Neural tube closure requires the endocytic receptor Lrp2 and its functional interaction with intracellular scaffolds

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MS TITLE: Neural tube closure requires the endocytic receptor Lrp2 and its functional interaction with intracellular scaffolds

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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. Both Referees 2 and 3 raise questions about the importance of convergent extension to explain the phenotype and the mechanism linking Lrp2 to PCP. The referees would like to see stronger evidence of an interaction of Lrp2 with Shroom3 and Gipc1 and further evidence of a CE defect. Addressing these questions will likely require additional experiments and analysis.If you are able to revise the manuscript along the lines suggested, 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 currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost

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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

Summary: The authors have identified that LRP2 is essential for neural tube closure in Xenopus and mice.
They also show that known intracellular adaptor proteins for LRP2, NHERF1 and GIPC1, act as scaffolds for the LRP2 receptor to mediate endocytosis of LRP2 and cell membrane, in conjunction with Shroom3. This reduction of membrane via LRP2/Shroom3/Gipc1 enables apical constriction of the neural epithelium for cranial NT closure. The results add a new level of understanding of the importance of apical constriction and new mechanisms by which this is achieved during embryonic neurulation.

Comments for the author

Major concerns:
-In the Intro, it is described that humans with LRP2 mutations resemble mouse Lrp2 mutants and that is why the mouse is a good model to study this. However, in the last paragraph you describe using Xenopus as well but with no reason as to why. Then to start the results you address “the feasibility of using Xenopus to study the etiology of LRP2-related NTDs”. Clearer logic in the intro as to why you chose both mice and Xenopus would be helpful.

-It is confusing in Figure 2 I, the authors show the green as “injected” but then in Figure 2 J,K,L,M, the red dotted line represents the injected side, and the green is extent of neural fold on uninjected side. It would be clearer if green meant injected for both the experimental design schematic as well as the dotted line schematic. Then make the uninjected a different color.

-Supplemental figure 3 should be included with the main figures of the manuscript. It describes the cellular phenotype and is used to explain how Lrp deficiency results in aberrant AC, so it is important to include as a regular figure, not supplemental data.

-The schematic at the end is very well done and informative. It would be nice to expand this figure to include a cartoon of how the membrane is being endocytosed in a systematic way to reduce the apical membrane surface area over time. This will help the reader visualize the mechanism.

Minor concerns:
-In line 36, delete “Recent studies have revealed that”. It is unnecessary.

-I prefer to not use the language “higher vertebrate” because it is unclear what you really mean and abides by an anthropocentric view of biology. It is better to just say “vertebrates”.

-Delete adjectives that do not add meaning to the statement. For example, instead of saying “a wide range”, just say “a range”. Adding the word “wide” does not improve the quality of the sentence or more accurately describe your thought. Another example “a key gene”, just say “a gene”. Another example; delete
“interestingly” and “conspicuously” when describing data. Delete “severe” in line 158. Line 227,302 delete
“strongly.

-Lines 83, 132-133- What do you mean by “either severely dilated (65%, 22 / 133 34; Fig. S2B) or open neural tubes”. Please describe what a “dilated” neural tube means.

-Line 134-136: The authors write “At E18.5, when a proper skin covered skull had formed in WT embryos (Fig. S2E), we observed exencephaly and anencephaly in Lrp2 null mutants (Fig. S2F), presumably a consequence of failed neural tube closure.” They should not say both exencephaly and anencephaly, at this stage it is more correct to say anencephaly. And it seems logical to

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“presume” anencephaly as a consequence of failed neural tube closure unless the incidence is higher than the 35% NTD seen earlier. Do the authors think that the 65% “dilated” neural tube ultimately “ruptures” exposing the neural tissue?

-Line 254- is “caudalward” a word?

-Line 310: since you technically cannot “prove” anything, chose a different word than “proving”, such as “indicated that”.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Kowalczyk et al. describes the function of Lrp2 in regulation of apical constriction and planar cell polarity (PCP) protein Vangl2 during neural tube closure in mouse and Xenopus. The work is initiated based on clinical association of LRP2 mutations with neural tube closure defects (NTDs) in human patients. The authors aim to use frog and mouse vertebrate models to dissect molecular mechanisms responsible for the function of Lrp2 in NTDs. The advances made by the study include: 1) Lrp2 is required for neural tube closure (NTC) in both models; 2) Lrp2 controls apical constriction of neuroepithelial cells by modulating apical membrane remodeling; 3) Lrp2 regulates Vangl2 localization during neural morphogenesis; and 4) Lrp2 interacts functionally with the intracellular molecules Shroom3 and Gipc1 to affect apical constriction. The authors propose that via interacting with intracellular adaptors and scaffold proteins, Lrp2 can orchestrate apical constriction and planar cell polarity to control neural tube closure.

Comments for the author

The study is generally well controlled with high quality data, and the results provide important insight into the function of Lrp2 in neural morphogenesis.
However, the conclusion about PCP requires additional data and further analysis.

Specific comments:
Fig.1 shows periciliary localization of LRP2, but Fig. 4 shows at least 2 apical clusters of LRP2 per cell. How does the distribution compare? Does loss of Lrp2 affect cilia morphology?

Much of the data on the mechanism of Lrp2 in apical constriction, such as its control of endocytosis in Xenopus, is included in the supplemental figure 3.
As the data support a crucial conclusion in the manuscript, they should be moved into the dataset shown as a real figure.

Does the medial-anterior localization of Lrp2 in Xenopus overlap with that of Vangl2? Does Lrp2 overlap with Vangl2 in the vesicles prior to membrane localization of Vangl2 (proposed in the model)?

Although Vangl2/VANGL2 protein distribution is altered in lrp2/Lrp2 mutants how this affects cell polarity or directional movement is not shown or discussed. Further analysis of the supplemental movies may help to shed light on this issue.

Does Shroom3 bind to Lrp2 or any of the intracellular adaptors analyzed here?
Do Shrrom3 and Lrp2 act in parallel pathways rather than in the same pathway with one regulating actomyosin and the other controlling endocytosis? This should be discussed in more depth.

The functional interaction between Lrp2 and Shroom3/Gipc1 seems to be in the context of apical constriction only. Is there any evidence that Shroom3 and/or Gipc1 also control Vangl2 localization? Since the final model includes Gipc1 bridging Lrp2 and Vangl2, this should be substantiated.

The PBD deletion mutant can affect binding of Lrp2 to other interactors in addition to Gipc1. The results in Fig. 6J-N are not strictly conclusive regarding to Gipc1.

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The temporal succession model implies that cells undergoing apical constriction early on will proceed to undertake CE at a later stage. However more cells seem to undertake CE than those that are apically constricting early on. The model may apply only to a subpopulation of the cells in the neuroepithelium. This should be stated more clearly.

Reviewer 3

Advance summary and potential significance to field

The authors have analysed the function of lrp2 in the neuroepithelium during morphogenesis of the neural tube. This group previously described the presence of forebrain patterning defects and NT closure defects in Lrp2 null mice. Here, they have made use of both this mouse model and Xenopus (using morpholinos and a targeted mutant) to examine the function of Lrp2, in particular in apical constriction of neuroepithelial cells.

The authors describe a series of experiments which implicate Lrp2 in apical constriction and propose a model in which Lrp2 is needed for removal for endocytic removal of apical membrane. It is established that cells need to endocytose their apical membrane to constrict (the appropriate papers in Xenopus are referenced in the discussion and this is also shown in Drosophila) – the current study adds Lrp2 to the components needed for this activity, provides evidence that this is required for neural tube morphogenesis and proposes mechansisms involving interaction with intracellular proteins.

Comments for the author

1. Live imaging of apical constriction (line 202) is shown for a representative cells (3J) but I cannot see the quantification of this ('Quantitative analysis of cell surface size demonstrated size fluctuation over time in control cells that ultimately finalized AC')?

It's also not very clear what is meant but the description that size fluctuations were unaffected by Lrp2 MO - does this mean they constricted but then did not stabilise in a constricted state? This should be clarified.

2. Actin staining is shown to delineate cell shape but the analysis would be enhanced by addition of staining for non-muscle myosin of the types involved in cell-level shape change (eg, phospho myo-II – particularly relevant as a cell polarity defect is also invoked in the Lrp2 MO model) and in cargo transport (myo-V). I note that the model includes myo-6 based on the previous work, but the discussion could include prediction about myo-II levels?

3. The authors describe a caudal NT closure defect in lrp2 MO injected Xenopus (although closure does appear compete in Fig2L,M) and propose that a PCP defect leads to impaired CE movements. This mechanism would be consistent with known cause of NT closure defects in Xenopus and mice. However, there are some issues with ascribing such a mechanism for Lrp2.

First, is there a CE defect? In J and K the neural fold appears further from the midline on the MO injected side, which the authors take to indicate a CE defect – the terminology is important here (convergence could mean movement of the fold towards the midline (eg, line 261) whereas CE refers to a narrowing and lengthening with characteristic cell intercalation). A CE defects is one possible interpretation of the 2J,K phenotype but it is not clear from a static dorsal view whether this could also be due to impaired folding. If live imaging with cell tracing is not possible then a transverse section would help show if the fold is present but laterally displaced.

As well as a wider neural plate, CE defects result in lack of elongation but this is less obvious here (were length measurements made?). Similarly, crispr mediated targeting is describing as causing impaired neural plate narrowing and lengthening (Fig S2L-O).While the neural folds appear further apart in the targeted embryos (I and K) it is less easy to agree that lengthening is impaired – if anything in these images the marked region of neural plate is longer.
Nevertheless, there are indications of possible altered cell planar polarity eg, disruption of the anterior localisation of pigment granules (3B) and possible altered Vangl2 localisation dynamics.

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This may be due to the altered apical surface properties but whether it contributes to NT closure defects is less clear (see comments on CE in Xenopus and mouse).

4. The relationship of CE/PCP to the mouse model: The mouse NT phenotype does not resemble PCP mutants in which CE defects affect the spinal and hindbrain region with the most anterior NT generally spared. The equivalence to caudal neurulation defects in mouse and humans (line 240) to seems over-stated. Spinal NTDs aren't reported by the authors in the mouse model and the cited human study is a linkage study suggestive of a genetic association.

5. Mouse NT phenotype: A proportion of Lrp2-/- embryos are predicted to fail in cranial NT closure in accordance with the previous report (Kur et al 2014). Given the variable NT phenotype (e.g. 12/34 have open cranial NT), how did the authors select embryos for apical constriction analysis at E8.5 (which is before closure defects are apparent)? Does the neuroepithelium of all embryos show the AC defect? If so, how do the authors reconcile the 2 differing phenotypes (eg, Fig. S2B & D). A comment on the potential variability and how this is handled in data analysis should be included.

Additional comments:
The details of the forebrain phenotypes in Lrp2 mutant mice are included in the text (page 5) and supplementary figure but the description would benefit from being more precise. The observations at E8.5 and 9.5 indicate that some embryos will go on to fail in NT closure but the illustration of these subsequent defects are minimal (one fetus at E18.5). Specifically:

- In FigS2 22/34 embryos are described as having a 'dilated dorsal forebrain'. The authors should define how this cut-off was made and preferably present the quantitative measurements (as shown in panel B).
- A section through the regions of wild-type vs dilated vs failed NT closure embryos would help to compare the phenotypes, however, as cranial NTDs have been previously shown in this mutant it is not essential.
- Panel F has a very unusual appearance, differing from the usual appearance of anencephaly or exencephaly – the lesion appears skin covered and there also appears to be facial clefting (a front view would clarify this). Was this typical appearance of fetuses that 'failed NT'?
- Inclusion of images at a stage that is definitely after the stage of closure (even if delayed) eg. 10.5 onwards, but earlier than E18.5 would help clarify the nature of the defect (as would sections).
- Fig1C would benefit from a low magnification panel to orientate the reader on the angle of view.
- The authors previously demonstrated a role for Lrp2 in folate uptake. It would be relevant to speculate whether this function is separate or in some related to the effect they observe on apical constriction.
- Line 238 'In addition to the impairment in AC, we had noticed widening of the caudal NP upon lrp2 LOF in Xenopus (Fig. 2L, M)' – this is not clear as the NT appears closed in these panels and perhaps the authors are referring to 2J,K?
- 4F,G should include some indication of the axial level of the section – cranial neural fold?

First revision

Author response to reviewers' comments

We thank all three reviewers for their insightful comments and suggestions, which we believe have helped to increase our understanding of the morphogenetic events contributing to anterior neural tube closure.

Reviewer 1
Advance Summary and Potential Significance to Field:

Summary: The authors have identified that LRP2 is essential for neural tube closure in Xenopus and mice. They also show that known intracellular adaptor proteins for LRP2, NHERF1 and GIPC1, act as

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scaffolds for the LRP2 receptor to mediate endocytosis of LRP2 and cell membrane, in conjunction with Shroom3. This reduction of membrane via LRP2/Shroom3/Gipc1 enables apical constriction of the neural epithelium for cranial NT closure. The results add a new level of understanding of the importance of apical constriction and new mechanisms by which this is achieved during embryonic neurulation.

Reviewer 1
Comments for the Author:

Major concerns:

1) In the Intro, it is described that humans with LRP2 mutations resemble mouse Lrp2 mutants and that is why the mouse is a good model to study this. However, in the last paragraph you describe using Xenopus as well, but with no reason as to why. Then to start the results you address "the feasibility of using Xenopus to study the etiology of LRP2-related NTDs". Clearer logic in the intro as to why you chose both mice and Xenopus would be helpful.

Arguments why *Xenopus* is an excellent model to use alongside the mouse for this analysis have been added. The respective part of the introduction now reads: "We used the African Clawed Frog *Xenopus laevis* to study morphogenetic events during neurulation, a process which closely resembles that of humans and mice, but can be manipulated and observed in the petri dish *in vivo*."

2) It is confusing in Figure 2 I, the authors show the green as "injected" but then in Figure 2 J,K,L,M, the red dotted line represents the injected side, and the green is extent of neural fold on uninjected side. It would be clearer if green meant injected for both the experimental design schematic as well as the dotted line schematic. Then make the uninjected a different color.

The "color of the lineage tracer" in Fig. 2I has been adjusted so that red now stands for "injection site" as well as for "morphologically affected". As reviewer #3 also had questions about the effect of Lrp2 LOF on posterior neural plate narrowing, three pictures in figure have been removed and replaced by a control and a bilaterally injected embryo plus sections through the closing posterior neural plate (see reviewer #3, comment **3)**).

3) Supplemental figure 3 should be included with the main figures of the manuscript. It describes the cellular phenotype and is used to explain how Lrp deficiency results in aberrant AC, so it is important to include as a regular figure, not supplemental data.

This was a point raised by both reviewer #1 and #2 - as suggested, Fig. S3 was moved to the main figures section and is now Fig. 4.

4) The schematic at the end is very well done and informative. It would be nice to expand this figure to include a cartoon of how the membrane is being endocytosed in a systematic way to reduce the apical membrane surface area over time. This will help the reader visualize the mechanism.

We have extended the hypothetical model figure (now Fig. 8) to include a cartoon of how we envisage a repetitive mechanism of apical constriction and membrane removal during apical constriction.

5) Minor concerns:

In line 36, delete "Recent studies have revealed that". It is unnecessary.

I prefer to not use the language "higher vertebrate" because it is unclear what you really mean and abides by an anthropocentric view of biology. It is better to just say "vertebrates".

Delete adjectives that do not add meaning to the statement. For example, instead of saying "a wide range", just say "a range". Adding the word "wide" does not improve the quality of the sentence or more accurately describe your thought. Another example "a key gene", just say "a gene". Another example; delete "interestingly" and "conspicuously" when describing data. Delete "severe" in line 158. Line 227,302 delete "strongly.

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We agree - the text was edited according to the reviewer's suggestions.

6) Lines 83, 132-133- What do you mean by "either severely dilated (65%, 22 / 133 34; Fig. S2B) or open neural tubes". Please describe what a "dilated" neural tube means.

As suggested by reviewer #3, Fig. S2 has been modified to include further embryonic stages, with which we try to clarify the development of the open vs. "dilated" neural tube phenotypes. Embryos that fall in the "closed and dilated" category have a closed neural tube with a malformed and small, yet inflated-looking forebrain vesicle. As indicated by the red dotted line on the E9.5 whole mount embryo as well as on the newly included sections of an E12.5 embryo (Fig. S2A), this inflation can be traced back to a "dilation" or widening of the neural tube's frontodorsal midline (i.e. the roof plate).
Our results strongly suggest that defective AC of neuroepithelial cells (in the dorsolateral neural folds) impairs neural plate folding and consequently hampers neural fold elevation, apposition and fusion. Depending on the severity of the defect, two phenotypes can arise: 1) **open** neural tube; the neural folds do not get in contact with each other such that neural fold fusion and tube closure fails; 2) **closed, dilated** neural tube; the folds do get in touch and fuse, creating a roof plate, but roof plate morphogenesis is impaired. This could be due to Lrp2's expression in the roof plate and its importance for further roof plate development. This is also reflected by the later appearance of the dorsal brain and skull of such embryos, in which roof plate-derived parts of the brain such as the choroid plexus protrude through the skull's incompletely closed sutures (Fig. S2A, E18.5). We thus think that "dilated" is actually a good term to describe this phenotype.

7) Line 134-136: The authors write "At E18.5, when a proper skin covered skull had formed in WT embryos (Fig. S2E), we observed exencephaly and anencephaly in Lrp2 null mutants (Fig. S2F), presumably a consequence of failed neural tube closure." They should not say both exencephaly and anencephaly, at this stage it is more correct to say anencephaly. And it seems logical to "presume" anencephaly as a consequence of failed neural tube closure unless the incidence is higher than the 35% NTD seen earlier. Do the authors think that the 65% "dilated" neural tube ultimately "ruptures" exposing the neural tissue?

We had indeed assumed that the 35 % of embryos at E9.5 that present with an open neural tube would be the ones that go on to develop exencephaly / anencephaly in later stages. Exencephaly in Lrp2 null mutants is described (Willnow, PNAS 1996; Spoelgen, Development 2005) and the (admittedly quite untypical looking) anencephaly is something that we have seen consistently at E18.5 over the years, with a frequency that was similar or lower than the initial 35 % of open neural tubes.
Reviewer #3 asked us to add embryos of other stages between E9.5 and E18.5 to Fig. S2, which has completed the picture and has enhanced our understanding of the development of NTDs in the mutants. Similar to the ratio at E9.5, 35 % of E10.5 mutants have an open neural tube. However, these embryos are also growth-retarded and, given the observation that intrauterine death frequently occurs at mid-gestation, are most probably the ones that die of defects such as cardiovascular failure (Baardman et al. DMM 2016, Christ et al., Hum Mol Genet 2020) and are resorbed. Among the remaining 65 % of embryos with a closed and dilated dorsal midline, some embryos had an open anterior (telencephalic) neuropore (ANP). This persistent ANP appeared to worsen over time, leading to progressive enlargement of the ANP (maybe some sort of "rupture") and eventually to exposure or extrusion of brain tissue.
Please also refer to the new legend of Fig. S2; the main text has been amended to read: "In WT embryos at E9.5, the anterior neural tube is closed and midline separation of the forebrain vesicles starts. Compared to the WT (Fig. S2A), *Lrp2* null mutants at this stage had either severely dilated or open neural tubes. At E18.5, when a proper skin-covered skull had formed in WT embryos, *Lrp2* null mutants had either a small skull and a dilated fontanelle through which dorsal midline (dML) tissue such as choroid plexus protruded, or an atypical form of anencephaly (cf. Willnow et al., 1996). Evaluation of mutants between E9.5 and E18.5 suggested that embryos with dilated neural tubes at E9.5 could follow two developmental paths: 1) they could develop an increasingly dilated dML, culminating in defective dML-derived organs and impaired fontanelle closure; 2) they could show impaired anterior neuropore (ANP) closure in addition, leading to further opening of the ANP, eventually exposing anterior neural tissue and culminating in tissue atrophy and atypical anencephaly. Few embryos with open neural tubes at E9.5 might catch up and continue down path 2), however, numbers suggested that they die mid-gestation due to cardiovascular defects (Baardman et al., 2016; Christ et al., 2020), as resorption of embryos was frequently observed."

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8) Line 254- is “caudalward” a word?

To make the nomenclature clearer, the sentence was rewritten.

9) Line 310: since you technically cannot “prove” anything, chose a different word than “proving”, such as “indicated that”.

We agree, the sentence has been rewritten.

Reviewer 2
Advance Summary and Potential Significance to Field:

The manuscript by Kowalczyk et al. describes the function of Lrp2 in regulation of apical constriction and planar cell polarity (PCP) protein Vangl2 during neural tube closure in mouse and Xenopus. The work is initiated based on clinical association of LRP2 mutations with neural tube closure defects (NTDs) in human patients. The authors aim to use frog and mouse vertebrate models to dissect molecular mechanisms responsible for the function of Lrp2 in NTDs. The advances made by the study include:
1) Lrp2 is required for neural tube closure (NTC) in both models;
2) Lrp2 controls apical constriction of neuroepithelial cells by modulating apical membrane remodeling; 3) Lrp2 regulates Vangl2 localization during neural morphogenesis; and 4) Lrp2 interacts functionally with the intracellular molecules Shroom3 and Gipc1 to affect apical constriction. The authors propose that via interacting with intracellular adaptors and scaffold proteins, Lrp2 can orchestrate apical constriction and planar cell polarity to control neural tube closure.

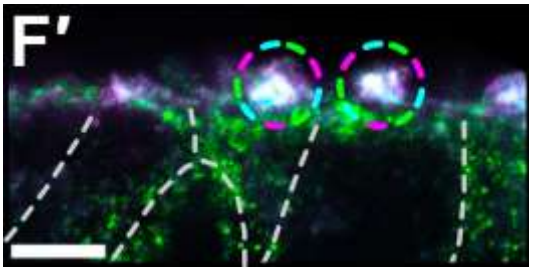
Reviewer 2
Comments for the Author:

The study is generally well controlled with high quality data, and the results provide important insight into the function of Lrp2 in neural morphogenesis. However, the conclusion about PCP requires additional data and further analysis.

Specific comments:

1) Fig.1 shows periciliary localization of LRP2, but Fig. 4 shows at least 2 apical clusters of LRP2 per cell. How does the distribution compare? Does loss of Lrp2 affect cilia morphology?

While Fig. 1D shows an *en face* view on the neuroepithelium with LRP2 concentrated in the periciliary region, Fig. 4F (now Fig. 5G) shows LRP2 localization on coronal sections through the neuroepithelium (now more clearly indicated by a graphic in Fig. 5F). The super-resolution *en face* view focuses on the apical surface of cells and easily allows distinction between single primary cilia, i.e. single cell surfaces. Coronal sections through the curved and pseudostratified neuroepithelium, however, tend to produce oblique cross sections through multiple cells, making a clear allocation of an apical surface to its respective lateral borders difficult. The two apical clusters shown in Fig. 5G likely represent staining on the apical surface of two different cells, thus matching the distribution shown in Fig. 1D. The figure added here for the reviewer demonstrates the putative cell borders, but we chose not to add these in the manuscript, as we cannot be absolutely sure about the course of the cell boundaries and corresponding apical surfaces.



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As to cilia morphology: so far, there is no evidence that LRP2 deficiency affects the morphology of the primary cilium (see Fig. 4A in Christ et al., Dev Cell 2012). A clear statistical analysis of e.g. cilia length, positioning and structure is beyond the scope of this manuscript and being planned in following studies.

2) Much of the data on the mechanism of Lrp2 in apical constriction, such as its control of endocytosis in Xenopus, is included in the supplemental figure 3. As the data support a crucial conclusion in the manuscript, they should be moved into the dataset shown as a real figure.

This was a point raised by both reviewer #1 and #2 - as suggested, Fig. S3 was moved to the main figures section and is now Fig. 4.

3) Does the medial-anterior localization of Lrp2 in Xenopus overlap with that of Vangl2? Does Lrp2 overlap with Vangl2 in the vesicles prior to membrane localization of Vangl2 (proposed in the model)?

Prior to membrane localization of Vangl2, vesicular Vangl2 frequently appears to be in contact with Lrp2-positive intracellular compartments, as immunofluorescent staining for Vangl2 and Lrp2 overlaps in several instance within single cells. We observe, however, that Lrp2 and Vangl2 overlap partially, which might indicate that they are both part of Rab11-positive recycling endosomes (see Fig. 5G), but are sorted differently in a dynamic manner within this compartment. Medial-anterior localization of Lrp2 precedes that of Vangl2 by about one stage and the re- localization process appears to be very dynamic. At the time when Vangl2 appears at the membrane but before it is overtly asymmetric, few Lrp2-positive vesicular structures can be found at the membrane together with Vangl2, however they mostly appear mutually exclusive, as the remainder of Lrp2 vesicles in the cytoplasm does not contain any Vangl2. These data have been added as Fig. S5C and S5D.

4) Although Vangl2/VANGL2 protein distribution is altered in lrp2/Lrp2 mutants, how this affects cell polarity or directional movement is not shown or discussed. Further analysis of the supplemental movies may help to shed light on this issue.

It is true that we had not addressed the question of how Vangl2 mislocalization upon loss of *lrp2* function affects cell polarity or directional cell movement. This analysis has actually been very much complicated by the overall lack of knowledge about cell polarity and cell dynamics at the forebrain level. One obvious reason for that is that the forebrain area remains wide and does not undergo convergent extension (CE) at the time when PCP-mediated mediolateral cell intercalation brings about CE in the caudal neural plate. Our observation of a temporally dynamic Vangl2 relocalization from vesicular stores to the lateral membrane has as such been a first step towards describing PCP dynamics in the forebrain area.
In order to address the above concern of reviewer #2, we tried to identify processes taking place in the posterior neural plate of *Xenopus* that could also be analyzed in the forebrain area. Process **1) Cell anisotropy and long axis orientation.** At the **hindbrain / spinal cord level**, neuroepithelial cells are anisotropic, in the sense that they feature a long axis and a shorter axis perpendicular to it. At first, the long axis is oriented in anteroposterior direction, and during posterior neural fold convergence this orientation changes to a mediolateral alignment (Butler and Wallingford, eLife 2018). We have now analyzed axis orientation and anisotropy **at the forebrain level** from st. 15 onwards (i.e. before anterior neural fold convergence). At st. 15, cells were anisotropic with their long axis predominantly oriented in mediolateral direction (Fig. S5F). Concomitant with anterior neural fold convergence and apical surface reduction during st. 16 and 17, anisotropy persisted but orientation of the long axis shifted from mediolateral to anteroposterior. At st. 17, *lrp2*MO-injected cells, however, had failed not only to reduce their surface area but showed reduced anisotropy and did not adopt a preferential planar orientation (Fig. S5G, H). Process **2) T-transitions**: Despite the failure to establish anisotropy and planar alignment, *lrp2*MO cells nevertheless underwent so-called T1-transitions, i.e. here: the shrinkage of a mediolaterally oriented cell-cell junction into a vertex and its resolution into an anteroposteriorly oriented junction. This is one of the mechanisms leading to CE and requires the orchestrated shrinking and elongation of junctions (Bertet et al., Nature 2004), a process that did not seem to be overtly impaired by loss of Lrp2. This makes sense in the light that Vangl2 is a factor that accumulates at **shrinking** junctions (Butler and Wallingford, eLife 2018) and that T1-transitions are less frequent upon functional loss of Vangl2 in *Vangl2*$^{lp/lp}$ mice.

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Together, these data strongly suggest that the premature mislocalization of Vangl2 to the membrane in Lrp2- deficient cells impairs polarity on the cellular level, but does not prevent overall directional cell movement as judged from cell junction motility and the T1-transition-mediated exchange of cellular neighbors.
The text has been adapted accordingly.

5) Does Shroom3 bind to Lrp2 or any of the intracellular adaptors analyzed here? Do Shrrom3 and Lrp2 act in parallel pathways rather than in the same pathway, with one regulating actomyosin and the other controlling endocytosis? This should be discussed in more depth.

Addressing the question of whether Shroom3 binds to Lrp2 or any of the other adaptors analyzed here requires further experiments such as co-immunoprecipitation assays and *in vivo* as well as super-resolution microscopy. We definitely want to do these analyses in the future. However, we feel that at the moment, especially with the current restrictions that our labs are facing due to the pandemic, these experiments are beyond the scope of the manuscript.

Our data suggest that Shroom3 initiates AC and recruits LRP2 which acts on the steps of AC involving endocytosis. The Shroom3 pathway has been described before to control the planar distribution of apical contractile actomyosin networks. We hypothesize that the pathways of Lrp2-mediated endocytosis and Shroom3-mediated actomyosin recruitment cooperate to integrate these processes crucial for AC.
The discussion has been amended to read: “Thus, Gipc1-mediated guidance of Lrp2-positive endocytic vesicles through the apical actin meshwork could account for efficient removal of apical membrane upon Shroom3-induced AC in anterior NP cells. In such a setting, the pathways of Lrp2-mediated endocytosis and Shroom3-mediated actomyosin recruitment cooperate to integrate processes which are crucial for AC.

6) The functional interaction between Lrp2 and Shroom3/Gipc1 seems to be in the context of apical constriction only. Is there any evidence that Shroom3 and/or Gipc1 also control Vangl2 localization? Since the final model includes Gipc1 bridging Lrp2 and Vangl2, this should be substantiated.

We tried to substantiate whether the Lrp2-associated intracellular adaptors identified here in the process of apical constriction also control Vangl2 localization. For this, we chose to concentrate on Gipc1, as a recruitment and co- localization of Vangl2 with Shroom3 upon Shroom3 GOF has already been demonstrated in apically constricting cells of *Xenopus* (Ossipova et al., *Nature Communications* 2014).
Lrp2 and Gipc1 appear to act differentially on Vangl2: while Lrp2 was required for the temporospatial control of Vangl2 localization, our new results suggest that Gipc1 was necessary to maintain the protein expression levels of Vangl2. Instead of inducing the premature relocalization of Vangl2 to the basolateral membrane, *gipc1*MO-mediated LOF led to an overall reduction of eYFP-Vangl2 (now included in the manuscript as Fig. S7J). This is in line with a role for Gipc1 as a positive regulator of apical protein recycling as also shown by Burk et al. (*Nature Communications* 2017) for Gipc1-mediated PlexinD1 sorting. These results demonstrate an even more complex integration of Lrp2 and Gipc1 functional interaction. We suggest that upon *gipc1* LOF, Lrp2-mediated transport of Vangl2 is wrongly sorted to the lysosomal degradation pathway, whereas upon *lrp2* LOF, Vangl2 is prematurely mislocalized from Rab11-positive compartments to the basolateral membrane.
We would also like to point out that we have taken much care throughout the text not to suggest that the data we show are evidence for a direct physical interaction between Lrp2 and its adaptors. Thus, Fig. 8 (formerly Fig. 7) was always meant to be a “hypothetical model” of Lrp2 interactions and as such intended to summarize both the results obtained here and integrate them with other published work - also to create ideas on how to further address Lrp2’s molecular function during NTC. However, a question mark was added to the intracellular scaffold to clearly state that this putative direct interaction is yet to be analyzed in real time and super resolution approaches. Gipc1 could also facilitate the sorting process of endocytic vesicles without direct physical interaction with Lrp2.

7) The PBD deletion mutant can affect binding of Lrp2 to other interactors in addition to Gipc1. The results in Fig. 6J-N are not strictly conclusive regarding to Gipc1.

We agree that these results are not strictly conclusive regarding Gipc1. Our intention here was to show that the distal PBD - the region known to mediate binding between Lrp2 and Gipc1 - is indeed

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necessary for normal neurulation. We believe that together with the localization / mislocalization data and the functional interaction between Lrp2 and Gipc1, these data strongly indicate that Gipc1 is indeed involved in mediating neural morphogenesis together with Lrp2.

8) The temporal succession model implies that cells undergoing apical constriction early on will proceed to undertake CE at a later stage. However, more cells seem to undertake CE than those that are apically constricting early on. The model may apply only to a subpopulation of the cells in the neuroepithelium. This should be stated more clearly.

We agree that in this manuscript, we are focusing only on a subpopulation of the neuroepithelium, i.e. the anterior neural plate, the future forebrain. To state this more clearly, the part of the discussion that describes the idea of a temporal succession was changed to read "anterior neurulation" and "a temporal succession of AC and PCP in the forebrain area".
From our observations and measurements in the forebrain area, we can conclude that basically all cells reduce their apical surface to a certain extent during the time window analyzed here. While some cells, especially in the midline and lateral margins of the neural plate, constrict more rapidly and to a greater extent, the remainder of anterior neural plate cells also constricts and probably finalizes constriction after st. 17/18, i.e. after the time window of our analysis. We did not assess how many cells in the forebrain area actually end up being planar polarized, but this population consists of either the same or a smaller number of cells compared to those undergoing AC. We would thus conclude that within the forebrain area, all cells that end up being planar polarized have undergone some degree of apical constriction before.

Reviewer 3
Advance Summary and Potential Significance to Field:

The authors have analysed the function of lrp2 in the neuroepithelium during morphogenesis of the neural tube. This group previously described the presence of forebrain patterning defects and NT closure defects in Lrp2 null mice. Here, they have made use of both this mouse model and Xenopus (using morpholinos and a targeted mutant) to examine the function of Lrp2, in particular in apical constriction of neuroepithelial cells.
The authors describe a series of experiments which implicate Lrp2 in apical constriction and propose a model in which Lrp2 is needed for removal for endocytic removal of apical membrane. It is established that cells need to endocytose their apical membrane to constrict (the appropriate papers in Xenopus are referenced in the discussion and this is also shown in Drosophila) – the current study adds Lrp2 to the components needed for this activity, provides evidence that this is required for neural tube morphogenesis and proposes mechansisms involving interaction with intracellular proteins.

Reviewer 3
Comments for the Author:

1) Live imaging of apical constriction (line 202) is shown for a representative cells (3J) but I cannot see the quantification of this ('Quantitative analysis of cell surface size demonstrated size fluctuation over time in control cells that ultimately finalized AC')?

We are sorry about that - the right term here should probably have been "Measurement of cell surface areas over time demonstrated size fluctuation in control cells that finalized AC". This has been adjusted in the text. We have also added more cell size measurements in supplementary figure S3. Here, size measurements of several cells from two representative live-imaged *Xenopus* embryos were plotted to visualize the overall decrease in surface area and the size fluctuations during the process.

1.1) It's also not very clear what is meant but the description that size fluctuations were unaffected by Lrp2 MO - does this mean they constricted but then did not stabilise in a constricted state? This should be clarified.

Yes indeed: our data support the idea that Lrp2-deficient cells are actually able to constrict apically

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per actomyosin contractions but then fail to stabilize in a constricted state. To clarify how we envisage a repetitive mechanism of apical constriction and membrane removal as a means to stabilize a constricted state, we have depicted the process in the hypothetical model figure Fig. 8 as also requested by reviewer #1.

2) Actin staining is shown to delineate cell shape but the analysis would be enhanced by addition of staining for non-muscle myosin of the types involved in cell-level shape change (eg, phospho myo-II – particularly relevant as a cell polarity defect is also invoked in the Lrp2 MO model) and in cargo transport (myo-V). I note that the model includes myo-6 based on the previous work, but the discussion could include prediction about myo-II levels?

We have attempted pMyoII IF staining in both mouse and frog. In this figure for the reviewer, representative images from the forebrain region of mouse and *Xenopus* were assembled. In both models, pMyoII was detected in vesicular structures as well as at the cell borders with varying intensity. In some cells of *Lrp2* mutants and in some targeted cells in *Xenopus* it appeared as if Lrp2-deficient cells have slightly less pMyoII at their cell borders, yet, we did not recognize a striking difference comparing control and Lrp2-deficient cells. We acknowledge that analysis of non- muscle myosins will enhance our understanding of the molecular mechanisms behind Lrp2-mediated AC. As the numbers of our current analysis are still too low for reliable interpretation and further in-depth analysis will be required, we would like to suggest that this will be addressed in the future with better antibodies that are now available in our labs.

We have removed unpublished data provided for the referees in confidence.

3) The authors describe a caudal NT closure defect in lrp2 MO injected Xenopus (although closure does appear compete in Fig2L,M) and propose that a PCP defect leads to impaired CE movements. This mechanism would be consistent with known cause of NT closure defects in Xenopus and mice. However, there are some issues with ascribing such a mechanism for Lrp2.
First, is there a CE defect? In J and K the neural fold appears further from the midline on the MO injected side, which the authors take to indicate a CE defect – the terminology is important here (convergence could mean movement of the fold towards the midline (eg, line 261) whereas CE refers to a narrowing and lengthening with characteristic cell intercalation). A CE defects is one possible interpretation of the 2J,K phenotype but it is not clear from a static dorsal view whether this could also be due to impaired folding. If live imaging with cell tracing is not possible then a transverse section would help show if the fold is present but laterally displaced.

First, Fig. 2L, M have been removed, as they appear to have been misleading: Fig. 2M was an embryo which had been injected unilaterally to highlight the phenotype by direct comparison to the uninjected side. The neural fold on the uninjected side basically advances so much towards and even across the midline, that it reaches the lagging neural fold of the injected side, thereby seemingly closing the neural tube, which, looking at the inside morphology, is not true. Instead, we put in a representative embryo that has been injected with *lrp2*MO bilaterally together with an uninjected control from the same clutch. This embryo shows the extent to which both the anterior and posterior neural plate are affected by loss of Lrp2 - the neural plate is wider and at the same time shorter (both embryos were imaged at the same magnification!). However, we agree that we should use the term CE more carefully, as even though widening and shortening are hallmarks of disrupted CE in the neural plate, they might actually stem from other morphogenetic defects, too, as highlighted by the transversal sections at the trunk level: in the WT, the neural folds are elevated and apposed, made possible by the maximal apical constriction basal elongation of midline cells (Fig. 2J'1). In the morphant, however, the elevated neural folds appear to be separated due to the extremely large apical surfaces of midline cells, which appear to be in poor contact and not intercalated with the underlying deep cells of the neural plate. It appears that neural fold convergence is indeed impeded by the large surfaces of floor plate cells. During revision, we also did record more movies, however, since the focus of the work is really on the analysis of the forebrain defects upon Lrp2 deficiency, we just could not analyze the posterior neural plate with the detail that would be needed to give a definite answer about mediolateral cell intercalation (especially since in *Xenopus*, intercalation occurs both in the superficial as well as the deep layer of the neuroepithelium). Our results from the forebrain region indicate, however, that Lrp2-deficient neuroepithelial cells are still able to undergo

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neighbor exchange by T1-transitions (see reply to comment **4)** by reviewer #2 and updated Fig. S5J, K), suggesting that characteristic cell intercalation (and thus CE in its original sense?) is not impaired. We have tried to revise the text towards a careful use of the term CE and replaced it by "neural fold convergence" where advised.

3.1) As well as a wider neural plate, CE defects result in lack of elongation but this is less obvious here (were length measurements made?). Similarly, crispr mediated targeting is describing as causing impaired neural plate narrowing and lengthening (Fig S2L-O).While the neural folds appear further apart in the targeted embryos (I and K) it is less easy to agree that lengthening is impaired – if anything in these images the marked region of neural plate is longer.

As described above and shown in Fig. 2J, elongation of the neural plate was definitely impaired in *lrp2* morphants. Even though it might be less obvious, but the neural plates of crispants are always shorter as well. Admittedly, this is not readily visible in the pictures in Fig. S2, probably due to the fact that the *Xenopus* embryo is a sphere and that the elongated neural plate, spanning the dorsal side from the anteriormost to the posteriormost point, is therefore not easy to depict in its entire length. This is also the reason why length measurement are hard to do at these stages. To visualize this a bit better, we have added two pictures of frontal views for the reviewer, which hopefully help to better illustrate the impaired elongation.

In the control, the caudal neural plate continues in the back of the spherical embryo and the posterior limit can thus not be exactly determined (unfortunately, this is also the case when a dorsal view is imaged, see Fig. 2J). In the crispant, the wide neural plate however can be seen in its entire length. The phenotype of crispants is actually often more severe - possibly because all tissues (also e.g. the dorsal mesoderm / notochord, which crucially determines axis elongation) are targeted by injection at the one cell- stage, while *lrp2*MO has been targeted to the neural plate only, to prevent such additional phenotypes.

3.2) Nevertheless, there are indications of possible altered cell planar polarity eg, disruption of the anterior localisation of pigment granules (3B) and possible altered Vangl2 localisation dynamics. This may be due to the altered apical surface properties but whether it contributes to NT closure defects is less clear (see comments on CE in Xenopus and mouse).

Since reviewer #2 also asked us to show the effect of mislocalized Vangl2 on cell polarity, we identified suitable "descriptors of planar cell polarity" for the forebrain and do show more evidence now that (planar) cell polarity is indeed altered upon loss of Lrp2-mediated endocytic activity (Fig. S5F-I). Since endocytosis-mediated localization of core PCP components has been identified as crucial for cell polarity by others, we are quite confident that the loss of Lrp2 indeed affects tissue remodeling on a planar level in the neural plate.

4) The relationship of CE/PCP to the mouse model: The mouse NT phenotype does not resemble PCP mutants in which CE defects affect the spinal and hindbrain region with the most anterior NT generally spared.

We absolutely agree that the Lrp2$^{-/-}$ phenotype does not entirely resemble the phenotype of PCP mutants: neither do Lrp2 mutants develop craniorachischisis, nor is the anterior NT region spared from NTDs. Our results on Vangl2 localization suggest a straightforward explanation for this divergence

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in phenotypes: while in Vangl2 loss-of-function mutants such as Lp, Vangl2 protein is stuck in the ER and fails to reach the plasma membrane (Merte et al., Nat Cell Biol 2010), Vangl2 protein is preferentially mislocalized to the plasma membrane in Lrp2 mutants, suggesting that the cellular outcome has to be different from a loss of function. Even though it should also affect PCP-dependent processes, it apparently induces a somewhat milder NTD.
The additional defects in anterior NT closure can be ascribed to the impairment of apical constriction, which has been clearly linked to anterior NTDs (Wallingford 2005). In addition, facial clefting together with holoprosencephaly (i.e. impaired separation of telencephalic vesicles) in Lrp2 mutants are phenotypes which arise from Lrp2- dependent misregulation of Shh signaling in the ventral midline of the forebrain.

4.1) The equivalence to caudal neurulation defects in mouse and humans (line 240) to seems over-stated. Spinal NTDs aren't reported by the authors in the mouse model and the cited human study is a linkage study suggestive of a genetic association.

It is correct that we have not observed open spina bifida in *Lrp2* mutants. However, as we focused on forebrain development, we did not investigate the caudal NT in detail. The research lab of Prof. Aldskogius has previously reported morphological malformations of *Lrp2*$^{-/-}$ spinal cords in the caudal region. Even though they are not described as such, these malformations (absence of vertebral arches, sunken dorsal midline) clearly look like spina bifida occulta, a closed caudal NTD (see below, Fig. 1 from Wicher and Aldskogius, Developmental Neuroscience 2008).

We have removed unpublished data provided for the referees in confidence.

Also, the cited studies on human NTDs (Rebekah Prasoona et al., 2018; Renard et al., 2019) are based on linkage analyses and LRP2 variants were identified to be associated with increased risk of NTDs affecting brain and spinal cord. Such caudal NTDs are also present in patients with certain Vangl2 variants (Kibar, Clin Genet 2010). Together, these literature data suggest that loss of Lrp2 and possibly the effect this has on Vangl2 localization impact on caudal neural tube closure and further development. These are interesting aspects we want to address in the future. We have nevertheless removed both references from the main text to make this less over-stated and instead focused on the observations in *Xenopus*. The paragraph now reads: "In addition to the impairment in hingepoint formation, we had noticed that the caudal NP remained wide and short upon *lrp2* LOF in *Xenopus* (Fig. 2I, J). Narrowing and lengthening are hallmarks of caudal neurulation, a consequence of convergent extension (CE) movements mediated by PCP signaling (Sutherland et al., 2020). We thus asked whether cell polarity was affected upon *lrp2* LOF."

5) Mouse NT phenotype: A proportion of Lrp2-/- embryos are predicted to fail in cranial NT closure in accordance with the previous report (Kur et al 2014). Given the variable NT phenotype (e.g. 12/34 have open cranial NT), how did the authors select embryos for apical constriction analysis at E8.5 (which is before closure defects are apparent)?

Embryos for these studies were not preselected by phenotypic appearance and the experimental approach was unbiased as noted in the revised Methods section which now reads: "*Lrp2*$^{-/-}$ embryos at E8.5, i.e. before neural tube closure, were included in the studies in an unbiased way and not preselected by phenotypic appearance".

5.1) Does the neuroepithelium of all embryos show the AC defect?
Yes - in all E8.5 embryos evaluated by either scanning electron microscopy or by whole mount immunofluorescence, we see defects in AC.

5.2) If so, how do the authors reconcile the 2 differing phenotypes (eg, Fig. S2B & D). A comment on the potential variability and how this is handled in data analysis should be included.

So far, we have no experimental data set explaining the two differing phenotypes in *Lrp2*$^{-/-}$ embryos at E9.5. However, variability in phenotypic expressivity is widely described in mouse models with NTD. (Heussler et al., 2002, Hong and Krauss 2018.). We can only speculate that beside the genetic contribution to the process of neural tube morphogenesis in the mutants, external (environmental) factors such as nutritional status of the embryo in the uterus play a role. Micronutrients such as folate

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have an important impact on the intricate balance of morphogenetic processes during the critical time window of neural tube closure and can cause additional variability in the severity of defects in mutants. A section has been added in the discussion about how folate could affect apical constriction in our model (as has also been asked for by this reviewer in comment **12)**). The addition of further stages to Fig. S2 has enhanced our understanding of what potentially becomes of the embryos with open vs. closed / dilated neural tubes at E9.5.

Additional comments:
6) The details of the forebrain phenotypes in Lrp2 mutant mice are included in the text (page 5) and supplementary figure but the description would benefit from being more precise. The observations at E8.5 and 9.5 indicate that some embryos will go on to fail in NT closure but the illustration of these subsequent defects are minimal (one fetus at E18.5).

As indicated above, more embryos from additional stages have been added to Fig. S2, please see the figure legend for a more detailed description of the development of the phenotypes over time. The description in the main body of the text has been amended to read: "In WT embryos at E9.5, the anterior neural tube is closed and midline separation of the forebrain vesicles starts. Compared to the WT (Fig. S2A), *Lrp2* null mutants at this stage had either severely dilated or open neural tubes. At E18.5, when a proper skin-covered skull had formed in WT embryos, *Lrp2* null mutants had either a small skull and a dilated fontanelle through which dorsal midline (dML) tissue such as choroid plexus protruded, or an atypical form of anencephaly (cf. Willnow et al., 1996). Evaluation of mutants between E9.5 and E18.5 suggested that embryos with dilated neural tubes at E9.5 could follow two developmental paths: 1) they could develop an increasingly dilated dML, culminating in defective dML-derived organs and impaired fontanelle closure; 2) they could show impaired anterior neuropore (ANP) closure in addition, leading to further opening of the ANP, eventually exposing anterior neural tissue and culminating in tissue atrophy and atypical anencephaly. Few embryos with open neural tubes at E9.5 might catch up and continue down path 2), however, numbers suggested that they die mid-gestation due to cardiovascular defects (Baardman et al., 2016; Christ et al., 2020), as resorption of embryos was frequently observed.

7) Specifically: In FigS2 22/34 embryos are described as having a 'dilated dorsal forebrain'. The authors should define how this cut-off was made and preferably present the quantitative measurements (as shown in panel B).

For our studies we used E9.5 embryos that were not preselected. We never saw the need to perform measurements on the dilated forebrains since at E9.5, there are only two phenotypes: either neural tubes are open or they are closed, but very obviously and consistently dilated in comparison to control littermates. This has been very consistent over the years in these mutants (Spoelgen et al., 2005, Christ et al., 2012).

8) A section through the regions of wild-type vs dilated vs failed NT closure embryos would help to compare the phenotypes, however, as cranial NTDs have been previously shown in this mutant it is not essential.

Images of coronal sections of the forebrain at E12.5 were included in Fig. S2A to more clearly demonstrate the dilated dorsal forebrain phenotype of *Lrp2*$^{-/-}$ embryos.

9) Panel F has a very unusual appearance, differing from the usual appearance of anencephaly or exencephaly – the lesion appears skin covered and there also appears to be facial clefting (a front view would clarify this). Was this typical appearance of fetuses that 'failed NT'?

We agree that the embryos that have presented as anencephalic are not typical for anencephalic embryos. Analysis of further embryonic stages has helped to pinpoint that this phenotype probably results from improper closure and subsequent re-opening of the anterior neuropore (see new Fig. S2).
As to facial clefting: this is indeed typical for Lrp2 mutants on a C57BL/6N background and occurs both in embryos with open as well as with closed, dilated neural tubes. We have previously shown that this phenotype is caused by impaired SHH signaling in the ventral midline of the developing forebrain (Spoelgen et al., Development 2005, Christ et al., Dev Cell 2012).

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10) Inclusion of images at a stage that is definitely after the stage of closure (even if delayed) eg. 10.5 onwards, but earlier than E18.5 would help clarify the nature of the defect (as would sections).

We now included images of embryos at E9.5, E10.5, E12.5, E13.0 and E18.5 as well as coronal sections of the forebrain at E12.5 in Figure S2 to demonstrate the phenotypes of *Lrp2*$^{-/-}$ embryos and help clarify the nature of the defect. We are thankful to the reviewer for suggesting this, as it has been a tremendous step forward in understanding the putative development of NTDs in the Lrp2 null mice. Please refer to the new legend of Fig. S2, the amended main text (cited above under comment **6)**) and also the response to reviewer #1's comment **7)**.

11) Fig1C would benefit from a low magnification panel to orientate the reader on the angle of view.

Fig. 1C has been exchanged for a lower magnification overview of the entire anterior neural folds of that embryo.

12) The authors previously demonstrated a role for Lrp2 in folate uptake. It would be relevant to speculate whether this function is separate or in some way related to the effect they observe on apical constriction.

The discussion has been amended to include speculation about how the function of folate might converge with the processes identified in this study.
"In addition to membrane removal, ligand uptake by LRP2 can also be relevant for AC. One physiological ligand for LRP2 in the neural plate is folate bound to its receptor FOLR1 (Kur et al., 2014). FOLR1 is required for AC and consequently neurulation in *Xenopus* (Balashova et al., 2017) and acts on actomyosin-dependent AC alongside Shroom3 (Martin et al., 2019). At this point, Lrp2-mediated membrane removal identified here and folate-dependent intracellular processes might very well interact."

13) Line 238 'In addition to the impairment in AC, we had noticed widening of the caudal NP upon lrp2 LOF in Xenopus (Fig. 2L, M)' – this is not clear as the NT appears closed in these panels and perhaps the authors are referring to 2J,K?

This has been corrected as Fig. 2 was also adapted to more clearly address the caudal neural plate phenotype in
Xenopus.

14) 4F,G should include some indication of the axial level of the section – cranial neural fold?

A schematic was included as Fig. 5F (formerly Fig. 4) to help indicate the level of the sections - yes, they are in the cranial neural fold region.

Second decision letter

MS ID#: DEVELOP/2020/195008

MS TITLE: Neural tube closure requires the endocytic receptor Lrp2 and its functional interaction with intracellular scaffolds

AUTHORS: Izabela Kowalczyk, Chanjae Lee, Elisabeth Schuster, Josefine Hoeren, Valentina Trivigno, Levin Riedel, Jessica Goerne, John B Wallingford, Annette Hammes-Lewin, and Kerstin Feistel
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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Reviewer 1

Advance summary and potential significance to field

The authors have identified that LRP2 is essential for neural tube closure in Xenopus and mice. They also show that known intracellular adaptor proteins for LRP2, NHERF1 and GIPC1, act as scaffolds for the LRP2 receptor to mediate endocytosis of LRP2 and cell membrane, in conjunction with Shroom3. This reduction of membrane via LRP2/Shroom3/Gipc1 enables apical constriction of the neural epithelium for cranial NT closure. The results add a new level of understanding of the importance of apical constriction and new mechanisms by which this is achieved during embryonic neurulation.

Comments for the author

The authors have done an excellent job in responding to my review and to the other two reviewers. The data are strong and interesting. The response and revised manuscript are acceptable to me.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Kowalczyk et al. describes the function of Lrp2 in regulation of apical constriction and planar cell polarity (PCP) protein Vangl2 during neural tube closure in mouse and Xenopus. The work is initiated based on clinical association of LRP2 mutations with neural tube closure defects (NTDs) in human patients. The authors aim to use frog and mouse vertebrate models to dissect molecular mechanisms responsible for the function of Lrp2 in NTDs. The advances made by the study include: 1) Lrp2 is required for neural tube closure (NTC) in both models; 2) Lrp2 controls apical constriction of neuroepithelial cells by modulating apical membrane remodeling; 3) Lrp2 regulates Vangl2 localization during neural morphogenesis;
and 4) Lrp2 interacts functionally with the intracellular molecules Shroom3 and Gipc1 to affect apical constriction. The authors propose that via interacting with intracellular adaptors and scaffold proteins, Lrp2 can orchestrate apical constriction and planar cell polarity to control neural tube closure.

Comments for the author

The authors have performed additional experiments and revised the manuscript to address all the concerns that I have raised. The manuscript is now appropriate for publication in Development.

Reviewer 3

Advance summary and potential significance to field

(As original review)
The authors have analysed the function of lrp2 in the neuroepithelium during morphogenesis of the neural tube.
This group previously described the presence of forebrain patterning defects and NT closure defects in Lrp2 null mice. Here, they have made use of both this mouse model and Xenopus (using morpholinos and a targeted mutant) to examine the function of Lrp2, in particular in apical constriction of neuroepithelial cells.
The authors describe a series of experiments which implicate Lrp2 in apical constriction and propose a model in which Lrp2 is needed for removal for endocytic removal of apical membrane. It is established that cells need to endocytose their apical membrane to constrict (the appropriate papers in Xenopus are referenced in the discussion and this is also shown in Drosophila) – the current study adds Lrp2 to the components needed for this activity, provides evidence that this is required for neural tube morphogenesis and proposes mechansisms involving interaction with intracellular proteins.

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Comments for the author

The revised manuscript and response to the comments on the orginal manuscript have addressed my questions/comments. There are several points where the explanation has been clarified or expanded with addition of data and figure panels which I believe will help the accessibility to the reader and/or help interpretation of the findings (eg, Fig. S2 and elsewhere). I congratulate the authors on their paper and on clearly explaining their responses to the reviewer comments.

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