Brahma-related gene 1 has time-specific roles during brain and eye development

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MS TITLE: *Brahma-related gene 1* has time-specific roles during brain and eye development

AUTHORS: Doerthe Holdhof, Melanie Schoof, Michael Spohn, Malte Hellwig, Daniela Indenbirken, Natalia Moreno, Kornelius Kerl, and Ulrich Schueller

I have now received all the referees' reports on the above manuscript, and 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.

As you will see, the referees have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which will have to involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by all three of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers'

box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This paper describes the effects of conditional deletions in the Sox2 expression domain during stages of neurogenesis in the mouse embryo of the Brg1 gene, which functions in the SWI/SNF chromatin remodelling complex.
Tamoxifen-induced deletions were conducted at various ages from E6.5 to E14.5. However, most of the data presented focuses on deletions induced at E7.5 and E9.5 as these displayed differences in phenotypes suggesting different temporal roles for BRG1. Comparison with a deletion of another SW/SNF gene (Ini1) revealed that the phenotypes in the SVZ were similar though a transdifferentiation phenotype of the retinal RPE to neural retina was specific to Brg1. These studies extend knowledge of the role of the SWI/SNF complex during specific stages of neuronal development and suggest indirect context-specific roles of this gene in ocular development. As mutations in this gene have been associated with various neurological conditions, tumours and rare dysmorphic syndromes, understanding the function of this gene is important.

Comments for the author

While the manuscript is quite well written and provides sound and interesting data, my main concern about the data presented in this paper is the fact that all the analyses were done numerous days (5, 7, 9 or 11 days)
after the Cre induction with Tamoxifen. There is no documentation of when the expression of BRG1 protein is lost, nor a timecourse of the phenotype development. Similarly, the main RNA expression studies are done on cells collected at E14.5 after Tamoxifen induction at E7.5 and E9.5. The concern is that the phenotypes described and the changes in expression are at a late stage and may reflect secondary effects from cellular changes that have occurred in the preceding 5-7 days.
The fact that both normal and mutant cells contribute to the various pathologies (e.g. the rosettes) suggests that many of the effects are non-
cell autonomous and likely reflect loss of mutant cells in the preceding period. In this regard the authors have not examined whether there is increased loss of the Brg1-mutant cells by cell death (e.g. TUNEL). As a result they are left to speculate in the Discussion about the occurrence of cell death, based on other studies, rather than document this and can only suggest that proliferation is either normal (as in the neocortex) or reduced
(as in the SVZ ki67-negative clusters and in the retina). While the Ki67 analyses suggested loss of cells this was never quantified, and I wonder whether a BrdU assay for S phase or phospho-histone-3 IHC for M-phase would have been better approaches to quantify changes in cell proliferation. The lack of quantification leads to rather vague conclusions (e.g. p7, para3).
The expression analyses have delivered broad categories of changed gene ontologies but further analyses of the pathway involved are lacking.
Software such as Ingenuity pathway Analysis or similar would have been useful to mine this data.
The authors have not performed any validation studies of the RNA-Seq data. Confirmation of changes in gene expression
(e.g. QPCR or digital droplet PCR) by would be useful, as would defining when these changes in expression occur after the induction of the gene deletion with Tamoxifen? Also, can the authors be certain that these changes are due to loss of Brg1 and not just due to loss of different populations of cells in the period following the Tamoxifen induction? Ideally, the RNA-Seq should have been conducted soon after the loss of Brg1 protein and at or just prior to when the phenotype started to appear. However, having established changes in certain ontologies and pathways a week following deletion, it may be more cost-effective to backtrack and examine when these changes occur by more targeted approaches (QPCR). The risk with this approach is that it may miss the precipitating changes that resulted in the gene expression observed in this paper.
If there are changes in certain pathways (BMP, TGFβ, FGF) as speculated (p9 para 2 and p10, para 1-2) then it would be sensible to demonstrate actual changes in those pathway activities (Smad phosphorylation or ERK and FRS2 phosphorylation).

The results of the neurosphere assay are intriguing but are potentially confounded if the mutant cells have a greater propensity for undergoing apoptosis. The methods for this technique need to be expanded to provide details of the numbers of cells initially used and whether there was any evidence of differential death or proliferation, other than number or size of the neurospeheres. In other words, are the differential responses in this assay due to altered responsiveness to the neurosphere induction or just altered cell population dynamics? It is also not clear how relevant the assays of gene expression in brain cells, isolated at E14.5, are for the retinal cells (p10, para 1; Figure 5).
Importantly, the authors must detail the statistical methods used to determine significance of any quantitative data. No descriptions were provided at all.
Minor points Page 5, para 3: It was not clear to me from the descriptions whether the authors considered all the morphological phenotypes observed in different parts of the brain and retina to be 'rosette-like' structures. They certainly looked similar to this untrained eye. Rosette formation in the retina is commonly associated with altered cell-cell interactions; see for example the Crb1 mutants. In these Brg1 mutants, the appearance of the rosettes does seem to be an effect of loss of cells/neurons and it is not clear that this is a primary direct effect of the Brg1 deletion.
Page 7, para 1, last sentence: Clarify sentence "The in H&E stains more cell dense structure was Brg1 positive and surrounded by a Brg1 negative cell population..."
Page 7, para 3, last sentence: Where are the data to support this statement about the retina-like region near the basal cerebrum?
Page 8, para 1, last sentence: Please provide more detail about the lslRFPfl/fl strain of mice. as my understanding is that the RFP construct is in the ROSA locus as described by Luche et al and requires a 2-step action by Cre to cause expression of RFP. This may also reduce the efficiency of recombination by the Sox2-Cre transgene. The notation used her suggests the floxed RFP is in the Isl allele?
P10, para 3: Other RGC markers to consider would include NR2F1, or TUBB3 antibodies at E14.5 and RHO for photoreceptors at E18.5?
P15, para 1: Note that conceptuses collected at E14.5 are actually fetal and not embryonic.
Figure 3: I did find the figure label background squares for images B,C,D,E,J,K,L etc rather distracting. Would suggest removing those squares and just making the figure labels bolder and closer to the corner of the image. They take up almost the quarter of a small image. I also found the crosses for empty panels disconcerting and initially wondered whether there had been an error in image rendering. Minor journal style issue.
Figure 3, image H: the Ki67 stain appears very weak and indistinct particularly when compared to stain in image Q.
Figure 5, image K: Is this section truly representative of the phenotype?
There is only one rather incomplete eye present in this section, suggesting a peripheral section. How penetrant was this phenotype? There did not appear to be an RPE transdifferentiation in Figure 1H and only one eye in Figure 1I appears to show this. Do these mutant eyes have lenses?
Figure S2: The volcano plots appear to show highly selected datasets, which I am not convinced is appropriate.

Reviewer 2

Advance summary and potential significance to field

The authors of this paper investigated the roles of Brg1 in Sox2-positive neural progenitor/stem cells (NSCs)
in mice. They found morphological defects in brain subregions and eyes, which exhibit rosette-like structures.
The authors compared mRNA of Brg1-deficient NSCs with those of wild-type NSCs, which were isolated from the embryos and grown in neurospheres. They found the changes of growth factors and ECM component genes in the cultured Brg1-deficient NSCs. The authors then tried to relate the alterations to the transdifferentiation of RPE to retina in the mice. Unfortunately, the study failed to scratch out the roles of Brg1 in NSC clearly. The results are descriptive but do not provide mechanistic interpretations.

Comments for the author

Major comments Given the use of CreER system, the authors should provide Cre reporter patterns. Without this, it is impossible to clarify whether the phenotypes are autonomous effects of Brg1 deletion or not.
Figure 1: They identified the lesion in basal part of the brain. What is the identity of basal lesion? Based on the position, the structure can be the optic stalk, which connect the brain and retina.
Figure 2: Are there rosettes in Ini1-cko mouse retina, too? The H&E image of Ini1-cko mouse retina in Figure 2E(ii) looks less compact, although it does not show clear rosettes.
Figure 3: The authors should co-stain Brg1 and Ki67 to delineate direct relationship between cell proliferation and Brg1 loss. They also need to quantify the results.
Figure 4: Interpretation of FACS data is incomplete. What is the mechanism resulting in the decrease of Brg1(+) population in Brg1-cko mouse brain? The authors show no significant decrease in cell proliferation in Brg1-cko mouse brain. However, they did not examine the death of the cells, which also can cause the loss of the cells. The authors need to examine cell death by TUNEL or caspase-3 staining. The authors may examine as they refer a previous work in Discussion (page 11).
Figure 5:
- Neurospheres were generated using brain NSCs, but the authors interpreted retinal phenotypes using the results. To do that, they need to check whether the changes of gene expression were also happened in the retina.
- The authors tried to relate the increase of Fgf15, which is observed in brain neurospheres, to RPE-to-
retina transdifferentiation in Brg1-cko mouse eyes. It is necessary to show the elevation of Fgf15 in the retina by ISH.
- The results in Figure 1J show that RPE is maintained by E14.5. Therefore, it is likely that the transdifferentiation that the authors claim might happen between E14.5 and E18.5. Otherwise, retina might have expanded into RPE territory upon the loss of RPE. Again, Cre reporter analysis is essential to clarify this.
- At E18.5, Sox2-positive cells should be RPCs, not Muller glia, astrocytes, and ChAT amacrine subset.
Those three cell types are not developed yet in the embryonic mouse retina.

Minor Delete ‘in’ between ‘The’ and ‘H&E’ in page 7 (6th line).

Reviewer 3

Advance summary and potential significance to field

This manuscript from the Schüller group examines how of the chromatin remodeling component, Brahma-related gene 1 (Brg1) influences neurodevelopment during the critical time window of E7.5-E12.5, a time during which the forebrain acquires telencephalic and diencephalic character, neurulation is completed, and retinae are forming. For the study, the authors used a combination of time-specific knock-outs, cultured neurospheres, and RNA-Seq analysis. These approaches generated a handful of genes that seem to be differentially regulated by Brg1 during this particular phase of development, including the stem cell marker sox2 and many genes that encode components of the extra cellular matrix (ECM) and some proteins that interact with the ECM. The authors did not examine a direct relationship between these genes and Brg1 expression/activity. Instead, the authors provide a histological characterization of nervous tissues and share their RNA-Seq dataset. The authors focused their discussion on a group of differentially expressed genes (DEGs) that have been implicated in forebrain and eye development.

Comments for the author

The work provides some interesting data, characterizing a partially-penetrant phenotype and potentially providing insight into how Brg1 acts during neural and neuronal development. That said, I have a number of concerns about the manuscript, its suitability for Development since I don't see a clear developmental mechanistic advance. I’ve listed some of my concerns/questions and susgestions below.

(1) In Figures 1 and 2, the authors show that Tg(sox2-cre/ERT2)-mediated removal of brg1 or ini1 leads to some striking phenotypes including tumor-like rosettes and, in the case of the brg1 ko, ectopic retina. In Figure 1, the authors point out this ectopic retina region but don't characterize it with immunohistochemistry until Figure 5. Moreover, the authors provide no explanation of the tissue that it potentially lost/converted into these ectopic retinae.
(2) In Figures 1 and 2, the authors examine the phenotypes that occur when cre induction occurs at specific times and then depletion of either brg1 or ini1 commences. They use the data in these figures to argue that the ectopic retina formation they observe may be a function that is specifically mediated by loss of brg1. In my mind, the likely conclusion from this is that Brg1 itself (and not the SWI/SNF complex) may be required to repress retinal fate. Did the authors check to see if the brg1 or ini1-deficient cells in vivo had upregulated other components of the SWI/SNF complex? Did the authors check to see if genes required for early eye formation (e.g., pax6 or rax) are upregulated a day or two after the loss of the brg1? The authors do include the necessary controls to show the level at which the Brg1 and Ini1 proteins are depleted after tamoxifen treatment, but they do not explore whether other components of the SWI/SNF complex are altered in these conditions.
(3) The authors do not explore how the Brg1+ or Ini1+ cells that remain after tamoxifen treatment could impact the phenotypes they observe. What I'm really asking here is whether the phenotype observed is because of the mix of cell types (with regard to their brg1 or ini1 levels),
(4) In Figure 3 and supplemental data, the authors show that the morphological alterations that they observe within the SVZ seem to be confined to regions that continue to express Brg1 or Ini1. It seems that what the authors have uncovered is an alteration that arises because the cells within the SVZ and other regions of the forebrain do not uniformly express Brg1 (or Ini1). I'm curious about what would have happened if they had been able to drive Brg1 expression instead of depleting it. If the cells are somehow sensing an imbalance among the Brg1+ cells and the Brg1- cells, one would expect the over-
expression of Brg1 to have a similar phenotype as the Tg(sox2-cre/ERT2); brg1fl/fl experiments.
(5) One of my biggest concerns (and perhaps hopefully misplaced) is that from my reading it seems that the authors performed bulk RNA-Seq analyses on FAC-sorted RFP+ cells that were heterogeneous in their expression of Brg1. If the cre efficiency is comparable from experiment to experiment, then it is likely that the RNA the authors sequenced came from a cell population that they are calling brg1-negative but could have been 14%-52% wild-type. Moreover, the analysis of changes in gene expression in the temporal knock-outs provide an average overview of the changes that occur but doesn't account for individual differences in cells, as it could be that differences in cell location within the tissue could dramatically influence DGE.
(6) The authors use their data presented in Figures 1, 2, and 5 to suggest that loss of brg1 from E7.5 causes RPE to transdifferentiate into retina. I'm not sure that this is the case and a number of experiments would need to be performed to definitively test this hypothesis, including but certainly not limited to (1) more careful examination of early and later markers of RPE in their brg1 ko animals (the one image of the retina shown in Figure 5B and C is small and lacks immunohistochemistry) and (2) the generation of an eye specific brg1 ko (using the Tg(rax:creERT2) mice, for example).
(7) The authors overlook literature from other organisms that have examined the consequences of brg1 loss-of-function. For example, in zebrafish, an allele of brg1 prevents differentiation of retinal progenitor cells in the developing retina. Interestingly, the brg1 mutant zebrafish retinae contain an RPE.

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

While the manuscript is quite well written and provides sound and interesting data, my main concern about the data presented in this paper is the fact that all the analyses were done numerous days (5, 7, 9 or 11 days) after the Cre induction with Tamoxifen. There is no documentation of when the expression of BRG1 protein is lost, nor a timecourse of the phenotype development. Similarly, the main RNA expression studies

are done on cells collected at E14.5 after Tamoxifen induction at E7.5 and E9.5. The concern is that the phenotypes described and the changes in expression are at a late stage and may reflect secondary effects from cellular changes that have occurred in the preceding 5-7 days.

Response:

Thank you for your constructive criticism. We followed your advice and examined earlier timepoints after tamoxifen induced activation of cre in order to investigate short term effects of Brg1 deficiency. In detail, we examined embryos 3 days post tamoxifen administration (Fig. 3), i. e. at E10.5 after tamoxifen injections at E7.5 and at E12.5 after tamoxifen injections at E9.5. These results show that the main phenotypic alterations do not occur immediately after loss of the Brg1 protein, but at later stages of embryonic development (Fig. 4). It certainly appears possible that slight changes in gene expression appear prior to the major phenotypic alterations. However, we thought that the report on gene expression analysis would be more instructive from time points of phenotypic alterations. Therefore, we decided not to replace our analyses by analyses of earlier timepoints.

The fact that both normal and mutant cells contribute to the various pathologies (e.g. the rosettes) suggests that many of the effects are non-cell autonomous and likely reflect loss of mutant cells in the preceding period. In this regard the authors have not examined whether there is increased loss of the Brg1-mutant cells by cell death (e.g. TUNEL). As a result they are left to speculate in the Discussion about the occurrence of cell death, based on other studies, rather than document this and can only suggest that proliferation is either normal (as in the neocortex) or reduced (as in the SVZ ki67-negative clusters and in the retina). While the Ki67 analyses suggested loss of cells this was never quantified, and I wonder whether a BrdU assay for S phase or phospho-histone-3 IHC for M-phase would have been better approaches to quantify changes in cell proliferation. The lack of quantification leads to rather vague conclusions (e.g. p7, para3).

Response:

We highly appreciate your suggestions. Therefore, we stained for cleaved caspase 3 and pHH3 and performed quantifications thereof (Fig. 3).

The expression analyses have delivered broad categories of changed gene ontologies but further analyses of the pathway involved are lacking. Software such as Ingenuity pathway Analysis or similar would have been useful to mine this data. The authors have not performed any validation studies of the RNA-Seq data. Confirmation of changes in gene expression (e.g. QPCR or digital droplet PCR) by would be useful, as would defining when these changes in expression occur after the induction of the gene deletion with Tamoxifen? Also, can the authors be certain that these changes are due to loss of Brg1 and not just due to loss of different populations of cells in the period following the Tamoxifen induction? Ideally, the RNA-Seq should have been conducted soon after the loss of Brg1 protein and at or just prior to when the phenotype started to appear. However, having established changes in certain ontologies and pathways a week following deletion, it may be more cost-effective to backtrack and examine when these changes occur by more targeted approaches (QPCR). The risk with this approach is that it may miss the precipitating changes that resulted in the gene expression observed in this paper.

Response

Thank you for your comments. In the revised manuscript, we included KEGG pathway analyses (Fig. S4), which fit quite well to the GO terms analyses we have already showed in the first submission. Unfortunately, there was no RNA left for performing qPCR for selected genes, but we were able to stain for the Fgf15 protein to validate the changes in Fgf15 gene expression. We also took a closer look on the time point, at which the phenotypic alterations first appear. Since they are not present immediately after loss of the Brg1 protein, we believe that E14.5 is the right time for studying changes in gene expression.

If there are changes in certain pathways (BMP, TGFß, FGF) as speculated (p9, para 2 and p10, para 1-2) then it would be sensible to demonstrate actual changes in those pathway activities (Smad phosphorylation or ERK and FRS2 phosphorylation).

Response

We thank you for your suggestions. We added heatmaps of MAPK signaling and TGFß signaling pathways in the supplement (Fig. S5, S7). They show alterations in the respective pathways. Furthermore, we conducted immunohistochemistry of phosphorylated Erk1/2 and phosphorylated Smad1/5/8 to examine changes in the pathways' activities upon Fgf15 and Bmp-7 upregulation, respectively.

The results of the neurosphere assay are intriguing but are potentially confounded if the mutant cells have a greater propensity for undergoing apoptosis. The methods for this technique need to be expanded to provide details of the numbers of cells initially used and whether there was any evidence of differential

death or proliferation, other than number or size of the neurospheres. In other words, are the differential responses in this assay due to altered responsiveness to the neurosphere induction or just altered cell population dynamics? It is also not clear how relevant the assays of gene expression in brain cells, isolated at E14.5, are for the retinal cells (p10, para 1; Figure 5).

Response

We appreciate your comment and added the volume we used to culture the cells in addition to the cell concentration. Since the Brg1 deficient cells did not survive in culture, we were not able to examine, whether this was due to increased cell death, inability to proliferate or the incompetence to form neurospheres. Based on our own results and results from literature, we are certain that the Brg1 deprived cells die in culture. Concerning the gene expression analyses, we are certain that we did not only isolate the brain, but also the cell accumulation that we assume to be enlarged retina. To clarify this, we included a sentence explicitly stating that cells of the mutant retina were part of our bulk RNA sequencing analysis.

Importantly, the authors must detail the statistical methods used to determine significance of any quantitative data. No descriptions were provided at all.

Response

Thank you for pointing that out. We included the section "Statistics" in materials and methods.

Minor points
Page 5, para 3: It was not clear to me from the descriptions whether the authors considered all the morphological phenotypes observed in different parts of the brain and retina to be 'rosette-like' structures. They certainly looked similar to this untrained eye. Rosette formation in the retina is commonly associated with altered cell-cell interactions; see for example the Crb1 mutants. In these Brg1 mutants, the appearance of the rosettes does seem to be an effect of loss of cells/neurons and it is not clear that this is a primary direct effect of the Brg1 deletion.

Response:

We appreciate this comment and added an explanation for the presence of rosettes in the retina of mutants due to loss of Brg1 at E9.5

Page 7, para 1, last sentence: Clarify sentence "The in H&E stains more cell dense structure was Brg1 positive and surrounded by a Brg1 negative cell population..."

Response:

We have deleted this potentially confusing sentence in the revised manuscript.
Page 7, para 3, last sentence: Where are the data to support this statement about the retina-like region near the basal cerebrum?

Response:

We have deleted the statement in the revised manuscript.
Page 8, para 1, last sentence: Please provide more detail about the lslRFPfl/fl strain of mice. as my understanding is that the RFP construct is in the ROSA locus as described by Luche et al and requires a 2-step action by Cre to cause expression of RFP. This may also reduce the efficiency of recombination by the Sox2-Cre transgene. The notation used her suggests the floxed RFP is in the Isl allele?

Response:

We followed your advice and included a more detailed description of our fate-mapping mice.
P10, para 3: Other RGC markers to consider would include NR2F1, or TUBB3 antibodies at E14.5 and RHO for photoreceptors at E18.5?

Response

We included immunohistochemistry for Nr2f1.

P15, para 1: Note that conceptuses collected at E14.5 are actually fetal and not embryonic.

Response

Thank you for pointing that out. We used the term "embryo" throughout the manuscript for all developmental stages, as the gestation period in mice is so much shorter than in humans making it less important to distinct between embryonic and fetal stages. This simplification has been suggested previously by others (e.g. Chen et al. 2017).

Chen, V. S., Morrison, J. P., Southwell, M. F., Foley, J. F., Bolon, B., & Elmore, S. A. (2017). Histology Atlas of the Developing Prenatal and Postnatal Mouse Central Nervous System, with Emphasis on Prenatal Days E7.5 to E18.5. Toxicol Pathol, 45(6), 705-744. https://doi.org/10.1177/0192623317728134

Figure 3: I did find the figure label background squares for images B,C,D,E,J,K,L etc rather distracting. Would suggest removing those squares and just making the figure labels bolder and closer to the corner of the image. They take up almost the quarter of a small image. I also found the crosses for empty panels disconcerting and initially wondered whether there had been an error in image rendering. Minor journal style issue.

Response

We followed your suggestion and reduced the size of the squares and replaced the crosses with text. Please note that Fig 3 of the first submission is now Fig S1.

Figure 3, image H: the Ki67 stain appears very weak and indistinct, particularly when compared to stain in image Q.

Response

We have removed the Ki67 staining and chosen to show pHH3 stainings instead.

Figure 5, image K: Is this section truly representative of the phenotype? There is only one rather incomplete eye present in this section, suggesting a peripheral section. How penetrant was this phenotype? There did not appear to be an RPE transdifferentiation in Figure 1H and only one eye in Figure 1I appears to show this. Do these mutant eyes have lenses?

Response

In order to clarify this, we have changed the orders of our figures and included new images. The phenotype in Fig 4 H (Fig. 5K in the first version of the manuscript) is present in 100 % of all mutant embryos after Brg1 loss at E7.5, but occurs first after E14.5 (Fig. 4 O-V). The mutants have lenses.

Figure S2: The volcano plots appear to show highly selected datasets, which I am not convinced is appropriate.

Response

The volcano plots in Fig. S3 show all valid genes per dataset. Valid genes are those that:
1) do not contain outliers and are determined via Cook's distance
2) are in average strong enough express (independent filtering).
Only differentially expressed genes (DEGs) are marked in red and defined by log2 fold change and by false discovery rate to ensure that they are selected in an objective manner.

Reviewer 2 Comments for the Author:
Major comments

Given the use of CreER system, the authors should provide Cre reporter patterns. Without this, it is impossible to clarify whether the phenotypes are autonomous effects of Brg1 deletion or not.

Response

Thank you for your suggestion. We included an RFP staining as a reporter in Fig. S7.

Figure 1: They identified the lesion in basal part of the brain. What is the identity of basal lesion? Based on the position, the structure can be the optic stalk, which connect the brain and retina.

Response

Thank you for your comment. We are certain that the cell accumulation at the basal brain parts are part of an enlarged retina due to RPE transdifferentiation and therefore not part of the brain but of the embryonic eye (Fig. 4).

Figure 2: Are there rosettes in Ini1-cko mouse retina, too? The H&E image of Ini1-cko mouse retina in Figure 2E(ii) looks less compact, although it does not show clear rosettes.

Response

We did not find any rosettes in the Ini1 knockout mice.

Figure 3: The authors should co-stain Brg1 and Ki67 to delineate direct relationship between cell proliferation and Brg1 loss. They also need to quantify the results.

Response

Thank you for this suggestion. We added quantifications of proliferating and apoptotic cells in Fig. 3 und Fig. S2.

Figure 4: Interpretation of FACS data is incomplete. What is the mechanism resulting in the decrease of Brg1(+) population in Brg1-cko mouse brain? The authors show no significant decrease in cell proliferation in Brg1-cko mouse brain. However, they did not examine the death of the cells, which also can cause the loss of the cells. The authors need to examine cell death by TUNEL or caspase-3 staining. The authors may examine as they refer a previous work in Discussion (page 11).

Response

Thank you for this suggestion. We added quantifications of proliferating and apoptotic cells (Fig. 3 and Fig. S2). As the number of apoptotic cells *in vivo* is increased, we infer that increased apoptosis is also the explanation, why there are no Brg1 positive cells after cultivation detectable.

Figure 5: Neurospheres were generated using brain NSCs, but the authors interpreted retinal phenotypes using the results. To do that, they need to check whether the changes of gene expression were also happened in the retina.

Response:

The region containing the enlarged retina is attached to the embryonic brain and was therefore isolated for both, the neurosphere assay and the gene expression analysis. To prevent misunderstandings in this regard, we included a respective sentence in the results section and thank the reviewer for pointing this out.

The authors tried to relate the increase of Fgf15, which is observed in brain neurospheres, to RPE-to-retina transdifferentiation in Brg1-cko mouse eyes. It is necessary to show the elevation of Fgf15 in the retina by ISH.

Response:

Thank you for your comment. We have verified the expression of Fgf15 by immunohistochemistry in Fig. 6.

The results in Figure 1J show that RPE is maintained by E14.5. Therefore, it is likely that the transdifferentiation that the authors claim might happen between E14.5 and E18.5. Otherwise, retina might have expanded into RPE territory upon the loss of RPE. Again, Cre reporter analysis is essential to clarify this.

Response:

We appreciate this comment. We now included a detailed description on the occurrence of the transdifferentiated retina in Fig. 4 O-V. Furthermore, we performed cre reporter analysis (Fig. S7) and discussed the possibility of an alternative cause for the presence of the transdifferentiated RPE.

At E18.5, Sox2-positive cells should be RPCs, not Muller glia, astrocytes, and ChAT amacrine subset. Those three cell types are not developed yet in the embryonic mouse retina.

Response:

Thank you. We changed the sentence accordingly.

Minor
Delete 'in' between 'The' and 'H&E' in page 7 (6th line).

Response:

Thank you. We deleted the sentence entirely in the revised manuscript.

Reviewer 3 Comments for the Author:
The work provides some interesting data, characterizing a partially-penetrant phenotype and potentially providing insight into how Brg1 acts during neural and neuronal development. That said, I have a number of concerns about the manuscript, its suitability for Development since I don't see a clear developmental mechanistic advance. I've listed some of my concerns/questions and suggestions below.

(1) In Figures 1 and 2, the authors show that Tg(sox2-cre/ERT2)-mediated removal of brg1 or ini1 leads to some striking phenotypes including tumor-like rosettes and, in the case of the brg1 ko, ectopic retina. In Figure 1, the authors point out this ectopic retina region but don't characterize it with immunohistochemistry until Figure 5. Moreover, the authors provide no explanation of the tissue that it potentially lost/converted into these ectopic retinae.

Response

Thank you for your comment. In order to make it more comprehensible to follow our line of presenting evidence for the basal cell accumulation being ectopically localized retina, we moved the characterization by IHC from figure 5 to figure 4. Furthermore, we included an improved explanation of the phenomenon of "RPE to retina transdifferentiation" in the discussion section to better explain, which cells are likely converted into retina.

(2) In Figures 1 and 2, the authors examine the phenotypes that occur when cre induction occurs at specific times and then depletion of either brg1 or ini1 commences. They use the data in these figures to argue that the ectopic retina formation they observe may be a function that is specifically mediated by loss of brg1. In my mind, the likely conclusion from this is that Brg1 itself (and not the SWI/SNF complex) may be required to repress retinal fate. Did the authors check to see if the brg1 or ini1-deficient cells in vivo had upregulated other components of the SWI/SNF complex? Did the authors check to see if genes required for early eye formation (e.g., pax6 or rax) are upregulated a day or two after the loss of the brg1? The authors do include the necessary controls to show the level at which the Brg1 and Ini1 proteins are depleted after tamoxifen treatment, but they do not explore whether other components of the SWI/SNF complex are altered in these conditions.

Response

Thank you for these comments. We agree with the reviewer that Brg1 is the important component to repress retinal fate and not the SWI/SNF complex in general. Therefore, we included a sentence addressing this in the discussion section. In order to examine how Brg1 loss influences the expression of other SWI/SNF members, we examined the RNA sequencing data (Fig. S6). Since the ectopic retina was first detectable at E16.5, we did not check the expression of genes immediately after loss of Brg1. However, we examined the morphology of embryos 3 days after tamoxifen treatment (Fig. 3) and did not find any abnormalities in H&E stains.

(3) The authors do not explore how the Brg1+ or Ini1+ cells that remain after tamoxifen treatment could impact the phenotypes they observe. What I'm really asking here is whether the phenotype observed is because of the mix of cell types (with regard to their brg1 or ini1 levels),

Response

Yes, we think this is likely. Many of the cells that have not been recombined and still express Brg1, clearly contribute to the phenotype. This is obvious in rosette-like formations including many Brg1 positive cells as well as in enlarged retina that only partially lacks Brg1. As discussed, we think that paracrine signals of neighboring Brg1 (or Ini1) deficient cells and/or the lack of cells that have died due to the induced Brg1 (or Ini1) deprivation, significantly contribute to the alterations observed in our mutant embryos.

(4) In Figure 3 and supplemental data, the authors show that the morphological alterations that they observe within the SVZ seem to be confined to regions that continue to express Brg1 or Ini1. It seems that what the authors have uncovered is an alteration that arises because the cells within the SVZ and other regions of the forebrain do not uniformly express Brg1 (or Ini1). I'm curious about what would have happened if they had been able to drive Brg1 expression instead of depleting it. If the cells are somehow sensing an imbalance among the Brg1+ cells and the Brg1- cells, one would expect the over-expression of Brg1 to have a similar phenotype as the Tg(sox2-cre/ERT2); brg1fl/fl experiments.

Response

Thank you for this interesting idea. Following your suggestion, we generated a BRG1 overexpressing lentivirus and transduced primary wild type neural stem cells cultured as neurospheres. However, our preliminary

data suggest that BRG1 overexpression does not interfere with the formation and survival of neural stem cells *in vitro* like Brg1 deficiency does as presented in our manuscript. Furthermore, studies from other groups have shown that BRG1 overexpression and knockdown has different/opposing effects in other settings. For example, Yang et al. 2019 (*Cancers*) showed that knockdown of BRG1 in ATRT cells promoted migration of the tumor cells, whereas overexpression inhibited migration. Li et al. 2018 (*Biomedicine and Pharmacotherapy*) reported the opposing effects of Brg1 knockdown and overexpression regarding neuronal cell viability in an *in vitro* model of cerebral ischemia and reperfusion injury. Based on our preliminary data and these published results, we decided not to follow up any further on the idea that over expression of Brg1 might have a similar phenotype as its deletion.

Li, F., Liang, J., & Tang, D. (2018). Brahma-related gene 1 ameliorates the neuronal apoptosis and oxidative stress induced by oxygen-glucose deprivation/reoxygenation through activation of Nrf2/HO-1 signaling. Biomed Pharmacother, 108, 1216-1224. https://doi.org/10.1016/j.biopha.2018.09.144

Yang, Y. P., Nguyen, P. N. N., Ma, H. I., Ho, W. J., Chen, Y. W., Chien, Y., Yarmishyn, A. A., Huang, P. I., Lo, W. L., Wang, C. Y., Liu, Y. Y., Lee, Y. Y., Lin, C. M., Chen, M. T., & Wang, M. L. (2019). Tumor Mesenchymal Stromal Cells Regulate Cell Migration of Atypical Teratoid Rhabdoid Tumor through Exosome-Mediated miR155/SMARCA4 Pathway. Cancers (Basel), 11(5). https://doi.org/10.3390/cancers11050720

(5) One of my biggest concerns (and perhaps hopefully misplaced) is that from my reading it seems that the authors performed bulk RNA-Seq analyses on FAC-sorted RFP+ cells that were heterogeneous in their expression of Brg1. If the cre efficiency is comparable from experiment to experiment, then it is likely that the RNA the authors sequenced came from a cell population that they are calling brg1-negative but could have been 14%-52% wild- type. Moreover, the analysis of changes in gene expression in the temporal knock-outs provide an average overview of the changes that occur but doesn't account for individual differences in cells, as it could be that differences in cell location within the tissue could dramatically influence DGE.

Response

We certainly agree with the reviewer that the reduced efficiency of the cre recombinase in the presence of two transgenes is a limitation of this study. In order to make this more transparent, we added it to the discussion section. Still, we don't see any argument, why the changes in gene expression that we observed should not be due to the loss of Brg1 (even if it's only a partial loss). We also agree with this reviewer that our results only represent an average overview of the changes. However, it should still be valid to speculate that observed changes in gene expression are associated with the most prominent phenotypic alterations observed in the mutant mice. Finally, the upregulation of Fgf15, which we have now confirmed by immunohistochemistry in the enlarged mutant retina, is a nice example for the gene expression analyses to point out important changes.

(6) *The authors use their data presented in Figures 1, 2, and 5 to suggest that loss of brg1 from E7.5 causes RPE to transdifferentiate into retina. I'm not sure that this is the case and a number of experiments would need to be performed to definitively test this hypothesis, including but certainly not limited to (1) more* careful examination of early and later markers of RPE in their brg1 ko animals (the one image of the retina shown in Figure 5B and C is small and lacks immunohistochemistry) and (2) the generation of an eye specific brg1 ko (using the Tg(rax:creERT2) mice, for example).

Response

Thank you for your suggestions. In the revised manuscript, we have added larger images of the mutant retina (Fig. 6 D) and also included images of the eyes at different timepoints (Fig. 4 O-V). Furthermore, we stained for Ezrin as an RPE marker (Fig. 6 G, J). Despite the fact that we agree with the reviewer that the examination of *Rax- creER^{T2}::Brg1$^{fl/fl}$* mice would be of high interest, the time needed to generate this model (import of *Rax-creER^{T2}* mice (time unknown, also due to the Covid-19 pandemic), breeding of at least 2 generations (6 months)) unfortunately exceeds the time to submit a revised manuscript.

(7) The authors overlook literature from other organisms that have examined the consequences of brg1 loss-of- function. For example, in zebrafish, an allele of brg1 prevents differentiation of retinal progenitor cells in the developing retina. Interestingly, the brg1 mutant zebrafish retinae contain an RPE.

Response

We have incorporated information regarding Brg1 loss in other model organisms than the mouse in the discussion section.

Second decision letter

MS ID#: DEVELOP/2020/196147

MS TITLE: *Brahma-related gene 1* has time-specific roles during brain and eye development

AUTHORS: Doerthe Holdhof, Melanie Schoof, Sina Al-Kershi, Michael Spohn, Catena Kresbach, Carolin Goebel, Malte Hellwig, Daniela Indenbirken, Natalia Moreno, Kornelius Kerl, and Ulrich Schueller

I have now received all the referees reports on the above manuscript, and 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 overall evaluation is more positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. One of the reviewers still has some reservations about parts of the new analysis. I would be grateful if you could please address his/her concerns. If you do not agree with any of their criticisms or suggestions explain clearly why this is so in your rebuttal letter.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

In the revised manuscript the authors appear to have carefully addressed all my previous concerns and questions. I have commented on only minor editorial issues that the authors may wish to consider. As stated in my earlier review the paper makes an important contribution to understanding the function of the Brg1 gene separately and as part of the SWI/SNF complex in brain and eye development.

Comments for the author

Minor points
Abstract, first sentence and throughout the document: Subtle issue of usage for 'which' and 'that'. The word 'which' is always preceded by a comma and is commonly used for non-restrictive phrases that are not intrinsic to the sentence
(i.e. phrase is separated by commas from rest of the sentence). By contrast, the word 'that' is not preceded by a comma and used for restrictive phrases that are central to the sentence (no commas). In parts, the writing in the manuscript was somewhat cumbersome and could have been simplified.
As Cre recombinase is a bacterial gene/protein, the gene name is lower case italicized (cre) but the protein name is not italicized and the first letter is upper-case (Cre). There is a lot of inconsistency in nomenclature of genes and proteins throughout the manuscript. Note that mouse proteins are all capitalized and not italicized (e.g. BRG1); genes have first letter capitalized and are italicized (e.g. Brg1).
Correct typographical error "GABAergic synpase" to "GABAergic synapse"
P10, First paragraph and elsewhere. Presumably "…tamoxifen treatment…" in last sentence represents the Brg1 deletion not just Tam treatment per se? The term "tamoxifen treatment" is used as a proxy for Brg1 conditional deletion.
P12. Adjust nonsensical sentence that contains the phrase "…less cells are abundant…"

Reviewer 3

Advance summary and potential significance to field

I appreciate that we are in very challenging times due to the pandemic, but I'm not convinced that this revision alleviates my concerns, especially with regard to (1) the indirect nature of the phenotype(s) observed and (2) the lack of insight into which of the genes that are misregulated upon loss of Brg1 in some neural progenitors are the ones responsible for the phenotypes observed. To me, the main finding of the paper is that the authors have performed experiments that point to a window of developmental time during which Brg1 function is especially important in sox2+ neural progenitor cells and that Brg1 knock-out cells as well as cells in close proximity to Brg1 ko cells exhibit dysregulation of gene expression.

Comments for the author

The authors have included a new figure, Figure 3, which helps to strengthen their characterization of the Brg1 knock-out in sox2+ cells, but they use general terms like "macroscopic" and "high power" to describe the magnification used. I'd prefer some mention of actual magnification (e.g., 0.5 x, 10X, 100X).

The new quantitation and statistics throughout are improved but still need a bit more information. For example, it was unclear to me if the n in the figure legends was for number of animals or number of fields of cells.

The phenotypes in Figure 4, especially for the E16.5 and E18.5 retinae in which the cell layers appear to be folded on and around each other, a phenotype often due to hyperproliferation, is difficult to reconcile with the pH3 data in Figure 3.

The authors make claims about RPE cells directly transdifferentiating into NR cells, which I think they are supporting with data from Supplemental Figure S7, but given the high levels of apoptosis that is induced by loss of Brg1 in sox2+ cells (which include RPE progenitors), I'm still not sure they have substantiated the claim that RPE cells transdifferentiate into NR, only that some RPE progenitors could have acquired the loss of Brg1.

The authors included a new figure, Figure 6 to address concerns about whether it is valid to speculate about the genes that are upregulated upon loss of Brg1 in sox2+ cells. While these data provide validation for the idea that some of the genes identified in their RNA-Seq experiment are indeed upregulated, it does not address how/whether some/all of these genes cause the phenotype.

In Figure 6, the authors provide immunostaining of sections with Fgf15. I'm not exactly sure which cells are stained. Did the authors perform a secondary-antibody only control to make sure that much of what we are seeing is not due to antibody trapping? It is that more cells express Fgf15 or simply that there are more cells (due to the phenotype of Brg1) that are the type that typically express.

Some minor concerns:
Supp Fig 2, the heading over the image of the section of the whole embryo should read E7.5 instead of E7,5.

Some of the letters on Supp Fig 5 seem to be missing/out of order. For example there are no i and ii on the boxes in B, C, D, F (and it seems like it should have been B, C, D, E). The legend says "now difference", which should, I think, be "no difference". Did the authors actually quantify the number of p-SMAD+ cells?

Second revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

Minor points

Abstract, first sentence and throughout the document: Subtle issue of usage for 'which' and 'that'. The word 'which' is always preceded by a comma and is commonly used for non-restrictive phrases that are not intrinsic to the sentence (i.e. phrase is separated by commas from rest of the sentence). By contrast, the word 'that' is not preceded by a comma and used for restrictive phrases that are central to the sentence (no commas). In parts, the writing in the manuscript was somewhat cumbersome and could have been simplified.

Response:

We thank the reviewers for critically re-evaluating our manuscript. We apologize for the incorrect use of "which" and "that" and thank the reviewer for the helpful explanation. We corrected the manuscript according to the mentioned rule. To overall simplify the manuscript, we changed several expressions and sentences throughout the whole manuscript.

As Cre recombinase is a bacterial gene/protein, the gene name is lower case italicized (cre) but the protein name is not italicized and the first letter is upper-case (Cre). There is a lot of inconsistency in nomenclature of genes and proteins throughout the manuscript. Note that mouse proteins are all capitalized and not italicized (e.g. BRG1); genes have first letter capitalized and are italicized (e.g. Brg1).

Response:

We apologize for the inconsistent nomenclature. We corrected several gene and protein names in the manuscript.

Correct typographical error "GABAergic synpase" to "GABAergic synapse"

Response:

We corrected the misspelling and thank the reviewer for this attentive observation.

P10, First paragraph and elsewhere. Presumably "…tamoxifen treatment…" in last sentence represents the Brg1 deletion not just Tam treatment per se? The term "tamoxifen treatment" is used as a proxy for Brg1 conditional deletion.

Response:

We agree with Reviewer 1 that it might be confusing to the reader to use "tamoxifen treatment" as a proxy for Brg1 deletion. Therefore, we changed this term throughout the whole manuscript.

P12. Adjust nonsensical sentence that contains the phrase "…less cells are abundant…"
Response:
We apologize for this inaccurate sentence. We changed the wording, accordingly.

Reviewer 3 Comments for the Author:

The authors have included a new figure, Figure 3, which helps to strengthen their characterization of the Brg1 knock-out in sox2+ cells, but they use general terms like "macroscopic" and "high power" to describe the magnification used. I'd prefer some mention of actual magnification (e.g., 0.5 x, 10X, 100X).

Response:
We appreciate the comment and agree that the exact description of the used magnification would be helpful. Therefore, we added this information to all figures.

The new quantitation and statistics throughout are improved but still need a bit more information. For example, it was unclear to me if the n in the figure legends was for number of animals or number of fields of cells.

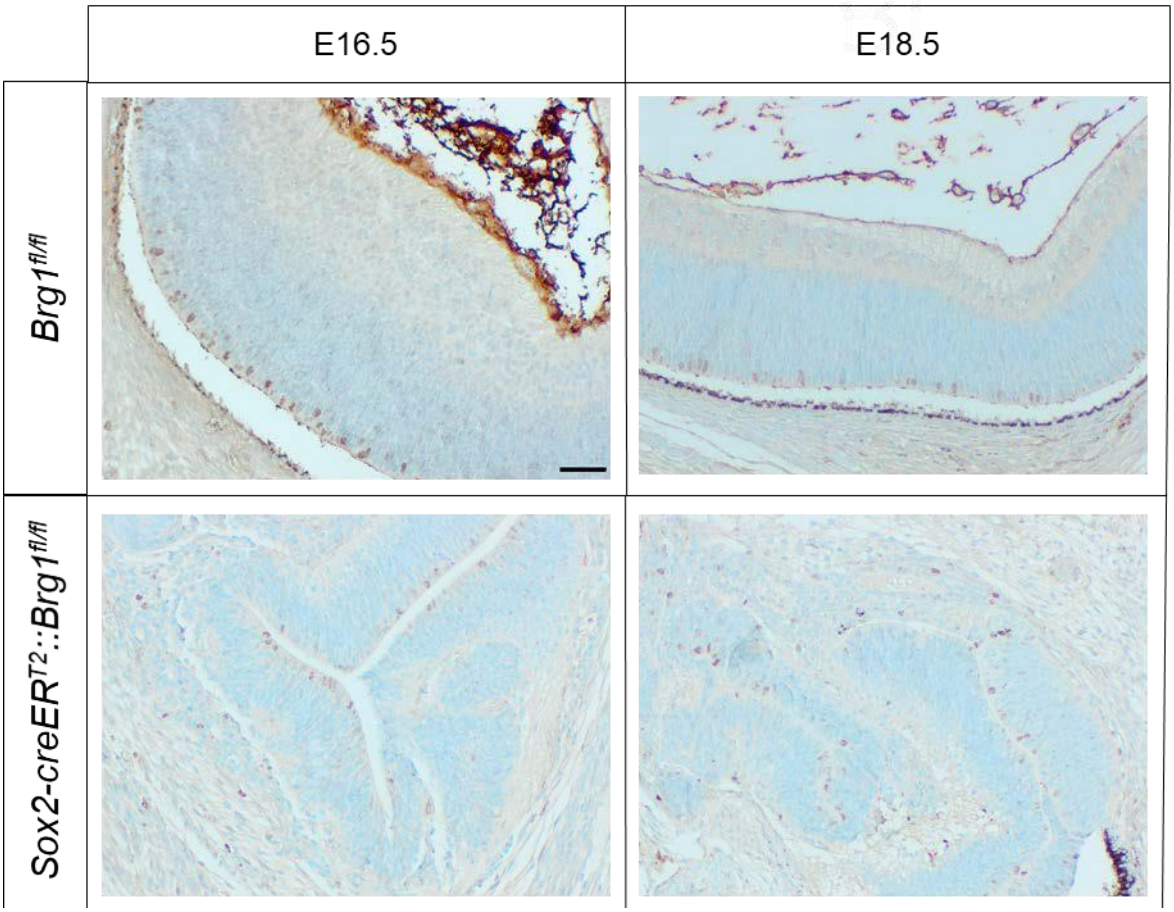
Response:

We apologize for the unclear presentation of data. The n represents the number of included animals. We added this information to the figure legends.

The phenotypes in Figure 4, especially for the E16.5 and E18.5 retinae in which the cell layers appear to be folded on and around each other, a phenotype often due to hyperproliferation, is difficult to reconcile with the pH3 data in Figure 3.

Response:

To address this valid concern and rule out that the phenotype is caused by hyperproliferation, we performed additional pHH3 staining of animals sacrificed at E16.5 and E18.5. No increase in proliferation, as indicated by pHH3 staining, was observed (Rebuttal Fig. 1). This further underlines the results presented in Figure 3, which could show an increase in apoptosis at E12.5 upon Brg1 loss at E7.5.



Rebuttal Fig. 1: PHH3-staining of frontal sections showing the retina of control and mutant mice on E16.5 and E18.5 (100x magnification, scale bar corresponds to 50µm).

The authors make claims about RPE cells directly transdifferentiating into NR cells, which I think they are supporting with data from Supplemental Figure S7, but given the high levels of apoptosis that is induced by loss of Brg1 in sox2+ cells (which include RPE progenitors), I'm still not sure they have substantiated the claim that RPE cells transdifferentiate into NR, only that some RPE progenitors could have acquired the loss of Brg1.

Response:

We thank the reviewer for this comment. In our manuscript, we present results from different experimental setups that support the hypothesis that the altered NR might be the result of transdifferentiated RPE cells:

- Expression of *Fgf15* is significantly upregulated upon *Brg1* loss (Fig. 6A,I). Increased FGF signalling has been shown to induce transdifferentiation of RPE cells to NR *in vitro* (Zhao et al., 1995)
- In animals with *Brg1* loss, the normal RPE was only visible in some regions (Fig. 6D green line). In addition, the RPE layer partly seems to directly transition into the enlarged NR (Fig. 6D white arrow). BRG1 negative cells were predominantly found in locations where the RPE is normally present (Fig 6 C, E, blue arrows) and close to these cells, single pigmented cells were present (Fig. 6 E, blue arrowheads).
- Cells within the enlarged NR stained positive for Ezrin, a marker that normally identifies the microvilli of the RPE (Fig. 6J).
- Results of a fatemapping experiment with new IF-P stainings, which had been included into the first revision at the request of the reviewers, confirm that NSCs targeted by the Cre-recombinase develop into RPE cells (Fig. S7). Therefore, RPE cells could be possible cells of origin of the altered NR.

Nevertheless, we agree with the reviewer that our manuscript provides no final proof for the hypothesis that the enlarged NR results from a transdifferentiation of RPE cells. Therefore, we have tried to make it clearer that this is only a hypothesis by removing corresponding statements from the abstract (p 3) and the paragraph title and by rephrasing statements in the text (p 10). Furthermore, we clearly mention in the discussion that we cannot rule out that the enlarged NR is (partially) the result of uncontrolled proliferative activity of the NR itself (p 14).

The authors included a new figure, Figure 6 to address concerns about whether it is valid to speculate about the genes that are upregulated upon loss of Brg1 in sox2+ cells. While these data provide validation for the idea that some of the genes identified in their RNA-Seq experiment are indeed upregulated, it does not address how/whether some/all of these genes cause the phenotype.

Response:

We agree with the reviewer that the additionally included data cannot explain the exact mechanism, by which the deletion of Brg1 leads to the upregulation of the mentioned genes. By adding these stainings to our manuscript, we aimed to strengthen the hypothesis that the dysregulation of several genes that are involved in proper retinal development, such as Fgf15, contributes to the observed morphological abnormalities in eye development. We believe that the additionally required experiments that would further elucidate the mechanisms causing the upregulation of these genes rather exceed the scope of our current project.

In Figure 6, the authors provide immunostaining of sections with Fgf15. I'm not exactly sure which cells are stained. Did the authors perform a secondary-antibody only control to make sure that much of what we are seeing is not due to antibody trapping? It is that more cells express Fgf15 or simply that there are more cells (due to the phenotype of Brg1) that are the type that typically express.

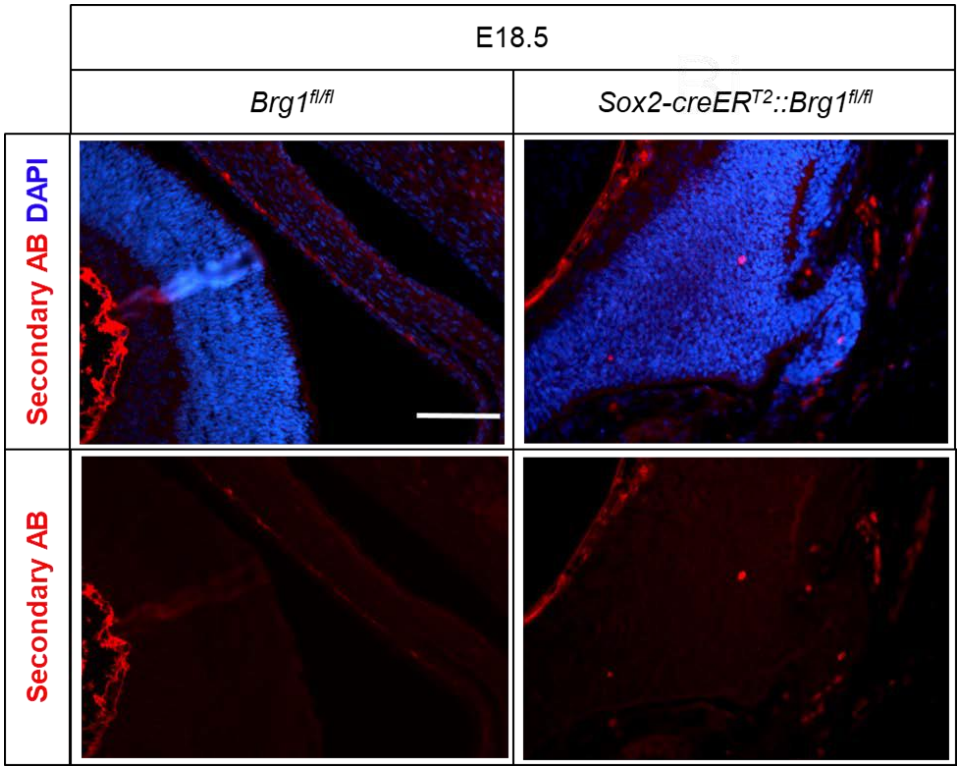
Response:

We performed a secondary antibody-only staining, which showed no signs of unspecific staining, thereby confirming a reliable FGF15 staining signal (Rebuttal Fig. 2). Thus, increased FGF15 protein levels support the observation of an increased Fgf15 expression revealed by RNA-sequencing (Fig. 6A,F). Previous studies showed that Fgf15 expression is typically downregulated during murine retinal development (Kurose et al., Gene Expr Patterns 2004;4(6):687-93). At E18.5, Fgf15 is normally only expressed in the outer neuroblastic layer of the retina (Kurose et al., 2004), which we could confirm in the Fgf15 staining of $Brg1^{fl/fl}$ control mice (Fig. 6F).
Concerning the reviewer's last question, we believe that the increased FGF15 signal is the reason of both factors, more cells expressing Fgf15 at all, as well as more cells that typically express Fgf15:

1. *Brg1* loss leads to a more immature expression profile of cells, which we could confirm by increased NR2F1 expression (Fig. 6K). Kurose et al. showed that in early retinal stages, *Fgf15* is expressed in larger parts of the retina than in later embryonal stages. Hence, the increased

FGF15 signal in our mouse model can be explained by a generally more immature cell type in the altered NR, which leads to more FGF15 positive cells.

2. Due to the enlargement and layering of the altered NR, there are more cells that belong to the outer neuroblastic layer, which is the murine retinal area that typically expresses *Fgf15* at E18.5 (Kurose et al., 2004).



Rebuttal Fig. 2: IF-staining with secondary anti-mouse antibody and DAPI of frontal sections showing the retina of control and mutant mice on E18.5 (400x magnification, scale bar represents 100µm), corresponding to FGF15 staining in Fig. 6.

Some minor concerns:

Supp Fig 2, the heading over the image of the section of the whole embryo should read E7.5 instead of E7,5.

Response:

We thank the reviewer for this remark. We changed the heading, accordingly.

Some of the letters on Supp Fig 5 seem to be missing/out of order. For example there are no i and ii on the boxes in B, C, D, F (and it seems like it should have been B, C, D, E). The legend says "now difference", which should, I think, be "no difference". Did the authors actually quantify the number of p- SMAD+ cells?

Response:

We appreciate this comment. We corrected the figure marks as well as the misspelling. We indeed quantified the proportion of SMAD+ cells, which showed no difference between mice with or without Brg1 deletion.

Third decision letter

MS ID#: DEVELOP/2020/196147

MS TITLE: *Brahma-related gene 1* has time-specific roles during brain and eye development

AUTHORS: Doerthe Holdhof, Melanie Schoof, Sina Al-Kershi, Michael Spohn, Catena Kresbach, Carolin Goebel, Malte Hellwig, Daniela Indenbirken, Natalia Moreno, Kornelius Kerl, and Ulrich Schueller

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.