ZFP423 regulates early patterning and multiciliogenesis in the hindbrain choroid plexus

Filippo Casoni, Laura Croci, Francesca Vincenti, Paola Podini, Michela Riba, Luca Massimino, Ottavio Cremona and G. Giacomo Consalez
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MS TITLE: ZFP423 regulates early patterning and multiciliogenesis in the hindbrain choroid plexus

AUTHORS: Filippo Casoni, Laura Croci, Francesca Vincenti, Paola Podini, Luca Massimino, Ottavio Cremona, and G. Giacomo Consalez

I have now received the reports of two referees on 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.

As you will see, both referees express great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they ask that you provide illustrations for the hindbrain expression of Zfp432 at E9.5, and that you analyse cell death in the Zfp432-mutant hindbrain choroid plexus. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

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.

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

In this manuscript, the authors showed that Zfp432 is essential to hChP development. In its absence, the hChP failed to form. The rudimentary hChP epithelium lacked characteristic multiciliated cells and showed regional loss of Lmx1a and Otx2 expression from E11.5 to E13.5. While transcriptome analysis suggests dysregulation of Wnt and Bmp pathways, it is unclear how these pathways are affected spatially and how they integrate with Zfp432 to control hChP development. Overall, the findings are novel and interesting, but the study needs to be strengthened by addressing several outstanding issues listed below.

Comments for the author

What is the functional significance of cell fate alteration from multiciliated cells to monociliated cells?

Does hChP formation depend on ependymal cells being multiciliated?

Does Zfp432 cell autonomously regulate hChP development?

What is the expression patterns of Zfp432 at E9.5 hindbrain/RP?

Does the mutant hChP undergo apoptosis?

Is expression pattern of Lmx1a, Otx2, Wnt1, Msx2 altered in the mutant at E9.5?

Can authors validate that Wnt and Bmp signaling are dysregulated in E9.5 mutants by using Axin 2 (Wnt signaling) RNA in situ and pSmad1 (Bmp signaling) staining?

Reviewer 2

Advance summary and potential significance to field

The manuscript ‘ZFP423 regulates early patterning and multiciliogenesis in the hindbrain choroid plexus” investigates the necessity of the Joubert syndrome associated kreuppel-type zinc finger transcription factor Zfp423 in the maturation of the hindbrain ChP. While ZFP423 has been shown to act generally as a transcriptional repressor (Cho, et al. PLoS One, 2013), and specifically required for cell cycle progression and maturation of cerebellar Purkinje neuron progenitors (e.g. Casoni, et al. Dev. 2017); the authors contribute new findings to understand developmental roles of this critical gene product by investigating the generation and maturation (specifically epithelial cell multiciliogenesis) of hindbrain choroid plexus (hChP). The authors also perform tissue restricted comparative bulk RNA sequencing in WT vs. a zfp423 mutant (zfp Δ 28-30) to identify potential gene regulatory roles for ZFP423 in hChP progenitors and immature tissue—identifying known ChP regulatory genes validated by qPCR (e.g. Lmx1a, Otx2, Wnt1, GemC, and FoxJ1) as well as novel candidates (e.g. Wnt8a). The authors find that Zfp423 regulates hChP generation with only a lateral vestigial portion forming in the zfp Δ 28-30 mutant. They determine severe disruptions in epithelial cell maturation including cell morphology and organization, polarization, elaboration of microvilli and multiciliogenesis. They also characterize non-epithelial (mesnchymal, endothelial, pericyte) components of the ChP and identify disruptions in endothelial continuity. This manuscript is relevant to the readership of Development and the conclusions are well-supported by the data presented.

Comments for the author

The intersection with the extensive literature on Zfp423 in other cell types in not leveraged for a detailed explanation of the developmental roles of Zfp423 in ChP— particularly on separable roles in processes of proliferation, differentiation, and maturation. For developmental roles, readers are interested in processes of patterning, surivival, proliferation, differentiation, and maturation. The results should be presented less as phenomenology and more in the context of developmental processes and the known roles for Zfp423 that the authors already published for cerebellum (e.g. zfp Δ 9-20 altered mitotic spindle orientation, reduced proliferation, stalled cell cycle progression, increased DNA damage and reduced postmitotic cells and zfp Δ 28-30 increased DNA damage and reduced postmitotic cells, but with an increase in proliferating progenitor cells. Even without data on survival, mitosis, spindle, and DNA damage, relating all conclusions to this known construct would make the existing data more broadly relevant.

Other concerns:
Major Concerns:
1. The authors claim (pg 5) that this manuscript reports the expression of ZFP423 in roof plate. This was already shown by Casoni et al Dev 2017. Please focus on novel conclusions.
2. The authors show (pg 10, fig 3B) that ZFP423 expression shown a rostral-caudal gradient in hChP epithelium. Is this a true R-C gradient, or consistent with a developmental role in immature and progenitor cells, a developmental gradient?
3. On page 12 (figure 7) the authors conclude that Zfp423 mutant epithelial cells take on a squamous phenotype. While the organization can be determined as stratified (or pseudostratified) by nuclear staining), the cell shape is less apparent. Since this observation is critical to the conclusions (page 16), the authors should use stains that completely outline the cell body, even H&E could characterize the organization better than a nuclear stain alone.
4. On page 13 (figure Sup 3) the authors present, but do not emphasize data indicating that the multiciliognesis is partially recued by E18.5. This finding is interesting a suggested delayed differentiation rather than complete disruption in specification.
5. The very early expression of Zfp423 further support a master-regulatory role in the hindbrain for ChP formation. The authors indicate E8.5-E9.5 expression, but do not show the data (age 15). The inclusion of such expression data would further support the conclusion as should be considered, if available.
6. While the authors do not perform any assays on survival or proliferation, they suggest (page 17), based on the Gmnc expression, that the mutation results in altered conversion from proliferating epithelium into differentiated cells. Analysis of progenitor ratios over developmental time could indicate proliferative roles.
Cell survival/death should be evaluated. The authors should discuss these data.

Minor Concerns:
7. On page 3, in the Introduction, please cite primary sources when economical do so (eg Lun, et al 2015;
rather than Lehtinen et al 2013- predating review— for differential transcriptomes of ChP).
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10. Page 13, include citation for Watanabe et al 2012 as critical publications understanding ChP epithelial development.
11. Page15 discussion. The authors should not use the term “completely absent… except” as it is misleading. The structure is “almost completely absent”
12. Wording:
a. Usage of “ependymal” and “epithelial” are confused in the manuscript. When addressing ChP epithelial cells, the term “epithelial” is standard, as these cells are distinct from ependymal cells (as the author notes), for example on pages 2 ,16, 17.
b. Some undefined and perhaps unnecessary abbreviations were used, e.g. Abs or Abs. rather than antibodies (pg 12).
c. Mixed usage of ‘oligociliated’ and ‘multiciliated’ suggests different meaning. If the authors mean the same thing, please choose one term… ‘multiciliated’ is the most accepted.

d. The authors sometime abbreviate and sometime do not abbreviate ChP, choroid plexus. Please be consistent.
e. In multiple places, references in text are preceded by 'inter alia…'

First revision

Author response to reviewers' comments

RESPONSES TO THE REVIEWERS

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript, the authors showed that Zfp423 is essential to hChP development. In its absence, the hChP failed to form. The rudimentary hChP epithelium lacked characteristic multiciliated cells and showed regional loss of Lmx1a and Otx2 expression from E11.5 to E13.5. While transcriptome analysis suggests dysregulation of Wnt and Bmp pathways, it is unclear how these pathways are affected spatially and how they integrate with Zfp423 to control hChP development. Overall, the findings are novel and interesting, but the study needs to be strengthened by addressing several outstanding issues listed below.

Reviewer 1 Comments for the Author:

1- What is the functional significance of cell fate alterations from multiciliated cells to monociliated cells?
To answer this we have mainly referred to a paper (Banizs et al., 2005) in which this issue is addressed and discussed. This paper shows evidence that cilia function is necessary for regulating ion transport and CSF production, rather than CSF flow through the ventricles. Cilia dysfunction observed in Tg737orpk mutants leads to altered ion transport across the CP epithelium and an increase in the production of CSF due to an altered distribution of a transporter/channel/exchanger in the cilia axoneme, similar to what was previously established by the case of polycystin 1 (Liu et al., 2005). This is now discussed on page 21 in the section titled *"Zfp423 and the specification of multiciliated hChP cells"*.

2- Does hChP **formation** depend on ependymal cells being multiciliated?
Our data do not allow us to state that multiciliated cells are a requisite for ChP formation. Wherever we do not see multiciliated cells, we also cannot see anything resembling a ChP and we observe a persistence of squamous cells. Zfp423 is a master gene in hChP formation and its lack causes a global defect in the specification of hChP cell fate. However we do not know if multiciliogenesis is a prerequisite or just a component of a broader cell fate specification program. Besides *Zfp423*, other multiciliogenesis genes, e.g. Foxj1 (Hagenlocher et al., 2013) are expressed at very early stages, suggesting that multiciliogenesis is inherent in the specification of ChP fates, together with microvillus formation and the expression of transport proteins such as transthyretin.

3- Does Zfp423 cell autonomously regulate hChP development?
Zfp423 is expressed in the roof plate and in order to best address this point, the gene should be inactivated selectively in roof plate derivatives. Unfortunately, the Gdf7-Cre line has been discontinued, and other lines are not as specific, plus this series of experiments would require several months of work between obtaining permits and breeding/analyzing. However, our recent results clearly indicate that at E10.5 ZFP423 is expressed almost exclusively in the ChP epithelium, not in the underlying mesenchyme (**Fig. 3D**). The ChP epithelium is lineally related to the roof plate. Because the phenotype has a very early onset and the main changes are observed in the ChP epithelium, we argue that the early stages of development are likely controlled cell-autonomously by *Zfp423*. One gene in particular, namely *Lmx1a*, which is expressed in the ChP epithelium starting at E9.5, is significantly downregulated (**Fig. 7 A-C'** and **Fig. S4A-D'**). Many of the DEGs altered at E9.5 are certainly epithelial genes (Fig. S5 and data not shown).

4- What is the expression pattern of Zfp423 at E9.5 hindbrain/RP?
In the new version of the paper we changed **Fig. 3**. In the new **Fig. 3** we show wholemount LacZ staining of *Zfp423* expression (**A-C**) obtained in gene trap mutants (Wurst et al., 1995) at early developmental stages (E7.5-E9.5). These images show that the gene is transcribed starting at open neurula in the prospective hindbrain (Fig. 3B) and remains expressed thereafter, at key stages of ChP development. Moreover, the new figure now contains two panels (D,E) that show expression of ZFP423 protein in the ChP epithelium, which contains proliferating basal progenitors, and lack of expression in the postmitotic territories of the nascent ChP. Accordingly, we changed the text at page 11 in the section named "*ZFP423 protein is expressed in early epithelial progenitors of the prospective hChP.*".

5- Does the mutant hChP undergo apoptosis?
We have done immunofluorescence stainings with an activated Casp3 antibody. Our results failed to reveal any evidence of cell death in the mutant and wt alike in the ChP primordium. These experiments were performed at E10.5 (**Fig. S1E-G'**), and at E11.5 and E12.5 (not shown). We also tested cell death with a tunel kit at E12.5, and again we found no significant differences between the wt and mutant ChP (data not shown). These negative results are in keeping with what we found in the cerebellar VZ and published in Casoni et al, 2017. Then we assessed the presence of DNA damage signalling, as in Casoni et al 2017. To this end, we immunostained sagittal sections with a γH2AX antibody at E10.5, demonstrating in three independent experiments a slight increase in the number of cells positive for this marker. This evidence is shown in **Fig. S1C-D**.

Based on this outcome, we sought to determine whether cell cycle progression stalls in the mutant hChP, as previously observed in the VZ. We employed an antibody against PHH3 to detect cells in G2 phase, which display a dotted staining, and cells in M phase, which display a full nuclear staining (**Fig. S1A,A'**). The ratio between G2 positive cell and M positive cells (**Fig. S1B**) is indicative of a stall in G2 phase in mutants, leading to a delay of the cell cycle progression. Taken together, these data strengthen the notion that Zfp423 participates in DNA damage repair.

All these results are described in the first paragraph of the results sections, named "*ZFP423 protein is expressed in early epithelial progenitors of the prospective hChP*" on page 11 and 12.

6- Is expression pattern of Lmx1a, Otx2, Wnt1, Msx2 altered in the mutant at E9.5?
We have perfomed new experiments to answer this question at earlier stages of development starting at E9.5. RTqPCR and immunofluorescence at E9.5 show that *Lmx1a* is significantly downregulated in the mutant at E9.5. Conversely, *Otx2* is unchanged at the same stage, but declines thereafter. Our most recent findings strongly suggest that Zfp423 is required to promote or maintain the expression of *Lmx1a* at the onset of hChP development. These data are now shown in **Fig. 7** and **Fig. S4**.
Wnt1 and *Msx2* transcripts are also reduced at E9.5 by RTqPCR, confirming RNA-seq results. These data are now shown in **Fig. 8B,C**.

7- Can the authors validate that Wnt and Bmp signaling are dysregulated in E9.5 mutants by using Axin 2 (Wnt signaling) RNA in situ and pSmad1 (Bmp signaling) staining?
With regard to BMP signaling, we investigated it because of the robust and persistent upregulation of the extracellular BMP antagonist gene *Grem1* at all stages analyzed (**Fig. 8 B,C**). However, immunofluorescence results for SMAD1,5,8 phosphorylation are not grossly altered in the mutant at E10.5.

Axin2 mRNA is not significantly altered at E9.5 or E11.5 by RTqPCR (not shown). While mRNA levels in the hindbrain are unchanged, we have not been able to conduct in situ hybridization experiments for this gene due to time constraints. However the normal *Axin2* expression domain at E11.5 is shown below:

Fig 1. In situ hybridisation shows *Axin2* expression at the level of upper and lower Rhombic Lip and of the ChP (black arrows) in a parasagittal section of a mouse embryo at E11.5. Image credit: Allen Institute. © 2008 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas. Available from: https://developingmouse.brain-map.org/

This image shows that Axin2 at E11.5 is highly expresses at the level of the upper and lower rhombic lip, as well as in the nascent ChP (black arrows), suggesting a role in the development of the ChP.

Conversly, *Wnt3* is robustly upregulated. We quantitated this transcript by RTqPCR and the results are shown in **Fig 8. D**. Wnt3 does not activate canonical Wnt signaling. Instead, it activates a MAPK pathway (Anne et al., 2013). Accordingly, our IF results indicate that p- ERK is upregulated in the mutant LRL and hChP primordium at E10.5 as shown in **Fig. 8 E,E'** and **Fig. 8 F,F'**.

All these new results are described on page 16 in the paragraph titled "*Genes critically involved in hindbrain patterning are dysregulated in the Zfp423 mutant*".
Fig. S5 (RNAseq analysis) has been implemented with a schematic representation of the tissue specimens analyzed by RNA-seq and by RT-qPCR at E9.5 (**Fig. S5A**).

Reviewer 2 Advance Summary and Potential Significance to Field:

The manuscript 'ZFP423 regulates early patterning and multiciliogenesis in the hindbrain choroid plexus" investigates the necessity of the Joubert syndrome associated ruppel-type zinc finger transcription factor Zfp423 in the maturation of the hindbrain ChP. While ZFP423 has been shown to act generally as a transcriptional repressor (Cho, et al. PLoS One, 2013), and specifically required for cell cycle progression and maturation of cerebellar Purkinje neuron progenitors (e.g. Casoni, et al. Dev. 2017); the authors contribute new findings to understand developmental roles of this critical gene product by investigating the generation and maturation (specifically epithelial cell multiciliogenesis) of hindbrain choroid plexus (hChP). The authors also perform tissue restricted comparative bulk RNA sequencing in WT vs. a zfp423 mutant (zfp Δ 28-30) to identify potential gene regulatory roles for ZFP423 in hChP progenitors and immature tissue—identifying known ChP regulatory genes validated by qPCR (e.g. Lmx1a, Otx2, Wnt1, GemC, and FoxJ1) as well as novel candidates (e.g. Wnt8a). The authors find that Zfp423 regulates hChP generation with only a lateral vestigial portion forming in the zfp Δ 28-30 mutant. They determine severe disruptions in epithelial cell maturation including cell morphology and organization, polarization, elaboration of microvilli, and multiciliogenesis. They also characterize non-epithelial (mesenchymal, endothelial, pericyte) components of the ChP and identify disruptions in endothelial continuity. This manuscript is relevant to the readership of Development and the conclusions are well-supported by the data presented.

Reviewer 2 Comments for the Author:

The intersection with the extensive literature on Zfp423 in other cell types in not leveraged for a detailed explanation of the developmental roles of Zfp423 in ChP— particularly on separable roles in processes of proliferation, differentiation, and maturation. For developmental roles, readers are interested in processes of patterning, survival, proliferation, differentiation, and maturation. The results should be presented less as phenomenology and more in the context of developmental processes and the known roles for Zfp423 that the authors already published for cerebellum (e.g. zfp Δ 9-20 altered mitotic spindle orientation, reduced proliferation, stalled cell cycle progression, increased DNA damage and reduced postmitotic cells and zfp Δ28-30 increased DNA damage and reduced postmitotic cells, but with an increase in proliferating progenitor cells. Even without data on survival, mitosis, spindle, and DNA damage, relating all conclusions to this known construct would make the existing data more broadly relevant.

We have rearranged the results section by describing the alterations first, and focusing on their molecular basis afterwards. We value the quality and detail of the phenomenological description presented in this paper, which provides the reader with convincing evidence of the gross alterations, as well as some of the subtler ones, observed in Δ28-30 mutants. However we fully agree with the reviewer that the mechanistic analysis of those abnormalities is of prime importance for the Development readership, and we have now tried to make it more exhaustive and to concentrate it at the end of the Results section, since it provides novel handles to the analysis of this developmental process.

Other concerns:
Major Concerns:
1. The authors claim (pg 5) that this manuscript reports the expression of ZFP423 in roof plate. This was already shown by Casoni et al Dev 2017. Please focus on novel conclusions.
We have replaced the original figure 3 with a totally new one focusing on the ChP. It contains wholemount (**Fig. 3A-C**) and high resolution images (**Fig. 3 D,E**) revealing the early and specific expression of Zfp423 in the prospective hindbrain and hChP epithelium.

2. The authors show (pg 10, fig 3B) that ZFP423 expression shows a rostral-caudal gradient in hChP epithelium. Is this a true R-C gradient, or consistent with a developmental role in immature and progenitor cells, a developmental gradient?
Excellent point! The new **Fig. 3** now contains two panels (**D,E**) that show expression of ZFP423 protein in the ChP epithelium, which contains proliferating basal progenitors, and lack of expression in the postmitotic territories of the nascent ChP, suggesting a developmental role in immature progenitor cells

3. On page 12 (figure 7) the authors conclude that Zfp423 mutant epithelial cells take on a squamous phenotype. While the organization can be determined as stratified (or pseudostratified) by nuclear staining), the cell shape is less apparent. Since this observation is critical to the conclusions (page 16), the authors should use stains that completely outline the cell body, even H&E could characterize the organization better than a nuclear stain alone.
We performed H&E staining on E12.5 wt and Δ28-30 embryos. The new panel in **Fig. 7K,L** shows that the epithelial cells in the wt are cuboidal while the mutant cells are squamous.

4. On page 13 (**Fig. S3**) the authors present, but do not emphasize data indicating that the multiciliogenesis is partially rescued by E18.5. This finding is interesting and suggests delayed differentiation rather than complete disruption in specification.
In fact, a paper from the Dymecki group (Hunter and Dymecki, 2007) has demonstrated that medial and lateral territories of the 4th ventricle choroid plexus are specified by different combinations of extracellular signals from the roof plate. Thus, it is conceivable that ZFP423 may play a more critical role in the medial territory. At any rate, the lateral stump that is maintained in the mutant only accounts for ~5% of the total hindbrain ChP. Based on this reasoning, we have replaced the verb "rescued" with "preserved" (page 21).

5. The very early expression of Zfp423 further supports a master-regulatory role in the hindbrain for ChP formation. The authors indicate E8.5-E9.5 expression, but do not show the data (page 15). The inclusion of such expression data would further support the conclusion as should be considered,

if available.
We have included these data. Please see response to reviewer 1, items 3 and 4

6. While the authors do not perform any assays on survival or proliferation, they suggest (page 17), based on the *Gmnc* expression, that the mutation results in altered conversion from proliferating epithelium into differentiated cells. Analysis of progenitor ratios over developmental time could indicate proliferative roles. Cell survival/death should be evaluated. The authors should discuss these data.
The same concern has been raised by reviewer 1. Here is the answer to reviewer 1, item 5 "We have done immunofluorescence stainings with an activated Casp3 antibody. Our results failed to reveal any evidence of cell death in the mutant and wt alike in the ChP primordium. These experiments were performed at E10.5 (**Fig. S1E-G'**), and at E11.5 and E12.5 (not shown). We also tested cell death with a tunel kit at E12.5, and again we found no significant differences between the wt and mutant ChP (data not shown). These negative results are in keeping with what we found in the cerebellar VZ and published in Casoni et al, 2017.
Then we assessed the presence of DNA damage signalling, as in Casoni et al 2017. To this end, we immunostained sagittal sections with a γH2AX antibody at E10.5, demonstrating in three independent experiments a slight increase in the number of cells positive for this marker. This evidence is shown in **Fig. S1C-D**.

Based on this outcome, we sought to determine whether cell cycle progression stalls in the mutant hChP, as previously observed in the VZ. We employed an antibody against PHH3 to detect cells in G2 phase, which display a dotted staining, and cells in M phase, which display a full nuclear staining (**Fig. S1A,A'**). The ratio between G2 positive cell and M positive cells (**Fig. S1B**) is indicative of a stall in G2 phase in mutants, leading to a delay of the cell cycle progression. Taken together, these data strengthen the notion that Zfp423 participates in DNA damage repair.

All these results are described in the first paragraph of the results sections, named "*ZFP423 protein is expressed in early epithelial progenitors of the prospective hChP*" on page 11 and 12. "

Minor Concerns:
On page 3, in the Introduction, please cite primary sources when economical do so (eg Lun, et al 2015; rather than Lehtinen et al 2013- predating review— for differential transcriptomes of ChP).

We added the suggested references on page 3.

Page 9 (Fig 2): please clarify what tissue was used for RT-PCR. In other locations and figures, hindbrain in specified, but not here. Also not specified on pg 11 (although clarified in Fig 5 legend).
To better clarify this issue, we are providing a schematic of the sectioning done to obtain the specimens analòyzed by RT-qPCR. The sketch is now shown in **Fig. S5A** and we have modified the text accordingly.

9. On page 11, please clarify the statement "This marker is downregulated in medial and lateral sections of the Zfp423 mutant hChP…". Is this meant to emphasize that the entire tissue is affected, or only the medal and lateral-most regions, not in between?
We meant that the whole tissue is affected, both in the lateral and in the medial sections.

10. Page 13, include citation for Watanabe et al 2012 as critical publications understanding ChP epithelial development.
We apologize for overlooking this key paper. We have the corresponding reference on page 19.

11. Page 15 discussion. The authors should not use the term "completely absent… except" as it is misleading. The structure is "almost completely absent"
We corrected the text accordingly. thank you.

12. Wording:
We have corrected the wording mistakes pointed out by the reviewer.

a. sage of “ependymal” and “epithelial” are confused in the manuscript. When addressing ChP epithelial cells, the term “epithelial” is standard, as these cells are distinct from ependymal cells (as the author notes), for example on pages 2 ,16, 17.
We have replaced ependymal with epithelial.

b. Some undefined and perhaps unnecessary abbreviations were used, e.g. Abs or Abs. rather than antibodies (pg 12).
Abbreviations removed

c. Mixed usage of ‘oligociliated’ and ‘multiciliated’ suggests different meaning. If the authors mean the same thing, please choose one term… ‘multiciliated’ is the most accepted.
We have adopted “multiciliated” throughout the paper

d. The authors sometime abbreviate and sometime do not abbreviate ChP, choroid plexus. Please be consistent.
Corrected

e. In multiple places, references in text are preceded by ‘inter alia…’ Removed

REFERENCES CITED IN THE TEXT

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Second decision letter

MS ID#: DEVELOP/2020/190173

MS TITLE: ZFP423 regulates early patterning and multiciliogenesis in the hindbrain choroid plexus

AUTHORS: Filippo Casoni, Laura Croci, Francesca Vincenti, Paola Podini, michela riba, Luca Massimino, Ottavio Cremona, and G. Giacomo Consalez

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.

Reviewer 1

Advance summary and potential significance to field

This study offers a previously uncharacterized role of Zfp423 in hindbrain choroid plexus development. They provide a detailed description of novel phenotypes and associated changes in gene expression, including several key transcriptional regulators implicated in choroid plexus epithelium (ChP) development. The finding that Zfp423 is a master regulator of ChP development is interesting and appeals to the audience of Development.

Comments for the author

In this revised manuscript, the authors have included additional data to clarify many of the concerns raised previously. Significance of altered Wnt or Bmp expression is still lacking. Nevertheless, the study provides strong evidence that Zfp423 is a key player in the initiation and maintenance of hChP development.

Reviewer 2

Advance summary and potential significance to field

The manuscript 'ZFP423 regulates early patterning and multiciliogenesis in the hindbrain choroid plexus" investigates the necessity of the Joubert syndrome associated kreuppel-type zinc finger transcription factor Zfp423 in the maturation of the hindbrain ChP. While ZFP423 has been shown to act generally as a transcriptional repressor (Cho, et al. PLoS One, 2013), and specifically required for cell cycle progression and maturation of cerebellar Purkinje neuron progenitors (e.g. Casoni, et al. Dev. 2017); the authors contribute new findings to understand developmental roles of this critical gene product by investigating the generation and maturation (specifically epithelial cell multiciliogenesis) of hindbrain choroid plexus (hChP).
The authors also perform tissue restricted comparative bulk RNA sequencing in WT vs. a zfp423 mutant (zfp Δ 28-30) to identify potential gene regulatory roles for ZFP423 in hChP progenitors and immature tissue—identifying known ChP regulatory genes validated by qPCR (e.g. Lmx1a, Otx2, Wnt1, GemC, and FoxJ1) as well as novel candidates (e.g. Wnt8a). The authors find that Zfp423 regulates hChP generation with only a lateral vestigial portion forming in the zfp Δ 28-30 mutant. They determine severe disruptions in epithelial cell maturation including cell morphology and organization, polarization, elaboration of microvilli and multiciliogenesis. They also characterize non-epithelial (mesnchymal, endothelial, pericyte)
components of the ChP and identify disruptions in endothelial continuity. This manuscript is relevant to the readership of Development and the conclusions are well-supported by the data presented.

Comments for the author

The authors have thoroughly addressed previous concerns and the revised manuscript is an important study ready for publication.