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Transcription co-factor LBH is necessary for survival of cochlear hair cells

Huizhan Liu, Kimberlee P. Giffen, M'Hamed Grati, Seth W. Morrill, Yi Li, Xuezhong Liu, Karoline J. Briegel and David Z. He
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Original submission

First decision letter

MS ID#: JOCES/2020/254458

MS TITLE: Transcription Co-Factor LBH Is Necessary for Survival of Cochlear Hair Cells

AUTHORS: huizhan liu, Kimberlee P Giffen, M'hamed Grati, Seth W Morrill, Yi Li, Xue Zhong Liu, Karoline J Briegel, and David Z He
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. There were concerns with appropriate images for controls, the relative lack of statistical analysis, and the writing throughout the manuscript. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes 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 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. Please attend to all of the reviewers' comments. 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

The manuscript by Liu et al., investigates the function of the transcription co-factor, Lbh, in the development, function and survival of HCs in the cochlea. Lbh was deleted using Rosa26-Cre which targets all cells very early and thus should create a complete knockout of Lbh. The authors found that cochlear HCs develop normally but they begin to degenerate at ~P12 and eventually most OHCs and many IHCs die, resulting in hearing loss. The authors also perform RNA-seq analysis and show changes in over 5,000 genes in the HCs of mice lacking Lbh.
The data in the manuscript is high quality and provides interesting and novel results. While the field has quite extensive knowledge of genes that regulate HC differentiation, far less is known about the molecular mechanisms that govern survival and maintenance of HCs.

Comments for the author

However the writing is sloppy often making conclusions that are not supported by the data and my biggest concern is the lack of statistical analysis in several figures. Before publication, I suggest the following changes:

Major concerns
1) The introduction of Lbh expression in the inner ear is missing details from several papers. Scheffer et al., 2015 is cited as a reference for Lbh expression in vestibular HCs, and since previous sentences refer to the adult cochlea, the reader assumes Lbh expression has been detected in adult vestibular HCs too. However that is not the case, Scheffer et al., only used peri-natal tissue and found Lbh expression in both HCs and supporting cells in the utricle and also in both cell types in the cochlea. Data from Kolla et al., 2020 and Elkon et al., 2015 (both Nat Comm) also show that Lbh is expressed in both HCs and SCs in the P0-1 cochlea. All of these details need to be incorporated into the Introduction to give the reader a better understanding of what is known about Lbh already. In addition, the difference in Lbh level between IHCs and OHCs was measured in the adult cochlea and that needs to be clarified in the last sentence of the 1st paragraph. The fact that Lbh is NOT HC-specific in the developing cochlea is important, because it begs the question as to why the authors only focused on HCs in this manuscript. What about its role in supporting cells? At the least, it would be nice to add counts of supporting cell nuclei at various ages to Figure 2.
2) In the 2nd paragraph of the Introduction, it is not clear what was done by Lindley and Briegel 2013 versus this paper. It appears that both used Rosa26-Cre mice to delete Lbh but I don't have access to the Lindley and Briegel 2013 paper to see if they examined the inner ear.
3) In the 2nd paragraph of the Introduction and 2nd paragraph of the Results, the authors need to explain their mouse model better so that the reader understands when Lbh was deleted and in what cell types. Rosa26-Cre needs to be specifically named in both of these paragraphs and its expression pattern described. It should result in ubiquitous Lbh deletion very early – perhaps generating a complete knockout of the gene similar to a germline LbH KO mouse.
The LbhΔ2 abbreviation also needs to be explained. Some of this detail can (and should) be moved from the methods section.
4) Figure 2 and 2nd paragraph of the Results: It does not appear that any statistical analysis was performed on the ABR or DPOAE data. This is needed before any conclusions about ABR or DPOAE threshold shifts can be made. Based on the graph in Fig. 2A, I doubt that there are differences between LbhΔ2 heterozygous and control mice at low frequencies but that is not clear in the text. Also in Fig.2B I am not confident that there are difference in DPOAE thresholds for any genotype at 8 or 16kHz. Please run stats on all data in this figure, add asterisks showing the significant differences to all graphs, revise the text to only describe what is statistically significant from controls, and add details for what statistical test was used to the Figure legend.

5) No control data or statistical analysis was used in Figure 3 to show that LbhΔ2 homozygous mice have HC loss. The figure legend says that control counts are presented in 3B, but I don't see it in the graph. In addition please add error bars for each bar and asterisks showing the significant differences to the graphs. Also make sure that the Results text matches the data. For example the text says that at 1 month, there is 50% OHCs lost in the base of LbhΔ2 homozygous mice, but the graph only shows 20% loss at this age.
6) No statistical analysis was used in the qPCR validation of the RNA-seq dataset (Fig 7G). Please add error bars for each bar and asterisks showing the significant differences to the graphs.
7) Discussion paragraph 4 and 5 are focused on changes in the Notch and Wnt pathways however there are many other pathways that are altered in LbhΔ2 homozygous HCs that could be the cause of HC degeneration and death. Why are Notch and Wnt, pathways that many papers have shown to be downregulated to low levels in the cochlea by 1 week of age, highlighted here? It seems to me that genes involved in metabolic processes, DNA damage/repair, and autophagy (pathways also identified in the RNA-seq dataset) are far more likely to be responsible for HC death than Notch and Wnt pathways. I suggest that the discussion of Notch and Wnt is reduced and discussion of the other pathways are increased.
8) Summary statement: Similar to point #7, the author conclude that LbH function is mediated by Notch and Wnt pathways but there are many other pathways that are altered in LbhΔ2 homozygous HCs based on their RNA-seq data and the focus on Notch and Wnt is inappropriate here.
9) End of Methods: The section about statistics states that Student t-tests were used for data analysis. This is only appropriate for a small number of figures where only 2 genotypes and 1 parameter are compared. For Figures 2 and 3 ANOVAs are needed since there are either 3 genotypes and multiple frequencies or 2 genotypes and multiple ages and cochlear turns.

Minor concerns
1) The explanation of a "transcription co-factor" does not occur until the 6th paragraph of the Discussion. Please briefly introduce this term and the difference from a transcription factor in the Introduction when Lbh is described as having this function.
2) Figure 1A: what is nSC in the graph? Why not just SC?
3) Figure 1B: Higher magnification images are needed to demonstrate that Lbh is not detected in SCs at this age. It looks to me like there is some staining in pillar cells in the image presented.
4) Figure 1B-G: Please add a label stating the age of the sample in each image.
5) Figure 1C and E: It would be very helpful for the reader to see Lbh alone in these panels to better assess the expression in IHCs.
6) Last sentence of Results paragraph 1: Please revise to better match the data -- expression of Lbh was only examined in the utricle at P12. You cannot conclude that Lbh is "not expressed in vestibular HCs" – in fact the Scheffer et al., paper and your Fig1 A shows expression in vestibular HCs at E16 & P7 and there could be differences between the utricle and other vestibular organs. You only examined 1 age and 1 organ.
7) 2nd paragraph of the Results: Please define CM and briefly explain how that is different from ABR and DPOAE measurements. What does it tell you?
8) 2nd paragraph of the Results: Fig 2D shows an increased EP in the LbhΔ2 homozygous mice. What does that mean? The text only explained what an EP reduction would mean.
9) Figure 3A: Please provide better images for 3A – the myo7a and HC loss is hard to see. Perhaps higher mag images would be better.
10) Figure 4: Please add a label stating the age of the sample in each image.
11) Figure 5: Please add the statistical test used to the figure legend.
12) Section 4 of the Results refers to Lbh expression as limited to HCs, but in Fig. 1A, it is also expressed in supporting cells too.
13) Figure 6: Please add the statistical test used to the figure legend.
14) Figure 6: Please add labels to 6D for upregulated genes and 6E for downregulated genes so the reader can easily see the differences between these 2 very similar plots.
15) Methods: Please state where the Lbh-floxed and Rosa26-Cre mice were obtained from. Were they purchased from a commercial vendor or gotten from a PI at another university? Please also state the strain background of these mice and whether both genders were used.
16) Methods: Please add dilution factor used for the 2 primary antibodies for the immunostaining. In addition phalloidin is shown in Figure 1 but not included in the methods.

Reviewer 2

Advance summary and potential significance to field

This study by Liu and colleagues provides a solid description of the phenotype in a novel mouse mutant with a defect in outer hair cell survival. Overall the findings are reasonable, the manuscript is clearly written and the figures are fine. Two consistent omissions in many figures are comparative images from WT samples and indications of statistical significance, see below for specific images. While the phenotypic differences appear convincing, it is important to provide both WT comparisons and proof of significant differences. This study will provide valuable data regarding factors relevant to outer hair cell survival.

Comments for the author

The following specific issues should be addressed.

Title page, I believe that the names for the third author are reversed, should be M'hamed Grati.

Page 5, I assume CM stands for cochlear microphonic? It is not defined.

Figure 2A, which of the shown values are significantly different?

Figure 2B, same point, which, if any of these values are significantly different? They all appear, with the possible exception of the mutant value at 32 KHz, as if they could be non-significant changes.

Figure 2E and F, are the changes significant relative to the one month time point? I agree that the changes look larger but without statistics it is not appropriate to conclude progressive hearing loss.

Figure 3B it is important to show the data for WT animals, at a minimum, as a comparison. Especially because most mutant mouse lines are on a C57Bl/6 background which carries its own age-related hearing loss.

Figure 4, again some control data would be nice for comparison.

First revision

Author response to reviewers' comments

Reviewer 1

Major concerns:

1) The introduction of Lbh expression in the inner ear is missing details from several papers. Scheffer et al., 2015 is cited as a reference for Lbh expression in vestibular HCs, and since previous sentences refer to the adult cochlea, the reader assumes Lbh expression has been detected in adult vestibular HCs too. However that is not the case, Scheffer et al., only used peri-natal tissue and found Lbh expression in both HCs and supporting cells in the utricle and also in both cell types in the cochlea. Data from Kolla et al., 2020 and Elkon et al., 2015 (both Nat Comm) also show that Lbh is expressed in both HCs and SCs in the P0-1 cochlea. All of these details need to be incorporated into the Introduction to give the reader a better understanding of what is known about Lbh already. In addition, the difference in Lbh level between IHCs and OHCs was measured in the adult cochlea and that needs to be clarified in the last sentence of the 1st paragraph. The fact that Lbh is NOT HC-specific in the developing cochlea is important, because it begs the question as to why the authors only focused on HCs in this manuscript. What about its role in supporting cells? At the least, it would be nice to add counts of supporting cell nuclei at various ages to Figure 2.

While Lbh mRNA is expressed at low levels in supporting cells during embryonic development and early postnatal stages, we did not detect any LBH protein in supporting cells (SCs) of adult cochleae, consistent with RNA-Seq data by Ranum et al. (2019) and Kolla et al. (2020). In contrast, both LBH mRNA and protein were highly expressed in cochlear HCs both at developmental and adult stages. To clarify this, we expanded on the RNA and protein expression data in Fig. 1, as well as included a better clarification in the Results part, paragraph 1.
We also would like to point out that Lbh-deficient mice did not show any supporting cell abnormalities (Fig. 3) whereas degeneration of hair cells and stereocilia bundles occurs as early as P12 in those mice. Hence, the focus of this manuscript is on HCs.

2) In the 2nd paragraph of the Introduction, it is not clear what was done by Lindley and Briegel 2013 versus this paper. It appears that both used Rosa26-Cre mice to delete Lbh but I don't have access to the Lindley and Briegel 2013 paper to see if they examined the inner ear.

Lbh expression in adult cochlear hair cells was first reported by us (Liu et al., 2014). Briegel and colleagues (Lindley and Briegel, 2013) generated Lbh-KO mice to study mammary gland development and cancer.

3) In the 2nd paragraph of the Introduction and 2nd paragraph of the Results, the authors need to explain their mouse model better so that the reader understands when Lbh was deleted and in what cell types. Rosa26-Cre needs to be specifically named in both of these paragraphs and its expression pattern described. It should result in ubiquitous Lbh deletion very early – perhaps generating a complete knockout of the gene similar to a germline LbH KO mouse. The LbhΔ2 abbreviation also needs to be explained. Some of this detail can (and should) be moved from the methods section.

Included as suggested.

4) Figure 2 and 2nd paragraph of the Results: It does not appear that any statistical analysis was performed on the ABR or DPOAE data. This is needed before any conclusions about ABR or DPOAE threshold shifts can be made. Based on the graph in Fig. 2A, I doubt that there are differences between LbhΔ2 heterozygous and control mice at low frequencies but that is not clear in the text. Also in Fig.2B I am not confident that there are difference in DPOAE thresholds for any genotype at 8 or 16kHz. Please run stats on all data in this figure, add asterisks showing the significant differences to all graphs, revise the text to only describe what is statistically significant from controls, and add details for what statistical test was used to the Figure legend.

Statistical analysis has been conducted and asterisks marking statistical significance are included in the text, figures and legend as suggested.

5) No control data or statistical analysis was used in Figure 3 to show that LbhΔ2 homozygous mice have HC loss. The figure legend says that control counts are presented in 3B, but I don't see it in the graph. In addition please add error bars for each bar and asterisks showing the significant differences to the graphs. Also make sure that the Results text matches the data. For example the text says that at 1 month, there is 50% OHCs lost in the base of LbhΔ2 homozygous mice, but the graph only shows 20% loss at this age.

In Fig. 3, the percentage of surviving hair cells was calculated based on cell count from wildtype mice (as 100%). We now replotted the figure in the revision. Error bars are also presented. We also corrected inconsistency in the text and figure caption.

6) No statistical analysis was used in the qPCR validation of the RNA-seq dataset (Fig 7G). Please add error bars for each bar and asterisks showing the significant differences to the graphs.

These are fold changes in mean gene expression (n = 3; normalized to Gapdh) compared between WT and Lbh-KO OHCs. The plot was intended to show the same trend of change with two different methods. No statistical analysis can be made between the two groups.

7) Discussion paragraph 4 and 5 are focused on changes in the Notch and Wnt pathways however there are many other pathways that are altered in LbhΔ2 homozygous HCs that could be the cause of HC degeneration and death. Why are Notch and Wnt, pathways that many papers have shown to be downregulated to low levels in the cochlea by 1 week of age, highlighted here? It seems to me that genes involved in metabolic processes, DNA damage/repair, and autophagy (pathways also identified in the RNA-seq dataset) are far more likely to be responsible for HC death than Notch and Wnt pathways. I suggest that the
discussion of Notch and Wnt is reduced and discussion of the other pathways are increased.
8) Summary statement: Similar to point #7, the author conclude that LbH function is mediated by Notch and Wnt pathways but there are many other pathways that are altered in LbhΔ2 homozygous HCs based on their RNA-seq data and the focus on Notch and Wnt is inappropriate here.

We focused on Notch and Wnt since several previous studies showed that LBH is involved in Notch and Wnt pathways. We are aware that Notch and Wnt pathways are downregulated after birth. However, low level expression of Notch and Wnt is necessary for survival of hair cells. One study showed that LBH is involved in DNA repair/damage. In the revision, we trimmed discussion of Notch and Wnt and included more discussion of other potential mechanisms such as damage/repair and autophagy as Reviewer 1 suggested. The sentence in Summary Statement was also modified.

9) End of Methods: The section about statistics states that Student t-tests were used for data analysis. This is only appropriate for a small number of figures where only 2 genotypes and 1 parameter are compared. For Figures 2 and 3 ANOVAs are needed since there are either 3 genotypes and multiple frequencies or 2 genotypes and multiple ages and cochlear turns.

Analysis using ANOVA is now included.

Minor concerns:

1) The explanation of a “transcription co-factor” does not occur until the 6th paragraph of the Discussion. Please briefly introduce this term and the difference from a transcription factor in the Introduction when Lbh is described as having this function.

Included as suggested.

2) Figure 1A: what is nSC in the graph? Why not just SC?

nSC stands for non-sensory cells. Previous studies used GFP-positive hair cells and GFP-negative non-sensory surrounding cells for bulk RNA-seq (Scheffer et al., 2015). Thee GFP-negative cells described in previous study are not necessarily supporting cells. Based on definition of supporting cells, only those non-sensory cells in the organ of Corti are supporting cells. That was why we used non-sensory cells to describe them. Since the distinction is not important for the present study, we now used supporting cells in the text.

3) Figure 1B: Higher magnification images are needed to demonstrate that Lbh is not detected in SCs at this age. It looks to me like there is some staining in pillar cells in the image presented.

This is a survey picture to show LBH expression in the cochlea. LBH may be weakly expressed in some supporting cells at this age. But high magnification images showed no LBH expression in pillar or Deiters’ cells at P8 and onwards.

4) Figure 1B-G: Please add a label stating the age of the sample in each image.

Added as suggested.

5) Figure 1C and E: It would be very helpful for the reader to see Lbh alone in these panels to better assess the expression in IHCs.

An image with DAPI staining is included in the figure.

6) Last sentence of Results paragraph 1: Please revise to better match the data -- expression of Lbh was only examined in the utricle at P12. You cannot conclude that Lbh is “not expressed in vestibular HCs” – in fact the Scheffer et al., paper and your Fig1 A shows expression in vestibular HCs at E16 & P7 and there could be differences between the utricle and other vestibular organs. You only examined 1 age and 1 organ.

We examined LBH expression in all three vestibular end organs at P12 and P30 and did not see any positive staining in hair cells or supporting cells at either age. We included two new panels in the revised figure.

7) 2nd paragraph of the Results: Please define CM and briefly explain how that is different from ABR and DPOAE measurements. What does it tell you?

CM is now defined in the revision. What CM measurement reflects is also included in the text.

8) 2nd paragraph of the Results: Fig 2D shows an increased EP in the LbhΔ2 homozygous mice. What does that mean? The text only explained what an EP reduction would mean.

What EP measurement reflects is now provided in the text. We also explained why EP magnitude increased in LBH-null mice.

9) Figure 3A: Please provide better images for 3A – the myo7a and HC loss is hard to see. Perhaps higher mag images would be better.

Low magnification (10x or 20x) is often used for cell count as low magnification allows to survey (cover) a larger area. They are not intended to show details of hair cells but are sufficient for hair cell count.

10) Figure 4: Please add a label stating the age of the sample in each image.

Included as suggested.

11) Figure 5: Please add the statistical test used to the figure legend.

Added as suggested.

12) Section 4 of the Results refers to Lbh expression as limited to HCs, but in Fig. 1A, it is also expressed in supporting cells too.

We rewrote the text as suggested.

13) Figure 6: Please add the statistical test used to the figure legend.

Added as suggested.

14) Figure 6: Please add labels to 6D for upregulated genes and 6E for downregulated genes so the reader can easily see the differences between these 2 very similar plots.

Added.

15) Methods: Please state where the Lbh-floxed and Rosa26-Cre mice were obtained from. Were they purchased from a commercial vendor or gotten from a PI at another university? Please also state the strain background of these mice and whether both genders were used.

Information is now provided.

16) Methods: Please add dilution factor used for the 2 primary antibodies for the immunostaining. In addition phalloidin is shown in Figure 1 but not included in the methods.

Included as suggested.

Reviewer 2:

The following specific issues should be addressed.

Title page, I believe that the names for the third author are reversed, should be M'hamed Grati.

Corrected.

Page 5, I assume CM stands for cochlear microphonic? It is not defined.

Defined.

Figure 2A, which of the shown values are significantly different? Figure 2B, same point, which, if any of these values are significantly different? They all appear, with the possible exception of the mutant value at 32 KHz, as if they could be non-significant changes. Figure 2E and F, are the changes significant relative to the one month time point? I agree that the changes look larger but without statistics it is not appropriate to conclude progressive hearing loss.

We made some changes to the figure as suggested and statistic significance is now indicated.

Figure 3B it is important to show the data for WT animals, at a minimum, as a comparison. Especially because most mutant mouse lines are on a C57Bl/6 background which carries its own age-related hearing loss.

Aged-marched wildtype mice were used to estimate hair cell loss in the previous figure. We replotted the figure (together with revision of figure caption) and added additional panels in the revision.

Figure 4, again some control data would be nice for comparison.

Included as suggested.

Second decision letter

MS ID#: JOCES/2020/254458

MS TITLE: Transcription co-factor LBH is necessary for survival of cochlear hair cells

AUTHORS: huizhan liu, Kimberlee P Giffen, M'hamed Grati, Seth W Morrill, Yi Li, Xue Zhong Liu, Karoline J Briegel, and David Z He
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

I apologize for the long delay. One reviewer dropped out and the other needed extra time due to the pandemic. As you will see, the reviewer gave a highly favourable reports and made only one simple request related to the significance of a data point. Please either address or rebut. I hope that you will be able to carry this out, because I would like to be able to accept your paper.

We are aware that you may be experiencing disruption to the normal running of your lab that makes 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.

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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. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 2

Advance summary and potential significance to field

This is a solid paper that demonstrates the role of Lbh in outer hair cell survival. It provides valuable new data regarding another gene that is required for outer hair cell survival.

Comments for the author

The authors have addressed all of my concerns with one exception. In Figure 2A it seems unlikely that there is a significant difference between the mutant and wildtype at 8 kHz. and certainly the level of significance seems unlikely to be equivalent to the levels at other frequencies as suggested by the same asterisks.

Second revision

Author response to reviewers' comments

We are pleased to submit our revised manuscript for reconsideration. We appreciate Reviewer 2's comment regarding statistical significance of the ABR threshold at 8 kHz. We checked all values and confirmed that the difference between WT and homozygous mice is statistically significant ($p = 0.00151$) as shown in Figure 2A. The difference between WT and heterozygous mice is also significant ($p = 0.03490$). The mean and SD of the ABR threshold are 27.1 ± 3.9 dB for WT (n=7), 32.1 ± 3.9 dB for heterozygotes and 38.6 ± 6.3 dB for homozygotes. We appreciate that he/she read the manuscript so carefully. This is important for the quality of research and publication. Please convey our thanks.

Third decision letter

MS ID#: JOCES/2020/254458

MS TITLE: Transcription co-factor LBH is necessary for survival of cochlear hair cells

AUTHORS: huizhan liu, Kimberlee P Giffen, M'hamed Grati, Seth W Morrill, Yi Li, Xue Zhong Liu, Karoline J Briegel, and David Z He
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.