh7 on the localization and processing of Sufu and Gli3 (Fig. 6). The results of additional experimental controls for these experiments are shown in Fig. S8-S13 in the Supplementary Materials.

Furthermore, we have largely expanded previous quantitative data with several new analyses to provide a quantitative readout for all of these experiments. In particular, both Fig. 5 and Fig. 6 now report a quantitative analysis of IF assays, while the quantification of Western Blot assays shown in Fig. 4 and Fig. 6 is reported in Table S5 and Table S6 in the Supplementary Materials.

Finally, both co-IP assays in Fig. 4 and IF assays in Fig. 5 document the interaction of Cdh7/Ncdh with Shh pathway components and the different subcellular localisation of Cdh7 and Ncdh both in cell cultures and in the neural tube in vivo. The dataset shown in Fig. 6 on the effects of Cdh7 on Sufu and Gli3 localisation and processing is still based on in vitro assays, but these are very challenging assays to carry out in vivo and it was hardly possible to perform them within the deadline to submit this revised manuscript.

Specific Points.
Figure 1: (panel A) Shows over-expression of Shh by electroporation at HH10. Figure should indicate hours post electroporation, the representative section shown appears to be older than the indicated stage HH21.

We have indicated the 48 hours post-electroporation in the figure legends, but we prefer to maintain HH staging of the embryos in the figures, due to slight batch-to-batch variations in the final stage of embryos incubated for the same amount of time.

We have checked again the morphology of the embryos used for the
experiments shown in Fig. 1 and we agree with the Reviewer that embryos previously indicated as HH st.21 were actually slightly older (HH st. 23). This information has now been corrected in the revised manuscript.

**Why is Shh-EP restricted to the ventral NT?**
**What happened to Cadh7 expression in cases of more “extensive” Shh-EP?**

As requested by the Reviewer, we have performed additional experiments to overexpress Shh in a broad domain extending from the intermediate to the dorsal spinal cord region. The results of these experiments show that Cdh7 expression is repressed within the electroporated domain, but it is ectopically induced in adjacent non-electroporated dorsal region (Fig. 1R-W). Along with the results of SmoM2 electroporation and of treatments of spinal cord explants with different doses of N-Shh and SAG, these data suggest that Cdh7 expression is activated by moderate levels of Shh signalling, and repressed by high levels of Shh signalling.

**What is the identity of cells not expressing Shh/Cadh7?**

To facilitate the interpretation of these data, we have now included a diagram in Fig. 1K. As shown in this diagram, Shh is produced by floor plate cells, while Cdh7 is expressed in p2, p1 and p0 domains. Therefore, the region located between the floor plate and p2, which does not express Shh and Cdh7, corresponds to the p3 and pMN progenitor domains.

**Panel E** shows an explants assay in which authors claim a Shh dose-dependent induction of Cadh7 expression. Quantitative data is required in these experiments.

As requested by the Reviewer, we now report the fraction of cells expressing each marker at different doses of N-Shh (Fig. 1AE). Furthermore, we have performed additional experiments showing that similar results can be obtained by treating explants with different doses of the Smo agonist SAG (Fig. 1AF).

**Panels F-J** show Cadh7 over-expression and Pax7 regulation. Quantification of the GFP (Cadh7 reporter vs/Pax7 (red immuno) fluorescence intensity ratio would be much more informative. In addition fluorescence ratio of individual cells should be represented by dots at either dorsal or ventral NT.

As requested by the Reviewer, we have a performed a quantitative analysis of fluorescence intensities for cells located at different DV levels of the NT and report it as surface plots in Fig. S1. This analysis confirms that cells that were scored as Pax7+ or Pax7- based on microscopic analysis do show very different levels of Pax7 signal upon quantification of fluorescence intensities.

**Moreover, dorsal and ventral NT should be identified by marker expression ie: Olig3, pax6, Nkx6, Dbx, etc, and not randomly.**

As requested by the Reviewer, we report that Cdh7 overexpression results in
cells ectopically expressing the p0 marker Dbx1 dorsal to its endogenous expression domain (Fig. 2I-M and Fig. S1X-AC). This suggests that Pax7 repression by Cdh7 can alter DV cell identities in the intermediate spinal cord region.

Figures 2 and 4: By coIPs experiments, authors show the direct Shh/Cadh7 interaction, with a Kd=4.8nM, and map the Shh binding domain within the cadherin repeat 1-2 (CDR1, CDR2). Figure 4 could be pooled together with Figure 2.

Following the Reviewer’s suggestion, the data shown in Fig. 2 and Fig. 4 of the previously submitted manuscript have been combined together in Fig. 4 of the revised manuscript.

Figure 3: Next authors test for a functional Shh/Cadh7 interaction by luciferase assays performed in chick intermediate NT explants that are in vitro treated with various doses of recombinant N-Shh. Authors should show whether explants respond to Shh addition as expected (statistical significance of increased Luc-activity) 4nM increase statistical should be shown in the main figure (not is supplementary).

As suggested by the Reviewer, doses of 4 nM N-Shh are indeed able to significantly upregulate reporter activity in comparison with untreated explants. Tables 1-4 in the Supplementary Materials report the p values for each possible pairwise comparison in Fig. 3 and in Fig. S2-S4. We could not find a neat way to show all the statistically significant p values in the main Figures and for this reason we are showing only those that are directly relevant to Cdh7 function. However, the significant effect of 4 nM N-Shh treatments is clearly mentioned in the Results text.

Figures 5 and 6: These analyses require a combination with a membrane marker to measure fluorescence intensity in relation to such marker. As shown in Figure 5, Figure 6, Supplementary Figure S11 and S13, analyses are inconclusive. These figures should be combined, and as much as possible performed in the NT. Experiments performed in the NT are far more relevant for the model that authors want to propose, than those performed in an exogenous system such as NIT3T3 cells, and therefore should go in the main body of the paper.

As explained above, we have combined these figures as suggested by the Reviewer, we have performed a much more extensive quantification of these experiments and we have been able to obtain similar results in cultured cells and in the neural tube for a large part of these experiments. Unfortunately, we could not include the analysis of membrane markers in these experiments due to the lack of good antibodies. However, the fact that Cdh7 can directly interact with soluble N-Shh presented in the extra-cellular media (Fig. 4A, E) strongly suggests that the interaction between Cdh7 and Shh takes place at the cell membrane. Given the limited time available for this submission, and considering the vast amount of data that are already included in this manuscript, we believe that a more detailed characterisation of Cdh7 function using different
assays should be continued in future investigations.

Authors claim that Shh activation (by electroporation of SmoM2) promotes Cadh7 expression. On the one hand, SmoM2 electroporation shown in Supplementary Fig S11 only causes a minor ectopic expression of Cadh7, which needs quantitative data in order to be conclusive; is this ectopic Cadh7 expression cell autonomous? This is an important point, since in Figure 1 of this study, authors claim that Shh electroporation in the NT caused a dorsal shift in Cadh7 expression, but they do not show ectopic expression. How do authors explain the different phenotypes obtained by Shh- and/or SmoM2- over-activation in the NT?

As explained above, we have included new data to show that both Shh and SmoM2 affect Cdh7 expression in a similar, location-dependent manner and that, in particular, Shh electroporation in the intermediate-dorsal neural tube can promote ectopic Cdh7 expression in the dorsal spinal cord region.

Figure 7 shows a model of Cdh7-dependent specification of the Pax7+/Pax7- NT boundary via regulation of Gli3R production and Pax7 expression. As previously published Wnt activity directly regulates Gli3 expression in the dorsal spinal cord, which should be indicated in the model.


Li et al (Development. 2009 Dec;136(23):4055-63)

We have corrected this mistake in Fig. 7 of the revised manuscript.