LATS1/2 control TGFB-directed epithelial-to-mesenchymal transition in the murine dorsal cranial neuroepithelium through YAP regulation

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MS TITLE: Lats1/2 control TGF-beta directed EMT in the dorsal cranial neuroepithelium through YAP regulation

AUTHORS: Idaliz M Martinez Traverso, Jeffery D Steimle, Xiaolei Zhao, Jun Wang, and James F Martin
ARTICLE TYPE: Research Article

Dear Dr. Martin,

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 from their reports, the referees recognise the potential of your work, but they also raise significant concerns about it. Several controls are thought to be missing, the state of the embryos is under question, the phenotypes sometimes unclear, and the relationship to EMT also not evident. Given the nature of these concerns, I am afraid I have little choice other than to reject the paper at this stage.

However, having evaluated the paper, I do recognise the potential importance of this work. I would therefore be prepared to consider as a new submission an extension of this study that contains new experiments, data and discussions and that address fully the major concerns of the referees. The work required goes beyond a standard revision of the paper. Please bear in mind that the referees (who may be different from the present reviewers) will assess the novelty of your work in the context of all previous publications, including those published between now and the time of resubmission.

If you decide to resubmit, please go to BenchPress and click on the 'Submit a new manuscript' link within the Author Area.

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Please ensure that you click the 'This is a resubmission' checkbox, and enter the manuscript identification number shown above. I would also ask you to provide in the cover letter an explanation of the key ways in which the manuscript differs from the current submission, followed by a point-by-point response to the referees' concerns.
We do understand that the work entailed in a potential new submission is significant, and that you may prefer to submit elsewhere without further delay. Please do let us know if you decide not to resubmit to Development, so that we can close our file.

Many thanks for sending your work to Development.

Yours sincerely,

Benoit Bruneau
Handling Editor
Development

Reviewer 1 Advance Summary and Potential Significance to Field:

In this study, Traverso and colleagues continued their study on Hippo signaling in regulating EMT of neuroepithelium and neural tube closure during early embryonic development. Specifically, they investigated the function of Hippo components Lats1 and Lats2 in the forming neural crest cells and discovered that loss of Lats1/2 (DCKO) led to delayed neural tube closure and disrupted cellular polarity and integrity. At the molecular level, loss of Lats1/2 resulted in an increased Tgf-β signaling, which may be responsible for altered EMT. Finally, inactivation of Hippo signaling mediator Yap/Taz led to restored Tgf-β signaling and restored neuroepithelial defects in Lats1/2 DCKO mice. Overall, this study is interesting and expands knowledge of the mechanism of Hippo signaling in regulating early neural crest cell development. It fits well within the scope of Development.

Reviewer 1 Comments for the Author:
Here are some specific comments:

1. In the Introduction section, the authors stated that neural tube defects (NTDs) are among the most common birth defects worldwide (page 4, line 69). It is not clear what the supporting evidence is for this statement. According to the CDC, NTDs are not the most common birth defects worldwide. Please clarify this statement.
2. Is pYap present in the neural folds prior to neural tube (NT) closure? It is difficult to see this in Figure 2A.
3. It is very interesting that only cranial neural tube closure is affected in Lat1/2 DCKO mice. Can the authors provide any insight into why the cranial neural tube is so dependent on Lats1/2?
4. Are the cells that infiltrated into the ventricle of Lats1/2 DCKO mice derived from cranial neural crest cells?
5. In the cell polarity study, it would be helpful to examine β-catenin and n-cadherin expression in the neural folds prior to neural tube fusion.
6. To investigate the mechanisms underlying the phenotypes of Lats1/2 DCKO mice, the authors performed RNA-seq analysis and focused on Tgf-β signaling as the downstream pathway regulated by Lats1/2-Yap signaling and genes related to EMT, such as Snai1 and Snai2. It would be informative to know if the embryos analyzed in Figure 4 were collected prior to cranial neural crest cell migration.
7. Do the 3CKO-Y mice (with Yap haploinsufficiency) survive to birth? Do they show any developmental defects?
8. The authors stated that Snai2 transcript levels were restored to the control level in 3CKO-T embryos. However, Snai2 was hardly detectable in the neuroepithelium in 3CKO-T sample (Figure 5C).

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Other comments:

1. In Figure 1H, it is difficult to see the open NT.
2. Figures 1J, M, P should be rotated to show a dorsal view of the NT.
3. Please add some arrows to indicate the differences among Figures 1K, N and Q.
4. In Figure 2D, how did the authors perform the statistical analysis?
5. Please correct the labeling inconsistencies: Figure 2: N-cad labeling in the Figure, vs n-cad labeling in the figure legend and the manuscript text. Figure 3: Z-score labeling in the Figure, vs z-score labeling the figure legend.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this manuscript, the authors present an interesting characterization of the roles of Hippo signaling kinases LATS1 and LATS2 in the phosphorylation of downstream effectors YAP/TAZ to regulate TGF-beta induced EMT in the pre-migratory neural crest from the dorsal cranial neural tube.

The Authors report the following main findings and conclusions:

ϖInactivation of Hippo components Lats1 and Lats2 in the cranial neuroepithelium of mouse embryos using a Wnt1CreSOR driver resulted in neural tube and craniofacial defects in double conditional knock out (DCKO) mice for Lats1/2.
ϖLats1/2 DCKO mutant embryos had microcephaly with delayed and defective neural tube closure.
ϖNeuroepithelial cell polarity and cell integrity were disrupted within the cranial neural tube in Lats1/2 DCKO mutants.
ϖEmbryonic neural tube RNA-sequencing revealed increased TGF-beta signaling in Lats1/2 DCKO mutants, as well as upregulation of markers of epithelial-to-mesenchymal transition (EMT) in the cranial neural tube.
ϖInactivation of Hippo signaling downstream effectors Yap and Taz suppressed neuroepithelial defects, aberrant EMT, and TGF-beta upregulation in Lats1/2 DCKO embryos.
ϖLats1/2 function via YAP and TAZ.
ϖHippo signaling modulates TGF-beta in pre-migratory neural crest EMT.
General Comment:
Previous studies (McPherson et al., EMBO J. 2004) reported that Lats1 and Lats2 are expressed in tissues of ectodermal and mesodermal origin, respectively, and essential for normal development. Specifically, McPherson described that at E8.5–9.5, Lats1 is highly expressed in the neural tube and head fold neuroepithelium, with prominent expression in the mesencephalon at E10.5. In contrast, Lats2 was detected prominently in lateral mesodermal plate, somites, and cardiac outflow tract at E8.5, and in the heart field by E10.5. Moderate Lats2 expression was also observed in the head mesenchyme, developing gut, lungs and dermatome of the somites. Overlapping expressions of Lats1 and Lats2 was observed in branchial arches and limb buds, as well as the cardiogenic crescent. In the same manuscript, McPherson also reported that constitutive Lats2−/−embryos show overgrowth in restricted tissues of mesodermal lineage and undergo death in utero on or before E12.5 preceded by defective proliferation. In contrast, St John et al. reported that constitutive loss of Lats1 (Lats1–/–) in the mouse results in infertility and growth retardation, as well as lack of mammary gland development. The large majority of Lats1 -/- mice died within their first postnatal day, and their demise was associated with internal haemorrhage (St John et al., Nature Genetics 1999).

In summary, while essential roles for Lats1 and Lats2 have already been reported in mouse embryonic development and Lats1 expression patterns described in head domains (like the neural tube and the cephalic neuroepithelium), their potential functions in neural crest cell behaviors, neural tube and craniofacial morphogenesis are still unknown. Therefore, the overall message emerging from this manuscript is novel and the study is of interest.

Overall, the findings presented in this study contribute to our knowledge of the Hippo pathway in developmental processes and uncover new roles of LATS1 and LATS2 in early craniofacial morphogenesis.

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That said, some of the reported findings would benefit from additional controls and/or further details, as described below. Lastly, the very interesting and important genetic rescue experiments would benefit from further evaluation.

Reviewer 2 Comments for the Author:
Specific Critiques:

-Using the Wnt1-CreSOR driver, the authors show that Lats1/2 deficiency results in neuroepithelial disorganization and defective cellular migration. Overall, the study uncovers important roles of Lats1/2 in TGF-beta induced EMT in the pre-migratory neural crest in the dorsal cranial neural tube. Interestingly, the authors report that Lats1 or Lats2 haploinsufficiency is enough to circumvent embryonic lethality caused by deletion of both Lats1 and Lats2, strongly suggesting "functional redundancy" between Lats1 and Lats2 in neural crest. In consideration of these results, it would appears critical to show here expression patterns of Lats1 and Lats2 in early craniofacial development with a special focus on pre-migratory neural crest in the dorsal cranial neural tube and craniofacial epithelium. Indeed, if the reported results are due to "functional redundancy" between Lats1 and Lats2, as the authors indicate, then the two genes must be co-expressed in the same embryonic craniofacial domains.

Interestingly, published results (McPherson et al., EMBO J. 2004) appear to indicate that Lats1 is highly expressed in the neural tube and head fold neuroepithelium, while only moderate expression of Lats2 was observed in the developing head, and only in the head mesenchyme. In view of the published results, it is difficult to explain functional overlapping roles between Lats1 and Lats2 in neural crest/early head domains (or to invoke "functional redundancy" -as the authors define it- a term that this reviewer does not particularly appreciate). This discrepancy could be explained considering, for example, that the analyses reported in EMBO J 2004 might lack the necessary level of detail needed to rigorously establish gene expression patterns, or they could possibly be inaccurate. In light of all the above, it is critical to implement thorough expression analyses for Lats1 and Lats2 in early embryonic heads of mice to the present study. Are both Lats1 and Lats2 expressed in both neural crest and neural crest-derived mesenchyme and cephalic epithelium? Or is only one of them expressed in these tissues? Or is one gene expressed at higher levels than the other one in these tissues? All of these questions should be thoroughly addressed here, even before describing the phenotype resulting for the loss of function (LOF) of the two genes and even before invoking overlapping functions of the two genes. It is also strange that the authors do not discuss, or even cite in their References, either the EMBO J. 2004 or the Nature Genetics 1999 paper, since these two studies report expression patterns in early mouse embryos and also findings from LOF of Lats1 and Lats2.
-Fig. 1. As discussed above, exhaustive expression patterns of Lats1 and Lats2 should be included to this figure.

-Fig. S2. Quality control for RNA-Seq analysis shows a plot displaying the principal component analysis (PCA) along PC1 and PC2 for the four samples (2 controls and 2 DCKO). It is somewhat concerning that, unlike the controls, DCKO1 and DCKO2 segregate very far apart. This could possibly result from processing and analyzing only 2 biological replicates in the RNA-Seq experiments. The authors should discuss the result from the PCA and the lack of reproducibility between DCKO1 and DCKO2.

-Fig. 4. It is strongly suggested to bring up the contrast for pSMAD3 staining, which is hardly visible, in the present rendering in Panel F (top right quadrant). In addition, it would be advisable to show also a merged image, including DAPI staining in addition to pSMAD3 staining in order to provide unequivocal proof that the pSMAD3 signal is nuclear.

-Fig. S4. The genetic rescue experiments (truly heroic genetics!) are of great importance, as they can convincingly demonstrate that LATS1/2 control neural crest EMT through YAP/TAZ. Indeed, the authors aim to show that Yap/Taz haploinsufficiency reduces cell infiltration and partially recovers neuroepithelial cell architecture in Lats1/2-deficient neural tubes. However, rendering of these critical experiments requires further evaluation and improvement. Indeed, the essential "control" needed to unequivocally prove the presence of partial rescue in the "haplo" embryo is the DCKO embryo, which is not shown in this figure but should be included. The authors need to incorporate

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images of the DCKO embryo (for each assay: gross morphology, histology, and immunostaining for pHH3).

-Fig. S4. In Panel C of this figure the authors show immunostaining of proliferation marker pHH3 and claim that mitotic cells appear in the apical edge of control neural tubes and also at the apical edge of the polarized neuroepithelium in haplo (partially rescued) neural tubes. In order to convincingly demonstrate partial rescue of neuroepithelial cell architecture and cell polarity, it is essential to include stainings for beta catenin and N cad, which can adequately and unequivocally demonstrate whether there is a rescue (even partial) of cell polarity.

Lastly, given that FigS4 shows results that are very important and germane to the overall message of the study, in the opinion of this reviewer this figure should be moved to the main text.

-Fig. 5. In Panel A, as in Fig.S4, the essential “control” needed to unequivocally prove the presence of partial rescue is the DCKO embryo, which is not shown in this figure and should be included.

-Fig. 5. In Panel D, signal for pSMAD3 does not appear to be nuclear but mostly cytoplasmatic. Is this an artifact generated by the Ab used here? Is this background noise? Regrettably, these results are not fully convincing. The authors should try a different Ab for pSMAD3, or alternatively they could stain for pSMAD2. One explanation could be that the Ab for pSMAD3 used here recognizes also other forms of pSMADs (like pSMAD5 or pSMAD7) that can also localize to the cell’s cytoplasm. This experiment needs additional work and improvement.

-Inclusion of model or effective closing illustration. In order to summarize the results of the study in a clear and easy-to-remember message, the authors should include a cartoon or schematic representation as last figure of the paper. This cartoon should deliver the take-home message by illustrating the most salient conclusions of the study on roles of LATS1 and LATS2 in early craniofacial development.

Minor Critiques:

-Given the myriad review articles on neural crest that have been recently published (e.g. Nature Reviews Neuroscience, 2021; Annual Review of Genetics, 2021) it would be good to add at least a couple of recent reviews to the References, in addition to the older -and excellent- ones that have been cited in the current manuscript (Santagati and Rijli, 2003; Theveneau and Mayor, 2012; and Bronner and Simões-Costa, 2016).

-Substantial editing of the nomenclature is needed. Throughout the text, proteins are listed either as “Yap and Taz” (e.g. in the Abstract, line 14; in the Introduction, page 5, line 89 and line 91; and in multiple other locations) or as “YAP and TAZ”. The former nomenclature is not correct according to the latest nomenclature accepted for mouse proteins. “YAP and TAZ” should be used consistently throughout the text. The same editing is necessary for the kinases “LATS1 and LATS2”, which are often -but not always-listed as Lats1 and Lats2 (e.g. in the Introduction, page 5, line 93 and line 96; and in multiple other locations). Consistency would be highly desirable.

-Multiple figures would greatly benefit from the addition of arrows or arrowheads to better highlight defects and/or to underscore details of the observed phenotypes. Just to provide a few examples: in Fig.1, arrows should be added in Panels N and especially Q to point to the abnormal structures; in Fig.2, arrows should be included in Panel A to highlight the unchanged levels of phosphorylation in the head epithelium.
These additions would greatly help those who might not be experts in craniofacial anatomy/embryology.

-Page 10, line 198 and line 201: the authors cite Figure S1 while they should cite Figure S2.

Reviewer 3 Comments for the Author:

The manuscript by Traverso et al., entitled "Lats1/2 control TGF-beta directed EMT in the dorsal cranial neuroepithelium through YAP regulation" does not in fact have anything to do with the EMT.

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EMT was simply not examined in any of the embryos. All the analyses including RNAseq were performed in deformed and visibly dysmorphic (and likely dying) embryos, therefore, it is highly likely that molecular and cellular defects observed in the mutants are the consequence (and not causes) of dysmorphic and possibly dying tissue.

Major:

I) The finding that "that Lats1/2 are required for proper neural tube closure during cranial development" is not supported by the data:
   - **Figure 1**. The open NT is not obvious from the panels shown in Fig. 1D and 1H. Please show pictures taken from the dorsal side. The abnormal NT closure is not obvious in microCT images either. In fact, in all panels shown, the NT appears to be closed. The authors need to show imaged from the dorsal view as well as sagittal sections. NT closure defects is not obvious in the coronal sections shown.
   - The development of DCKO embryos is delayed relative to controls. The authors should use controls of comparable somite stage to the mutants. E.g., use ~23-somite controls for E9,5 and 29-somite controls for E10. Otherwise, embryo phenotypes from different genotypes are not directly comparable.

II) Polarity, assayed in **Figure 2**, is analyzed in in severely dysmorphic embryos. Therefore, changes in the expression patterns noted by the authors are likely to be the consequences and not the cause of defective morphology. The authors should perform their analyses at least one day earlier (or before gross morphologic defects are notable) to establish the causality.

III) This reviewer does not see apico-basal polarity defects, e.g., laminin is distributed on the basal side. The described "defects" in b-cat and WGA localizations are only seen in dysmorphic tissue. There may be defects in cell shape.

IV) Figure 4 . Embryo stages are not listed. Panels in 4F are of poor quality.

V) Figure 5. Differences in embryo morphology could be due to variability in the genetic background. Fig. 5D. pSMAD3 staining may be an artifact. pSMAD3 should be nuclear

Table S4. The penetrance of NT, Fb, and PA defects in DKO animals is not stated. The number of embryos analyzed is too small to conclude rescue

Minor:
1) The designation of Wnt1CreSOR strain is not consistent among various subsections of the paper, the text, legend, and methods. In some places it's Wnt1^{CreSOR} and in others, it's Wnt1^{Cre2SOR}. It would be best to designate the transgenic Wnt1-Cre2 strain as is designated in the paper describing the strain and the Jackson labs. Authors should also mention in the text that it is a transgenic line.

2) Manuscript should be checked for typos

Figure 1. Write out the genotypes of Controls

<u>Author response to reviewers' comments</u>

Reviewer 1
Major:
1. In the Introduction section, the authors stated that neural tube defects (NTDs) are among the most common birth defects worldwide (page 4, line 69). It is not clear what the supporting evidence is for this statement. According to the CDC, NTDs are not the most common birth defects worldwide. Please clarify this statement.

> **Response:** We thank the reviewer for pointing this. The most common severe congenital anomalies are heart defects, neural tube defects, and Down syndrome (WHO, 2020). To

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address this, the wording was changed to "NTDs are among the most common birth defects of the central nervous system worldwide, with a prevalence that varies according to ethnic and racial background, geographic location, and surveillance program accessibility (Blencowe et al., 2018; Wallingford et al., 2013; Williams et al., 2016; Zaganjor et al., 2016)." (page 4, lines 60-63).

2. Is pYap present in the neural folds prior to neural tube (NT) closure? It is difficult to see this in Figure 2A.

Response: We thank the reviewer for this question. At E10.5, neural folds have already fused. To address this question, we evaluated transcriptomic data from E8.5 embryos. According to transcriptomic profiling of mouse neural tubes, *Lats1/2* are expressed in neural folds at E8.5 (Fig. S1B; Yu at al. 2017), suggesting the phosphorylation of Yap at this stage. Neural tube tissue collected from E8.5 embryos included the edge of the elevated neural plates, further trimmed to eliminate any non-neural tissues (Yu at al. 2017).

Reference:

Yu, J., Mu, J., Guo, Q., Yang, L., Zhang, J., Liu, Z., Yu, B., Zhang, T. and Xie, J. (2017). Transcriptomic profile analysis of mouse neural tube development by RNA-Seq. *IUBMB Life* **69**, 706–719.

3. It is very interesting that only cranial neural tube closure is affected in Lat1/2 DCKO mice. Can the authors provide any insight into why the cranial neural tube is so dependent on Lats1/2?

Response: We thank the reviewer for this comment. LATS1/2 kinases likely have essential and specific roles in cranial neural tube closure due to the unique ectomesenchymal potential and gene regulatory network of cranial neural crest cells. Hippo signaling might be involved in the cranial neural crest gene regulatory network but not in that of the trunk crest. Furthermore, tissue mechanical forces required for craniofacial morphogenesis could also be mediated through the Hippo pathway. This has been added and expanded in the Discussion section (pages 19- 20, lines 393-423).

4. Are the cells that infiltrated into the ventricle of Lats1/2 DCKO mice derived from cranial neural crest cells?

Response: We thank the reviewer for this question. Yes, we used an mTmG reporter to track recombined cells and found that infiltrating cells were GFP+, indicating that these cells are derived from Wnt1-cre recombined cells (Fig. S2A). Wnt1 is a marker of the dorsal neural tube and derived neural crest cells; therefore, GFP+ infiltrating cells are derived from either neuroepithelial or neural crest cells.

5. In the cell polarity study, it would be helpful to examine β-catenin and n-cadherin expression in the neural folds prior to neural tube fusion.

Response: We thank the reviewer for this suggestion. We were unable to obtain publication quality data of neural folds at E8.5, but added new data at E9.5.
Previously published data has shown the expression of both Beta-catenin and N- cadherin at the apical edge of neural folds in mouse embryos at E8.5 (Pieters et al. 2020). Consistently, our new data indicated that by E9.5, Beta-catenin and N- cadherin are expressed in the apical edge of the neural tube of control embryos and *Lats1/2* DCKO mutants (now included in Fig. S2F). Beta-catenin and N-cadherin expression can also be appreciated in ventricular infiltrating cells (asterisk) in *Lats1/2* DCKO mutants.

Reference:

Pieters, T., Sanders, E., Tian, H., van Hengel, J. and van Roy, F. (2020). Neural defects caused by total and Wnt1-Cre mediated ablation of p120ctn in mice.
BMC Dev. Biol. **20**, 17.

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6. To investigate the mechanisms underlying the phenotypes of Lats1/2 DCKO mice, the authors performed RNA-seq analysis and focused on Tgf- β signaling as the downstream pathway regulated by Lats1/2-Yap signaling and genes related to EMT, such as Snai1 and Snai2. It would be informative to know if the embryos analyzed in Figure 4 were collected prior to cranial neural crest cell migration.

Response: We thank the reviewer for this comment. We have revised our manuscript to provide more clear information on collection timepoints. RNA-seq was performed at E10.5, after neural crest cells have started migrating. We performed RNA-seq at this stage because phenotypes caused by loss of *Lats1* and *Lats2* were observed at E10.5. We have also validated these findings by *Tgfb1* RNAscope at E9.5 and E10.5 in the revised manuscript.

7. Do the 3CKO-Y mice (with Yap haploinsufficiency) survive to birth? Do they show any developmental defects?

Response: We thank the reviewer for this question. The 3CKO-Y embryo had a closed neural tube without any obvious craniofacial phenotypes when compared with control embryos. However, we don't know if 3CKO-Y mice survive to birth since only E10.5 embryos were collected. Since we were focusing on craniofacial development in this study, we haven't completed a detailed analysis of any developmental defects in other tissues/organs. Other studies are underway.

8. The authors stated that Snai2 transcript levels were restored to the control level in 3CKO-T embryos. However, Snai2 was hardly detectable in the neuroepithelium in 3CKO-T sample (Figure 5C).

Response: We thank the reviewer for pointing this. A more representative image was included in our revised manuscript (now Fig. 8A).

Minor:

1. In Figure 1H, it is difficult to see the open NT.

Response: We thank the reviewer for pointing this. In the revised manuscript, dorsal views of embryos were added, along with higher magnification views of *Lats1/2* DCKO mutant cranial regions.

2. Figures 1J, M, P should be rotated to show a dorsal view of the NT.

Response: We thank the reviewer for this suggestion. Dorsal views of the 3D reconstruction were added in the revised manuscript.

3. Please add some arrows to indicate the differences among Figures 1K, N and Q.

Response: We thank the reviewer for this suggestion. Arrows were added pointing to the open neural tube in the *Lats1/2* DCKO embryo. Higher magnification views of the cranial neural tubes (with the neural tube outlined) were also included for better comparison in the revised manuscript.

4. In Figure 2D, how did the authors perform the statistical analysis?

Response: We thank the reviewer for this question. An unpaired t-test was used to determine statistical significance between the width of the pseudostratified neuroepithelium around the ventricle of both control and *Lats1/2* DCKO neural tubes.

5. Please correct the labeling inconsistencies: Figure 2: N-cad labeling in the Figure, vs n-cad labeling in the figure legend and the manuscript text. Figure 3: Z-score labeling in the Figure, vs z-score labeling the figure legend.

Response: We thank the reviewer for pointing this. We have corrected the labeling inconsistencies between figures and figure legends in the revised manuscript.

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Reviewer 2
Major:
1. Using the Wnt1-CreSOR driver, the authors show that Lats1/2 deficiency results in neuroepithelial disorganization and defective cellular migration. Overall, the study uncovers important roles of Lats1/2 in TGF-beta induced EMT in the pre-migratory neural crest in the dorsal cranial neural tube. Interestingly, the authors report that Lats1 or Lats2 haploinsufficiency is enough to circumvent embryonic lethality caused by deletion of both Lats1 and Lats2, strongly suggesting "functional redundancy" between Lats1 and Lats2 in neural crest. In consideration of these results, it would appears critical to show here expression patterns of Lats1 and Lats2 in early craniofacial development with a special focus on pre-migratory neural crest in the dorsal cranial neural tube and craniofacial epithelium. Indeed, if the reported results are due to "functional redundancy" between Lats1 and Lats2, as the authors indicate, then the two genes must be co-expressed in the same embryonic craniofacial domains.

Interestingly, published results (McPherson et al., EMBO J. 2004) appear to indicate that Lats1 is highly expressed in the neural tube and head fold neuroepithelium, while only moderate expression of Lats2 was observed in the developing head, and only in the head mesenchyme. In view of the published results, it is difficult to explain functional overlapping roles between Lats1 and Lats2 in neural crest/early head domains (or to invoke "functional redundancy" -as the authors define it- a term that this reviewer does not particularly appreciate). This discrepancy could be explained considering, for example, that the analyses reported in EMBO J 2004 might lack the necessary level of detail needed to rigorously establish gene expression patterns, or they could possibly be inaccurate. In light of all the above, it is critical to implement thorough expression analyses for Lats1 and Lats2 in early embryonic heads of mice to the present study. Are both Lats1 and Lats2 expressed in both neural crest and neural crest-derived mesenchyme and cephalic epithelium? Or is only one of them expressed in these tissues? Or is one gene expressed at higher levels than the other one in these tissues? All of these questions should be thoroughly addressed here, even before describing the phenotype resulting for the loss of function (LOF) of the two genes and even before invoking overlapping functions of the two genes. It is also strange that the authors do not discuss, or even cite in their References, either the EMBO J. 2004 or the Nature Genetics 1999 paper, since these two studies report expression patterns in early mouse embryos and also findings from LOF of Lats1 and Lats2.

Response: We thank the reviewer for these suggestions. *Lats1* and *Lats2* RNA expression patterns were included in a new supplemental figure and have been described in the Results section in the revised manuscript. Based on our RNAscope data, both *Lats1* and *Lats2* are expressed in the cranial neuroepithelium at E10.5, and *Lats1* expression was more enriched than *Lats2* expression (Fig. S1A).
Furthermore, we analyzed available RNA-seq data from embryonic mouse neural tubes at E8.5, E9.5, and E10.5 (Yu at al. 2017) and found that both *Lats1* and *Lats2* transcripts are detected in the neural folds/neural tube during neurulation, although *Lats2* is expressed at lower levels than *Lats1* (Fig. S1B), which can explain why *Lats2* was not detected in the study by McPherson et al., 2004. We have added these results and removed the term "functional redundancy" to prevent any overinterpretations. In addition, the following studies have now been included and described in the revised manuscript:

McPherson, J. P., Tamblyn, L., Elia, A., Migon, E., Shehabeldin, A., Matysiak- Zablocki, E., Lemmers, B., Salmena, L., Hakem, A., Fish, J., et al. (2004). Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. *EMBO J.* **23**, 3677–3688.

St John, M. A. R., Tao, W., Fei, X., Fukumoto, R., Carcangiu, M. L., Brownstein, D. G., Parlow, A. F., McGrath, J. and Xu, T. (1999). Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. *Nat. Genet.* **21**, 182–186.

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Reference:

Yu, J., Mu, J., Guo, Q., Yang, L., Zhang, J., Liu, Z., Yu, B., Zhang, T. and Xie, J. (2017). Transcriptomic profile analysis of mouse neural tube development by RNA-Seq. *IUBMB Life* **69**, 706–719.

2. Fig. 1. As discussed above, exhaustive expression patterns of Lats1 and Lats2 should be included to this figure.

Response: We thank the reviewer for this suggestion. In this revised manuscript, *Lats1* and *Lats2* RNA expression patterns were included in a new supplemental figure (Fig. S1). We also tried our best with immunofluorescence staining of LATS1 and LATS2 but unfortunately were not able to obtain publication quality data of that.

3. Fig. S2. Quality control for RNA-Seq analysis shows a plot displaying the principal component analysis (PCA) along PC1 and PC2 for the four samples (2 controls and 2 DCKO). It is somewhat concerning that, unlike the controls, DCKO1 and DCKO2 segregate very far apart. This could possibly result from processing and analyzing only 2 biological replicates in the RNA-Seq experiments. The authors should discuss the result from the PCA and the lack of reproducibility between DCKO1 and DCKO2.

Response: We thank the reviewer for this comment. Quality control data for RNA- Seq analysis are now presented in Fig. S3. The two DCKO replicates strongly correlate with each other based on Spearsman's Rho (95%, Fig. S3D).
Experimental results, such as *Snai2* and *Tgfb1* upregulation, are also consistent and reproducible across different DCKO samples. PCA analysis is designed to look at the largest sources of variation in the dataset. Control and DCKO samples separate well along PC1, explaining 67.3% of variation between samples. PC2 explains only 20.9% of sample variation. Separation of the 2 DCKO samples along PC2 possibly resulted from variations in phenotype severity, dissected material, and/or low replicate number (as suggested). A brief discussion was included in the revised manuscript (page 10, lines 197-199) and the PCA plot was revised to include the percentages of variance explained per component (Fig. S3E).

4. Fig. 4. It is strongly suggested to bring up the contrast for pSMAD3 staining, which is hardly visible, in the present rendering in Panel F (top right quadrant). In addition, it would be advisable to show also a merged image, including DAPI staining in addition to pSMAD3 staining in order to provide unequivocal proof that the pSMAD3 signal is nuclear.

Response: We thank the reviewer for this suggestion. In this revised manuscript, contrast was increased, and merged images were included for all experimental conditions (now Fig. S4B). We also outlined some of the nuclei for easier examination.

5. Fig. S4. The genetic rescue experiments (truly heroic genetics!) are of great importance, as they can convincingly demonstrate that LATS1/2 control neural crest EMT through YAP/TAZ. Indeed, the authors aim to show that Yap/Taz haploinsufficiency reduces cell infiltration and partially recovers neuroepithelial cell architecture in Lats1/2-deficient neural tubes. However, rendering of these critical experiments requires further evaluation and improvement. Indeed, the essential "control" needed to unequivocally prove the presence of partial rescue in the "haplo" embryo is the DCKO embryo, which is not shown in this figure but should be included. The authors need to incorporate images of the DCKO embryo (for each assay: gross morphology, histology, and immunostaining for pHH3).

Response: We thank the reviewer for this suggestion. DCKO bright-field images, histology, and immunostaining panels were included for easy comparison in the revised manuscript (now Fig. 6).

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6. Fig. S4. In Panel C of this figure the authors show immunostaining of proliferation marker pHH3 and claim that mitotic cells appear in the apical edge of control neural tubes and also at the apical edge of the polarized neuroepithelium in haplo (partially rescued) neural tubes. In order to convincingly demonstrate partial rescue of neuroepithelial cell architecture and cell polarity, it is essential to include stainings for beta catenin and N cad, which can adequately and unequivocally demonstrate whether there is a rescue (even partial) of cell polarity.

Response: We thank the reviewer for this suggestion. According to Reviewers' concerns, conclusions regarding cell polarity have been revised or removed in the revised manuscript to avoid overinterpretations. In the revised manuscript, partial rescue of neuroepithelial cell architecture and ventricular space in haplo embryos can be appreciated against DCKO embryos in histology panels (Fig. 6B). In addition, Fig. 6C shows the difference in mitotic cell distribution, although no change in proliferation was detected.

7. Lastly, given that FigS4 shows results that are very important and germane to the overall message of the study, in the opinion of this reviewer this figure should be moved to the main text.

Response: We thank the reviewer for this suggestion. We agree that the results from the haploinsufficient embryos are very important to the progression of the study and have moved the haploinsufficient data to the main text (now Fig. 6).

8. Fig. 5. In Panel A, as in Fig.S4, the essential "control" needed to unequivocally prove the presence of partial rescue is the DCKO embryo, which is not shown in this figure and should be included.

Response: We thank the reviewer for this suggestion. DCKO bright-field images, histology, and immunostaining panels were included for easy comparison in the revised manuscript (now Fig. 7).

9. Fig. 5. In Panel D, signal for pSMAD3 does not appear to be nuclear but mostly cytoplasmatic. Is this an artifact generated by the Ab used here? Is this background noise? Regrettably, these results are not fully convincing. The authors should try a different Ab for pSMAD3, or alternatively they could stain for pSMAD2. One explanation could be that the Ab for pSMAD3 used here recognizes also other forms of pSMADs (like pSMAD5 or pSMAD7) that can also localize to the cell's cytoplasm. This experiment needs additional work and improvement.

Response: We thank the reviewer for pointing this. We used a different antibody to detect a pSMAD3 nuclear signal, although some cytoplasmic background is present. New immunostaining results are included in Fig. 5B, with magnified views that show nuclear pSMAD3 in DCKO folds but not in control neural tubes. To further validate the upregulation of TGF-beta in *Lats1/2* DCKO neural tubes, we also used RNAscope to examine the TGF-beta ligand *Tgfb1* (Fig. 5A) in the revised manuscript.

10. Inclusion of model or effective closing illustration. In order to summarize the results of the study in a clear and easy-to-remember message, the authors should include a cartoon or schematic representation as last figure of the paper. This cartoon should deliver the take-home message by illustrating the most salient conclusions of the study on roles of LATS1 and LATS2 in early craniofacial development.

Response: We thank the reviewer for this suggestion. A schematic model was added in the revised manuscript (Fig. 9).

Minor:

1. *Given the myriad review articles on neural crest that have been recently published (e.g. Nature Reviews Neuroscience, 2021; Annual Review of Genetics, 2021) it would be good to add at least a couple of recent reviews to the References, in addition to the older -and excellent- ones that have been cited in the current manuscript (Santagati and Rijli, 2003; Theveneau and Mayor, 2012; and Bronner and Simões-Costa, 2016).*

Response: We thank the reviewer for this suggestion. In the revised manuscript, the following recently published review articles were added to our cited references:

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Du, W., Bhojwani, A. and Hu, J. K. (2021). FACEts of mechanical regulation in the morphogenesis of craniofacial structures. *Int. J. Oral Sci.* **13**, 1–16.

Gandhi, S. and Bronner, M. E. (2021). Seq Your Destiny: Neural Crest Fate Determination in the Genomic Era. *Annu. Rev. Genet.* **55**, 349–376.

Martik, M. L. and Bronner, M. E. (2021). Riding the crest to get a head: neural crest evolution in vertebrates. *Nat. Rev. Neurosci.* **22**, 616-626.

2. Substantial editing of the nomenclature is needed. Throughout the text, proteins are listed either as "Yap and Taz" (e.g. in the Abstract, line 14; in the Introduction, page 5, line 89 and line 91; and in multiple other locations) or as "YAP and TAZ". The former nomenclature is not correct according to the latest nomenclature accepted for mouse proteins. "YAP and TAZ" should be used consistently throughout the text. The same editing is necessary for the kinases "LATS1 and LATS2", which are often -but not always- listed as Lats1 and Lats2 (e.g. in the Introduction, page 5, line 93 and line 96; and in multiple other locations). Consistency would be highly desirable.

Response: We thank the reviewer for pointing this. The nomenclature was revised and corrected.

3. Multiple figures would greatly benefit from the addition of arrows or arrowheads to better highlight defects and/or to underscore details of the observed phenotypes. Just to provide a few examples: in Fig.1, arrows should be added in Panels N and especially Q to point to the abnormal structures; in Fig.2, arrows should be included in Panel A to highlight the unchanged levels of phosphorylation in the head epithelium. These additions would greatly help those who might not be experts in craniofacial anatomy/embryology.

Response: We thank the reviewer for this suggestion. Arrows, arrowheads, outlines, and image magnifications have been added to multiple panels in different figures to highlight defects and specific details in the revised manuscript.

4. Page 10, line 198 and line 201: the authors cite Figure S1 while they should cite Figure S2.

Response: We thank the reviewer for pointing this. Figure citations were revised and corrected.

Reviewer 3

Major:

I. The finding that "that Lats1/2 are required for proper neural tube closure during cranial development" is not supported by the data:

- *Figure 1. The open NT is not obvious from the panels shown in Fig. 1D and 1H. Please show pictures taken from the dorsal side. The abnormal NT closure is not obvious in microCT images either. In fact, in all panels shown, the NT appears to be closed. The authors need to show imaged from the dorsal view as well as sagittal sections. NT closure defects is not obvious in the coronal sections shown.*

Response: We thank the reviewer for pointing this. In the revised manuscript, bright-field and 3D reconstruction dorsal views of embryos were added to Fig. 1. In Fig. 1B, arrows were added pointing to the open neural tube in the *Lats1/2* DCKO embryo. Higher magnifications of the cranial neural tubes (with the neural tube outlined) were included for better comparison.

- *The development of DCKO embryos is delayed relative to controls. The authors should use controls of comparable somite stage to the mutants. E.g., use ~23-somite controls for E9,5 and 29-somite controls for E10. Otherwise, embryo phenotypes from different genotypes are not directly comparable.*

Response: We thank the reviewer for this suggestion. We used cre-negative controls from

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litters including *Lats1/2* DCKOs, so that *Lats1/2* DCKO mutants and control embryos were from the same respective litters. At E9.5, the *Lats1/2* DCKO mutants and littermate control embryos analyzed were somite matched, and we tried to match the somite number when possible at E10.5 (>30 somites for *Lats1/2* DCKO mutant embryos). E10.5 DCKO embryos with less than 30 somites were removed from our analyses.

II. Polarity, assayed in Figure 2, is analyzed in in severely dysmorphic embryos. Therefore, changes in the expression patterns noted by the authors are likely to be the consequences and not the cause of defective morphology. The authors should perform their analyses at least one day earlier (or before gross morphologic defects are notable) to establish the causality.

Response: We thank the reviewer for pointing this. We have added new data and modified the polarity study results and conclusions in the revised manuscript. One day earlier at E9.5, Beta-catenin and N-cadherin were expressed in the apical edge of the neural tube of control embryos and *Lats1/2* DCKO mutant embryos (now included in Fig. S2F). Beta-catenin and N-cadherin expression could also be appreciated in ventricular infiltrating cells in *Lats1/2* DCKO mutant embryos.
Upregulation of *Snai2* and TGF-beta signaling ligand *Tgfb1* was also detected in *Lats1/2* DCKO neural tubes by E9.5 (now included in Fig. S4A). Polarity defects were described as part of the resulting phenotypes seen in the absence of *Lats1/2* at E10.5. Further analyses indicated the upregulation of EMT transcription factors and EMT inducer TGF-beta signaling. Therefore, the loss of polarity and the apical
detachment of neuroepithelial cells in *Lats1/2* DCKO neural tubes could be due to the aberrant EMT process in the absence of *Lats1/2*.

III. This reviewer does not see apico-basal polarity defects, e.g., laminin is distributed on the basal side. The described "defects" in b-cat and WGA localizations are only seen in dysmorphic tissue. There may be defects in cell shape.

Response: We thank the reviewer for pointing this. According to the Reviewer's concerns, the polarity study results were revised, and the associated conclusions were modified. We now describe changes in the apical attachment and shape of neuroepithelial cells rather than apicobasal polarity defects.

IV. Figure 4. Embryo stages are not listed. Panels in 4F are of poor quality.

Response: We thank the reviewer for pointing this. Embryo stages have been added to all panels across figures. Panels included in Fig. 4F (now Fig. S4B) have been revised: contrast was increased and merged images were included for all experimental conditions.

V. Figure 5. Differences in embryo morphology could be due to variability in the genetic background. Fig. 5D. pSMAD3 staining may be an artifact. pSMAD3 should be nuclear

Response: We thank the reviewer for pointing this. All embryos studied are in a similar mixed genetic background. Additionally, we used a different antibody to detect a pSMAD3 nuclear signal, although some cytosplasmic background is present. New immunostaining results are included in Fig. 5B, with magnified views that show nuclear pSMAD3 in DCKO folds but not in control neural tubes. To further validate the upregulation of TGF-beta in *Lats1/2* DCKO neural tubes, we also used RNAscope to examine the TGF-beta ligand *Tgfb1* (Fig. 5A) in the revised manuscript.

VI. Table S4. The penetrance of NT, Fb, and PA defects in DKO animals is not stated. The number of embryos analyzed is too small to conclude rescue

Response: We thank the reviewer for this comment. Table S4 describes the craniofacial defects found in E10.5 rescue embryos. We made it clearer in the text that DCKO mutant embryos have 100% penetrance of NT, Fb, and PA defects (page 16, lines 317-320). We collected over 10 litters of rescue embryos, and all 3CKO-T rescue embryos (n=6) had a neural tube comparable to that of controls.

Minor:

1. The designation of Wnt1CreSOR strain is not consistent among various subsections of the

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paper, the text, legend, and methods. In some places it's Wnt1CreSOR and in others, it's Wnt1Cre2SOR. It would be best to designate the transgenic Wnt1-Cre2 strain as is designated in the paper describing the strain and the Jackson labs. Authors should also mention in the text that it is a transgenic line.

> **Response:** We thank the reviewer for pointing this. We have corrected the labeling inconsistencies. *Wnt1*^{Cre2} was used to designate the cre driver strain, and we have now mentioned that it is a transgenic line (page 6, line 100-101).

2. *Manuscript should be checked for typos*

> **Response:** We thank the reviewer for pointing this. We have proofread and corrected typos in the manuscript.

3. *Figure 1. Write out the genotypes of Controls*

> **Response:** We thank the reviewer for pointing this. Genotypes for controls include all cre-negative embryos (from litters with DCKO mutant embryos present). We added this note to the text (page 6, line 108), in addition to mentioning it in Table S1.

Resubmission

First decision letter

MS ID#: DEVELOP/2022/200860

MS TITLE: LATS1/2 control TGF-beta directed EMT in the dorsal cranial neuroepithelium through YAP regulation

AUTHORS: Idaliz Michelle Martinez Traverso, Jeffrey D Steimle, Xiaolei Zhao, Jun Wang, and James F Martin

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 positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Reviewer 1 raises a concern about the Cre line used driving recombination in the male germline. This is seems an important issue and I suggest it needs to be addressed and/or acknowledged in a revision. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

In this manuscript from Martinez Traverso et al., the authors explore the function of the Hippo signaling pathway kinases Lats1 and Lats2 in the dorsal neuroectoderm and neural crest EMT. Upon conditional disruption of these genes using Wnt1CreSor in mice, the authors observe failed neurulation and dramatic tissue dysmorphology of the neuroepithelium. Transcriptomic analysis revealed that these phenotypes relate to changes in EMT and cell adhesion regulating genes as well as increased expression of Tgfb1, leading to hyperactivation of TGFB/SMAD signaling. Manipulation of TGFB signaling in cells and in Lats1/2 conditional mutant embryos confirms the functional

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relevance of these changes. Finally, conditional loss of function of Yap/Taz in the context of Lats1/2 disruption confirm that LATS1/2 operate through the canonical Hippo signaling pathway in this context.

Comments for the author

Unfortunately, I find one potentially significant issue with this manuscript that must somehow be addressed: It should be noted that the Wnt1-Cre2 (Wnt1-CreSor) mouse line consistently mediates recombination in the male germline. This fact is noted in the MGI entry for this allele and also in a recent paper (Dinsmore et al., Genesis, 2022). The inclusion of the mTmG reporter may partially address this concern, but indeed this appeared to yield a widespread recombination pattern (rather than a highly restricted dorsal pattern) in e.g. Fig. 2B. According to methods, crosses indeed relied on transmission of the Cre and Lox alleles from the father and the resulting embryos are likely to be haploinsufficient for Lats1/2 outside of the neuroepithelium/NCCs. This caveat may not substantially affect the conclusions of the paper, but should be considered and mentioned.
In addition to this potentially major concern, one minor concern might be considered.
The claim that that loss of Lats1/2 impacts NT morphogenesis by impacts on cell migration may overreach possible interpretations from a DEG IPA analysis. Though many genes involved in cell migration are changed in their expression, these are crucial genes for many aspects of tissue morphogenesis and integrity and an assertion on cell migration specifically cannot be made without direct observation.

Reviewer 2

Advance summary and potential significance to field

Neural tube defects can be severe birth defects and the cellular and molecular mechanisms that cause them need to be better understood. In this manuscript the authors have expanded our knowledge of the role Hippo signaling plays in NC migration and NTDs. It is well-known that Lats1/2 control the Hippo components Yap and Tap to regulate gene expression. This report shows how these factors control EMT and cell shape to effect neural tube closure.
Comments for the author

Development manuscript review (Dev-2022, 200860v1-Martin)
LATS1/2 control TGF-beta directed E 1 MT in the dorsal cranial neuroepithelium 2 through YAP regulation by Traverso et al.
This manuscript seeks to understand the role of Hippo signaling in early craniofacial development and in neural crest signaling, migration and cell specification. Conditional KO of Lats1/2 resulted in neural tube and craniofacial defects. Increased Yap and Taz nuclear function caused aberrant EMT and TGF-beta signaling giving rise to neural tube defects. This study was performed by established Hippo pathway investigators.
After reading the reviewer's comments and author responses to the initial submission of the manuscript, it is clear that a detailed review was accomplished and the authors have performed more experiments and clarified some of the issues we found in the manuscript. While some interpretation of the data was not convincing, it appears that the authors have successfully corrected these statements and provided new data.
The previous reviewer's comments and author responses are appropriate and any more comments I have would not greatly improve the manuscript.

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First revision

Author response to reviewers' comments

We have responded to the following comments and criticisms by the reviewers.

Reviewer 1 Comments for the Author:

1. **It should be noted that the Wnt1-Cre2 (Wnt1-CreSor) mouse line consistently mediates recombination in the male germline. This fact is noted in the MGI entry for this allele and also in a recent paper (Dinsmore et al., Genesis, 2022). The inclusion of the mTmG reporter may partially address this concern, but indeed this appeared to yield a widespread recombination pattern (rather than a highly restricted dorsal pattern) in e.g. Fig. 2B. This caveat may not substantially affect the conclusions of the paper, but should be considered and mentioned.**

After careful review of the manuscript by Philippe Soriano and colleagues (Dinsmore et al., 2022) and our mouse breeding scheme, we have added additional description to the manuscript and within the methods. Specifically, we have added "The *Wnt1*^{Cre2} transgene has been reported to be active in the male germline, and therefore, the male mouse passes on recombined (Δ) alleles irrespective of *Wnt1*^{Cre2} inheritance (Dinsmore et al., 2022)." at lines 527-530 within the **Material and Methods**, under subheading *Mice*. Additionally, the genotypes and labels throughout the text and Tables S1 and S2 have been adjusted accordingly.

The male mice used throughout the manuscript were either Wnt1-Cre2$^{Tg/+}$; Lats1$^{F/+}$; Lats2$^{F/+}$ or Wnt1-Cre2$^{Tg/+}$; Lats1$^{F/+}$; Lats2$^{F/+}$; Yap$^{F/+}$; Taz$^{F/+}$ in the case of the rescue experiments. The male germline activity of the Wnt1-Cre2 results in the male either passing on a wildtype (+) allele or a recombined flox allele (Δ) irrespective of Wnt1-Cre2 inheritance based on the recent report (Dinsmore et al., 2022). Therefore, for example, the genotype of our mutant embryos is Wnt1-Cre2$^{Tg/+}$; Lats1$^{F/\Delta}$; Lats2$^{F/\Delta}$ and exhibit complete removal of *Lats1* and *Lats2* in the neural crest on a background of germline heterozygosity.

With respect to pattern of GFP, such as that shown in Fig. 2B, the R26^{mTmG} reporter is inherited from the female mouse (Lats1$^{F/+}$; Lats2$^{F/+}$; R26$^{mTmG/mTmG}$) and therefore is unaffected by the male germline recombination.

Although anecdotal, we have previously collected Wnt1-Cre2$^{Tg/+}$; Lats1$^{F/F}$; Lats2$^{F/F}$ embryos when we reversed the cross, i.e., the female carrying the Wnt1-Cre2 instead of the male. The cranial neural tube phenotype described in this manuscript was consistent with the embryos recovered when the female carried Wnt1-Cre2.

2. **The claim that that loss of Lats1/2 impacts NT morphogenesis by impacts on cell migration may overreach possible interpretations from a DEG IPA analysis. Though many genes involved in cell migration are changed in their expression, these are crucial genes for many aspects of tissue morphogenesis and integrity and an assertion on cell migration specifically cannot be made without direct observation.**

Our conclusion that loss of *Lats1/2* impact NT morphogenesis may be due to migration defects was based on changes in gene expression and observed neural crest cells in the ventricular space. Furthermore, previous work, including our own, have demonstrated that cell migration is directly affected by loss of Yap/Taz signaling (Hindley et al., 2016; Kumar et al., 2019; Manderfield et al., 2014; Wang et al., 2016; Zhao et al., 2021), suggesting that loss of Yap/Taz regulation by loss of *Lats1/2* could have a similar but opposite effect.

Nevertheless, we have changed the language, e.g., **Introduction** lines 84-85 and **Results** lines 185 and 227, to suggest that the morphogenesis defects may be due to migration defects. As the reviewer points out, the cell migration related genes identified in our transcriptional profiling datasets, e.g., the EMT master regulators *Snai1/2* and*Twist1*, are pleiotropic and could be affecting multiple steps during morphogenesis of the neural tube to give us the observed structure. However, our phenotypic observations of neural crest derived cells infiltrating the ventricle certainly suggests that aberrant migration is occurring, but how this ultimately affects neural tube folding and closure was not explored.

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Second decision letter

MS ID#: DEVELOP/2022/200860

MS TITLE: LATS1/2 control TGF-beta directed EMT in the dorsal cranial neuroepithelium through YAP regulation

AUTHORS: Idaliz Michelle Martinez Traverso, Jeffrey D Steimle, Xiaolei Zhao, Jun Wang, and James F Martin
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.

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