Mirror-symmetric microtubule assembly and cell interactions drive lumen formation in the zebrafish neural rod

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 08 May 2012

Thank you for the submission of your manuscript to The EMBO Journal. We have now received the reports from the two referees that were asked to evaluate your study, which I copy below. As both referees think that your manuscript is highly interesting and their comments are quite positive, I would like to invite you to revise it according to the referees' comments.

Without going into all the details that you will find below, both referees think that the concept of the midline as a polarization signal is very interesting. However, as both point out, the key question remains how cells are able to sense the midline and translate that information to the cell polarization machinery. While a full answer to this question is obviously out of the scope of a single study, some mechanistic insight into this process needs to be provided in a revised version of your manuscript. The rest of the concerns can be considered peripheral compared to this one and will not fundamentally influence our decision to accept or reject the study. Obviously, every effort to address them will be only in the best interest of your manuscript.

Please be aware that acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is 'The EMBO Journal' policy to allow a single round of major revision only.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html
We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact me as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let me know in advance and I will be able to grant an extension.

Do not hesitate to contact me by e-mail or on the phone in case you have any questions.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

The ms by Clarke and colleagues analyzes the molecular and cellular mechanisms underlying neural rod midline formation. It has previously been reported that midline formation within the zebrafish neural rod relies on a mirror-symmetric division that localizes Par3 at the abscission site and allow one of the daughter cell to cross the midline. However, when cell divisions are inhibited, the neural tube still forms a midline, suggesting that c-divisions are not mandatory for cell polarization and midline formation. The present study addresses mechanisms by which neural rod midline formation is achieved in the presence and absence of cell divisions. The author show that cells extend over the future midline and accumulate Par3, their centrosome and Rab11 in the part of their cortex adjacent to the future midline. These cells can then either undergo c-divisions or polarize independently of c-divisions. In the absence of c-divisions, the cells retract their contra-lateral cell extensions to the side where the nucleus lies. The authors show that although the neural tube can form without cell divisions, the c-divisions facilitate the formation of a continuous lumen. The authors further show that expression of a dominant negative form of Rab11 prevent lumen opening without visibly affecting cells polarization. The results clarify the respective roles of cell polarization and cell division in zebrafish neural tube development. Moreover the Rab11 loss of function experiment very nicely confirms previous findings on the importance of membrane traffic in lumen formation.

The ms contains a number of potentially very interesting observations. However, more mechanistic insight into the proposed processes is required before the manuscript is suitable for publication in a high-ranking journal such as EMBO Journal.

1. The most surprising observation is the accumulation of apical markers at the region of cells intersecting the midline. While this is interesting as such, some information as to the mechanisms by which these cells ‘recognize’ the midline would be required.

2. The experiment showing that a locally restricted knock-down of rab11 locally affects lumen opening is interesting, but only loosely connected to the process of apical polarization of these cells. The authors need to better characterize the molecular and cellular basis of this phenotype (c-division, abscission, microtubules organization, nature of the cargo).

Referee #2:

This manuscript describes studies on lumen formation in the neural rod of zebrafish embryos. Cavitation of rod-like structures to generate lumens is a common and important mechanism in
various developmental processes. Zebrafish neural tube formation is an excellent subject to study this phenomenon as it allows high-resolution imaging in vivo. The authors take full advantage of the opportunities available in this system, making extensive use of transgenic fish for in vivo time laps imaging, supplemented by immunocytochemistry in several cases.

The main conclusion is that cells that cross the midline before lumen formation is initiated form a distinct structure at the organ midline, not at any specific point relative to the extent of the cell. This apical specialization thus arises along the length of the cell rather than its end, a novel observation. A mirror-symmetrical cytoskeletal organization forms around this apical focus. While so-called crossing divisions, previously invoked as critical in lumen formation, are not necessary for this polarization, such divisions provide a major advantage for tissue organization, and their role in lumen formation is not eliminated. The great unanswered question in this study is how cells know where along their length the organ midline lies, and what signals initiate the polarization centered on this point. While the study presented here leaves this question open, defining this remarkable phenomenon is a very substantial contribution to our understanding of neural tube formation with implications for lumen formation in general.

The experiments are very well executed and the work is well presented. I have rather few specific concerns or questions.

Fig. 3. The point of mirror symmetry is much clearer in B than in C and D. Can this be improved?

p.8. In the nocodazole experiments, the point that longer treatment is more effective is, of course, true but also raises the question whether this longer treatment may become toxic. The wash-out experiment (Fig. 4D) is thus critical to this point. I suggest that the data represented by Fig. 4D should be quantified.

p. 9 and Fig. 5. The result on RAB11A are interesting but not as well connected to the rest of the data as one might wish. This could probably be achieved by some changes or additions to the text. One point, however, is not compelling to me: RAB11ADN is said not to inhibit apical localization of Pard3. This rests on comparing Fig. 5D and G, using C and F as guide for the DN expression (red). I see a substantial reduction of Pard3 (green) in these regions. I believe that the text needs to be revised, unless the picture shown is not typical (but, of course, we assume that it is). The conclusions and discussion should be adjusted to reflect the image.

Response to reviewers' comments

We thank both reviewers for their encouraging comments that our work “contains potentially very interesting observations” and “is a very substantial contribution to our understanding of neural tube formation”. Our responses to specific points are below.

Reviewer 1:

Point 1: “The most surprising observation is the accumulation of apical markers at the region of cells intersecting the midline. While this is interesting as such, some information as to the mechanisms by which these cells 'recognize' the midline would be required.”

We have addressed this deficiency by two new experiments (with new figure and results section) that help to propose a mechanism for midline position specification (Figure 7). We show that when neural cells from the left and right hand sides of the neural keel are prevented from interacting with each other, they will assemble apical complexes at their most anti-basal tip. However when left and right cells are allowed to interdigitate as normally do in the neural rod the apical complexes are assembled within the zone of interdigitation rather than at their most anti-basal tips. We also show that the ECM component Laminin provides at least part of the anti-basal signal. Thus the position where apical markers accumulate is determined by integrating anti-basal signals with cell-cell interactions in the interdigitation zone.

Point 2: “The experiment showing that a locally restricted knock-down of rab11 locally affects
lumen opening is interesting, but only loosely connected to the process of apical polarization of these cells.”

We have re-written both the Rab1la results section and the discussion to make our reasoning behind including this data and the implications of our results clearer. Specifically, since it was previously suggested that Rab1la endosomes are necessary to target Pard3 to the apical domain (Bryant et al, 2010), we decided to test whether Rab1la has a role in apical protein targeting in zebra fish, whether it also accumulates in cells at the intersection of the midline and whether it is a necessary component for lumen formation in our system. We show that, perhaps surprisingly given the Bryant et al 2010 suggestion, Rab1la is not necessary for the initial midline localisation of junctional proteins such as ZO1 and Pard3-GFP at rod stages. It is, however, necessary for maintaining the coherent planar organisation of the apical surface at the midline. We also show that RAB11A is absolutely required for lumen formation and this role is independent of its role in completion of cytokinesis. Therefore, Rab1la trafficking provides a strong example of a process that responds to the organising machinery at the neural midline and is critical for neural tube formation. We show that Rab1la trafficking is dependent on the microtubule cytoskeleton, which reinforces our suggestion that the mirror-reversal of microtubule polarity at the neural midline is a key principle in lumen formation.

“The authors need to better characterize the molecular and cellular basis of this phenotype (c-division, abscission, microtubules organization, nature of the cargo)”.

We have now included more data (Figure S2), as discussed above, showing the effects that Rab1ladn expression has on ZO1, aPKC and Crb2a protein localisation, as well as determining that the lack of lumen opening is not specific to a defect in cytokinesis (Figure 5I). We have expanded our discussion of the potential mechanisms for the RAB11ADN phenotype that we see. However, a complete description of Rab1la’s function is not the aim of our current work and we suspect will require a full paper on its own.

Reviewer 2:
Point 1: “The great unanswered question in this study is how cells know where along their length the organ midline lies, and what signals initiate the polarization centered on this point.”
See response to reviewer 1, point 1.

Point 2: “fig. 3. The point of mirror symmetry is much clearer in B than in C and D. Can this be improved?”
We have repeated this experiment and replaced figure 3B and C with a much clearer example of a wild type cell before, during and after division.

Point 3: “p.8. In the nocodazole experiments, the point that longer treatment is more effective is, of course, true but also raises the question whether this longer treatment may become toxic. The wash-out experiment (Fig. 4D) is thus critical to this point. I suggest that the data represented by Fig. 4D should be quantified.”
We have repeated the nocodazole experiment and quantified the number of cells in which we see this result. See figure 4D legend: “16 out of 26 cells from 4 embryos re-positioned Pard3-GFP from a basal to apical position following nocodazole wash out. The remaining 10 cells had an unclear morphology (3), became different cell types (4) or died (3)”. The majority of cells thus recover from nocodazole treatment and this suggests toxicity is not an issue here.

Point 4: p. 9 and Fig. 5:
“The result on RAB11A are interesting but not as well connected to the rest of the data as one might wish. This could probably be achieved by some changes or additions to the text.”
Please see response to reviewer 1, point 2.

“ One point, however, is not compelling to me: RAB11ADN is said not to inhibit apical localization of Pard3. This rests on comparing Fig. 5D and G, using C and F as guide for the DN expression (red). I see a substantial reduction of Pard3 (green) in these regions. I believe that the text needs to be revised, unless the picture shown is not typical (but, of course, we assume that it is). The conclusions and discussion should be adjusted to reflect the image.”

We have now analyzed this phenotype more carefully. We believe our data shows it is safe to conclude that the localization of Pard3 to the midline in Rab1ladn tissue is normal (Figure 5D, G and J) and our attempts at comparing the intensity of staining between experimental and control
segments have not shown any significant difference, however there is variability between embryos (possibly due to variability in injected mRNA levels for the fusion protein Pard3-GFP). To help with this we have used antibodies against aPKC, ZO1 and Crb2a (we do not have an antibody to endogenous Pard3). The data suggest Crb2a is initially reduced compared to controls but levels of both aPKC and ZO1 at the midline look fairly normal to start with, but their distribution is rather more clumpy than normal and gets progressively worse with time (Supplementary Figure S2). We have rewritten this section accordingly.

2nd Editorial Decision 18 October 2012

Thank you for the submission of your revised manuscript. As you will see from the enclosed reports, referees now consider their main concerns solved and suggest only minor points that would need further discussion. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to formally accept your manuscript for publication once these few more minor details have been addressed.

Besides the comments from the referees, I would also like to suggest a change in the title of the manuscript. In our opinion, the current title "Intracellular organization and tissue interactions underlying lumen formation" does not do justice to the nice findings presented in your study. I would suggest something along the lines of "Mirror-symmetric microtubule assembly drives apical specification and lumen formation in the zebrafish neural rod". Of course, this is just a suggestion, but I think that not only it does better describe your findings, but also it is more likely to attract the interest of potential readers. In any case, other possible ideas are very welcome.

After these remaining points have been addressed and we agree on a new title for your manuscript, you will receive an official decision letter accepting your manuscript for publication in The EMBO Journal. This letter will also include details of the further steps you need to take for the publication process to continue.

Thank you for your contribution to The EMBO Journal and congratulations on a successful publication.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

The manuscript by Clarke and colleagues has been revised along the lines suggested by the referees. I still have a few questions that should ideally be addressed before the manuscript is ready for publication.

1. The proposed mechanism for neural rod cells recognizing the midline (cell interdigitation across the midline) is very interesting, but the authors should discuss how such a mechanism is consistent with a timer-mechanism (proposed elsewhere) and the observation of double midlines in embryos with reduced convergent extension movements.

2. It's not clear whether rab11a morphant embryos represent a complete loss of function of rab11. Have the authors attempted to knock down more rab11 paralogues? - the phenotype they observe with rab11a MOs might reveal a rather specific function of rab11a in zebrafish, but might no
provide much information about the global function of rab11 in this process.

Collectively, I think this manuscript is interesting and should be published after the points mentioned above have been addressed.

Referee #2:

In this revision the authors have responded in a satisfactory manner to the questions raised originally. In particular they have provided a model for midline determination that is plausible and, if perhaps not the last word on the subject, provides a good basis of discussion. I have only two points that I believe should be clarified, both concerning description of figures.

Fig. 4B is described in a confusing manner. The figure is labeled to suggest that the top two panels (one each in i and ii) are untreated and the bottom four are treated. The legend to Bi says "... in nocodazole treated embryos..." suggesting that all Bi panels are treated. The Bii legend does not say but implies treatment. The main text only confuses the issue further. More specific and clear labeling should be added.

Figure 9 is mentioned just once early in the Discussion, followed by several pages of text that appears to refer to different parts of the figure, without any further correlation with it. There is a legend that describes the figure, but this is not a substitute for citing specific parts of the figure when the text refers to issues modeled in that part. As presented, Figure 9 has lost much of its usefulness. I suggest that the authors cite different parts when the Discussion touches upon the subject, closely integrating text and figure.

2nd Revision - authors’ response 29 October 2012

We would like to thank both reviewers for their prompt comments on our manuscript revision. We have incorporated these changes into our manuscript and our responses to specific points are below.

We have also altered the title, as suggested but would like to include the addition of ‘and cell interactions’. Our title is now “Mirror-symmetric microtubule assembly and cell interactions drive lumen formation in the zebrafish neural rod”.

We have also submitted a revised version of Figure 1, since we noticed that a dotted line was out of place.

Reviewer 1:

Point 1: “The proposed mechanism for neural rod cells recognizing the midline (cell interdigitation across the midline) is very interesting, but the authors should discuss how such a mechanism is consistent with a timer-mechanism (proposed elsewhere) and the observation of double midlines in embryos with reduced convergent extension movements.”

We have incorporated a discussion of these points into pages 13 and 14 in the Discussion and included an extra diagram in our summary Figure 9. Although it is true that we have unpublished evidence that a cell autonomous timer-mechanism initiates the process of NP cell polarisation, because this is not yet published we do not feel it is appropriate to mention it directly here. Instead we refer to a previous publication from the lab (Tawk et al, 2007), which demonstrates that when convergence is delayed cell polarisation and division will occur on time but in ectopic locations on either side of the neural plate. Since cell division is a dominant morphogenetic force and results in the localisation of apical complexes to the cleavage furrow, this results in double midlines. However, when we block division at the same time as delaying convergence, the underlying propensity of cells to polarise at their anti-basal extremity is revealed. When cells are allowed to interdigitate, polarisation will occur at the same time as cell interdigititation. We propose that this
interdigitation refines the underlying anti-basal polarisation to the area of interdigitation at the
midline (as suggested by the localisation of a-catenin puncta to this area).

**Point 2:** “It's not clear whether rab11a morphant embryos represent a complete loss of function of
rab11. Have the authors attempted to knock down more rab11 paralogues? - the phenotype they
observe with rab11a MOs might reveal a rather specific function of rab11a in zebra fish, but might
not provide much information about the global function of rab11 in this process.”

We have not interfered with the expression of other Rab11 paralogs. We used Rab11a since this
paralog is the only one that shows ubiquitous expression and is the paralog that is consistently used
in other journal articles that focus on polarity defects. However, there is a very high protein identity
between different zebra fish paralogs. Therefore it is likely that our Rab11aDN results reflect a more
global abrogation of Rab11 function. We have added a paragraph to the discussion on page 16,
paragraph 2, detailing this point.

**Reviewer 2:**

**Point 1:** “Fig. 4B is described in a confusing manner. The figure is labeled to suggest that the top
two panels (one each in i and ii) are untreated and the bottom four are treated. The legend to Bi
says "... in nocodazole treated embryos...” suggesting that all Bi panels are treated. The Bii legend
does not say but implies treatment. The main text only confuses the issue further. More specific and
clear labeling should be added.”

We have altered the text on page 7, paragraph 3, line 6. It now reads: “Whilst Pard3 fusion protein
was present at the neural rod midline in many cells before and shortly after nocodazole treatment, by
the 10-somite stage 88% cells in treated embryos had localised some (Figure 4Bi) or all (Figure
4Bii) Pard3 fusion protein ectopically to their basal side (Figure 4C).”

We have also altered the legend for figure 4 B to read: “Bi. Low magnification dorsal view of right
hand side of neural rod. Some Pard3-RFP is present apically before treatment as well as 15 minutes
after treatment with nocodazole. However, Pard3-RFP appears at the basal end of cells (dashed line
to right) after 105 minutes of nocodazole treatment.

Bii. Pard3-GFP is initially located at the apical pole (arrow) of this individual cell before treatment
as well as 15 minutes after treatment with nocodazole. However, Pard3-GFP appears at the basal
pole (arrowhead) within 75 minutes of nocodazole treatment.”

We believe that the interpretation of this figure is now clear.

**Point 2:** “Figure 9 is mentioned just once early in the Discussion, followed by several pages of text
that appears to refer to different parts of the figure, without any further correlation with it. There is
a legend that describes the figure, but this is not a substitute for citing specific parts of the figure
when the text refers to issues modeled in that part. As presented, Figure 9 has lost much of its
usefulness. I suggest that the authors cite different parts when the Discussion touches upon the
subject, closely integrating text and figure.”

We have now cited specific parts of figure 9 several times in the Discussion on pages 13 and 14.