TMEM132A ensures mouse caudal neural tube closure and regulates integrin-based mesodermal migration

Binbin Li, Liza Brusman, Jacob Dahlka and Lee Niswander
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MS TITLE: TMEM132A ensures mouse caudal neural tube closure through regulating integrin-based mesodermal migration

AUTHORS: Binbin Li and Lee Niswander

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. The referees recognise the strength of the genetic data implicating TMEM132A but indicate that further evidence is needed to support the proposed mechanism of disrupted mesoderm migration causing the NTDs. In addition, each of the referees asks for clarifications and quantitation of specific experiments. Adding these will also strengthen your study and help readers understand the results.

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This manuscript by Li and Niswander documents exciting and novel findings relating to the role of TMEM132A in closure of the neural tube and development of mesodermal structures. They demonstrate that global deletion of TMEM132A in mice causes prevalent folate-resistant spina bifida and limb malformations. The extensive work presented here builds on this groupÂ's previous findings that TMEM132A is required for Wnt ligand secretion through its interactions with WLS. I have a small number of major concerns which I hope the authors can address easily.

Comments for the author

1) "here we show that the spina bifida phenotype appears to be unrelated to Wnt signaling defect…" It is not clear to me how the authors have excluded relevance of their previous findings that TMEM132A regulates Wnt ligand secretion.
There are many known interactions between Wnt and integrin signalling, including in the migration of other cell types. Additionally, Wnt5a/Wnt11 double knockout embryos show ectopic accumulation of T-expressing cells under the posterior neuropore (Andre et al Development 2015). Is it not likely that what the authors describe here is a down-stream consequence of their previous findings?
2) The authors state that TMEM132A-knockdown cells "did not have lamellipodia but instead had significantly thinner protrusions". Quantification and higher resolution images of this phenotype would be useful. Surface ectoderm cells are known to produce equivalent protrusions which are required for posterior neuropore closure. Is TMEM132A expressed in the surface ectoderm (the LacZ images suggest it is expressed by some SE cells) and if so, does its loss diminish their protrusions?
3) The methods describe the wound assays being performed on "glass-bottom dish (cell culture treated or Collagen1 coated)". Please clarify in the figure legends which experiments were on surfaces with and without Coll1. If migration of the epithelial cells analysed was diminished by TMEM132A KD on tissue culture coating without collagen, this would suggest this protein impacts migration more broadly not specifically through collagen-binding integrins. Please consider rephrasing the statement that "TMEM132A regulates Collagen binding integrins" given the demonstration that integrin B1 is markedly down-regulated. Figure 4C shows it is also involved in laminin and fibronectin binding. Being a regulator of cell adhesion to multiple substrates would make TMEM132A even more exciting.
4) Are the authors able to confirm reduction in the expression of integrin pathway components, or changes in ECM molecules, in vivo?
5) In Figure 2B panel 4', the neuroepithelium appears markedly abnormal (long everted) whereas the underlying tailbud structures seem largely normal relative to the WT section above it. Can the authors explain how this could arise in relation to their conclusion that mesoderm abnormalities cause the neuroepithelial phenotype?

Minor comments:
6) The authors comment that KO embryos appeared morphologically normal at equivalent somite stages. One may expect that if T132A diminishes PSM migration its KO may impact somite formation. Did they observe any defects of somitogenesis, or perhaps smaller somites?
7) "the length of the posterior neuropore (PNP) is significantly increased" – this is clear from the images provided but if the authors have quantified PNP length it would be useful to include this data, particularly given some KOs do not develop spina bifida.
8) "knockdown of TMEM132A caused the cells to move randomly with erratic trajectories": This data (and supplementary videos) does not appear to have been included in the manuscript. While not critical, it would be worth providing if available.

9) Supplementary figures showing quantification and replicability of key western blotting results would be very helpful.
10) "The neural tube forms the central nervous system from brain to spinal cord" – the authors probably mean to write "comprised of the brain and spinal cord".
11) Providing more detail on the limb malformations observed would be useful for groups in that field. When the limb phenotype was unilateral, was one side more commonly affected?
12) Can the authors comment on their use of epithelial cell lines to model mesodermal migration?
13) The brightfield images provided suggest the T132A-KD cells are rounder which is a known consequence of diminished cell adhesion. Quantifying this could further corroborate their implication of integrin components.
14) Another model which causes a neuroepithelial eversion phenotype similar to what the authors describe here is retinoic acid excess (e.g. Seegmiller et al Teratology 1991, Figure 4E).

Reviewer 2

Advance summary and potential significance to field

In this manuscript Li et coll. examines the function of TMEM132A, a gene coding for a transmembrane protein that is expressed during development. Analyzing the phenotype TMEM132A null mutant mice embryo they show that this protein is involved in neural tube closure and mesoderm tissue morphology. In relation to the phenotype they describe, they found, using cell culture experiments, that TMEM132A downregulation diminishes both cell migration and expression levels of integrins that bind to collagen.
This manuscript is interesting; it shows the implication of a gene of mostly unknown function in very essential developmental processes such as mesoderm morphogenesis and neural tube closing. The use of mouse genetics and subsequent phenotype analysis is convincing. The molecular mechanism putatively underlying the phenotype is also informative. However, I am concerned that some critical interpretations made by the authors are not backed up by the data at this point

Comments for the author

Major comments:
- The author's claim (title, abstract, text…) that the neural tube closure phenotype is due to a deficient mesodermal migration. They have strong evidence suggesting defects in mesoderm migration in the mutant but I do not see any demonstration of causality between mesodermal migration and neural tube closure.
TMEM132A is also expressed in the neural tube, why can't it have a function on the closure of this tissue independently of its action in the mesoderm?
- Along the same lines the gene is expressed in lateral mesoderm as well as paraxial mesoderm, does part of the mesodermal phenotype (Fig2B 2'-4') can be attributed to a function of this gene in lateral mesoderm tissue?
- Author's conclusion on a defect on persistent directional migration is also questionable. First, because it is assessed in cell lines that can be very different from the embryonic mutant cells. Second, because there is no quantification on directionality or persistence (and I could not access the movies). The aspect of the cells in the siT132A treated condition seems very different (smaller, more round, and more contrasted) compared to siCtrl conditions. Could the effect they see be due to an effect on cell shape, cell proliferation, or cell survival?
Does paraxial mesoderm morphogenesis actually depend on persistent directional migration in the mouse embryo at these stages/ A/P level? There is some evidence in the literature (see comment below) suggesting that it depends as well on non-directional migration…
Minor comments:
- I don't understand this sentence: "It is possible that the abnormal mesoderm mass in the center forces the NE to bend in the reverse direction and/or the mesoderm is not present laterally to help to elevate the neural folds. It is less likely that the everted NE "pulls" and arrests the mesoderm migration since there are not tight cellular connections between the NE and mesoderm."
How it is possible that the mesoderm forces the neural tube even if there is no connection between them?

- A large part of our knowledge about tissue morphogenesis in the posterior part of vertebrate embryos is coming from works done using bird or fish embryos as model systems; I understand that these mechanisms might differ in mammals but I do think that at least some of these papers should be introduced, discussed, and cited in this manuscript.

Reviewer 3

Advance summary and potential significance to field

The authors Li and Niswander describe within their manuscript a KO phenotype in TMEM132A mice. They show presence of neural tube defects in KO mice and a mis-localization of paraxial mesoderm. Following up on this observation, the authors investigate the role of TMEM132A in cellular migration using scratch assays and show a reduction in cologne specific focal adhesion pathway components in cells with reduced TMEM132A in vitro. Overall, this manuscript begins to tease out the pathways that have been mis-regulated with loss of TMEM132A, however the results are preliminary relying heavily on larger scale ISH staining or histology. Before publication the manuscript will require additional quantification and description of mutants as well as further investigation of the proposed pathways responsible for the migration defect in TMEM132A low cells (i.e. rescue experiments).

Comments for the author

MAJOR POINTS:
Generally, quantification needed for many figures for example. Authors claim the length of PNP "significantly increased" with no quantification or knowledge of number of embryos examined.
The claims made in the section "TMEM132A is expressed during neural tube closure and required for lateral migration of caudal mesodermal cells" Are reliant on histology images, which while very instructive on morphology do not well characterize the fates of cells. Thus, it is difficult with the paper worded as such to make the claim of location of different tissue types, without parallel marker staining or dissections and subsequent PCR to confirm that the abnormal structures are indeed mis-localized tissue types and not a mass of undifferentiated cells.
Please quantify migration trajectories (Fig 3) to show claimed change in directional motility.
Additionally, the TMEM132A siRNA treated cells in fig 3C look like they are dying. Is there a higher death rate with loss of TMEM132A that would contribute to the apparent lack of mirgration in the scratch test?
To show that the lack of directional migration in TMEM132A mutants or in cells that have lost TMEM132A, it would be beneficial to do a rescue experiment where components of the collagen specific focal adhesion pathway are overexpressed. Would this rescue the scratch assay phenotype?

MINOR POINTS
The ISH examining the presence of NMPs never looks at overlap of the two markers Sox2 and T, but rather at their individual overarching patterns. While this does indicate that both Sox2 and T expression occurs similarly, it does not directly address the presence or absence or change in pattern of NMPs. Co-staining for the markers would better prove this point along with quantified similarities/differences between the +/+ and -/- embryos.
The Aldh1a2 mRNA expression in mutants in the presented figure S2D seems to have lost the region of Aldh1a2 expression in the caudal epiblast compared to the wildtype embryos. Please mention this in the text.

In Figure 4, the protein level quantification is needed underneath all examined proteins not just the significant ones as the blots are at times difficult to interpret by eye.

First revision

Author response to reviewers' comments

Response to the Reviewers

Reviewer 1 Advance Summary and Potential Significance to Field:
This manuscript by Li and Niswander documents exciting and novel findings relating to the role of TMEM132A in closure of the neural tube and development of mesodermal structures. They demonstrate that global deletion of TMEM132A in mice causes prevalent folate-resistant spina bifida and limb malformations. The extensive work presented here builds on this group's previous findings that TMEM132A is required for Wnt ligand secretion through its interactions with WLS. I have a small number of major concerns which I hope the authors can address easily.

Response: We very much appreciate the positive assessments from the reviewer and his/her insightful suggestions for the improvement of our manuscript. We have seriously considered all the comments and revised the manuscript accordingly. We submit a marked-up version of the revised manuscript with the changes highlighted in yellow. A point-by-point response to the reviewer is listed below.

Reviewer 1 Comments for the Author:
1) "here we show that the spina bifida phenotype appears to be unrelated to Wnt signaling defect…" It is not clear to me how the authors have excluded relevance of their previous findings that TMEM132A regulates Wnt ligand secretion. There are many known interactions between Wnt and integrin signaling, including in the migration of other cell types. Additionally, Wnt5a/Wnt11 double knockout embryos show ectopic accumulation of T-expressing cells under the posterior neuropore (Andre et al Development 2015). Is it not likely that what the authors describe here is a down-stream consequence of their previous findings?

Response: We are thankful to the reviewer for pointing out our inappropriate wording. As a result of TMEM132A loss, we found no significant change in expression by visualizing whole-mount E9.5 mouse embryos by ISH with riboprobes targeting neuromesodermal progenitor (NMP) markers Sox2 and T, neural lineage markers Sox1 and Sox3, and mesodermal lineage makers Tbx6 and Msgn1 (Fig. S2B & C). This suggests that NMP differentiation was not disrupted in mutant embryos. Given Wnt signaling plays indispensable role in NPM self-renewal as well as subsequent lineage choice, our interpretation was that the phenotype is unrelated to Wnt signaling. However, as the reviewer points out, Wnt signaling also functions in many other ways including cell migration. It may be possible that the mesodermal migration defect is due in part to disrupted Wnt signaling. What our work does demonstrate is that integrin protein levels and integrin pathway are downregulated by TMEM132A loss, which to our knowledge is independent of Wnt signaling. Nonetheless, it is possible that the overall phenotype is due to combined effects of TMEM132A loss that disrupt several aspects of neural tube closure, leading to spina bifida in mutant embryos. We have carefully re-worded the related sentence in the manuscript.

2) The authors state that TMEM132A-knockdown cells "did not have lamellipodia but instead had significantly thinner protrusions". Quantification and higher resolution images of this phenotype would be useful. Surface ectoderm cells are known to produce equivalent protrusions which are required for posterior neuropore closure. Is TMEM132A expressed in the surface ectoderm (the LacZ images suggest it is expressed by some SE cells) and if so, does its loss diminish their protrusions?

Response: Thanks for the reviewer`s suggestion. New Fig. 3F provides quantification to support our finding that lamellipodia formation is significantly affected by TMEM132A loss in our *in vitro* scratch wound healing model. Due to technical limitation during wide field live imaging, we did not acquire images with higher magnification. It is true that cell protrusions produced by surface ectoderm (SE) cells is critical for neural tube closure zippering. There is some expression of TMEM132A in SE. However, based on multiple observations, the neural folds can still close in some regions despite grossly abnormal mesoderm mass and convex splayed neural tissue (e.g. Fig. 2D). This suggests that SE zippering is not the predominant effect and instead we suggest that the mesodermal migration defect is the predominant phenotype.

3) The methods describe the wound assays being performed on “glass-bottom dish (cell culture treated or Collagen1 coated)”. Please clarify in the figure legends which experiments were on surfaces with and without Coll1. If migration of the epithelial cells analysed was diminished by TMEM132A KD on tissue culture coating without collagen, this would suggest this protein impacts migration more broadly, not specifically through collagen-binding integrins. Please consider rephrasing the statement that “TMEM132A regulates Collagen binding integrins” given the demonstration that integrin B1 is markedly down-regulated. Figure 4C shows it is also involved in laminin and fibronectin binding. Being a regulator of cell adhesion to multiple substrates would make TMEM132A even more exciting.

Response: We tried both with or without Collagen 1 coated glass-bottom dish (which is tissue culture treated already) for wound healing assay in HEK 293 cells and HeLa cells, and we observed the same trend regardless of the coating. As such we ultimately applied Collagen 1 coating only for HEK 293 cells to ensure the firm attachment of cells. We have modified the description in the Method section. With the above phenomena observed, we agree with the reviewer that TMEM132A functions more broadly than only through collagen-binding integrin heterodimers. We appreciate the reviewer`s advice and have broadened the related statement in revised manuscript.

4) Are the authors able to confirm reduction in the expression of integrin pathway components, or changes in ECM molecules, in vivo?

Response: We tried Western blot for integrins using protein extracted from embryos but we observed huge variation even between wild type samples over the caudal neuropore region. Perhaps there is a temporal or spatial restriction, which deserves further investigation in our future plan.

5) In Figure 2B panel 4’, the neuroepithelium appears markedly abnormal (long, everted) whereas the underlying tailbud structures seem largely normal relative to the WT section above it. Can the authors explain how this could arise in relation to their conclusion that mesoderm abnormalities cause the neuroepithelial phenotype?

Response: The data are now shown in Fig. 2C, as quantification of relative PNP length is now provided in Fig. 2B. Because of the more extreme curvature of the caudal region (Fig. 2C whole embryo pictures), it is difficult to obtain comparable sections to wildtype. Thus, we believe the apparent elongation of neuroepithelium (NE) in the mutant tail bud is mainly due to the angle of sectioning (as also seen in panel 3’). Despite the everted MHP bending, other aspects such as dorsal-ventral patterning appear normal in the NE. Based on our current evidence, as well as recently published data on modeling of mesoderm expansion (see response to Reviewer 2, point #1), we believe that the abnormal paraxial mesoderm mass in the midline forces the NE to bend in the reverse direction and/or the mesoderm is not present laterally to help elevate the neural folds.

Minor comments:
6) The authors comment that KO embryos appeared morphologically normal at equivalent somite stages. One may expect that if T132A diminishes PSM migration its KO may impact somite formation. Did they observe any defects of somitogenesis, or perhaps smaller somites?

Response: We did not observe a defect in terms of somite patterning based on somite number or position/size as revealed by staining with the caudal somitic compartment marker *Uncx4.1* and by somatic marker *Meox1* (Fig. 2E). These markers are expressed, although the mesoderm is abnormally localized under the notochord/NE.

7) “the length of the posterior neuropore (PNP) is significantly increased” – this is clear from the images provided but if the authors have quantified PNP length it would be useful to include this data, particularly given some KOs do not develop spina bifida.

Response: Thanks for the reviewer`s suggestion. We now include quantification of PNP length in new Fig. 2B, which shows significant PNP elongation in *Tmem132a* mutants at E9.5. Since the cases of mutants without spina bifida is rare, we did not count those embryos in this dataset.

8) “knockdown of TMEM132A caused the cells to move randomly with erratic trajectories”: This data (and supplementary videos) does not appear to have been included in the manuscript. While not critical, it would be worth providing if available.

Response: We agree with the reviewer and have now added more detailed analysis of the wound healing assay in new Fig. 3 D & E to better support our conclusions.

9) Supplementary figures showing quantification and replicability of key western blotting results would be very helpful.

Response: Thanks for the reviewer`s suggestion. We have added the quantification for the key western blotting result of ITGB1 in new Fig. 4B.

10) “The neural tube forms the central nervous system from brain to spinal cord” – the authors probably mean to write “comprised of the brain and spinal cord”.

Response: We appreciate the reviewer`s advice and have corrected the wording.

11) Providing more detail on the limb malformations observed would be useful for groups in that field. When the limb phenotype was unilateral, was one side more commonly affected?

Response: We did not notice an obvious preference of left or right side when the limb phenotype was unilateral. The lack of digits in either fore or hind-limb or both was very common. We only observed the total loss of hindlimb(s) but not the forelimb.

12) Can the authors comment on their use of epithelial cell lines to model mesodermal migration?

Response: In wound healing assays, epithelial cells are often used. The methods are well established, the cells are easily handled, the morphology of epithelial cells are clear, and the results are consistent. We did try to use wildtype and TMEM132a mutant mouse embryo fibroblasts (MEFs) but the data were not reproducible enough and the morphology too irregular in time-lapse imaging, thus making the subsequent analysis extremely difficult. That was why we chose HEK 293 cells and HeLa cells to study the migration defect upon TMEM132a loss.

13) The brightfield images provided suggest the T132A-KD cells are rounder, which is a known consequence of diminished cell adhesion. Quantifying this could further corroborate their implication of integrin components.

Response: We appreciate the reviewer`s suggestion. We have added new analysis of wound healing assay to strengthen our findings. The reviewer raises an interesting point but we feel this is beyond the scope of the current project.

14) Another model which causes a neuroepithelial eversion phenotype similar to what the authors describe here is retinoic acid excess (e.g. See gmiller et al Teratology 1991, Figure 4E).

Response: Based on the brief citation mentioned by the reviewer, we could not identify this paper. However, we believe the reviewer was referring to Griffith CM, Wiley MJ. Effects of retinoic acid on chick tail bud development. Teratology. 1991 Mar;43(3):217-24. doi: 10.1002/tera.1420430305. PMID: 2014484. After careful reading of this paper we feel the histology in Fig 4 and the authors description of the results do not appear similar to the *Tmem132a* KO mouse phenotype. Moreover, in our current work we did not observe a significant change in RA signaling (Fig. S2D). This suggests that TMEM132A functions in a different way than RA signaling.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this manuscript Li et al. examines the function of TMEM132A, a gene coding for a transmembrane protein that is expressed during development. Analyzing the phenotype TMEM132A null mutant mice embryo they show that this protein is involved in neural tube closure and mesoderm tissue morphology. In relation to the phenotype they describe, they found, using

cell culture experiments, that TMEM132A downregulation diminishes both cell migration and expression levels of integrins that bind to collagen.
This manuscript is interesting; it shows the implication of a gene of mostly unknown function in very essential developmental processes such as mesoderm morphogenesis and neural tube closing. The use of mouse genetics and subsequent phenotype analysis is convincing. The molecular mechanism putatively underlying the phenotype is also informative. However, I am concerned that some critical interpretations made by the authors are not backed up by the data at this point.

Response: We are thankful for the reviewer's careful assessment of our manuscript, which has helped to improve of manuscript. We have seriously discussed all the comments and revised the manuscript accordingly. The revised manuscript with the changes are marked. Point-by-point responses to the reviewer are listed below.

Reviewer 2 Comments for the Author:
Major comments:
-The author's claim (title, abstract, text…) that the neural tube closure phenotype is due to a deficient mesodermal migration. They have strong evidence suggesting defects in mesoderm migration in the mutant but I do not see any demonstration of causality between mesodermal migration and neural tube closure. TMEM132A is also expressed in the neural tube, why can't it have a function on the closure of this tissue independently of its action in the mesoderm?

Response: Thanks for the reviewer`s assessment. It is true that TMEM132A is expressed in the neuroepithelium (NE) and could function in this tissue. Our data ruled out several possible NE defects, for example we saw no defect in dorsal-ventral patterning (Fig. S2). We now include information from a paper published after our submission that used mathematical modeling and quantitative image analysis to support the idea that extrinsic forces due to mesoderm expansion laterally is important for caudal neural tube closure (Fig. 4, Veerle de Goederen, *et al*. *PNAS*, 2022, doi:10.1073/pnas.2117075119). Overall, their conclusion is that curvature of the neural plate at the midline and borders is not sufficient for neural tube closure but instead that extrinsic forces due to paraxial mesoderm expansion (grossly abnormal in *Tmem132a* mutants), notochord adhesion to the neural plate (not disrupted in *Tmem132a* mutants) and NNE width expansion (not determined in *Tmem132a* mutants) are sufficient for MHP formation and NT closure. Furthermore, their modeling in Fig. 5E shows that low mesoderm expansion results in exaggerated dorsolateral hinge points (DLHP), and their modeling is strikingly similar to what we observe in *Tmem132a* mutants (see below which we now include as Supplemental Figure S2F)

Note: Figure provided for reviewer has been removed. It showed part of Figure 2 from de Goederen et al. (2022) Hinge point emergence in mammalian spinal neurulation. Proc Natl Acad Sci U S A, 2022. 119(20): p. e2117075119 (doi: https://doi.org/10.1073/pnas.2117075119)

Thus, although we cannot formally exclude a role for TMEM132A in the NE or the lateral mesoderm (next point raised by reviewer 2), our data show a predominant defect in mesoderm expansion due to misplacement of the paraxial mesoderm at the midline. This, in combination with the recent modeling publication, draws a more causal connection between mesoderm migration and neural tube closure.

-Along the same lines the gene is expressed in lateral mesoderm as well as paraxial mesoderm, does part of the mesodermal phenotype (Fig2B 2'-4') can be attributed to a function of this gene in lateral mesoderm tissue?

Response: We cannot rule out a role for TMEM132A in the lateral mesoderm. However, as pointed out above, the modeling studies indicate that paraxial mesoderm expansion is critical for NT closure and it is the paraxial mesoderm (based on molecular markers like *Meox1*) that is grossly misplaced in *Tmem132a* mutants.

-Author's conclusion on a defect on persistent directional migration is also questionable. First, because it is assessed in cell lines that can be very different from the embryonic mutant cells. Second, because there is no quantification on directionality or persistence (and I could not access the movies). The aspect of the cells in the siT132A treated condition seems very different (smaller,

more round, and more contrasted) compared to siCtrl conditions. Could the effect they see be due to an effect on cell shape, cell proliferation, or cell survival? Does paraxial mesoderm morphogenesis actually depend on persistent directional migration in the mouse embryo at these stages/ A/P level? There is some evidence in the literature (see comment below) suggesting that it depends as well on non-directional migration…

Response: In wound healing assays, epithelial cells are often used. The methods are well established, the cells are easily handled, the morphology of epithelial cells are clear, and the results are consistent. We did try to use wildtype and *Tmem132a* mutant mouse embryo fibroblasts (MEFs) but the data were not reproducible enough and the morphology too irregular in time-lapse imaging, thus making the subsequent analysis extremely difficult. That was why we chose HEK 293 cells and HeLa cells to study the migration defect upon TMEM132a loss.
We have re-uploaded the supplementary movies. We now include new quantitative analysis of the wound healing movies in revised Fig.3 D & E to further support our conclusion. We also noticed that TMEM132A loss changes the cell shape, and we found that the formation of lamellipodium is severely affected (revised Fig. 3F), which is crucial for cell attachment and spreading as well as movement.
As for the cell proliferation and survival, we did not notice an obvious change due to TMEM132A knock- down. We plated the same number of cells in the insert well in our wound healing assay, and the cells reached equal confluency the next day when the insert was removed. At the final time point of the wound healing assay, there was still not a significant difference in cell number between groups.
As for the paraxial mesoderm morphogenesis, our evidence demonstrates that lateral migration of paraxial mesoderm is affected by TMEM132A loss. Our current work does not answer whether the anterior migration of paraxial mesoderm is affected or not. However, based on our morphological data, the normal A-P length based on somite number, and continued elongation of the tailbud caudal to the onset of the spina bifida, suggests that the anterior migration is less influenced by TMEM132A loss. We agree with the reviewer that our wording of persistent directional migration relative to *in vivo* paraxial mesoderm migration may be too specific and hence we have simplified the wording to just indicate the lateral migration of the paraxial mesoderm.

Minor comments:
-I don't understand this sentence: "It is possible that the abnormal mesoderm mass in the center forces the NE to bend in the reverse direction and/or the mesoderm is not present laterally to help to elevate the neural folds. It is less likely that the everted NE "pulls" and arrests the mesoderm migration since there are not tight cellular connections between the NE and mesoderm." How it is possible that the mesoderm forces the neural tube even if there is no connection between them?

Response: We agree that this second sentence is confusing. Moreover, the Veerle de Goederen et al. 2022 paper further supports the first sentence. Therefore we have deleted the sentence "It is less likely that the everted NE "pulls" and arrests the mesoderm migration since there are not tight cellular connections between the NE and mesoderm."

-A large part of our knowledge about tissue morphogenesis in the posterior part of vertebrate embryos is coming from works done using bird or fish embryos as model systems; I understand that these mechanisms might differ in mammals but I do think that at least some of these papers should be introduced, discussed, and cited in this manuscript.

Response: We appreciate this comment. The new paper by de Goederen is a nice compilation of histological data of the mouse embryo at relevant positions and time points and modeling that we feel this is the most appropriate manuscript to cite in our revised manuscript.

Reviewer 3 Advance Summary and Potential Significance to Field:
The authors Li and Niswander describe within their manuscript a KO phenotype in TMEM132A mice. They show presence of neural tube defects in KO mice and a mis-localization of paraxial mesoderm. Following up on this observation, the authors investigate the role of TMEM132A in cellular migration using scratch assays and show a reduction in cologne specific focal adhesion pathway components in cells with reduced TMEM132A in vitro. Overall, this manuscript begins to tease out the pathways that have been mis-regulated with loss of TMEM132A, however the results

are preliminary relying heavily on larger scale ISH staining or histology. Before publication the manuscript will require additional quantification and description of mutants as well as further investigation of the proposed pathways responsible for the migration defect in TMEM132A low cells (i.e. rescue experiments).

Response: Thank you for your comments concerning our manuscript. The comments are all valuable and helpful for revising and improving our paper. We have studied these comments carefully and have included new data and made corrections which we feel address all points and we hope meets with approval. Revised portions are marked in the paper.

Reviewer 3 Comments for the Author:

MAJOR POINTS:
Generally, quantification needed for many figures for example. Authors claim the length of PNP "significantly increased" with no quantification or knowledge of number of embryos examined.

Response: We thank the reviewer for the suggestion. We added new statistical data of the PNP length in revised Fig. 2B. We also added new statistical analysis of wound healing assay to further support our conclusion in new Fig. 3. We also quantified all band intensity for all western blots.

The claims made in the section "TMEM132A is expressed during neural tube closure and required for lateral migration of caudal mesodermal cells" Are reliant on histology images, which while very instructive on morphology do not well characterize the fates of cells. Thus, it is difficult with the paper worded as such to make the claim of location of different tissue types, without parallel marker staining or dissections and subsequent PCR to confirm that the abnormal structures are indeed mis-localized tissue types and not a mass of undifferentiated cells.

Response: We provided evidence that the population of cells that are mislocalized under the mutant neural tube are paraxial mesodermal tissue using *Meox1* as a paraxial mesodermal marker (revised Fig. 2E). We also saw no change in the expression of NMP markers *Sox2* and *T* or mesodermal makers *Tbx6* and *Msgn1* or neural lineage markers *Sox1* and *Sox3* in TMEM132A loss (revised Fig. S2B & C).

Please quantify migration trajectories (Fig 3) to show claimed change in directional motility.
Additionally, the TMEM132A siRNA treated cells in fig 3C look like they are dying. Is there a higher death rate with loss of TMEM132A that would contribute to the apparent lack of migration in the scratch test?

Response: Thanks for the reviewer`s comment. We have not observed a change of cell viability following TMEM132A knock down. We plated the same number of cells in the insert well in our wound healing assay, and the cells reached equal confluency the next day before the insert was removed. At the final time point of wound healing assay, there was no significant difference in cell number between groups. We have added new quantitative analysis for the wound healing assay in revised Fig. 3. We tracked individual cells (all of which were alive throughout the tracking) and now demonstrate the different behavior of tracked cells in the different experimental groups.

To show that the lack of directional migration in TMEM132A mutants or in cells that have lost TMEM132A, it would be beneficial to do a rescue experiment where components of the collagen specific focal adhesion pathway are overexpressed. Would this rescue the scratch assay phenotype?

Response: We appreciate this question, which was also discussed for reviewer 1. For the wound healing assay in HEK 293 cells and HeLa cells we used glass-bottom dishes (which are tissue culture treated already) with or without Collagen 1 coating. We observed the same trend regardless of the coating, suggesting that TMEM132A functions more broadly than only through collagen-binding integrin heterodimers. We have broadened our statement in the manuscript. We did note that Collagen 1 coating caused a quicker narrowing of the wound gap suggesting there was a certain level of rescue effect but this trend was similar between Control and knockdown groups.

MINOR POINTS

The ISH examining the presence of NMPs never looks at overlap of the two markers Sox2 and T, but rather at their individual overarching patterns. While this does indicate that both Sox2 and T expression occurs similarly, it does not directly address the presence or absence or change in pattern of NMPs. Co- staining for the markers would better prove this point along with quantified similarities/differences between the +/+ and -/- embryos.

Response: Due to technical difficulties in double labeling with these antibodies, along with the move of the first author to another state and some difficulties in mouse breeding, we have not been able to accomplish this co-labeling experiment. We hope that our previous results and new data will satisfy the reviewer of our conclusion that NMP differentiation is not altered and instead the predominant defect is in the migration of paraxial mesoderm to lie lateral to the neural tube to help to elevate the neural folds.

The Aldh1a2 mRNA expression in mutants in the presented figure S2D seems to have lost the region of Aldh1a2 expression in the caudal epiblast compared to the wildtype embryos. Please mention this in the text.

Response: We appreciate the reviewer`s suggestion. We have added corresponding description in the manuscript.

In Figure 4, the protein level quantification is needed underneath all examined proteins not just the significant ones as the blots are at times difficult to interpret by eye.

Response: Thanks for the reviewer`s advice. We have added quantification for all band intensity in western blot.

Second decision letter

MS ID#: DEVELOP/2021/200442

MS TITLE: TMEM132A ensures mouse caudal neural tube closure through regulating integrin-based mesodermal migration

AUTHORS: Binbin Li, Liza Brusman, Jacob Dahlka, and Lee Niswander

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. As you will see, the referees have some suggestions for improving the clarity of your study. I would particularly draw your attention to the comments of Referee 2. I agree with the referee that the Title and the Abstract should be edited so that a reader is aware that the closure defects may not be solely due to the loss of TMEM132A in the paraxial mesoderm tissue. 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

The authors have provided new data and made changes to the text which largely address my initial comments. Timely publication of a modelling-based paper now referenced by the authors facilitates interpretation of the importance of mesodermal structures in neural tube folding. Additional minor comments are listed below.

Comments for the author

- Apologies for the autocorrect typo in "See gmiller", which should have read Seegmiller as one word (PMID 2014479). This reference remains worth discussing particularly given the authors' data on Aldh1a2.
- Please state the somite ranges used to compare PNP length. This is important given the difference in somite stage between WT and mutants observed. Sub-dividing analyses in somite stages would be more useful. If the mesoderm's role is in bending of the neural plate at the DLHPs, one might expect their mutant to diverge from WT during Mode 2 closure.
- Closer alignment with the recently published manuscript by de Goederen et al, now referenced by the authors, would benefit interpretation of this study as well a helping validate computational findings in the de Goederen paper. Quantifying and correlating DLHP angles with the mesodermal area parameter shown in Supp Figure 2F in WTs and Tmem132A mutants would be useful.
- Supp Fig 2D - the Fgf8 signal is very difficult to see on a black background
- One would have appreciated more statistical comparison of western blot quantifications in addition to that of Itgb1 provide.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, Li et al. examine the function of TMEM132A, a gene coding for a transmembrane protein that is expressed during development. Analyzing the phenotype TMEM132A null mutant mice embryo they show that this protein is involved in neural tube closure and mesoderm tissue morphology. In relation to the phenotype they describe, they found, using cell culture experiments, that TMEM132A downregulation diminishes both cell migration and expression levels of integrins that bind to collagen.

Comments for the author

I want to thank the authors for taking into account some of my remarks and providing answers. I think the manuscript is better, for instance concerning the quantification of the migration essays. I really like this work and I think it has its place in Development, however not in the present form. I have the exact same major concern about the over-interpretation of the data as I had during the first round of revision. The authors did not demonstrate that the neural tube closing defect is solely due to the loss of their gene function in the paraxial mesoderm tissue. If the manuscript is published as such that will be misleading.
By reading the title and the abstract, readers will assume that this is the paper that demonstrates the role of the PSM cell migration on the neural tube closure whereas this causality is actually never demonstrated by the data. The authors do not have a condition in which the mutation is present in the paraxial mesoderm only, and we know that the gene is expressed in the neural tube. The new modeling paper they now cite, (a fascinating paper, by the way) is a modeling paper, not a demonstration. For me to accept this paper, the authors have to tone down their interpretation. At the very least, they should change the title and the summary to something like :

"TMEM132A ensures mouse caudal neural tube closure and regulates integrin-based mesodermal migration"
" This ensures proper lateral migration of mesoderm and suggest a role of this process to elevate the neural folds and to allow successful closure of the caudal neural tube...."

I want to stress that I am fine with the fact that the authors elaborate on the possible major role of PSM cell migration in neural tube closure in the discussion section of the manuscript.

Reviewer 3

Advance summary and potential significance to field

The authors in Li et al. describe a novel method by which the gene TMEM132A regulates neural tube closure through the proposed directional migration of mesodermal cells in the caudal portion of the developing mouse embryo. The phenotype of TMEM132A null mice mutants is sufficiently demonstrated to result in neural tube closure defects. The text clearly provides relevant descriptions of possible mechanisms, focusing on the possibility of migration loss due to a disruption in the collagen specific focal adhesion pathway. In the revisions, the authors have addressed multiple reviewer requests for quantification. In particular, the authors definitively show a change in directional migration of cells that have lost expression of TMEM132A in a wound healing assay. Additionally, text has been added to ensure reader understanding of proposed mechanisms with supporting literature reference. Overall, the resubmitted manuscript demonstrates an advancement in the field of developmental biology by highlighting the role of TMEM132A control on population migration and a subsequent result in altered embryo morphogenesis and the authors put this in context within the field well in the introduction and in the body of the text.

Comments for the author

Minor point:
The conclusions of the paper would benefit a wider audience if a brief conclusion paragraph was added to put the findings in context of the overall field. The authors give explanations throughout the text on migration and proposed mechanisms for neural tube closure, but for a clear summary this would also be beneficial in the last paragraph as it is both interesting and the main point of the paper.

Second revision

Author response to reviewers' comments

Note to all reviewers: To conform to the word limits of a short report, in conjunction with new data and text provided to address the reviewer critiques, we have shortened the text in ways that have not changed the content. Only substantial new wording to address the reviewers comments have been highlighted in the revised manuscript.

Reviewer 1 Advance Summary and Potential Significance to Field:
The authors have provided new data and made changes to the text which largely address my initial comments. Timely publication of a modelling-based paper now referenced by the authors facilitates interpretation of the importance of mesodermal structures in neural tube folding. Additional minor comments are listed below.

Reviewer 1 Comments for the Author:

- Apologies for the autocorrect typo in "See gmiller", which should have read Seegmiller as one word (PMID 2014479). This reference remains worth discussing, particularly given the authors' data on Aldh1a2.
Response: We thank the reviewer for providing this reference. We have cited it and discussed it in the revised manuscript.

- Please state the somite ranges used to compare PNP length. This is important given the difference in somite stage between WT and mutants observed. Sub-dividing analyses in somite stages would be more useful. If the mesoderm's role is in bending of the neural plate at the DLHPs,

one might expect their mutant to diverge from WT during Mode 2 closure.
Response: As stated before, we always compared somite number matched WT and mutant embryos (less than 2 somite difference). For comparing PNP length in Fig. 2B, we have added the somite range of 22-24 to the figure ligand. We focused our attention and embryo dissections at E9.5 (mode 2 closure) when indeed the mutant begins to diverge from WT.

- Closer alignment with the recently published manuscript by de Goederen et al, now referenced by the authors, would benefit interpretation of this study as well a helping validate computational findings in the de Goederen paper. Quantifying and correlating DLHP angles with the mesodermal area parameter shown in Supp Figure 2F in WTs and Tmem132A mutants would be useful.
Response: We appreciate the reviewer`s suggestion. We calculated the mesoderm area, DLHP curvature and MHP curvature, and the new data is included in revised Fig. 2F. These parameters are all significantly different in the mutant versus wildtype and our data provide in vivo support for the computational data by de Goederen.

- Supp Fig 2D - the Fgf8 signal is very difficult to see on a black background
Response: We digitally changed the background and adjusted the contrast to make the signal more obvious, without changing the signal pattern.

- One would have appreciated more statistical comparison of western blot quantifications in addition to that of Itgb1 provide.
Response: Thanks for the reviewer`s suggestion. We now also added quantifications for ITGA1 and ITGA2 to Fig. 4B.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this manuscript, Li et al. examine the function of TMEM132A, a gene coding for a transmembrane protein that is expressed during development. Analyzing the phenotype TMEM132A null mutant mice embryo they show that this protein is involved in neural tube closure and mesoderm tissue morphology. In relation to the phenotype they describe, they found, using cell culture experiments, that TMEM132A downregulation diminishes both cell migration and expression levels of integrins that bind to collagen.

Reviewer 2 Comments for the Author:
I want to thank the authors for taking into account some of my remarks and providing answers. I think the manuscript is better, for instance concerning the quantification of the migration essays. I really like this work and I think it has its place in Development, however not in the present form. I have the exact same major concern about the over- interpretation of the data as I had during the first round of revision. The authors did not demonstrate that the neural tube closing defect is solely due to the loss of their gene function in the paraxial mesoderm tissue. If the manuscript is published as such that will be misleading. By reading the title and the abstract, readers will assume that this is the paper that demonstrates the role of the PSM cell migration on the neural tube closure whereas this causality is actually never demonstrated by the data. The authors do not have a condition in which the mutation is present in the paraxial mesoderm only, and we know that the gene is expressed in the neural tube. The new modeling paper they now cite, (a fascinating paper, by the way) is a modeling paper, not a demonstration. For me to accept this paper, the authors have to tone down their interpretation. At the very least, they should change the title and the summary to something like: "TMEM132A ensures mouse caudal neural tube closure and regulates integrin-based mesodermal migration". "This ensures proper lateral migration of mesoderm and suggest a role of this process to elevate the neural folds and to allow successful closure of the caudal neural tube "
I want to stress that I am fine with the fact that the authors elaborate on the possible major role of PSM cell migration in neural tube closure in the discussion section of the manuscript.
Response: We appreciate the reviewer`s comment and suggestions. We agree and have changed the title, the abstract, and added a sentence to the final summary indicating the need for a tissue-specific KO to confirm a specific role for *Tmem132a* in the paraxial mesoderm.

Reviewer 3 Advance Summary and Potential Significance to Field:
The authors in Li et al. describe a novel method by which the gene TMEM132A regulates neural tube closure through the proposed directional migration of mesodermal cells in the caudal portion of the developing mouse embryo. The phenotype of TMEM132A null mice mutants is sufficiently demonstrated to result in neural tube closure defects. The text clearly provides relevant descriptions of possible mechanisms, focusing on the possibility of migration loss due to a disruption in the collagen specific focal adhesion pathway. In the revisions, the authors have addressed multiple reviewer requests for quantification. In particular, the authors definitively show a change in directional migration of cells that have lost expression of TMEM132A in a wound healing assay. Additionally, text has been added to ensure reader understanding of proposed mechanisms with supporting literature reference. Overall, the resubmitted manuscript demonstrates an advancement in the field of developmental biology by highlighting the role of TMEM132A control on population migration and a subsequent result in altered embryo morphogenesis and the authors put this in context within the field well in the introduction and in the body of the text.

Reviewer 3 Comments for the Author:
Minor point:
The conclusions of the paper would benefit a wider audience if a brief conclusion paragraph was added to put the findings in context of the overall field. The authors give explanations throughout the text on migration and proposed mechanisms for neural tube closure, but for a clear summary this would also be beneficial in the last paragraph as it is both interesting and the main point of the paper.
Response: Thanks for the reviewer`s suggestion. We have added more details to the last paragraph to make the summary clearer.

Third decision letter

MS ID#: DEVELOP/2021/200442

MS TITLE: TMEM132A ensures mouse caudal neural tube closure and regulates integrin-based mesodermal migration

AUTHORS: Binbin Li, Liza Brusman, Jacob Dahlka, and Lee Niswander
ARTICLE TYPE: Research Report

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