THE
EMBO
JOURNAL

Manuscript EMBO-2013-86003

Cell segregation in the vertebrate hindbrain relies on actomyosin cables located at the interhombomeric boundaries

Simone Calzolari, Javier Terriente, and Cristina Pujades

Corresponding author: Cristina Pujades, University of Pompeu Fabra, Barcelona

Review timeline:

Submission date:	14 June 2013
Editorial Decision:	16 July 2013
Revision received:	18 October 2013
Editorial Decision:	19 November 2013
Additional correspondence:	22 December 2013
Additional correspondence:	27 January 2014
Revision received:	24 December 2013
Acceptance letter:	16 January 2014
Accepted:	16 January 2014

Editor: Karin Dumstrei

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 16 July 2013

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been reviewed by two referees and their comments are provided below.

As you can see, both referees find the analysis interesting and suitable for publication here. However, they also raise a number of different concerns that would have to be resolved for further consideration here. The referees are explicit in their reports and so I won't repeat all the raised issues here. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of the referees. I should add that it is EMBO Journal policy to allow only a single major round of revision and it is therefore important to resolve the raised concerns at this stage.

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

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

REFEREE REPORTS:

Referee #1:

The manuscript by Calzolari and colleagues analyzes the process of rhombomere boundary formation and maintenance in zebrafish embryogenesis. The authors show that the generation of rhombomere boundaries requires functional Eph4A signaling and, downstream of it, the formation of actomyosin cables at the boundaries.

The manuscript is well written and contains a number of potentially interesting observations. However, there are several, mostly minor points of criticism that need to be addressed before the manuscript is suitable for publication in EMBO Journal.

1. The results shown in Figure S1 are extensively described in the first paragraph of the text and thus should better be included within a main figure.
2. The hypothesis that actin and myosin are essential for boundary formation and maintenance is mainly addressed by changing actin dynamics using drugs. It would be interesting to supplement these data with clonal or mosaic genetic alterations of actomyosin cable formation to obtain spatially restricted information about the role of actomyosin cables for rhombomere boundary formation.
3. The combinatorial effect of Blebbistatin and Eph4a should not only be analyzed for boundary straightness but also for cell spreading.
4. Are spreading cells migratory or do they just passively intermix with surrounding tissues?
5. Does EphA4 co-localize with the actomyosin bundles?
6. The first part of the discussion focuses a lot on boundary formation in Drosophila. It might be good to extend the discussion to other model organisms where boundary formation has also been studied (eg boundaries between germ layer in Xenopus gastrulation).
7. The genetic background of the used reporter fish lines M¸4237 and Tg[elA:GFP] lines should be better explained.
8. Statistics of the actomyosin barriers (p10) are given in percentages. This is only useful when the sample size is also given. Please also include 'n'.

Referee #2 :

The manuscript by Calzolari et al., analyses the formation of cell boundaries during rhombomere development in vertebrates. The authors perform time lapse imagining to support the claim that the main mechanism involved in rhombomeric boundary formation relies on cell sorting, instead of changes in cell fates. In addition, they found that actomyosin cables are formed at the boundaries, and they propose that these cables play a major role, downstream of Eph signalling, in boundary formation.

The topic of this work is fascinating and it should be of interest for a wide audience of cell, molecular and developmental biologists, and in consequence appropriate for publication in EMBO J. Unfortunately the conclusions are not completely supported by the experiments, and some of the experimental tools are not adequate to solve this interesting problem. In addition no molecular mechanism to explain the link between Eph activity and actomyosin cable formation is proposed.

Specific comments:

1. A key experimental approach to analyse the mechanism that contribute to sharpening the rhombomere borders is the development of transgenic cell lines that recapitulate the expression of Krox20. Although some aspects of Krox20 expression are replicated in the M¸4127 and Tg[elA:GFP] lines, there is a facet of Krox20 that is not exactly reproduced in these lines and that is essential for the analysis: the jagged border of expression. In situ hybridization for Krox20 show multiple cells expressing the gene in prospective rhombomere 4 (Fig S1C, D; in inset shown in D. I can count between 15-20 Krox20 positive cells in the R4 domain). Importantly the expression of Krox20 in this "wrong" rhomobomere is very strong. However, the equivalent expression in the transgenic lines is much weaker; I can see in R4 only one cell in Fig S1N, and 4 or 5 cells in Fig SI. Consequently, the GFP florescence shown in Movies S2 and S3 does not show the same jagged

border that the one observed in Krox20 expression (Fig S1C, D). Without the appropriate levels of GFP expression these transgenic cells lines are not adequate for an accurate description of the cell movements associated to rhombomere boundaries sharpening.

2. Manual tracking shown in Fig 1M-R and Movie S4 is not convincing. I tried to follow the labelled nuclei, but many nuclei disappear or are very hard to follow.

3. The reasoning behind experiment shown in Fig 2 is correct. As GFP is more stable that Krox20 mRNA, the author expected to observe GFP positive cells in the wrong rhombomere if the main mechanism for boundary sharpening relies on Krox20 being switched off instead of cell sorting. Although the logic of the experiment is correct the experimental tools are not adequate. As I mentioned above the levels of expression in the transgenic lines are too low to be able to rule out a mechanism based on changes in gene expression. To support their claim, the authors should include younger embryos (11 hpf) in Figure 2 to show that they are able to visualize the jagged border using GFP florescence in the transgenic lines.

4. The authors mention that a "dividing cell at the boundary rounded up and transiently invaded the adjacent compartment, it was pushed back to the original rhombomere (Fig 3B-E), suggesting there was a physical barrier at boundary interfaces". I do not see how the normal rounding up during cell division and their consequent integration into the tissue could suggest a physical barrier, instead of changes in cell adhesion or cell rigidity.

5. The authors show that there is no particular accumulation of fibronectin or integrin at the rhomobomere boundaries and then they conclude that "These results show that extracellular matrix does not play a major role in keeping rhombomeric cells apart". This is an overstatement, as functional experiments are required to rule out the role of ECM on this process.

6. The presence of actomyosin cables at the rhombomeric boundaries is interesting; however the demonstration that these cables are effectors of boundary formation is not sound enough. The effect on rhombomeric boundaries after using actomysin inhibitors (Blebbistatin and Rockout) only shows a correlation between cables and borders, as these inhibitors could be affecting other cellular functions that are the direct regulators of boundary formation.

7. The observation that the loss of boundary formation in EphA4 MO is apparently rescued by Calyculin is interestingly, but no mechanism for this epistatic relation is established.

1st Revision - authors' response 18 October 2013

Referee #1:

We want to thank the Referee for the helpful comments and insights. We have made substantial revisions to address the comments and suggestions of the Referee. Our responses are the following:

1. The Referee suggests including Figure S1 as a main figure.

We followed the Referee's advice and Fig S1 has been improved and is now Fig 1. The modification consists in the exchange of pictures (H-I, M-N) for better ones, and the insertion of a new image (Fig 1R) in which jagged boundaries of GFP expression can be observed upon anti-GFP staining in 11hpf embryos.

2. The hypothesis that actin and myosin are essential for boundary formation and maintenance is mainly addressed by changing actin dynamics using drugs. It would be interesting to supplement these data with clonal or mosaic genetic alterations of actomyosin cable formation to obtain spatially restricted information about the role of actomyosin cables for rhombomere boundary formation.

We agree with the Referee, but unfortunately these clonal analysis experiments cannot be addressed as easily in zebrafish as they can be in other models such flies. However, to support our hypothesis that the disassembly of the actomyosin cable was the result of the direct targeting of our time-

controlled pharmacological treatments, and not a side effect of the drugs activity on other cellular processes, we made sure the dismantling of cables was reversible. Thus, we have complemented our studies with two alternative approaches. First, we injected the Rockout (together with an in vivo tracer such as DiO) directly in the lumen of the neural tube at the level of the hindbrain of Tg[myoII:mCherry] embryos and addressed its effects 1h after injection. This short and localized treatment resulted in loss of the actomyosin cable as fast as in 1h (data not shown), although the embryos were quite distorted due to the injection and this is why we did not include these data in the manuscript. At the time of injection, 18hpf, the lumen is very little opened (Gutzman and Sive, 2010), and it is difficult to assess that properly. Due to this, we took a second approach; we conditionally inhibited myosin II activity in Tg[myosinII:mCherry] embryos with Rockout during four hours; then the pharmacological agent was washed out and embryos let to develop for three additional hours 3h. Actomyosin cables were already dismantled after 4h of treatment; after washing out the drug, the cables were restored within 3h, strongly suggesting that the effect observed in the hindbrain is quite specific and reversible. Accordingly, we have included these results in Fig 6 with the rest of previously shown controls and discussed them in the manuscript.
Interestingly, there is a new tool, a photoinducible-blebbistatin (Kepiro et al, PNAs 2012) that could be used to address the local effect of the drugs. Although we have tried very hard to get this compound, we were unable to get some. Unfortunately, since it is not commercialized yet, we could not perform the desired experiments.

3. The Referee points to the combinatorial effect of Blebbistatin and Eph4a in cell spreading.
We did not include previously these experiments because we considered expectable and therefore dispensable. Now, we have included them in Fig 7 and discussed them accordingly in the manuscript.

4. *Are spreading cells migratory or do they just passively intermix with surrounding tissues?*
We think that our experiments unveil cells that were dividing close to the boundaries at the time we disrupt the cable, and are found in the neighboring territory because cannot be brought back to the rhombomere of origin. In vivo imaging experiments showed us that although this is not a passive mechanism, cells do not migrate within the neural tube since they are in a tight epithelium and remain attached to both sides of the neural tube –basal and apical-. The farther a cell is found from the territory of origin may depend on the number of divisions neighboring cells undergo. We included this discussion in page 14. The fact that cells do not migrate but mainly intermix can be observed in Movie S3 and Movie S5.

5. The Referee raised a very important question: in which rhombomere the cable is localized.
We have tried very hard to answer this question, but the optical resolution we can get is at the limit to detect structures within that range. However, the best we could get is the analysis of sagittal sections of Tg[myoII:mCherry] embryos immunostained with anti-EphA4 (Fig 4P-P’’’). The EphA4-staining colocalizes with the actomyosin cables in r3 and r5; however, since membranes are in close apposition we cannot completely rule out the presence of the cable in the even-numbered rhombomeres. Text describing this observation and discussing its implications has been added (see page 9 in Results and page 16 in Discussion).

6. The Referee proposed to extend the discussion to other model organisms where boundary formation has also been studied (eg boundaries between germ layer in Xenopus gastrulation).
We warmly thank the Referee for bringing our attention to these papers, we were not aware of some of them and they are really interesting for our work. We extended the discussion as suggested (see page 16-17) and included some new references (Rohani et al 2011, Hwang et al 2013, Fagotto et al 2013).

7. The genetic background of the used reporter fish lines Mü4127 and Tg[elA:GFP] lines should be better explained.
Better description of the Tg[elA:GFP] and two other references have been included (Stedman et al, 2009, Labalette et al, 2011) in Materials and Methods. As mentioned in point 1, we have changed the pictures in new Fig 1 (H-I, M-N), and included Fig 1R as mentioned before to provide better characterization of the lines. For a matter of space, we did not exhaustively describe the Mu4127 and 4xKaloop lines since they are well characterized in Distel et al 2009; however, we improved their description (see Results page 7, and Material and Methods).

© European Molecular Biology Organization

8. The Referee proposes to include the sample size in the functional experiments.
We have included "n" as the Referee suggested.

Referee #2:

We want to thank the Referee for the helpful comments and insights. We have made substantial revisions to address the comments and suggestions of the Referee. Our responses are the following:

1. The Referee is concerned about the replication of Krox20 expression in the Mü4127 and Tg[elA:GFP] lines, specially the jagged border of expression.
We agree with the Referee in this crucial aspect. In order to properly clarify this, we performed new in situ hybridizations for *kalTA4* and *gfp* in 11hpf embryos. As shown now in Fig 1H-I and M-N, positive cells for both probes are observed in the wrong rhombomere. As the Referee knows sometimes the strength or intensity of the staining does not correlate with the quantity of mRNA, as this is the case for *gfp* probe. However, it is true that if we claim that there is cell sorting at later stages, we should be able to show jagged boundaries of GFP-expression at early embryonic stages. Thus, we complemented the analysis with anti-GFP immunostainings in Tg[elA:GFP] embryos at 11hpf. As shown in Fig 1R, jagged boundaries of GFP-expression are displayed in r3, with GFP-positive cells found in the neighboring GFP-negative territories. We consider this as a strong evidence that support our previous ones, and the transgenic lines used in this study recapitulate *krx20* spatial expression, although a bit delayed in time as previously mentioned in the text.

2. The Referee is not convinced by the Manual tracking shown in Fig 1M-R and Movie S4.
As the Referee points out it is not easy to follow all the cell nuclei in the very same movie. In former Movie S4 we presented the Maximal Intensity Projection of several z stacks, with all the dataset. However, since there are many nuclei and these events happen in 3D, many of them overlap in the MIP. Therefore, to improve the visualization we have done the following: i) to slow down a bit the speed of the video and ii) to decrease the number of tracked-nuclei displayed in the MIP; in this case it contains fewer nuclei than the really tracked (dots in M'-R') but they can be better seen (now Movie S3). In addition we have simplified Fig 2 and displayed only time-lapse stacks showing cell nuclei in red and r3/r5 in green (Fig 2M-R), and the colored dots displaying the position of the tracked cells and r3/r5 in white (Fig M'-R´).

3. The Referee comes back to point 1 and wanted us to include younger embryos (11 hpf) displaying jagged border using GFP fluorescence in the transgenic lines.
Immunostaining for anti-GFP in 11hpf Tg[elA:GFP] embryos has been performed and included in Fig 1R. See comments on point 1 for further details.

4. The Referee comments: "I do not see how the normal rounding up during cell division and their consequent integration into the tissue could suggest a physical barrier, instead of changes in cell adhesion or cell rigidity".
To summarize, the observation made by in vivo imaging is that a cell upon rounding during cell division incurs into a neighboring territory; however, when the division is accomplished daughter cells are brought back to the territory of origin. This can be seen in Movie S5. We think that a plausible explanation, considering that cells remain attached to the apical and basal side of the neural tube all the time, is that a change in elasticity permits the dividing cell to incur in the adjacent rhombomere (see as well Discussion, page 14). We have explained better our thinking and changed a bit the text (see page 8).

5. The authors show that there is no particular accumulation of fibronectin or integrin at the rhomobomere boundaries and then they conclude that "These results show that extracellular matrix does not play a major role in keeping rhombomeric cells apart". This is an overstatement, as functional experiments are required to rule out the role of ECM on this process.
The referee is right and we did not observe accumulation of FN in the rhombomeric boundaries at different time stages and no functional experiments to inhibit production of ECM proteins were performed. Thus, we cannot conclude that ECM does not play a major role in keeping rhombomeric cells apart and we have softened a bit our statement.

6. The Referee thinks that the effect on rhombomeric boundaries after using actomysin inhibitors (Blebbistatin and Rockout) only shows a correlation between cables and borders, as these inhibitors could be affecting other cellular functions that are the direct regulators of boundary formation.
This is the same than point 2 raised by Referee 1. Although Fig 6 displays the performed control experiments to demonstrate that the experimental conditions used to modulate myosin II activity do not affect other cellular functions that can be direct regulators of boundary formation, we have extended our controls. As mentioned to Referee 1, to support our hypothesis that the disassembly of the actomyosin cable was the result of the direct targeting of our time-controlled pharmacological treatments, and not a side effect of the drugs activity on other cellular processes, we made sure the dismantling of cables was reversible. Thus, we have complemented our studies with two alternative approaches. First, we injected the Rockout (together with an in vivo tracer such as DiO) directly in the lumen of the neural tube at the level of the hindbrain of Tg[myoII:mCherry] embryos and addressed its effects 1h after injection. This short and localized treatment resulted in loss of the actomyosin cable as fast as in 1h (data not shown), although the embryos were quite distorted due to the injection and this is why we did not include these data in the manuscript. At the time of injection, 18hpf, the lumen is very little opened (Gutzman and Sive, 2010), and it is difficult to assess that properly. Due to this, we took a second approach; we conditionally inhibited myosin II activity in Tg[myosinII:mCherry] embryos with Rockout during four hours; then the pharmacological agent was washed out and embryos let to develop for three additional hours 3h. Actomyosin cables were already dismantled after 4h of treatment; after washing out the drug, the cables were restored within 3h, strongly suggesting that the effect observed in the hindbrain is quite specific and reversible. Accordingly, we have included these results in Fig 6 with the rest of previously shown controls and discussed them in the manuscript.
Interestingly, there is a new tool, a photoinducible-blebbistatin (Kepiro et al, PNAs 2012) that could be used to address the local effect of the drugs. Although we have tried very hard to get this compound, we were unable to get some. Unfortunately, since it is not commercialized yet, we could not perform the desired experiments.

7. The Referee thinks that a better discussion about the mechanism for the epistatic relation between Eph-ephrin signaling and actomyosin cables should be established.
We thank the Referee to invite us to properly discuss this issue. We think the rescue of the phenotype is due to the left overs of the cable in the knock-downs for EphA4. Calyculin A can rapidly assemble the remaining of actomyosin structures, due to its potent and quick effect (Filas et al 2012). We have discussed this better in page 15. In addition, new experiments showing EphA4-staining colocalizes with the actomyosin cables in r3 and r5 (Fig 4P-P''') and ectopic EphA4 in Ephrin-rhombomeres resulting in enrichment of actomyosin components around the EphA4-positive cell (Fig 8A-D) allowed us to better discuss the possible epistatic mechanism (see Discussion page 16-17).

2nd Editorial Decision 19 November 2013

Thank you for submitting your revised manuscript to The EMBO Journal. Your revision has now been re-reviewed by referee #1 and I am afraid that the outcome is not very positive.

The referee appreciates that some aspects have been strengthened. However s/he also finds that two original raised concerns have not been adequately addressed. The first issue concerns the need for better support for that actin and myosin are needed for boundary formation and maintenance. The second issue relates to the analysis of the cellular processes involved in cell mixing at the boundaries. The referee finds that both issues have not been sufficiently addressed. I recognize that these points are not easy to address, but we would need better experimental support for them in order to consider publication here. Given that it is not clear if they can be resolved, I see no other choice but to reject the manuscript at this stage. However, should further experiments allow you to address these last 2 remaining issues then I can offer to take a look at a resubmission.

For the present submission, I am very sorry that I can't be more positive on this occasion.

REFEREE REPORT

Referee #1:

The ms has been revised along the lines suggested by the referees. However, there still remain a few points of major criticism that have not yet been sufficiently addressed by the authors. Specifically, the authors mention in the rebuttal letter that mosaic genetic alterations for actin/myosin are very difficult in zebrafish, and they thus were unable to perform these experiments. I disagree with this view, as there are many reports which have used cell transplantation or early blastomere injections to obtain mosaic expression of certain genes or distribution of morpholinos. For interfering with myosin activity, there are various genetic tools available such as constitutive active or dominant negative versions of rhoA and rok. Myosin II activity can also be modulated by injection of myosin phosphatase mRNA or morpholinos targeted against this gene.

I am equally unconvinced about the analysis of the cellular processes involved in cell mixing at these boundaries. Cell divisions are certainly one possibility, but there are multiple other processes, such as junctional remodeling, apical constriction, cell extrusion and apoptosis, which might also be involved. If boundary violation would only be due to cell divisions, the authors could block cell divisions and then determine if cell mixing still occurs in embryos with compromised boundary formation.

Together, I do acknowledge that this is a potentially very interesting observation. However, the experimental analysis of this phenomenon is not yet at the level expected for an EMBO paper.

Additional correspondence 22 December 2013

I thank you again for the interest in our work. I feel that we can address the major points raised by the referee and return an amended manuscript suitable for publication in EMBOJ.

RESPONSE TO REVIEWER 1

We very much thank the constructive criticisms of the referee that helped us to substantially improve our manuscript. The referee acknowledges that the revised version has improved the original manuscript along the lines suggested. However, there are two points that in his/her view deserve further attention. I would like to comment on these issues raised by the reviewer in order to bring our alternative arguments and potential solutions.

Issue 1. The referee disagrees with our argument to justify that mosaic genetic alterations for actin/myosin are very difficult in zebrafish. He/she correctly points that there are several reports that have used cell transplantation of early blastomere injections to obtain mosaic expression of certain genes or distribution of morpholinos. Thus, the suggestions are: i) to interfere with MyosinII activity using various available genetic tools such as constitutive active or dominant negative versions of rhoA and rok; ii) Modulation of MyosinII activity by injection of myosin phosphatase mRNA or morpholinos targeted against this gene.

I agree with the referee that the best to support our functional data using pharmacological agents is to have a genetically based tool. This would allow to directly demonstrate the role of actomyosin cables in boundary formation and cell sorting in the hindbrain. Actually the only way I can think of doing so is to use the tools that the referee suggested, such as the dominant active/negative RhoA forms, and to conditionally activate their expression at the stage of interest. In my opinion, it is crucial to be able to interfere with MyosinII function late during embryonic development (15hpf onwards) to be able to conclude that the obtained phenotype is direct. We can do this experiment since we set up the conditional expression system as showed in the Fig. 8. We favored the experiment of ectopic expression of EphA4 because we were interested in getting information about the upstream regulators. But nevertheless, I do not have any objection to carry out the ectopic expression of dominant active/negative RhoA, with the expertise of this new conditional system.

However, I am somehow concerned about the suggestion about carrying out explant experiments to

address the function of actin/myosin. As pointed out by the referee in the first revision, it has been shown that the actomyosin system has pleiotropic functions that include the generation of germ-layer boundaries (Rohani et al, 2011; Fagotto et al, 2013). Therefore, it is very likely that any morphant or ectopic mRNA expression of these components at early stages (1 cell-stage injection) will disrupt cellular dynamics and compromise cell viability. Therefore, although cells from these embryos can be transplanted, most probably their viability will be seriously hampered before 16hpf, the stage at which we would like to make the transplants. The timing of the phenomenon is critical, and this is why we thought that it was the best to use pharmacological drugs that allowed us to incubate the embryos in a time-controlled manner, at the desired stages. Probably, I did not state this point clearly enough in the rebuttal, but I do think that perturbing MyosinII function at early stages will not bring us clear information on what happens in the hindbrain boundaries at late embryonic stages.

Issue 2. The Referee raises the very important question what are the cellular processes involved in cell mixing at boundaries when the actomyosin cable is compromised.

In the first revision the referee asked us whether spreading cells were migratory or did they just passively intermix with surrounding tissues. We answered to this question with our current thinking: the LOF experiments unveil cells that at the time we disrupted the cable were dividing close to the boundaries, and most probably because cannot be brought back to the rhombomere of origin, are found in the neighboring territory. This answer was based in our time-lapse experiments, which demonstrate that neuroepithelial cells are always attached to the apical and basal sides, even upon cell division (as shown in several previous articles), and that cell division challenges the boundaries. There is no cell migration in the neuroepithelium (except for neural crest cells exiting the neural tube) and we re-checked again this in all movies. To the best of our techniques, our data exclude the possibility of cell migration.

In this second review the referee argues that cell divisions are certainly one possibility, but there are multiple other processes, such as junctional remodeling, apical constriction, cell extrusion and apoptosis, which might also be involved. The referee points out that if boundary violation would only be due to cell divisions, we could block cell division and then determine if cell mixing still occurs in embryos with compromised boundary formation.

I fully agree with the referee that cell division is one of the possible mechanisms and we briefly mention this in the Discussion (p16), although never making it a main conclusion of the work. Since we felt that it was not the focus of the manuscript, we did not follow this line further. The suggestion of the referee to block cell proliferation and inhibit MyosinII activity in order to see whether this results in cell mixing/sorting is no doubt very sound. I agree that this will bring interesting observations about the mechanism by which cells of different identity mix upon loss of boundaries. However, most likely these observations by themselves will not be enough to substantiate a model on the underlying mechanism, but they will open other new questions. This is a feasible experiment and we have all the reagents to carry it out. However, it is difficult for me to foresee that this will help us to better understand the role of the actomyosin cable in segregating rhombomeric cells, which is the main goal of the paper. Having said that, if the referee thinks that this is necessary, we are open to do the experiment and to explore whether or not this brings more strength to our observations.

In summary, I thank again for the interest in our work. I feel that we can address the major points raised by the referee and return an amended manuscript suitable for publication in EMBOJ.

Additional correspondence (editor) 27 January 2014

As discussed, I have passed on your point-by-point response to referee #1 and I have now heard back from the referee. The referee is happy with the suggested experiments. Regarding the 2nd issue (cell mixing) the referee does suggest to inhibit cell divisions using drugs and then check whether boundary violation still takes place. I think that these are reasonable experiments that would add valuable insight and I would encourage you to carry them out.

2nd Revision - authors' response 24 December 2013

RESPONSE TO REVIEWER 1

We very much thank the constructive criticisms of the referee that helped us to substantially improve our manuscript. The referee acknowledges that the revised version has improved the original manuscript along the lines suggested. However, there are two points that in his/her view deserve further attention. We have performed the experiments we previously proposed and I will discuss the two main issues as follows.

Issue 1. The referee suggested interfering with myosin II activity using genetic tools to directly demonstrate the role of actomyosin cables in boundary formation and cell sorting in the hindbrain.

We generated the dominant negative/constitutive active RhoA forms, and conditionally activate their expression at 14hpf in Tg[myoII:mCherry] embryos for mosaic analysis as the Referee suggested. We included in the paper only the results obtained with the CA-RhoA (Fig 6J-O). When we induce CA-RhoA mosaic expression, we get an enrichment of myosin II structures in any ectopic location in the hindbrain (Fig 6M-o, yellow arrows). These results support the hypothesis that activation of RhoA is sufficient to assembly myosin II structures in the hindbrain. In the new version of the manuscript, these results are included in Fig 6J-O, explained in p12 and discussed in p15.
However, it is very difficult with our system to get ectopic expression at the apical side of the neural tube just where the cable is. We did several attempts to express DNRhoA there, but in none of them we got cells expressing the construct at the very same apical side of the boundary. Thus, experimentally this is very challenging due to the limitations of this conditional gain-of-function system. If we increase the time or concentration of tamoxifen to have more recombination events, the embryos do not recover from the treatment, and as we perform the experiment we are unable to drive or modulate the induction to the desired territories.
In spite of this, I feel confident we have genetic evidences to support our model, specifically: i) ectopic EphA4 expression experiments induce enrichment of actomyosin structures only in r4 and r6 (where Ephrins ligands are), (Fig 8), and ii) ectopic CA-RhoA is sufficient to assembly myosin II structures in any rhombomere, since this is a downstream effector and therefore when expressed ectopically is independent of EphA/Ephrin signalling.

Issue 2. The Referee raises the very important question what are the cellular processes involved in cell mixing at boundaries when the actomyosin cable is compromised. He/she suggested to block cell proliferation and inhibit myosinII activity in order to see whether this results in cell mixing/sorting.
We performed the suggested experiment, to determine if cell mixing still occurred in embryos with compromised boundary formation. For this, we inhibited myosin II activity and blocked cell proliferation at distinct cell cycle points using different pharmacological agents such: i) nocodazole, which interferes with the polymerization of microtubules inducing mitotic arrest; ii) aphidicolin and hydroxyurea, which block the cell cycle at early S-phase by different mechanisms. The results shown in Fig S9 demonstrate that cell mixing is reduced when cell proliferation is blocked, however we cannot avoid cell mixing in all embryos. This result suggests that cell division is one of the possible mechanisms although these observations are not enough to substantiate a model on the underlying mechanism. For this reason, we discus this issue briefly in the Discussion (p16) and we include a supplementary figure (Fig S9) with the results.
In summary, I thank again for the interest in our work. I feel that we have addressed the major points raised by the referee and hope that this new amended manuscript is suitable for publication in EMBOJ.

Acceptance letter 16 January 2014

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been re-reviewed by referee #1 who is as you can see below happy with the revised version. I am therefore very pleased to accept the paper for publication here.

Please see below for information on how to proceed.

REFEREE REPORT

Referee #1:

The authors have satisfactorily addressed all remaining points and the ms is now suitable for publication in EMBO Journal.

© European Molecular Biology Organization