CNKSR2, a downstream mediator of retinoic acid signaling, modulates the Ras/Raf/MEK pathway to regulate patterning and invagination of the chick forebrain roof plate

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Original submission

First decision letter

MS ID#: DEVELOP/2022/200857

MS TITLE: CNKSR2, a downstream mediator of retinoic acid signaling modulates the Ras/Raf/MEK pathway to regulate patterning and invagination of the chick forebrain roof plate

AUTHORS: Niveda Udaykumar, Mohd Ali Abbas Zaidi, and Jonaki Sen

Dear Dr. Sen,

I have now received the reports of three referees on your manuscript, and I have reached a decision. I am sorry to say that the outcome is not a positive one. The referees' comments are appended below, or you can access them online: please go to Development's submission site and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, all the referees express significant concerns about your paper and two of them are not in favour of publication. In particular, they comment on the incompleteness of some experiments, the insufficient quality of the in situ hybridisation images, the lack of sufficient quantification and statistical analysis of the data, and the need for further experiments to support the conclusions of the study (e.g. a rescue experiment with a knockdown resistant CNKSR2 construct). Having looked at the manuscript myself, I agree with their views and after careful consideration I have decided to reject your paper

I do realise this is disappointing news, but Development receives many more papers than we can publish, and we can only accept manuscripts that receive strong support from referees.

I do hope you find the comments of the referees helpful, and that this decision will not dissuade you from considering Development for publication of your future work. Many thanks for sending your manuscript to Development.

Reviewer 1

Advance summary and potential significance to field

In their manuscript, Udaykumar et al. investigate the function of the CNKSR2 gene in the invagination of the telencephalic roof plate in the chicken embryo. They show that CNKSR2 is a target gene of retinoic acid (RA) signalling that they had previously shown to be critical for roof plate invagination. Furthermore, CNKSR2 knock-down results in severe and moderate defects in roof plate invagination, increased proliferation, altered expression of several roof plate markers and in an increased number of pMEK1+ cells, an indicator of Ras/Raf/MEK signalling. Finally, interfering with MEK signalling rescues the roofplate defect of CNKSR2 knock-down.
Roof plate invagination is a critical step in the formation of the two telencephalic hemisphere. Defects in this process can lead to holoprosencephaly, a severe congenital malformation of the brain. Its aetiology is not very well understood, therefore there is a great interest in elucidating the underlying causes. This study identified a new molecular player in the field and characterized its upstream regulators and downstream effectors, thereby integrating it well into the existing framework of factors controlling roof plate invagination. Hence, this study will be of great interest for the readers of Development, there are, however, a few points the authors need to address before publication.

Comments for the author

Major points

1. The authors propose that CNKSR2 acts downstream of RA signalling to control roof plate invagination. While they provide strong evidence that RA controls CNKSR2 expression, they did not analyse whether CNKSR2 expression could rescue the invagination phenotype of embryos in which RA signalling was inhibited. That would be important to further consolidate CNKSR2‘s proposed role as a downstream effector of RA signalling.

2. The authors report that CNKSR2 knock-down leads to moderate and severe invagination phenotypes. They subsequently analyse changes in gene expression and proliferation but it remains open whether these parameters are differently affected in embryos in which roof plate invagination is moderately and severely affected. They also need to check the specificity of the roof plate invagination defect by rescue experiments using a CNKSR2 resistant construct.

3. The MKK1dn rescue experiments appear incomplete. The authors should include embryos electroporated with (i) the empty expression vector alone (control), (ii) the empty expression vector + CNKSR2 knock-down construct (to show that the invagination phenotype is present in this set of experiments), (iii) the empty expression vector + the MKK1dn construct (to test whether this construct on its own has an effect in this set of experiment) and (iv) the empty expression vector + CNKSR2 knock-down construct + the MKK1dn construct (to test for the rescue). The analysis of invagination, proliferation and gene expression need to be rigorously quantified. At this stage, this quantification is completely missing.

4. In mouse, apoptosis plays a crucial role in telencephalic roof plate invagination. The authors should investigate whether CNKSR2 knock-down affects apoptosis.

Minor points

1. The rationale for selecting CNKSR2 from the list of metabolism regulated genes remains unclear and needs to be explained.

2. The authors determine the CNKSR2 expression domain relative to that of active RA signalling in different sets of embryos. It would be more convincing, if they could perform a double labelling for CNKSR2 and for active RA signalling on the same or an adjacent sections.

3. The authors consistenly used the term “loss of CNKSR2”, but they are using a knock-down approach. Hence, they should replace the term loss with knock-down.

4. Figure 4F shows an EdU/GFP/DAPI triple stain, but the heading only indicates EdU and DAPI. Please remove the GFP channel from this figure.

5. Figure 5: better describe the altered expression patterns of Bmp7, Zic2, Wnt7b and Otx2 after loss of RA signalling. The in situ hybridisation of Zic2, Wnt7b and Otx2 in Fig. 5B-I are very weak and difficult to see. The shortening of the Zic2 and Otx2 expression domains need to be quantified.

6. Figure 6A-B: The electroporation of the control is not very widespread, could this figure be replaced? In knock-down embryos, pMEK1/2+GFP+ cells are very difficult to see. A higher resolution image is required.

7. Figure legends are overly long, but information on statistical tests are missing. Please shorten the text and include the statistical tests.

8. Discuss wider implications: have RA signalling and/or CNKSR2 been linked to holoprosencephaly in humans?

9. All gene names need to be written in italics.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Udaykumar et al presents a functional study of the CNKSR2 gene, encoding a scaffolding protein involved in modulating the Ras/Raf/Mek pathway, during roof plate patterning and morphogenesis in the chick forebrain. The same group had shown previously that RA signals from the dorsal mesenchyme were necessary for chick roof plate invagination and patterning (Gupta et al, Development 2015). Here the authors first show that CNKSR2 is expressed in the developing forebrain roof plate at the time of invagination and is positively regulated by retinoic acid (RA) signaling. They then use an RNAi electroporation strategy to show that CNKSR2 is necessary for roof plate invagination. CNKSR2 knock-down increases proliferation at the dorsal midline and impairs midline patterning. Conversely, CNKSR2 ectopic expression ectopically activates floor plate markers in the lateral forebrain, but does not reduce proliferation. The authors then set up experiments to show that CNKSR2 downregulates the Ras/Raf/Mek pathway in the roof plate and that it acts on RP invagination via modulating this pathway.
The manuscript identifies a potential new gene (CNKSR2) involved in forebrain roof plate invagination, a process which is not well understood and has implications for specific forms of human holoprosencephaly. Whether the pathway described here is specific for the chick or conserved in amniotes is not addressed and is unclear given that RA involvement in forebrain roof plate invagination is not conserved in mice. Moreover, some of the experiments are not totally convincing and the conclusions are not always supported by the data. See below my more specific comments.

Comments for the author

Major points

1 CNKSR2 expression and its regulation by RA
In Fig. 1A-5, CNKSR2 expression is detected as soon as HH16, although in Gupta and Sen (Development 2015), RALDH2 is detected in the dorsal mesenchyme only from HH20, and RARE in the roof plate at HH22. This suggests that RA signaling is not involved in the initial activation of CNKSR2. This should be discussed.

Figure 1G-J: The presence of RA pathway activity in the forebrain roof plate has been already very well documented in a previous paper (Gupta and Sen, Development 2015). Thus, the interest of this figure would be to demonstrate that the domains of RA pathway activity and CNKSR2 expression precisely coincide. However, in all three cases shown in Fig 1G-J, GFP (electroporation reporter) and AP (RARE activity reporter) overlap totally. Thus, the experiment does not show the total

extent of RARE activity, but rather the extent of the electroporated domain. To precisely compare the RARE activity and CNKSR2 expression domains, the electroporation should target boundaries between RARE-active and RARE-inactive regions.

Is CNKSR2 expression in the forebrain roof plate conserved in mice?

2 Roof plate patterning
The effect of CNKSR2 knock-down on roof plate patterning is difficult to evaluate in Fig 5 because the ISH staining is very faint. More convincing images should be shown.
In controls (Fig 5B'', D", F'' and H'') the limits of the Bmp7, Wnt7, Zic2 and Otx2 expression domains are very difficult to distinguish. For Zic2 (Fig. 5F-F'') and Otx2 (Fig. 5H-H''), I do not see the stronger expression in the lateral part of the W compared to the medial part.
In RNAi condition, the only clear result is the loss of Bmp7 (Fig. 5C''). In E'', what I see is a relatively strong ectopic expression of Wnt7 in the medial roof plate. It is not clear from the image shown whether there is also a downregulation of Wnt7 in the more lateral part. Moreover, the description of the result in the text does not fit with this observation: "Similarly, the midline expression domain of Wnt7 was absent, with only expression in the lateral arms of the W remaining" (Fig. 5E-E''). I actually see the contrary… For Zic2 (Fig.5G'') and Otx2 (Fig. 5 I'') expression, I hardly see a difference with the controls (F'' and H'').
In CNKSR2 ectopic expression experiments, again the clearest result is for Bmp7 (Fig.4K-L''). The ectopic expression of Zic2 (Fig. S4C-C''), Wnt 7b (Fig. S4E-E'') and Otx2 (Fig. S4G-G'') in CNKSR2-electoporated cells is also visible. However, for Otx2, it seems that control (pCAG-GFP) electroporation also leads to ectopic Otx2 expression (Fig. S4F-F'').

3 Role of CNKSR2 in MEK signaling and rescue of CNKSR2-RNAi by MKK1dn In Fig. 6B-B'', pMEK2 is stronger lateral to the electroporated region, while it remains very low in the electroporated (medial) region. This suggests a non-cell autonomous effect of the RNAi electroporation. Is it the case?

In the rescue experiment, a CNKSR2-RNAi control is missing. Moreover, the extent of the electroporated regions is very low in the medial roof plate in the examples shown (Fig. 6E-F'') suggesting that the expression of the electroporated constructs may not be sufficient to give a phenotype. More convincing samples should be shown.

The effect of MKK1-dn on MAPK signaling in this experiment should be clarified. In Fig S5F-F'' and Fig. S6C-D'', MKK1dn does not appear to repress pMEK1/2. Is it normal? MKK1/MEK1 is a MAPK kinase and thus acts on ERK phosphorylation and not on MEK1/2. Thus, in order to test the activity of the MKK1-dn construct in this experiment, the authors should analyze pERK and not pMEK1/2. However, it is possible that MKK1dn overexpression dilutes phosphorylable MEK1/2 and thus reduces pMEK1/2 levels in the electroporated cells. Could you clarify the effect of MKK1dn? It is important to verify properly whether MKK1-dn has an effect on MAPK signaling in these experiments.

If the altered roof plate invagination due to CNKSR2 knock-down can be rescued by downregulation of the Ras/Raf/Mek pathway, an overactivation of the Ras/Raf/Mek pathway in the roof plate of wild type embryos should impair invagination. Have the authors tested that?

4 Cell autonomy of the phenotypes
The question of the cell autonomy of the observed phenotypes should be addressed. It seems that CNKSR2 RNAi has effects on the most medial region of the roof plate but also on more lateral regions, which do not express the gene strongly. Moreover, sometimes the phenotypes are observed in regions that do not express GFP (or not strongly enough to be seen). To explain these observations, an indirect effect of CNKSR2 on lateral regions, for instance through BMP signaling, could be envisaged. In the experiments shown, the magnification and the quality of the images are not sufficient to assess cell autonomy of the phenotypes.

5 Cell death should be assayed in CNKSR2 RNAi to verify that the loss of the Bmp7-positive V-shaped roofplate region is not due to apoptosis.

Minor points

Page numbers would have been useful. I numbered the pages starting with p1 = title page.

Abstract, lines 2-3: it would be more accurate to state that defects in roof plate invagination lead to midline interhemispheric holoprosencephaly (MIH-HPE). The major forms of HPE involve defects of the ventral midline, which lead to more severe phenotypes.

Results section, p.7, in title “Knockdown of CNKSR2 affects cell proliferation in the invaginating roof plate”: “increases” would be more accurate than “affects”.

Fig 4F: remove green staining because it is very difficult to see the EdU staining under the GFP staining.

P10 (rescue experiment) third bottom line : Fig S5D-E’ should be Fig S5D-E’’.

Reviewer 3

Advance summary and potential significance to field

In this paper Udaykumar et al investigate the expression pattern, regulation and function of CNKSR2 (Connector enhancer kinase suppressor of Ras 2) in forebrain development, using the highly tractable chicken embryo as a model system. CNKSR2 was identified in a screen previously carried out by this group to uncover metabolism related genes in the forebrain. They find that expression of this gene is confined to the roof plate and coincides with the time when this structure invaginates as the forebrain expands. Using a transfected reporter for retinoic acid (RA) signalling they aim to show that CNKSR2 expression overlaps with that of RA activity and carry out gain and loss of function approaches which suggest that CNKSR2 is regulated by this signalling pathway. Using RNAi and mis-expression approaches they then assess the consequences of loss and gain of CNKSR2 on cell proliferation, MAPK signalling and patterning of the forebrain.

These are a relatively straight forward and logical series of experiments which provide some evidence for a novel mode of action of the RA signalling in promoting expression of an inhibitor of MAPK signalling and cell proliferation. However, the quality of the mRNA in situ hybridisation data is variable and, in some cases, too low to form conclusions from the figures provided. Some control electroporations appear to be much less efficient than the experimental plasmid and it is difficult to see expression of multiple markers in individual cells some images. Statistical analyses (t-test) is mentioned but not justified in the Methods, while numbers of sections assessed is provided numbers of cells and p-values for comparisons are not provided in the text or in the figure legends. In general, the Methods provided are very brief. The Discussion largely addresses their findings here and lacks reference to the wider literature on the roof plate and forebrain morphogenesis.

Comments for the author

Detailed comments which the author could address to improve the paper include:

1) In figures 1G-I is it possible to provide an overlay of the GFP and AP expression patterns – are high GFP expressing cells also high AP? - the AP level is varied, does this reflect level of transfection? The experiment has generated transfections largely in the roof plate region, it would be good to show that lateral transfections do not also lead to RARE-AP induction. Comparison with expression of an endogenous known RA target gene would strengthen this experiment.

2) In figure 2 overlay of GFP and mRNA ish would be helpful. The images in C and G suggest that the VP16 and DNRAR constructs are not acting cell autonomously (which is unexpected), can the authors provide evidence on a cell by cell basis for the relationship between CNKSR2 and construct expression.

3) In Figure 3 the RNAi knock down of CNKSR2 does not appear to be cell autonomous. The GFP (indicating transfected cells) is mosaic and so the KD should be too?

4) The conclusions drawn would be more compelling if the authors provided further details on how the % of roof plate phenotype was scored following KD of CNKSR2 (specific number of sections from specific number of embryos), it seems these phenotypes are just scored for the RNAi expressing embryos, are all controls “normal”?

5) In Figure 4g it is difficult to see GFP+ve / EdU +ve cells in the images provided. These images could be enlarged to provide evidence for such cells.

6) In figure 6, the control in A has very few GFP expressing cells, compared with the RNAi transfected cells in B. Is there an electroporation effect contributing to the phenotype (reduced roof plate/ invagination)? The region with more pMEK1/2in the experimental vs control does not appear to correlate with the GFP localisation in B, rather there is a general increase in pMEK1/2 +ve cells?
The authors need to explain this apparent non-cell autonomous effect.

7) For BMP7 expression analysis, is it possible to combine GFP and mRNA detection so the relationship between KD and BMP7 expression can be assessed in E’’’ and F’’’.

Rebuttal letter

Dear Prof. Guillemot,

This is with reference to the manuscript MS ID#: DEVELOP/2022/200857titled "CNKSR2, a downstream mediator of retinoic acid signaling modulates the Ras/Raf/MEK pathway to regulate patterning and invagination of the chick forebrain roof plate". We had submitted this manuscript for consideration for publication in Development and on 11th May, 2022, we received the reviews as well as the decision to reject the manuscript.

We would like to thank you and the reviewers for conducting a thorough review of the manuscript, for providing insightful comments, for pointing out the reasons for not recommending it for publication in the present form and also for suggesting ways to improve the quality of the manuscript. We were happy to note that all the reviewers commented on the importance of the findings and their novelty. However, the following were the key concerns expressed by all reviewers:

1. The quality of the images of RNA in situ hybridization showing the expression of roof plate markers Bmp7, Wnt 7b, Otx2 and Zic2 is poor as well as the domain of expression needs to be quantified.
2. In the experiment to rescue the CNKSR2-RNAi phenotype with co-electroporation of MKK1dn, electroporations of all the control constructs have not been carried out. Further, statistical analysis of cell proliferation, invagination and marker expression is missing.
3. The rescue of the phenotype observed with CNKSR2-RNAi with a knock-down resistant CNKSR2 construct has not been demonstrated.
4. The status of apoptosis in the roof plate following the knock-down of CNKSR2 has not been investigated.

After carefully going through all the comments of the reviewers we have come to the conclusion that we are in a position to address each one of the major concerns raised by reviewers. For example, we can improve the quality of the mRNA in situ hybridization data and also quantify the domain of expression of the roof plate markers in each case. We can attempt to rescue the CNKSR2 RNAi phenotype by co-expressing the mouse CNKSR2. We can carry out the required control electroporations for the rescue experiment with MKK1dn as well as provide quantification and statistical analysis of the data wherever it is missing. Further we can investigate the status of apoptosis in the roof plate following knockdown of CNKSR2.

Thus, we would like to resubmit a revised version of the manuscript after carrying out all of the required experiments and making the textual changes suggested by the reviewers. We request you to grant us time of three months to do this.

Looking forward to receiving your response.

Note: Point-by-point response to reviewers comments can be found under **First revision:** Author response to reviewers' comments.

Rebuttal decision letter

MS ID#: DEVELOP/2022/200857

MS TITLE: CNKSR2, a downstream mediator of retinoic acid signaling modulates the Ras/Raf/MEK pathway to regulate patterning and invagination of the chick forebrain roof plate

AUTHORS: Niveda Udaykumar, Mohd Ali Abbas Zaidi, and Jonaki Sen

Dear Dr. Sen,

Thank you for your appeal on your recently rejected manuscript. I do understand your disappointment, but given the opinions expressed by the reviewers, I saw little option other than to decline the paper.

However, I appreciate that in your rebuttal letter, you state that you will be able to address the concerns of the referees concerning the need for rescue experiments, the design of the experiments, their quantification and the need for better images. Therefore I am willing to reconsider a revised version of your manuscript that addresses the points raised by the reviewers. Upon resubmission, please provide a detailed response to the reviewers' comments and highlighting particularly any concerns that have not been included in the revised manuscript.

The revised manuscript and rebuttal will be sent to the original reviewers. If they are convinced by your revisions and arguments, then we would be able to consider the manuscript for publication.

To submit a revision, please go to your Author Area and click on the 'Submit a Revision' link.

First revision

Author response to reviewers' comments

Response to reviewers- Point by point

Reviewer 1 Comments for the Author:

Comment 1: The authors propose that *CNKSR2* acts downstream of RA signalling to control roof plate invagination. While they provide strong evidence that RA controls *CNKSR2* expression, they did not analyse whether *CNKSR2* expression could rescue the invagination phenotype of embryos in which RA signalling was inhibited. That would be important to further consolidate *CNKSR2*'s proposed role as a downstream effector of RA signalling.

Response: We thank the reviewer for suggesting this experiment. We have performed this experiment by co-electroporating RAR403 (dominant-negative receptor) with mouse *CNKSR2* (m*CNKSR2*) to see if this can rescue. These were compared to the pCAG-GFP, m*CNKSR2*, *CNKSR2*-RNAi, *CNKSR2*-RNAi+m*CNKSR2*, and RAR403 alone electroporated forebrains. We assessed for the

expression of m*CNKSR2* by mRNA *in situ* hybridization. However, our results do not show a rescue in the invagination defect suggesting that *CNKSR2* alone may not be the only factor acting downstream of RA signaling to regulate the invagination of the roof plate. We have added this data in the Supplementary Figure S9 for the data of this experiment.

Comment 2: The authors report that *CNKSR2* knock-down leads to moderate and severe invagination phenotypes. They subsequently analyze changes in gene expression and proliferation but it remains open whether these parameters are differently affected in embryos in which roof plate invagination is moderately and severely affected. They also need to check the specificity of the roof plate invagination defect by rescue experiments using a *CNKSR2* resistant construct.

Response 2a: We have examined cell proliferation on all the different phenotypes obtained upon knockdown of CNKSR2 in the midline and we find upon quantification of the cell proliferation in each case that there was no significant difference in the increase in cell proliferation in the roof plate midline between the moderately (U/V-shaped roof plate) affected and severely (flattened roof plate) affected embryos. This can be observed in Figure 1 where PH3 staining has been carried out on one example of the U/V shaped roof plate (Fig1 B-B") and on one example of a flattened roof plate (Fig1 C-C") when compared to control (Fig 1A-A").

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

(A) DAPI (blue) and PH3 staining (red) in control RNAi electroporated embryos at HH23. White arrowheads indicate the region of low cell proliferation. (A') GFP (green) and PH3 staining (red) in control RNAi electroporated embryos. (A") Merged images of DAPI,GFP and PH3 staining on control RNAi electroporated embryos. (B) DAPI (blue) and PH3 staining (red) in moderately affected *CNKSR2*-RNAi electroporated embryos at HH23. White arrowheads indicate the proliferating cells. (B') GFP (green) and PH3 staining (red) in moderately affected *CNKSR2*-RNAi electroporated embryos. (B") Merged images of DAPI, GFP and PH3 staining on moderately affected *CNKSR2*-RNAi electroporated embryos. (C) DAPI (blue) and PH3 staining (red) in severely affected *CNKSR2*-RNAi electroporated embryos at HH23. White arrowheads indicate the proliferating cells. (C') GFP (green) and PH3 staining (red) in severely affected *CNKSR2*-RNAi electroporated embryos. (C") Merged images of DAPI, GFP and PH3 staining on severely affected *CNKSR2*-RNAi electroporated embryos. Scale bars= 100μm.

Response 2b: We have performed a rescue experiment by co-electroporating *CNKSR2*-RNAi and m*CNKSR2* (mouse CNKSR2) and comparing it to embryos electroporated with m*CNKSR2* alone in the roof plate midline. Our results show that the co-electroporation of *CNKSR2*-RNAi and m*CNKSR2* partially rescues the invagination defect with lowered cell proliferation and *Bmp7* expression when compared to *CNKSR2*-RNAi alone. We have added this data on quantification of cell proliferation and Bmp7 expression in Fig 6.

Comment 3: MKK1dn rescue experiments appear incomplete. The authors should include embryos electroporated with (i) the empty expression vector alone (control), (ii) the empty expression vector + *CNKSR2* knock-down construct (to show that the invagination phenotype is present in this set of experiments), (iii) the empty expression vector + the MKK1dn construct (to test whether this construct on its own has an effect in this set of experiment) and (iv) the empty expression vector + *CNKSR2* knock-down construct + the MKK1dn construct (to test for the rescue). The analysis of invagination, proliferation and gene expression need to be rigorously quantified. At this stage, this quantification is completely missing.

Response: We agree with the reviewer and have added the missing controls. We have generated the following : 1) pCAG-GFP alone 2) *CNKSR2*-RNAi 3)MKK1dn alone as this construct has a IRES GFP in its backbone, 4) *CNKSR2*-RNAi+MKK1-dn followed by assessing cell proliferation and Bmp7 expression through RNA *in situ* hybridization. In addition to this, we have added the quantification of cell proliferation and the rescue data with m*CNKSR2*+*CNKSR2*-RNAi (Fig6) in the revised manuscript.

Comment 4: In mouse, apoptosis plays a crucial role in telencephalic roof plate invagination. The authors should investigate whether *CNKSR2* knock-down affects apoptosis.

Response: We have examined the status of apoptosis after the knockdown of *CNKSR2* at HH18 and HH23 . We found no significant change in the number of TUNEL positive cells between the control and knockdown of *CNKSR2*. This has been added in Supplementary Fig S3.

Minor points

Comment 1: The rationale for selecting *CNKSR2* from the list of metabolism regulated genes remains unclear and needs to be explained.

Response: We performed both whole mount *in situ* hybridization and section *in situ* hybridizations of all the metabolism related genes that were reported to be expressed in the HH18 and HH22 chick forebrain (Roy et.al.,2013). We have added the following in the introduction section of the revised manuscript to justify why we focused on CNKSR2 **"In this study, we have identified *CNKSR2* (Connector enhancer kinase suppressor of Ras 2), to be expressed in the similar domain to RA signaling in the forebrain roof plate midline. Thus, we investigated *CNKSR2* as a novel downstream mediator of RA signaling during forebrain roof plate invagination."**

Comment 2: The authors determine the *CNKSR2* expression domain relative to that of active RA signalling in different sets of embryos. It would be more convincing, if they could perform a double labelling for *CNKSR2* and for active RA signalling on the same or an adjacent section.

Response: For this experiment, we are faced with the limitation that the reporter constructs for RA signaling (RARE-AP) and the method of detection of mRNA in situ hybridization are the same, i.e., detection by alkaline phosphatase staining. Thus, simultaneous detection of *CNKSR2* expression through in situ hybridization and detection of active RA signaling is not possible in the same embryo or same section of the forebrain. Hence, we chose to perform these experiments on separate sets of embryos.

Comment 3: The authors consistently used the term "loss of *CNKSR2*", but they are using a knock-down approach. Hence, they should replace the term loss with knock-down.

Response: We have made the suggested change in the manuscript, wherever "loss of *CNKSR2*" appears.

Comment 4: Figure 4F shows an EdU/GFP/DAPI triple stain, but the heading only indicates EdU and DAPI. Please remove the GFP channel from this figure.

Response: We thank the reviewer for pointing out this error in the figure. We have removed the GFP channel and replaced the panel with the correct one.

Comment 5: Figure 5: better describe the altered expression patterns of Bmp7, Zic2, Wnt7b and Otx2 after loss of RA signalling. The in-situ hybridization of Zic2, Wnt7b and Otx2 in Fig. 5B-I are very weak and difficult to see. The shortening of the Zic2 and Otx2 expression domains need to be quantified.

Response: We have described the altered expression patterns of Bmp7, Zic2, Wnt7b and Otx2 after loss of RA signalling in the revised manuscript. We have added the following **"We have previously observed that inhibition of RA signaling in the forebrain roof plate leads to patterning defects with changes in expression of roof plate markers, *Bmp7*, *Wnt7b*, *Zic2*, and *Otx2*. For example, there was a loss of expression of *Bmp7* and a shortening of the expression domains of *Wnt7b*, *Zic2*, and *Otx2* upon inhibition of RA signaling** (Gupta & Sen, 2015)**. Thus, we investigated whether the knockdown of *CNKSR2* affects the expression of these markers similarly."**
We agree with the reviewer that the *in-situ* signal is hard to visualize. We have generated better images of the *in-situ* hybridization in Fig 5 in the revised manuscript. We have also done the quantification of the expression domains.

Comment 6: Figure 6A-B: The electroporation of the control is not very widespread, could this figure be replaced? In knock-down embryos, pMEK1/2+GFP+ cells are very difficult to see. A higher resolution image is required.

Response: We have replaced the panel with a better electroporated control embryo and also added a high-resolution image for better visualization of the data as suggested. The pMEK1/2 immunostaining in the knockdown embryos have been replaced and the higher magnification images have been added as well. Please refer to Fig 6 of the revised manuscript.

Comment 7: Figure legends are overly long, but information on statistical tests are missing. Please shorten the text and include the statistical tests.
Response: As the number of panels are relatively high and the number of figures is limited, we have attempted to reduce the text of the figure legends. The statistical test that we have used is student's t-test for the quantification and One-way ANOVA for Fig 6K. For all statistical tests, we used the software GraphPad Prism 8, which is mentioned in the materials and methods section under the subheading "statistical analysis", including the number of sections and embryos used in each case.

Comment 8: Discuss wider implications: have RA signalling and/or *CNKSR2* been linked to holoprosencephaly in humans?

Response: In the discussion section of the revised manuscript, we have added a section on the known literature for RA signaling, CNKSR2 and speculated on their possible role in the aetiology of HPE.

Comment 9: All gene names need to be written in italics.

Response: We have made the suggested changes to the text and figures in the manuscript.

Reviewer 2 Comments for the Author:

Major points

Comment 1: *CNKSR2* expression and its regulation by RA
In Fig. 1A-5, *CNKSR2* expression is detected as soon as HH16, although in Gupta and Sen (Development 2015), RALDH2 is detected in the dorsal mesenchyme only from HH20, and RARE in the roof plate at HH22. This suggests that RA signaling is not involved in the initial activation of *CNKSR2*. This should be discussed.

Response: One possible explanation for this could that the RALDH2 is present in very low levels in the chick forebrain roof plate at stages prior to HH20 and thus could not be detected via RNA *in situ* hybridization as reported in Gupta and Sen, 2015. Further, in this manuscript, we have provided data showing the presence of active RA signaling at stages HH18 onwards, indicating that RA signaling maybe operational in the roof plate at stages earlier than HH20. We have observed AP staining, a reporter for RA signaling at HH16 as well (Fig 2 in this document), indicating that RA signaling is operational in the roof plate since early stages.

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

(A) DAPI (blue) staining of the HH16 forebrain (B) GFP (green) indicating the extent of electroporation of the RA signaling reporter, RARE-AP. (C) Alkaline phosphatase staining indicating the presence of active RA signaling in the HH16 roof plate. Scale bars=100μm

Comment 2: Figure 1G-J: The presence of RA pathway activity in the forebrain roof plate has been already very well documented in a previous paper (Gupta and Sen, Development 2015). Thus, the interest of this figure would be to demonstrate that the domains of RA pathway activity and *CNKSR2* expression precisely coincide. However, in all three cases shown in Fig 1G-J, GFP (electroporation reporter) and AP (RARE activity reporter) overlap totally. Thus, the experiment does not show the total extent of RARE activity, but rather the extent of the electroporated domain. To precisely compare the RARE activity and *CNKSR2* expression domains, the

electroporation should target boundaries between RARE-active and RARE-inactive regions.
Response: We agree with the reviewer on this point; however, we are faced with limitation of the technique of electroporation which does not always span a broad domain. In this study, Fig 1G", H" and I'' the GFP is observed in a broad domain of the roof plate and there are large number of GFP positive cells that do not show the AP staining (white arrowheads in panels),indicating that the presence of RA signaling is only in the middle of the invaginating roof plate, at different stages of the chick embryo (Fig 3 in this document).

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

(G') DAPI (blue) staining of the HH18 forebrain. (G") GFP (green) indicating the extent of electroporation of the RA signaling reporter, RARE-AP, white arrowheads indicate the electroporation domain in the lateral roof plate devoid of AP staining. (G") Alkaline phosphatase staining indicating the presence of active RA signaling in the HH18 roof plate midline. (H') DAPI (blue) staining of the HH21 forebrain. (H") GFP (green) indicating the extent of electroporation of the RA signaling reporter, RARE-AP, white arrowheads indicate the electroporation domain in the lateral roof plate devoid of AP staining. (H") Alkaline phosphatase staining indicating the presence of active RA signaling in the HH21 roof plate midline. (I') DAPI (blue) staining of the HH23 forebrain. (I") GFP (green) indicating the extent of electroporation of the RA signaling reporter, RARE-AP, white arrowheads indicate the electroporation domain in the lateral roof plate devoid of AP staining. (I") Alkaline phosphatase staining indicating the presence of active RA signaling in the HH21 roof plate midline. Scalebars=100µm.

Comment 3: Is *CNKSR2* expression in the forebrain roof plate conserved in mice?

Response: We have observed that the expression of *CNKSR2* is present in the cortical plate from E14.5 to E18.5 in the mouse forebrain (Fig 4 in this document). The appropriate stage matched mouse embryo for HH23 would be E11.5, we have not carried out RNA in situ hybridization for *CNKSR2* in the mouse forebrain at this stage. Since we have found no expression of RALDH2 in the dorsal mesenchyme of the mouse forebrain at early stages and from literature we know that there is no LacZ reporter expression in the forebrain roof plate in the RARE-LacZ reporter mouse (Rossant et al., 1991), we hypothesize that RA signaling is not likely to be involved in regulating the invagination of the forebrain roof plate in mouse. Thus, we think it is unlikely that *CNKSR2* would be expressed in the mouse roof plate midline and perform the same function as in the chick.

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Rossant, J., Zirngibl, R., Cado, D., Shago, M., & Giguère, V. (1991). Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes & Development*, *5*(8), 1333–1344. https://doi.org/10.1101/gad.5.8.1333

Comment 4: Roof plate patterning

The effect of *CNKSR2* knock-down on roof plate patterning is difficult to evaluate in Fig 5 because the ISH staining is very faint. More convincing images should be shown. In controls (Fig 5B'', D", F'' and H'') the limits of the Bmp7, Wnt7, Zic2 and Otx2 expression domains are very difficult to distinguish. For Zic2 (Fig. 5F-F'') and Otx2 (Fig. 5H-H''), I do not see the stronger expression in the lateral part of the W compared to the medial part. In RNAi condition, the only clear result is the loss of Bmp7 (Fig. 5C''). In E'', what I see is a relatively strong ectopic expression of Wnt7 in the medial roof plate. It is not clear from the image shown whether there is also a downregulation of Wnt7 in the more lateral part. Moreover, the description of the result in the text does not fit with this observation: "Similarly, the midline expression domain of Wnt7 was absent, with only expression in the lateral arms of the W remaining" (Fig. 5E-E''). I actually see the contrary… For Zic2 (Fig.5G'') and Otx2 (Fig. 5 I'') expression, I hardly see a difference with the controls (F'' and H''). In *CNKSR2* ectopic expression experiments, again the clearest result is for Bmp7 (Fig.4K-L''). The ectopic expression of Zic2 (Fig. S4C-C''), Wnt 7b (Fig. S4E-E'') and Otx2 (Fig. S4G-G'') in *CNKSR2*- electoporated cells is also visible. However, for Otx2, it seems that control (pCAG-GFP) electroporation also leads to ectopic Otx2 expression (Fig. S4F-F'').

Response:

a) We have changed the panels in Fig 5B-I" as suggested by the reviewer.

b) For the change in the expression of Wnt7b upon knockdown of *CNKSR2*, we interpreted the result as follows; the Bmp7 expressing region of the roof plate (the middle loop of the W) appears to be absent upon knockdown of *CNKSR2* as shown by in situ hybridization of Bmp7. This leads to the formation of a U or V shaped invagination of the roof plate wherein the lateral arms of the W that express Wnt7b have joined together.

c) We have also measured the length of expression domains of Zic2 and Otx2 to show the change in the extent of expression of these markers upon knockdown of *CNKSR2*(Fig 5J).

d) Otx2 is also expressed in the ventral forebrain, as seen in the low magnification image of Otx2 RNA in situ hybridization in the chick forebrain (Fig 5, attached below). In the control electroporated samples, the electroporation has spanned part of the endogenous ventral expression domain of Otx2, which is being perceived as ectopic Otx2 expression in the control too. It can be appreciated that the intensity of Otx2 in the ectopic misexpression of m*CNKSR2* is much more robust when compared to the control, thereby indicating that misexpression of m*CNKSR2* is capable of inducing the expression of Otx2 in the lateral forebrain.

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Comment 5: Role of *CNKSR2* in MEK signaling and rescue of *CNKSR2*-RNAi by MKK1dn
In Fig. 6B-B'', pMEK2 is stronger lateral to the electroporated region, while it remains very low in the electroporated (medial) region. This suggests a non-cell autonomous effect of the RNAi electroporation. Is it the case?

Response: In this particular experiment, in the control, pMEK1/2 positive cells are low in the middle of the roof plate when compared to the flanks. Upon knockdown of *CNKSR2*, the middle region which has low number of pMEK1/2 positive cells, now shows an increase in the number of pMEK1/2 positive cells. We hypothesize that upon knockdown of *CNKSR2*, the middle loop of the W-shaped invaginating roof plate has now acquired characteristics of the flank which is manifested as increase in proliferating cells and pMEK1/2 positive cells. It is possible that there is a non-cell autonomous effect of the *CNKSR2* RNAi and we have discussed that possibility in the revised manuscript (Discussion section). We have provided higher magnification images to show that knockdown of *CNKSR2* leads to an upregulation of pMEK1/2 positive cells in the roof plate midline (Fig 6b-b" in revised manuscript).

Comment 6: In the rescue experiment, a *CNKSR2*-RNAi control is missing. Moreover, the extent of the electroporated regions is very low in the medial roof plate in the examples shown (Fig. 6E- F'') suggesting that the expression of the electroporated constructs may not be sufficient to give a phenotype. More convincing samples should be shown.
Response: We have added the controls to the revised figure (Fig 6) and changed the representative panel of pMEK1/2 immunostaining upon knockdown of *CNKSR2* in the roof plate.

Comment 7: The effect of MKK1-dn on MAPK signaling in this experiment should be clarified. In Fig S5F-F'' and Fig. S6C-D'', MKK1dn does not appear to repress pMEK1/2. Is it normal? MKK1/MEK1 is a MAPK kinase and thus acts on ERK phosphorylation and not on MEK1/2. Thus, in order to test the activity of the MKK1-dn construct in this experiment, the authors should analyze pERK and not pMEK1/2. However, it is possible that MKK1dn overexpression dilutes phosphorylable MEK1/2 and thus reduces pMEK1/2 levels in the electroporated cells. Could you clarify the effect of MKK1dn? It is important to verify properly whether MKK1-dn has an effect on MAPK signaling in these experiments.

Response: In MKK1-dn, the lysine amino acid at the 97th position in the ATP binding has been mutated to methionine. Therefore, this MKK1-dn can bind to Raf and prevent the downstream signaling cascade. The ideal readout for this signaling pathway is pMAPK or pERK, however we were unable to obtain immunostaining of the chicken forebrain tissues with the available antibodies for pMAPK. Hence, the repression of this pathway was represented by pMEK1/2 immunostaining. As this

construct was a gift from a laboratory that has already verified the ability of the construct to down regulate this particular pathway in the chick embryo (Mansour et.al., 1994), we proceeded to perform the functional analyses directly.

We have transfected this construct in HEK293T cells and performed pMAPK staining on them followed by quantification of the immunostaining to show downregulation compared to the control transfected cells. We have also removed the data of pMEK1/2 upon ectopic expression of MKK1-dn as in this case phosphorylation of MEK is not affected (numbers remain same between control and test), rather the downstream signaling cascade is inhibited.

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

(A) Immunostaining of pERK1/2 (red) and DAPI (blue)on HEK293T cells 44h post-transfection with pCAG-GFP, showing low numbers of pERK1/ 2 positive cells. (A') Immunostaining of pERK1/2 (red) on HEK293T cells 44h post-transfection with pCAG-GFP (green).(A")Merged images of A and A'.
(a) Magnified images of boxed region in A", white arrowheads indicate triple-positive cells. (B) Immunostaining of pERK1/2 (red) and DAPI (blue) on HEK293T cells 44h post-transfection with MKK1-dn-IRES-GFP, showing low numbers of pERK1/ 2 positive cells. (B') Immunostaining of pERK1/2 (red) on HEK293T cells 44h post-transfection with MKK1-dn-IRES-GFP (green).(B")Merged images of B and B'. (b) Magnified images of boxed region in B" white arrowheads indicate double-positive cells. (C) Immunostaining of pERK1/2 (red) and DAPI (blue) on HEK293T cells 44h post-transfection with HRASV12+GFP, showing high numbers of pERK1/ 2 positive cells. (B') Immunostaining of pERK1/2 (red) on HEK293T cells 44h post-transfection with HRASV12+GFP (green).(C")Merged images of C and C'. (c) Magnified images of boxed region in C", white arrowheads indicate triple-positive cells. (D) Immunostaining of pERK1/2 (red) and DAPI (blue) on HEK293T cells 44h post-transfection with HRASV12+MKK1-dn, showing reduced number of pERK1/ 2 positive cells. (D') Immunostaining of pERK1/2 (red) on HEK293T cells 44h post-transfection with HRASV12+MKK1-dn (green).(D")Merged images of D and D'. (d) Magnified images of boxed region in D" white arrowheads indicate double-positive cells. Quantification of the fluorescence intensity of pMEK1/2 across the four conditions. Statistical analysis was performed using One-way ANOVA in GraphPad Prism 8.

Comment 8: If the altered roof plate invagination due to *CNKSR2* knockdown can be rescued by downregulation of the Ras/Raf/Mek pathway, an overactivation of the Ras/Raf/Mek pathway in the roof plate of wild type embryos should impair invagination. Have the authors tested that?

Response: Yes, we have electroporated HRAsV12 in the chick forebrain roof plate, which is a constitutively active version of the Ras-GTPase protein in the pathway. We have observed that in this case too, invagination is impaired. We have added this data as supplementary to the manuscript as this provides additional support. Please refer Fig S8 of the revised manuscript and Supplementary Figures.

Comment 9: Cell autonomy of the phenotypes

The question of the cell autonomy of the observed phenotypes should be addressed. It seems that *CNKSR2* RNAi has effects on the most medial region of the roof plate but also on more lateral regions, which do not express the gene strongly. Moreover, sometimes the phenotypes are observed in regions that do not express GFP (or not strongly enough to be seen). To explain these observations, an indirect effect of *CNKSR2* on lateral regions, for instance through BMP signaling, could be envisaged. In the experiments shown, the magnification and the quality of the images are not sufficient to assess cell autonomy of the phenotypes.

Response: It is quite possible that there are non-cell autonomous effects of *CNKSR2* RNAi. We have discussed the possibility of this being mediated through BMP/Wnt signaling in the discussion section. However, we feel that investigating the autonomy of CNKSR2 is beyond the scope of the current study as it requires in-depth analyses.

Comment 10: Cell death should be assayed in *CNKSR2* RNAi to verify that the loss of the Bmp7-positive V-shaped roof plate region is not due to apoptosis.

Response: We have assessed this possibility by performing TUNEL assay for detecting apoptotic cells after knockdown of *CNKSR2* at HH18 and HH23. We have added this data as Supplementary Figure S3.

Minor points

Comment 1: Page numbers would have been useful. I numbered the pages starting with p1 = title page.

Response: We agree that this a lapse from our end. We have added the page numbers in the revised manuscript.

Comment 2: Abstract, lines 2-3: it would be more accurate to state that defects in roof plate invagination lead to midline interhemispheric holoprosencephaly (MIH-HPE). The major forms of HPE involve defects of the ventral midline, which lead to more severe phenotypes.

Response: We thank the reviewer for this suggestion, and we have made the change in the text in the revised manuscript.

Comment 3: Results section, p.7, in title "Knockdown of *CNKSR2* affects cell proliferation in the invaginating roof plate": increases" would be more accurate than "affects".

Response: We have made the change as suggested in the revised manuscript.

Comment 4: Fig 4F: remove green staining because it is very difficult to see the EdU staining under the GFP staining.

Response: We have made the suggested changes in the figure in the revised manuscript. Please refer Fig 4.

Comment 5: P10 (rescue experiment) third bottom line : Fig S5D-E' should be Fig S5D-E''.
Response: We thank the reviewer for pointing out this error in the labeling of the figure panels. We have rectified this error in the revised manuscript.

Reviewer 3 Comments for the Author:

Comment 1: In figures 1G-I is it possible to provide an overlay of the GFP and AP expression patterns – are high GFP expressing cells also high AP? - the AP level is varied, does this reflect level of transfection? The experiment has generated transfections largely in the roof plate region, it would be good to show that lateral transfections do not also lead to RARE-AP induction. Comparison with expression of an endogenous known RA target gene would strengthen this experiment.

Response: We have overlayed the GFP and the AP staining in this figure in the revised manuscript. Yes, high GFP expressing cells show high AP staining and this is being reflected by the extent of electroporation in this region. To date, we have detected the RARE-AP in the region of the roof plate only. It is possible that the lateral regions also may show some RARE-AP activity possibly through the expression of other RALDHs such as RALDH3 in this region. Therefore, it may not be appropriate to compare the lateral RARE-AP staining with that of the roof plate region. Also, to date we have do not know of any target gene expressed downstream of RA signaling in the context of the chick forebrain roof plate.

Comment 2: In figure 2 overlay of GFP and mRNA ish would be helpful. The images in C and G suggest that the VP16 and DNRAR constructs are not acting cell autonomously (which is unexpected), can the authors provide evidence on a cell-by-cell basis for the relationship between *CNKSR2* and construct expression.
Response: We have overlayed the GFP and the mRNA *in situ* hybridization in this figure at high magnification. In the figure C and G, the electroporation of each of the constructs span a broad extent of the lateral region of the forebrain and the roof plate respectively, leading to induction and absence of the *CNKSR2* transcript. In case of DNRAR all cells expressing GFP seem to have lost or downregulated *CNKSR2* expression. In the panels C-C'', there are few GFP positive cells

(expressing VP16-RAR) that do not show the expression of *CNKSR2* transcript, suggesting that higher levels of RA signaling are required for the induction of the *CNKSR2* transcript in this ectopic region which may not be possible with VP16-RAR.

Comment 3: In Figure 3 the RNAi knock down of *CNKSR2* does not appear to be cell autonomous. The GFP (indicating transfected cells) is mosaic and so the KD should be too?

Response: In figure 3, in all the *CNKSR2*-RNAi electroporated samples, the regions devoid of GFP show some expression of the *CNKSR2* mRNA. We have increased the brightness of the panel so that the faint expression pattern can be visualized. This is more appreciable in the higher magnification images of the boxed region indicating a mosaic pattern upon electroporation of the knockdown construct.

Comment 4: The conclusions drawn would be more compelling if the authors provided further details on how the % of roof plate phenotype was scored following KD of *CNKSR2* (specific number of sections from specific number of embryos), it seems these phenotypes are just scored for the RNAi expressing embryos, are all controls "normal"?

Response: We agree that the basis of quantification of the knockdown phenotypes were not provided in adequate detail in the materials and methods section. We have provided the required information in the revised manuscript under the "Materials and Methods "section. All the control RNAi electroporated samples were considered normal as they form the W shaped structure as shown in the representative images. We examined the morphology of the knockdown electroporated forebrains by DAPI staining. We assessed the morphology of forebrain roof plate from 29 embryos electroporated with *CNKSR2*-RNAi and characterized them as either flat roof plate or U/V shaped roof plate. We found 13 out of the 29 embryos showed flat roof plate and other 16 showed the U/V shaped roof plate. Based on this the percentage of occurrence was calculated and represented as a pie chart in the figure panel 3.

Comment 5: In Figure 4g it is difficult to see GFP+ve / EdU +ve cells in the images provided. These images could be enlarged to provide evidence for such cells.

Response: We have represented each channel separately with the enlarged images in the revised manuscript. We do agree with the reviewer that the EdU staining is masking the GFP positive cells.

Comment 6: In figure 6, the control in A has very few GFP expressing cells, compared with the RNAi transfected cells in B. Is there an electroporation effect contributing to the phenotype (reduced roof plate/ invagination)? The region with more pMEK1/2in the experimental vs control does not appear to correlate with the GFP localization in B, rather there is a general increase in pMEK1/2 +ve cells? The authors need to explain this apparent non-cell autonomous effect.

Response: We have replaced the control electroporated embryos in this figure panel. The control electroporation never showed any reduced roof plate invagination indicating that this not an effect of electroporation. With regard to this experiment, our interpretation of the result is that pMEK1/2 positive cells are appearing in this region upon knockdown of *CNKSR2*. This region when compared to control has a low number of pMEK1/2 positive cells. We have discussed if the possibility of *CNKSR2* working in a non-cell autonomous phenotype in the Discussion of the revised manuscript.

Comment 7: For BMP7 expression analysis, is it possible to combine GFP and mRNA detection so the relationship between KD and BMP7 expression can be assessed in E''' and F'''.

Response: We have not combined the GFP and the mRNA as the overlay of the two panels were not convincing as when they were represented separately. Also, in some cases as the adjacent sections were taken for analysis, perfect overlap could not be done.

Second decision letter

MS ID#: DEVELOP/2022/200857

MS TITLE: *CNKSR2*, a downstream mediator of retinoic acid signaling modulates the Ras/Raf/MEK pathway to regulate patterning and invagination of the chick forebrain roof plate

AUTHORS: Niveda Udaykumar, Mohd Ali Abbas Zaidi, Aishwarya Rai, and Jonaki Sen

I have now received the reports of two of the referees who reviewed the earlier version of your manuscript and I have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The reviewers' overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining suggestions and comments of the two referees. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

In their manuscript, Udaykumar et al. investigate the function of the CNKSR2 gene in the invagination of the telencephalic roof plate in the chicken embryo. They show that CNKSR2 is a target gene of retinoic acid (RA) signalling that they had previously shown to be critical for roof plate invagination. Furthermore, CNKSR2 knock-down results in severe and moderate defects in roof plate invagination, increased proliferation, altered expression of several roof plate markers and in an increased number of pMEK1+ cells, an indicator of Ras/Raf/MEK signalling. Finally, interfering with MEK signalling rescues the roofplate defect of CNKSR2 knock-down.
Roof plate invagination is a critical step in the formation of the two telencephalic hemisphere. Defects in this process can lead to holoprosencephaly, a severe congenital malformation of the brain. Its aetiology is not very well understood, therefore there is a great interest in elucidating the underlying causes. This study identified a new molecular player in the field and characterized its upstream regulators and downstream effectors, thereby integrating it well into the existing framework of factors controlling roof plate invagination.

Comments for the author

The authors addressed all my points and I support publication of their manuscript in Development. I only wonder whether Figure1 in their response letter might be referred to and included in the manuscript as a supplementary figure?

Reviewer 2

Advance summary and potential significance to field

The manuscript by Udaykumar et al presents a functional study of the CNKSR2 gene, encoding a scaffolding protein involved in modulating the Ras/Raf/Mek pathway, during roof plate patterning and morphogenesis in the chick forebrain. The same group had shown previously that RA signals from the dorsal mesenchyme were necessary for chick roof plate invagination and patterning (Gupta et al, Development 2015). Here the authors first show that CNKSR2 is expressed in the developing forebrain roof plate at the time of invagination and is positively regulated by retinoic acid (RA) signaling. They then use an RNAi electroporation strategy to show that CNKSR2 is necessary for roof plate invagination. CNKSR2 knock-down increases proliferation at the dorsal midline and impairs midline patterning. Conversely, CNKSR2 ectopic expression ectopically

activates floor plate markers in the lateral forebrain, but does not reduce proliferation. The authors then set up experiments to show that CNKSR2 downregulates the Ras/Raf/Mek pathway in the roof plate and that it acts on RP invagination via modulating this pathway.
The manuscript identifies a potential new gene (CNKSR2) involved in forebrain roof plate invagination, a process which is not well understood and has implications for specific forms of human holoprosencephaly. They show that CNKSR2 is activated by retinoic acid and acts on roof plate patterning and regulation of cell proliferation. They also show that the Ras/Raf/mapK pathway acts downstream of Cnksr2 in this process.
The authors have correctly answered my concerns and questions, adding a number of new data as well as several figures for the reviewers.

Comments for the author

The authors have correctly answered my concerns and questions, adding a number of new data as well as several figures for the reviewers.
I only have a few remarks left:
-In the chapter “CNKSR2 regulates the invagination of the forebrain roof plate by modulating “Ras/Raf/Mek signaling”, the authors added several data about the rescue of CNKSR2 knock-down by MKK1dn and mRNA CNKSR2 injection. The new data are more convincing. However, this new chapter is poorly organized and written and thus quite difficult to follow.
-As recommended, the authors have added in the abstract that defects in roof plate invagination lead to a specific form of HPE called midline interhemispheric holoprosencephaly (MIH-HPE). However, for consistency, MIH-HPE should also be mentioned in the introduction.

Second revision

Author response to reviewers' comments

Response to Reviewers – Point by Point

Reviewer 1:

Comment 1: The authors addressed all my points and I support publication of their manuscript in Development.

Response: We thank the reviewer for their suggestions for the significant improvement of our manuscript and for recommending its publication in Development.

Comment 2: I only wonder whether Figure1 in their response letter might be referred to and included in the manuscript as a supplementary figure?

Response: While this is a very good suggestion, unfortunately including Figure1 from the response letter in the manuscript as a supplementary figure would exceed the word limit. However, to address this, we will make the peer review comments and responses visible to the reader.

Reviewer 2:

Comment 1: In the chapter “CNKSR2 regulates the invagination of the forebrain roof plate by modulating “Ras/Raf/Mek signaling”, the authors added several data about the rescue of CNKSR2 knock-down by MKK1dn and mRNA CNKSR2 injection. The new data are more convincing. However, this new chapter is poorly organized and written and thus quite difficult to follow.

Response: We have rewritten this portion in the revised manuscript as suggested by the reviewer.

Comment 2: As recommended, the authors have added in the abstract that defects in roof plate invagination leads to a specific form of HPE called midline interhemispheric holoprosencephaly (MIH-HPE). However, for consistency, MIH-HPE should also be mentioned in the introduction.

Response: We have made the suggested changes in the revised manuscript.

Third decision letter

MS ID#: DEVELOP/2022/200857

MS TITLE: *CNKSR2*, a downstream mediator of retinoic acid signaling modulates the Ras/Raf/MEK pathway to regulate patterning and invagination of the chick forebrain roof plate

AUTHORS: Niveda Udaykumar, Mohd Ali Abbas Zaidi, Aishwarya Rai, and Jonaki Sen
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.