Admp regulates tail bending by controlling ventral epidermal cell polarity via phosphorylated myosin localization in *Ciona*

Yuki S. Kogure, Hiromochi Muraoka, Wataru C. Koizumi, Raphaël Gelin-alessi, Benoit Godard, Kotaro Oka, Carl-Philipp Heisenberg and Kohji Hotta
DOI: 10.1242/dev.200215

Editor: Thomas Lecuit

Review timeline

Original submission:	21 September 2021
Editorial decision:	26 October 2021
First revision received:	13 April 2022
Editorial decision:	6 May 2022
Second revision received:	15 August 2022
Accepted:	12 September 2022

Original submission

First decision letter

MS ID#: DEVELOP/2021/200215

MS TITLE: *Admp* regulates tail bending by controlling the intercalation of the ventral epidermis through myosin phosphorylation.

AUTHORS: Yuki S. Kogure, Hiromochi Muraoka, Wataru C. Koizumi, Raphael Gelin-alessi, Benoit Godard, Carl-Philipp Heisenberg, Kotaro Oka, and Kohji Hotta

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 strong interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. All reviewers concur that the manuscript requires extensive rewriting and clarification as well as additional experiments to strengthen the key conclusions. The characterization of mechanisms of MyosinII polarity is clearly beyond the scope of the present study. All in all the reviewers make a number of useful and constructive comments to improve the quality of the presentation. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

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

Reviewer 1

Advance summary and potential significance to field

This is an interesting manuscript showing that the BMP ligand ADMP plays a major role in controlling Ciona tail bending via effects on cell shape and behavior in the ventral epidermal midline. A transient but significant ventral bend in the tail is seen in many chordate embryos and provides an intriguing model for how interactions between tissues contribute to shape overall. The mechanisms are generally unknown and the only prior work in Ciona supported a completely different mechanism involving ventrally biased contractility in the notochord. The paper has some non-trivial issues, but the overall conclusions that ADMP is very important in tail bending and that it acts at least in part through effects on the ventral midline are well supported.

Comments for the author

The experiments are generally of a reasonable standard but the paper needs extensive editing to improve its clarity in English. This is not just a stylistic issue- the arguments and conclusions are not always clear. With some additional work, however, I think this could absolutely become a Development paper. My overarching concern at present is that the manuscript dances quite awkwardly around discrepancies between their observations and the conclusions from the Lu et al 2020 paper from Bo Dong's group. That paper proposes that DV asymmetries in actomyosin contractility in the notochord are the major cause of ventral tail bending. This manuscript tries to reconcile their very different conclusions by proposing that ventrally biased notochord contractility controls an early stage of bending from stage 18-20 whereas ADMP-dependent phenomena in the ventral midline dominate thereafter. Their data, however, contradict that. Figure 1 shows that ADMP MO embryos lack tail curvature as early as stage 20, indicating that ADMP-dependent phenomena act early as well as late. Supp Figure 2 shows that ADMP MO embryos with straight tails still have ventral biases in notochord actomyosin, indicating that biases within the notochord are not sufficient to bend the tail in and of themselves.

One straightforward explanation for these discrepancies would be if the Dong lab paper is wrong and DV differences in notochord actomyosin localization are not actually that important in ventral tail curvature. That paper presents strong evidence that there is a considerable DV bias in notochord actomyosin and that notochord contractility is important for tail bending, but there is no strong evidence that it is the bias in actomyosin localization that is actually driving the bending. An alternate view is that the ventral accumulation of actomyosin in the notochord might reflect earlier morphogenetic events such as the DV biases in notochord intercalation and resulting transient ventral groove described years ago by Ed Munro, and that the ventral bending of the tail may be powered by notochord elongation but ultimately controlled more by differences in cell/tissue properties and behaviors between the tissues above versus below the notochord.

I sympathize with the authors of the current manuscript who most likely do not want to stir up controversy about prior work from another group, but I think it is important that they address these discrepancies more directly. Fortunately, there are a couple of straightforward experiments that would shed a great deal of light. They may even have the needed images and just need to do new analyses. The first would be to more thoroughly document the time course of tail bending in ADMP morphants or dorsomorphin treated embryos. If they are right that the Dong phenomenon controls early curvature and that ADMP-dependent phenomena in the ventral midline control later curvature, then tail bending should be normal at stages 18 and 19. No quantitation is shown but the morphant tails look very straight as early as stage 20 in Figure 1, so I would be surprised if there wasn't a curvature defect from the beginning… The second experiment would be to better document the DV differences in notochord actomyosin at earlier stages in ADMP morphants and/or dorsomorphin treated embryos. Supp Figure 2 is intriguing but not entirely satisfying because it is at a relatively late stage when DV differences are no longer seen in wildtype embryos. What about earlier though?

If the ADMP MO blocks all tail curvature but has no effect on DV differences in notochord actomyosin accumulation, that would suggest that those DV differences aren't critical for tail curvature. Alternatively ADMP might be an upstream regulator of curvature-related phenomena in both the notochord AND the ventral midline epidermis.

My expectation here is not that the manuscript should be reorganized around attacking Lu et al 2020 but more that Lu et al conclusions should not be treated as incontrovertible fact and this manuscript should be a bit more direct about pointing out when they are in tension. I suspect that tail curvature may prove to be quite complicated with many tissues playing a role.
Smaller comments:

line 54: If terms like longitudinal are used then they need to be defined.

line 26, 79 etc: In many places, the ms seems to assume that there is a single upstream regulator of tail bending. I can't tell if this is an English issue or not but that does not seem like a reasonable assumption. Couldn't there be different upstream regulators of curvature in different tissues?

line 100: 'another molecular pathway' This may again be an English thing but isn't the issue here that ADMP may have different downstream targets in different tissues and not the use of different signaling pathways?

line 113: Is there any evidence that ADMP can act at a distance in Ciona embryos? Is this paragraph even needed?

section beginning line 179: The relationship between the triangular cells and intercalation is not at all clear. I appreciate that all of these midline cells intercalate and a subset of them appear apically constricted and thus triangular in some sections, but is there some deeper relationship being proposed here? If not, I think that the manuscript should be clearer in stating that the relationship between midline intercalation and midline apical constriction is not clear and they may just be two independent ADMP-driven behaviors of the ventral midline. Line 207 at the end of this section is particularly unclear: 'are suggested to invade from the basal plane side'???

lines 223-227 are similarly unclear. Is the hypothesis that apically constricting cells can't intercalate and this delays intercalation on the ventral side? That doesn't seem unreasonable but is there any evidence for that? The 'on the contrary' possibility isn't clear at all.

What is the point of lines 240-253? Is it just examples of where BMP signaling has been shown to influence cytoskeletal polarity? Is there some larger inference?

The model in lines 261-265 seems too strongly stated given that the functional relationship between pMLC apical contractility and intercalation are not clear.

line 266: 'bilateral side of the basal domain'???

The conclusion would also benefit from being open to tail bending potentially being a very complicated process. This manuscript makes a strong case that the ventral midline is important, but differential properties or behaviors between the CNS and the endodermal strand could well be involved too.

In Fig 1C, what stage was used for the quantitation? All these bar charts would benefit from showing the number of cells or embryos examined.

The model flowchart thing in Figure 5 is very busy and should be simplified.

The legend for Supp Fig 3 is hard to understand and needs to be improved.

Reviewer 2

Advance summary and potential significance to field

This paper describes that Admp regulates myosin phosphorylation in the ventral epidermis and controls the ventral vending, which constitutes a proposal to revise the preexisting model which explains the bending as the result of asymmetric distribution of actomyosin in the notochord.

Comments for the author

Major points
This paper describes that Admp regulates myosin phosphorylation in the ventral epidermis and controls the ventral vending, which constitutes a proposal to revise the preexisting model which explains the bending as the result of asymmetric distribution of actomyosin in the notochord. Although this reviewer appreciates the challenge and admit the relationship between Admp and myosin phosphorylation is a fact, their model does not sufficiently explain the morphological event as the function of Admp.

The authors' claims are based on the phenotype of embryo in which Admp is knocked-down by MO. Given Admp which is expressed endoderm and lateral epidermis (line, 112) and required for the expression of Bmp2/4 (line 121), it is not shown how the phosphorylation of Smad is affected in the ventral mid-line of the morphant; Fig.1D just shows the Smad signaling in normal embryos. Also, it is not examined how myosin phosphorylation is localized preferentially in the apical side and only in a limited number of ventral epidermal cells in normal embryo despite of BMP2/4 and Smad activities along the entire AP axis. If Admp regulates the intracellular localization of pMLC, the authors need to show that the total amount of pMLC is unchanged in control and the morphant embryo (Fig. 2) and how the localization is regulated. To examine the competence and threshold of the ventral epidermal cells for the apical constriction, over expression of Admp would be very important. Along this line, the phenotype of Y27632 embryo is inadequate to be compared with that of Admp morphant because Y27632 is expected to suppress total pMLC in the cells, and thus the similarity of the phenotype could not support the authors' claim.

Overall, this paper lacks insight into the mechanism how Admp regulates the localized myosin phosphorylation through cell-cell interaction and therefore it appears to be a descriptive piece of work, despite of the tissue mechanics experiments, quantitative data analyses, and 3D reconstruction of cell model. Furthermore, as the laser cutting points in the ventral side are too many and relaxation of bending only of the entire larva is observed, there is no clue to understand the relationship between cellular tension and myosin phosphorylation of the ventral epidermis cells that are assumed to drive the bending at a single cell level. The relaxation could also be explained by the combinatorial effect of ventrally-biased robust cell-to-cell adhesion and an internally generated force by notochord elongation.

In summary, the possible involvement of Admp in the event is potentially interesting, the current data are not sufficient to provide additional insight on the ventral bending mechanism and to convince readers that the authors' proposal is superior over the past model.

Other comments
Fig. 1A, it is difficult to distinguish dorsal and ventral sides of Admp morphants as

the neural tube was not visualized. In late tailbud stages, DV orientation appears to be reversed according to the statement, “bending to dorsal (dorsiflexion) was observed similar to WT (line, 84)”. At least D/V should be labeled in the photos.

Line 93, the Admp gene is, shoud be written as “The Admp encodes”.

Fig. 1B, it is quite strange that bending angle exceeds 90 for WT. Explanation for this is necessary. If the length of notochord is unchanged, the distance between the anterior most and posterior most cell may be useful for quantitation.

Fig. 1D, the right most of the bottom illustration must be representing lateral view but not ventral. As mentioned above, p-Smad staining of Admp morphant should be shown to demonstrate the effect of Admp.

Fig. 2D, A/B ratio of pMLC should be shown for non-triangular cells of control and morphants.

Fig. 3C, single cell level analysis and presentation of recoiling profile of both triangular and non-triangular cells, ideally with a kymograph, are informative.

Fig. 4, the description “Triangular-shaped cells” is adequate for 2D structure but not for 3D structure like cells. Are they triangular pyramid or triangular prism? line, 183, it is not clear what “anterior-posterior border” represents. Does it mean the anterior-posterior border of columnar cells? Does 3D reconstruction of Z stack images of the cell support either?

Fig. 5, in general, if intercalation is incomplete, it is expected that embryo becomes wider along L/R axis, as shown for the PCP mutants. Is this true for Admp morphant? If the width of morphant embryo is not affected, how is it explained?

Suppl. Fig. 2, the polarity (DV ratio of F-actin accumulation) is affected in Admp morphant and in fact the pattern is changed although the authors just stated that the asymmetric localization is still observed (line 106-107). Some explanation for the phenotype is necessary. If the change is true, it might affect the ventral bending.

Suppl. Fig. 3,
It is misleading that the plot number counts from the posterior side, while notochord cell number counted from the anterior side (line 405) for Fig. 1B. In addition, it is uncomfortable to see the head/tail position is reversed for this figure. The entire orientation should be reversed like others.

The data in many experiments should be properly quantitated. The number of independent experiments/observations and the number of data point per experiments must be indicated.

Fonts for figures seems to be randomly chosen without any rules, which disturbs readers’ understanding. Figures should be improved in many aspects, usage of colors, font size, boldness, resolution, etc.

Reviewer 3

Advance summary and potential significance to field

In this manuscript, Kogure et al. present an analysis of the role of the BMP-family member ADMP during the process of ventral tail bending during the tailbud stages of the ascidian Ciona robusta (previously known as Ciona intestinalis type A).

A previously published Development article (Lu et al., 2020), which established that tail bending is autonomous to the tail, requires a ventral activation of myosin II regulatory light chain in the notochord, as well as a synergy with other tail tissues.

Here the authors have focused on the role of the ventral epidermal midline, and the control of its morphogenesis by ADMP. They first report that morpholino- and small molecule-mediated inhibition of ADMP prevents ventral tail bending, and that this inhibition is associated with a change in the shape of some of the ventral epidermal cells, called “triangular cells” (see below why I put quotes…). These cells accumulate F-actin and activated myosin II regulatory light chain in an ADMP- and Rock-dependent manner. Using laser cutting experiments, the authors further demonstrate that the tension accumulated in ventral, but not dorsal, epidermal cells is necessary for tail bending. Finally, the authors argue that the “triangular cells” are undergoing intercalation, which they show is ADMP dependent.

Overall, the experiments appear rigorously carried out (but not properly described, see below) and the work is in principle of sufficient interest to be published in Development. It is complementary to the previous Lu et al. study, and of similar interest.

Comments for the author

I however have several major and minor issues that the authors need to address prior to publication (many of them should be addressed before publication in any journal.).

1) My first comment is purely methodological. The legends of the figures are simply not sufficient to understand what is shown. As will be seen below, there are several instances where lack of details in the description of what is shown severely confused me. In this paragraph, I will use figure 3 as an example, but all figure legends should be extensively revised and improved. In figure 3, it is nowhere said what the pictures in the panel represent: are these confocal pictures? Maximal projections? Single optical sections? Time is mentioned in panels A and C, but no scale is given. How much time elapsed between "before" and "after"? The heatmap is also not explained: what time scale are we looking at here: seconds, minutes, more? The explanation of "relaxation" in panels B and D leaves me in the fog. In the absence of critical experimental and image analysis details, this work will be very difficult to reproduce or even understand. The same comment applies to supplementary figures. For instance, the DV axis should be shown in Fig S2A. How the ratio in S2B is precisely calculated should be indicated (is this the peak intensity value? The intensity value integrated on several pixels flanking the peak value? The two measures may give different results as the dorsal and ventral intensity peaks have different shapes), etc.

2) I find the term "triangular cells" very confusing, as cells are 3D objects while a triangle is a 2D shape. If the 3D shapes shown in S5C and D represent a "triangular cell", then such a cell will not appear as triangular on all 2D optical sections. Many non triangular cells in a 2D section may turn up to be triangular in another and the 2D approach may be unsuitable. The lack of proper description of what the authors are showing makes me wonder whether my overall confusion is due to a fundamental flaw in the work presented of to its very poor presentation! I suggest to change the presentation of these cells, possibly by first describing their 3D shape and orientation in the context of the embryo and then explain how this translates on the 2D sections shown throughout the manuscript.

3) I understand that Figure S5C may describe the 3D shapes of a "triangular cell" (blue? Please confirm) and of a non-triangular one (mauve? Please confirm). But what is the orientation of these cells with respect to the embryo? Is anterior to the top and posterior to the bottom? How does this relate to intercalation?

4) I am puzzled by the relationships between apical myosin accumulation in the "triangular cells", intercalation of ventral epidermal cells and ventral tail bending. Cell intercalation, in convergent extension movements for instance, is generally a mechanism driving tissue elongation. The authors say that ventral epidermis midline cells fail to undergo intercalation when ADMP signalling is blocked. This sounds counter-intuitive to me, as this should favor tail bending! Indeed Figures 4C and S6 do not really show a defect in cell intercalation (there are 3 rows of cells with or without ADMP signalling) but rather a defect in the organisation of these rows, which become irregular in the absence of ADMP. This should be clarified.

5) This may not strictly be in line with the authors argumentation, but if intercalation during convergent extension is indeed a player during tail bending, I would expect: 1) that ventral cell intercalation is delayed compared with dorsal intercalation (this is indeed shown on Figure S7!); 2) that ADMP may be responsible for this delay. Testing this latter hypothesis would be easy and would much strengthen the manuscript.

6) The systemic use of the Rock-inhibitor Y27632 may not be informative concerning the role of myosin or ADMP in the epidermis, as this treatment inhibits Rock and myosin regulatory light chain activation in both epidermis and notochord. Using a more targeted approach, Lu et al. (2020) showed that myosin regulatory light chain activity in the notochord is needed for tail elongation. How do the authors differentiate the epidermal and notochord role of myosin in these experiments? I suggest either removing these experiments (Fig 3C) or replacing them with dorsomorphin-treated embryos (Fig S4).

7) Is ADMP only affecting the epidermis? Although the legend of Figure 1 is unclear on this point, I imaging that the P-smad staining only shows the epidermis. Are the authors sure that ADMP does not also signal to the notochord? Figure 2A reveals that the asymmetric distribution of actin and activated myosin in the notochord is affected by the inhibition of ADMP signalling... What happens at earlier stages?

8) Minor issues:
a. The English syntax should be checked throughout the manuscript. Some sections, presumably coming from one of the two labs involved, are very well written. This suggests that the authors should better coordinate to provide a uniformly satisfying text.
b. The author may want to comment why the effect of the laser cutting is much more subtle than the ADMP morpholino.
c. Line 80: scientists do not aim at "confirming their hypothesis" but at testing it rigorously and without preconception of the results, as formalised by Karl Popper.
d. I am not convinced that there are enough data in vertebrate systems to claim (in the abstract and in the discussion) that the identified role of ADMP may be conserved in bilaterians.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field...

This is an interesting manuscript showing that the BMP ligand ADMP plays a major role in controlling Ciona tail bending via effects on cell shape and behavior in the ventral epidermal midline. A transient but significant ventral bend in the tail is seen in many chordate embryos and provides an intriguing model for how interactions between tissues contribute to shaping overall. The mechanisms are generally unknown, and the only prior work in Ciona supported a completely different mechanism involving ventrally biased contractility in the notochord. The paper has some non-trivial issues, but the overall conclusions that ADMP is very important in a tail bending and that it acts at least in part through effects on the ventral midline are well supported.

ANS#1) Thank you for your comments. We have addressed all issues you suggested (details below). We have also added further experiments to strengthen our finding that ADMP affects the ventral midline and tail bending.

Reviewer 1 Comments for the Author...

The experiments are generally of a reasonable standard, but the paper needs extensive editing to improve its clarity in English. This is not just a stylistic issue- the arguments and conclusions are not always clear. With some additional work, however, I think this could absolutely become a Development paper.

ANS#2) According to your suggestion, the structure of the revised manuscript has been changed drastically to improve and clarify our conclusions, with the additional experiments, including the phenotype analysis of the early tailbud stages of Admp-MO, dorsomorphin, and the anti-pMLC staining of ectopically Bmp-expressed embryos.

My overarching concern at present is that the manuscript dances quite awkwardly around discrepancies between their observations and the conclusions from the Lu et al. 2020 paper from Bo Dong's group. That paper proposes that DV asymmetries in actomyosin contractility in the notochord are the major cause of ventral tail bending. This manuscript tries to reconcile their very different conclusions by proposing that ventrally biased notochord contractility controls an early stage of bending from stage 18-20, whereas ADMP-dependent phenomena in the ventral midline dominate thereafter. Their data, however, contradict that. Figure 1 shows that ADMP MO embryos lack tail curvature as early as stage 20, indicating that ADMP-dependent phenomena act early as well as late. Supp. Figure 2 shows that ADMP MO embryos with straight tails still have ventral biases in notochord actomyosin, indicating that biases within the notochord are not sufficient to bend the tail in and of themselves.

ANS#3) Thank you for your suggestion. We additionally examined the bending angle of Admp MO embryo at earlier stages st. 18 to st. 20 (Fig. 1 CD). The results supported your suggestion. ADMP-dependent phenomena act both early and late, and the morphants, Admp-MO and dorsomorphin-treated embryo, significantly reduced the tail bending angle even in the early stages.

Furthermore, with the data of Suppl. Fig. 2, these morphant embryos with straight tails still have ventral biases in notochord actomyosin, indicating that asymmetrical localization within the notochord is not sufficient to bend the tail in and of itself. Therefore, we have modified our proposals in the revised manuscript, as follows.

In line 92, When the bending angle of *Admp* MO embryos and WT at st. 18 to 22 (in which WT ventroflexion occurs) were compared (Fig. 1B), that of the *Admp* MO embryos was significantly reduced (Fig. 1C; N = 11/11). The tailbud embryos treated with dorsomorphin – an Admp/Bmp signaling inhibitor – also significantly reduced the bending angle (Fig. 1D; N = 5–12, Suppl. Fig. 1A and 1B). Therefore, it was suggested that Admp/BMP specifically regulates the ventroflexion of the ascidian tailbud embryos. Although a previous study has shown that notochord actomyosin asymmetry is responsible for tail bending during st. 18 to st. 20 (Lu et al., 2020), asymmetric localization of notochord actomyosin in the dorsoventral region was observed even in the *Admp* MO morphant at st. 18 to 22 (Suppl. Fig. 2).

One straightforward explanation for these discrepancies would be if the Dong lab paper is wrong and DV differences in notochord actomyosin localization are not actually that important in ventral tail curvature. That paper presents strong evidence that there is a considerable DV bias in notochord actomyosin and that notochord contractility is important for tail bending, but there is no strong evidence that it is the bias in actomyosin localization that is actually driving the bending. An alternate view is that the ventral accumulation of actomyosin in the notochord might reflect earlier morphogenetic events such as the DV biases in notochord intercalation and resulting transient ventral groove described years ago by Ed Munro, and that the ventral bending of the tail may be powered by notochord elongation but ultimately controlled more by differences in cell/tissue properties and behaviors between the tissues above versus below the notochord.

ANS#4) Thank you for your suggestion. We agree with your opinion about revising earlier Admp knock-down and dorsomorphin-treatment experiments. We did not completely rule out the possibility that the notochord DV bias drives the bending, but the role might be very weak because the DV bias remains in the Admp- MO/Dorsomorphin-treated embryos with straight tails (Supple. Fig. 2). We discuss these descriptions in the revised manuscript, following your advice.

In the discussion section, line 306, "A previous study indicated that the notochord generates asymmetric contraction force before st. 20 by the asymmetrical localization of actomyosin in the notochord (Lu et al., 2020). However, Admp MO embryos with straight tails still have ventral biases in notochord actomyosin (Suppl. Fig. 2). This indicated that Admp MO does not control asymmetrical localization of notochord actomyosin, and the localization seemed not enough to cause ventroflexion. The ventral accumulation of actomyosin in the notochord might reflect earlier morphogenetic events, such as the notochord intercalation, making a transient ventral groove (Munro and Odell, 2002)."

I sympathize with the authors of the current manuscript who most likely do not want to stir up controversy about prior work from another group, but I think it is important that they address these discrepancies more directly. Fortunately, there are a couple of straightforward experiments that would shed a great deal of light. They may even have the needed images and just need to do new analyses.

The first would be to more thoroughly document the time course of tail bending in ADMP morphants or dorsomorphin treated embryos. If they are right that the Dong phenomenon controls early curvature and that ADMP-dependent phenomena in the ventral midline control later curvature, then tail bending should be normal at stages 18 and 19. No quantification is shown but the morphant tails look very straight as early as stage 20 in Figure 1, so I would be surprised if there wasn't a curvature defect from the beginning…

ANS#5)Thank you for your advice. We described the time course of tail bending in ADMP morphants or dorsomorphin treated embryos. We added the picture of st. 18 and st. 19 to Figure 1A and quantified the bending angle of st.18–20 of WT and Admp-MO, dorsomorphin (Fig.1C and 1D). As expected, it showed Admp also controlled the early stage of tail bending.

The second experiment would be to better document the DV differences in notochord actomyosin at earlier stages in ADMP morphants and/or dorsomorphin treated embryos. Supp Figure 2 is intriguing but not entirely satisfying because it is at a relatively late stage when DV differences are no longer seen in wildtype embryos. What about earlier though? If the ADMP MO blocks all tail curvature but has no effect on DV differences in notochord actomyosin accumulation, that would suggest that those DV differences aren't critical for tail curvature. Alternatively, ADMP might be an upstream regulator of curvature-related phenomena in both the notochord AND the ventral midline epidermis.

ANS#6)We observed the Admp MO and dorsomorphine-treated embryo at st 18 to 22, and they remain asymmetrical accumulation of actomyosin in notochord as WT (Suppl. Fig. 2). From this result, it was suggested that the DV differences of actomyosin of notochord are not regulated by Admp. This would suggest that those DV differences are not critical for the ventroflexion.

My expectation here is not that the manuscript should be reorganized around attacking Lu et al. 2020, but more that Lu et al conclusions should not be treated as incontrovertible fact and this manuscript should be a bit more direct about pointing out when they are in tension. I suspect that tail curvature may prove to be quite complicated with many tissues playing a role.

ANS#7)We have taken your advice seriously and have made significant improvements to the structure of our manuscript.

Smaller comments:

Line 54: If terms like longitudinal are used, then they need to be defined.

ANS#8）We amended the term longitudinal, replacing it with AP.

line 26, 79 etc: In many places, the ms seems to assume that there is a single upstream regulator of tail bending. I can't tell if this is an English issue or not but that does not seem like a reasonable assumption. Couldn't there be different upstream regulators of curvature in different tissues?

ANS#9）We amended the following.

Line 26, "Here, we identify one of the upstream regulators of tail bending."

Line 85, "We focused on the *Admp* as one of the candidates for the upstream regulators of the tail bending."

line 100: 'another molecular pathway' This may again be an English thing but isn't the issue here that ADMP may have different downstream targets in different tissues and not the use of different signaling pathways?

ANS#10） We have changed the description as follows.

Line 110, "we hypothesized that *Admp* might control the morphology of tail bending, apart from the downstream pathway of *Msxb* that controls the differentiation of ventral peripheral neurons (Roure and Darras, 2016; Waki et al., 2015)."

line 113: Is there any evidence that ADMP can act at a distance in Ciona embryos? Is this paragraph even needed?

ANS#11) We add the reference to line 117: Since Admp is a morphogen, it can act at sites far from its original place expression (Imai et al., 2012).

section beginning line 179: The relationship between the triangular cells and intercalation is not at all clear. I appreciate that all of these midline cells intercalate and a subset of them appear apically constricted and thus triangular in some sections, but is there some deeper relationship being proposed here? If not, I think that the manuscript should be clearer in stating that the relationship between midline intercalation and midline apical constriction is not clear, and they may just be two independent ADMP-driven behaviors of the ventral midline.

ANS#12) Thank you for your suggestion. We also think there are likely "two independent ADMP-driven behaviors of the ventral midline."

We added this sentence to the discussion as follows. Line 282: "All midline epidermal cells intercalate and form apically constricted boat cells during early intercalation (Fig. 3). The relationship between midline intercalation and midline apical constriction is unclear, but both events are Admp-driven simultaneous behaviors of the ventral midline."

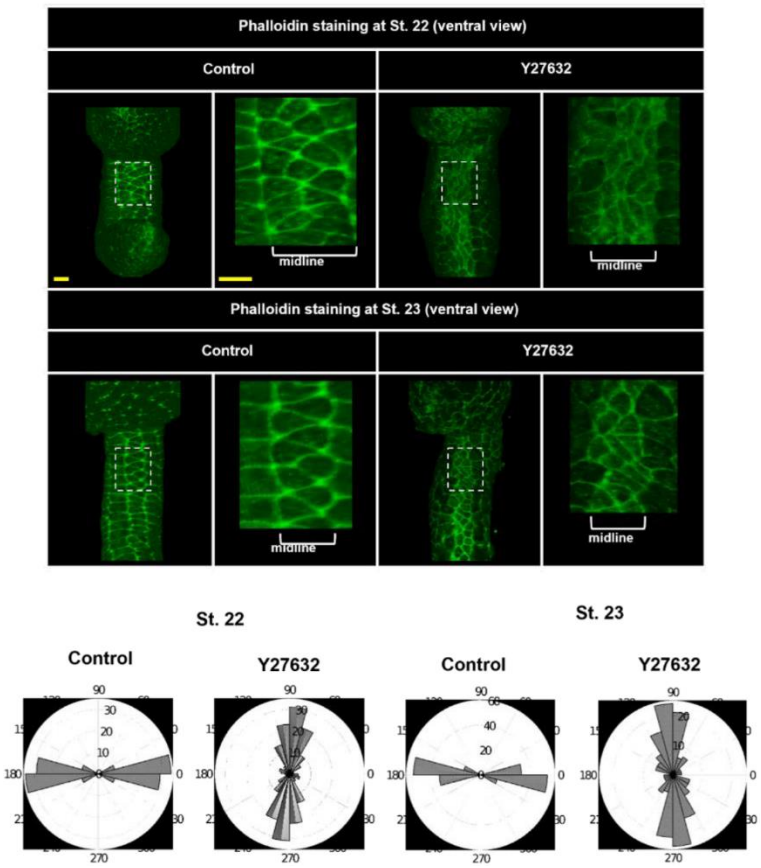
Line 207 at the end of this section is particularly unclear: 'are suggested to invade from the basal plane side'???

Ans#13) We deleted this section.

lines 223-227 are similarly unclear. Is the hypothesis that apically constricting cells can't intercalate and this delays intercalation on the ventral side? That doesn't seem unreasonable but is there any evidence for that? The 'on the contrary' possibility isn't clear at all.

ANS#14) Sorry if this was misleading. All ventral midline epidermal cells intercalate to form boat-shaped cells which look like "apically constricting cells" at a given plane (now we call "TSBC"). We hypothesized that boat cells are resistant to notochord AP elongation. Supporting this, the L-R bilateral polarity of boat cells is disrupted by the Y27632 treatment (see below

figures, for reviewer only). We believe that boat cells play a role in temporarily halting AP elongation during early intercalation.



(upper) Localization of actin by phalloidin staining at St. 22 and 23.

Ventral view of the ventral epidermis in WT and Y27632-treated embryo at St. 22 and 23. Note that cell intercalation was disrupted, and cell shape was elongated along with the AP axis in the Y27632-treated embryo. The scale bar indicates 10 μm.

(lower) Rose diagram of the major axis when cell morphology approaches an ellipse in WT and Y27632-treated embryos during St. 22 and 23.

We added the following explanations to line 258.

“How does the intercalation of ventral midline cells contribute to tail bending? The ventral epidermis undergoing intercalation would have relatively slow elongation during st. 20 to st. 22 compared to the already intercalated dorsal epidermis (Fig. 2B). Considering the notochord as the main force of elongation (Dong et al., 2011; Hara et al., 2013; Lu et al., 2019), ventral epidermal cells might resist the AP elongation force of notochord during st. 20 to st. 22 and generate resistance temporally in the ventral midline epidermal cells. Supporting this, even if the dorsal midline epidermis elongates linearly, the ventral midline epidermis did not change the AP length at the beginning of the intercalation (Fig. 2B) and showed AP resistance (Fig. 5). The apically accumulated pMLC of the boat cells during intercalation (Fig. 4A) might contribute to the halt of the AP elongation of the apical side (Fig. 6, green arrows) of adjacent cells during tail bending. Actually, the bilateral-polarized shape of boat cells disrupted the polarity by Y27632 treatment (data not shown).”

What is the point of lines 240-253? Is it just examples of where BMP signaling has been shown to influence cytoskeletal polarity? Is there some larger inference?

ANS#15) We changed the sentence on line 272, “How does Admp/BMP signaling regulate both the cell-cell intercalation and the apico-basal polarity of the ventral epidermis via pMLC? In this study, it was indicated that the localization of the pMLC was observed not only at the AP cell-cell border of intercalating cells (Suppl. Fig. 6) but also at the apical side (ventral side) translocated from the basal side (dorsal side) by pSmad signaling (Fig. 4C). Recent studies show that SMAD3 drives cell intercalation underlies secondary neural tube formation in the mouse embryo (Gonzalez-Gobartt et al., 2021). Moreover, it was known that the BMP-Rho-ROCK1 pathway targets MLC to control actin remodeling in fibroblasts (Konstantinidis et al., 2011). However, there have been no reports of Smad translocating MLC from the basal to the apical plane.”

The model in lines 261-265 seems too strongly stated, given that the functional relationship between pMLC, apical contractility and intercalation are not clear.

ANS#16) Intercalating cells and apically constricting cells are not separate; these are the same boat cells. However, their dependence on each other is unknown. So we discussed this as follows in line 298, “This process does not extend in the AP axial direction at the tissue level (Fig. 2B). We hypothesized that the myosin accumulation on the apical side of these boat-shaped cells resists the force of AP-axis notochord elongation in early intercalation and causes no ventral epidermis elongation (Fig. 6B).”

Line 266: ‘bilateral side of the basal domain’???

ANS#17) We deleted this sentence.

The conclusion would also benefit from being open to tail bending, potentially being a very complicated process. This manuscript makes a strong case that the ventral midline is important, but differential properties or behaviors between the CNS and the endodermal strand could well be involved too.

ANS#18) Thank you for your advice. In the discussion, we mentioned the other tissue contribution in line 256, as follows, “This is not to deny that genes other than Admp act on tissues other than the ventral epidermis to control flexion.”

In Fig 1C, what stage was used for the quantitation? All these bar charts would benefit from showing the number of cells or embryos examined.

ANS#19) St. 22 was used. The number of embryos is now indicated in the figure legends.

The model flowchart thing in Figure 5 is very busy and should be simplified.

ANS#20) In the revised manuscript, we simplified Figure 5 and made a new Figure 6C.

The legend for Supp Fig 3 is hard to understand and needs to be improved.

ANS#21) We have deleted Suppl. Fig. 3.

Reviewer 2 Advance Summary and Potential Significance to Field...

This paper describes that Admp regulates myosin phosphorylation in the ventral epidermis and controls the ventral vending, which constitutes a proposal to revise the preexisting model, which explains the bending as the result of asymmetric distribution of actomyosin in the notochord.

In the revised manuscript, we strengthen the ventral epidermis as a primary factor for the tail bending by additional experiments and the preexisting model is revised by testing notochord asymmetrical localization at the early stages. As Reviewer 1 mentioned, the ventral accumulation of actomyosin in the notochord might reflect earlier morphogenetic events, such as the notochord intercalation, making the transient ventral groove (Munro and Odell, 2002).

Reviewer 2 Comments for the Author...

Major points

This paper describes that Admp regulates myosin phosphorylation in the ventral epidermis and controls the ventral vending, which constitutes a proposal to revise the preexisting model, which explains the bending as the result of asymmetric distribution of actomyosin in the notochord. Although this reviewer appreciates the challenge and admits the relationship between Admp and myosin phosphorylation is a fact, their model does not sufficiently explain the morphological event as the function of Admp.

ANS#22) To answer the reviewer's requests, further experiments were performed, and figures were reconsidered for easy understanding. Based on these, the manuscript was substantially improved and the relationship between Admp and myosin phosphorylation was clarified. We hope the revised manuscript satisfies the reviewers.

The authors' claims are based on the phenotype of the embryo in which Admp is knocked down by MO. Given Admp, which is expressed endoderm and lateral epidermis (line 112) and required for the expression of Bmp2/4 (line 121), it is not shown how the phosphorylation of Smad is affected in the ventral mid-line of the morphant; Fig.1D just shows the Smad signaling in normal embryos.

ANS#23) We additionally observed the p-Smad staining of the Admp-MO embryo, and no positive signals were detected in the whole embryo (Fig.1E).

Also, it is not examined how myosin phosphorylation is localized preferentially on the apical side and only in a limited number of ventral epidermal cells in normal embryos despite BMP2/4 and Smad activities along the entire AP axis.

ANS#24) Sorry for our insufficient explanations. All the ventral epidermal cells become boat cells and have the apical accumulation of pMLC. The section that seems to have no accumulation of pMLC is one of the boat cell sections (SSBC; Fig. 3B).

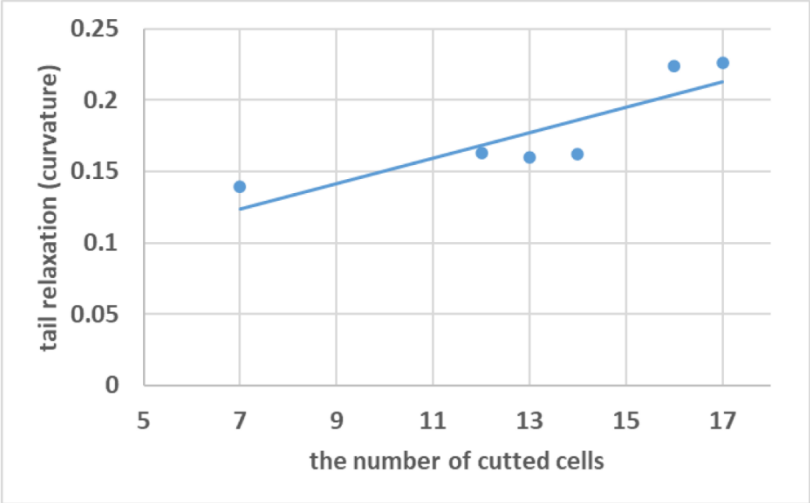
If Admp regulates the intracellular localization of pMLC, the authors need to show that the total amount of pMLC is unchanged in control and the morphant embryo (Fig. 2) and how the localization is regulated. To examine the competence and threshold of the ventral epidermal cells for the apical constriction, over-expression of Admp would be very important. Along this line, the phenotype of the Y27632 embryo is inadequate to be compared with that of the Admp morphant because Y27632 is expected to suppress total pMLC in the cells, and thus the similarity of the phenotype could not support the authors' claim.

ANS#25) We performed additional experiments of the ectopic expression of Bmp2/4 (Fig. 1E) as well as KD, and we found that ectopic pSmad positive cells also translocate the pMLC to the apical side (Fig. 4C). We cannot measure the difference in the total amount of pMLC in the ventral epidermis, but this experiment showed the Admp/BMP controls pMLC translocation from the basal to the apical side. Y27632 data was deleted.

Overall, this paper lacks insight into the mechanism of how Admp regulates the localized myosin phosphorylation through cell-cell interaction and therefore, it appears to be a descriptive piece of work, despite the tissue mechanics experiments and quantitative data analyses and 3D reconstruction of the cell model. Furthermore, as the laser cutting points in the ventral side are too many and relaxation of bending only of the entire larva is observed, there is no clue to understand the relationship between cellular tension and myosin phosphorylation of the ventral epidermis cells that are assumed to drive the bending at a single-cell level. The relaxation could also be explained by the combinatorial effect of ventrally-biased robust cell-to-cell adhesion and an internally generated force by notochord elongation.

ANS#26) Thank you for your comments. In addition to the tissue mechanics experiments, quantitative data analyses, and 3D reconstruction of cell models, we have shown how the localization of pMLC of the ventral epidermal cells was changed by the ectopic expression of

Bmp2/4. Although there are no clues to understanding the relationship between cell tension and pMLC in the ventral epidermal cells at the single cell level, as answered by ANS#24, both TSBC ("triangular cells" in the previous manuscript) and SSBC ("non-triangular cells" in the previous manuscript) are different cutting planes of the boat cell. So, the triangular cell and non-triangular cell are the same cell type, boat cells. All boat cells have apically localized pMLC. So, the observed relaxation in the laser-cut experiment is the result of the cut of the sum of successive cuts of the pMLC region of the ventral epidermal cells. For reviewers only, we show the correlation. There was a positive correlation between the tail relaxation and the number of cut cells of ventral midline cells below in different individuals (0.86, N=6).



We present a model in which this relaxation is generated by the resistance of the ventral epidermal cells to the extending force of the notochord (Fig. 6). In addition, this resistance might be generated by pMLC. The laser cutter experiments showed a relaxation of the ventral tail bending when the ventral midline epidermal cell was cut sequentially (Fig. 5). Considering the notochord as the main force of elongation (Dong et al., 2011; Hara et al., 2013; Lu et al., 2019), ventral epidermal cells might resist against the AP elongation force of notochord during st. 20 to st. 22 and generate resistance temporally in the ventral midline epidermal cells.

We described this as follows, line 294, "Actually, Y27632-treated embryos show cell shape elongation of ventral epidermal cells in the AP direction, supporting the possibility that pMLC exerts a resistance against notochord elongation in the AP direction (data not shown).

All midline epidermal cells undergoing early intercalation extend left and right and form boat-shaped cells. This process does not extend in the AP axial direction at the tissue level (Fig. 2B). We hypothesized that the myosin accumulation on the apical side of these boat-shaped cells resists the force of AP-axis notochord elongation in early intercalation and causes no ventral epidermis elongation (Fig. 6B). It has been reported that BMP regulates cell adhesion in early morphogenesis, such as neural tube closure and gastrulation (von der Hardt et al., 2007; Smith et al., 2021). Cell adhesion molecules might also be involved in the generation of resistance."

In summary, the possible involvement of Admp in the event is potentially interesting, the current data are not sufficient to provide additional insight into the ventral bending mechanism and to convince readers that the authors' proposal is superior to the past model.

ANS#27) We are not focusing on which model is superior. However, we have obtained additional data showing that ADMP controls tail bending without relying on the previous model in the notochord.

Other comments

Fig. 1A, it is difficult to distinguish the dorsal and ventral sides of Admp morphants as the neural tube was not visualized. In late tailbud stages, DV orientation appears to be reversed

according to the statement, “bending to dorsal (dorsiflexion) was observed similar to WT (line, 84)”. At least D/V should be labeled in the photos.

ANS#28) We labeled the position of the neuropore and the DV orientation in Figure 1A.

Line 93, the Admp gene is, should be written as “The Admp encodes”.

ANS#29) Now amended.

Fig. 1B, it is quite strange that the bending angle exceeds 90 for WT. The explanation for this is necessary. If the length of the notochord is unchanged, the distance between the anterior-most and posterior-most cell may be useful for quantitation.

ANS#30) The length of the notochord changes during the tail bend, so we measured the tail bending angle by the definition of Figure 1B. The angle exceeded 90° for WT, seen as follows.

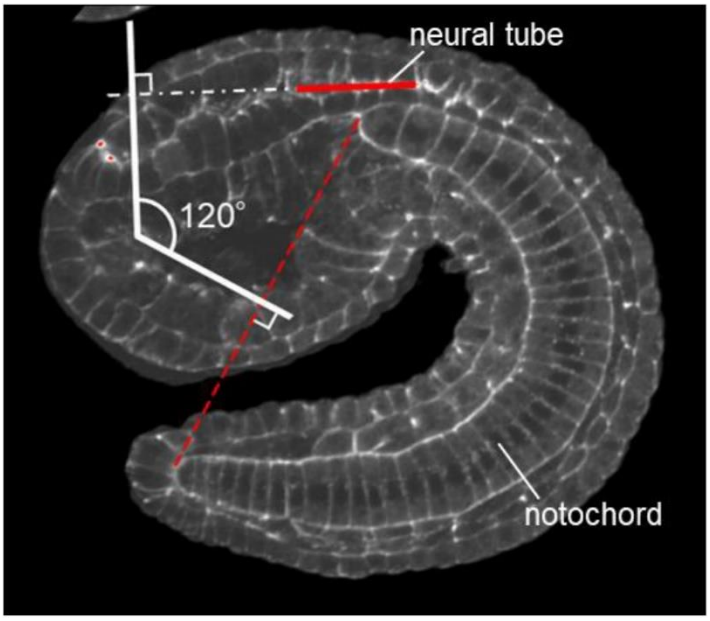
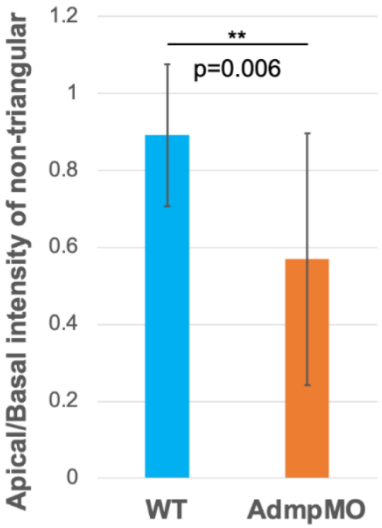


Fig. 1D, the right most of the bottom illustration must represent lateral view but not ventral. As mentioned above, p-Smad staining of Admp morphant should be shown to demonstrate the effect of Admp.

ANS#31) We deleted the schematic figure and added the p-Smad antibody staining of the Admp morphant (Fig. 1E).

Fig. 2D, the A/B ratio of pMLC should be shown for non-triangular cells of control and morphants.

ANS#32) Sorry for the misunderstanding. As shown in Figure 3B, the non-triangular cells of WT are just one of the boat cell ‘sections’ (SSBC).



For reviewers only, we compared with the non-triangular "section" of both WT and Admp MO (above graph). Similar to the result of the triangular section, the apical accumulation of pMLC was reduced in the Admp MO. This result also supports the argument of ANS#25, Admp controls the translocation of pMLC.

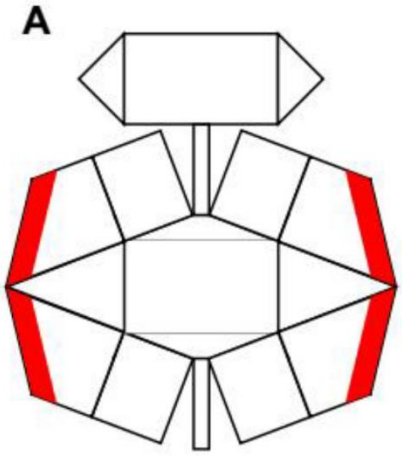
Fig. 3C, single cell level analysis and presentation of the recoiling profile of both triangular and non-triangular cells, ideally with a kymograph, are informative.

ANS#33) As answered by ANS#24, both TSBC ("triangular cells" in the previous manuscript) and SSBC ("non-triangular cells" in the previous manuscript) are different cutting planes of the boat cell. So, the triangular cell and non-triangular cell are the same cell type, boat cells. In this experiment, the laser cut each cell in an apico-basal direction. Since the triangular and square cross-sections are continuous (Fig. 3B), it is technically difficult to cut only the triangular cross-section or only the square cross-section.

Fig. 4, the description "Triangular-shaped cells" is adequate for 2D structure but not for 3D structure like cells. Are they triangular pyramid or triangular prism?

line, 183, it is not clear what "anterior-posterior border" represents. Does it mean the anterior-posterior border of columnar cells? Does 3D reconstruction of Z stack images of the cell support either?

ANS#34) We renamed the triangular cells as "boat cells (upside-down)" in Figure 2. We mean "the anterior-posterior border of ventral midline epidermal cells" in line 224. To help the reader understand how the 3D morphology of boat cells intercalates and contributes to ventroflexion at the tissue level, we prepared Suppl. Fig. 5 is as follows.



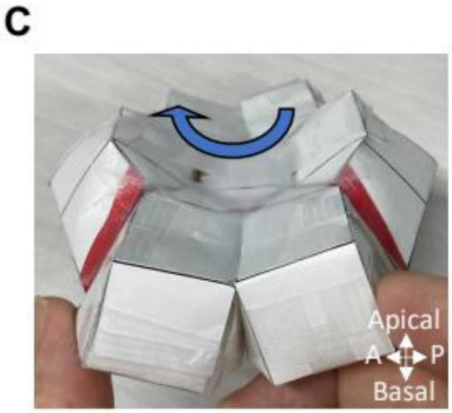


Fig. 5, in general, if intercalation is incomplete, it is expected that the embryo becomes wider along L/R axis, as shown for the PCP mutants. Is this true for Admp morphant? If the width of morphant embryo is not affected, how is it explained?

ANS#35) There is no difference between the width along the L/R axis of WT and Admp MO embryo at st. 24 (data not shown). We believe this is because the normal intercalation of the other five rows (except for the ventral epidermis) (Fig. 2D) and notochord elongation force help narrow the Admp MO tail.

Suppl. Fig. 2, the polarity (DV ratio of F-actin accumulation) is affected in Admp morphant and in fact, the pattern is changed, although the authors just stated that the asymmetric localization is still observed (line 106-107). Some explanation for the phenotype is necessary. If the change is true, it might affect the ventral bending.

ANS#36) We observed the Admp MO and Dorsomorphin-treated embryo at st. 18 to 22, and they remain asymmetrical accumulation of actomyosin in notochord as WT (Suppl. Fig. 2; The DV bias rather increased in the morphant of Suppl. Fig. 2B). Also, the timing of ventroflexion is later than the timing of DV bias in the notochord but rather coincides with the timing of the apical accumulation of the pMLC in the ventral epidermal cells (Suppl. Fig. 2B, Fig. 6A). From these results, we conclude that the DV bias of actomyosin of notochord is not regulated by Admp and is not critical for ventroflexion.

Suppl. Fig. 3,

It is misleading that the plot number counts from the posterior side, while notochord cell number counted from the anterior side (line 405) for Fig. 1B. In addition, it is uncomfortable to

see the head/tail position is reversed for this figure. The entire orientation should be reversed like others.

ANS#37) We deleted Suppl. Fig. 3.

The data in many experiments should be properly quantitated. The number of independent experiments/observations and the number of data point per experiments must be indicated.

ANS#38) The number of the samples has been added to each figure.

Fonts for figures seems to be randomly chosen without any rules, which disturbs readers' understanding. Figures should be improved in many aspects, including usage of colors, font size, boldness, resolution, etc.

ANS#39) All figures have been improved in the revised manuscript.

Reviewer 3 Advance Summary and Potential Significance to Field...

In this manuscript, Kogure et al. present an analysis of the role of the BMP-family member ADMP during the process of ventral tail bending during the tailbud stages of the ascidian Ciona robusta (previously known as Ciona intestinalis type A).

A previously published Development article (Lu et al., 2020), which established that tail bending is autonomous to the tail, and requires a ventral activation of the myosin II regulatory light chain in the notochord, as well as a synergy with other tail tissues.

Here the authors have focused on the role of the ventral epidermal midline and the control of its morphogenesis by ADMP. They first report that morpholino- and small molecule-mediated inhibition of ADMP prevents ventral tail bending, and that this inhibition is associated with a change in the shape of some of the ventral epidermal cells, called "triangular cells" (see below why I put quotes…). These cells accumulate F-actin and activated myosin II regulatory light chain in an ADMP- and Rock-dependent manner. Using laser cutting experiments, the authors further demonstrate that the tension accumulated in ventral, but not dorsal, epidermal cells is necessary for tail bending. Finally, the authors argue that the "triangular cells" are undergoing intercalation, which they show is ADMP dependent.

Overall, the experiments appear rigorously carried out (but not properly described, see below) and the work is in principle of sufficient interest to be published in Development. It is complementary to the previous Lu et al. study, and of similar interest.

Thank you for your comments. Further experiments strengthen our claim and do not support the findings of the Lu et al. study.

Reviewer 3 Comments for the Author...

I however have several major and minor issues that the authors need to address prior to publication (many of them should be addressed before publication in any journal.).

1) My first comment is purely methodological. The legends of the figures are simply not sufficient to understand what is shown. As will be seen below, there are several instances where lack of details in the description of what is shown severely confused me. In this paragraph, I will use figure 3 as an example, but all figure legends should be extensively revised and improved. In figure 3, it is nowhere said what the pictures in the panel represent: are these confocal pictures? Maximal projections? Single optical sections? Time is mentioned in panels A and C, but no scale is given. How much time elapsed between "before" and "after"? The heatmap is also not explained: what time scale are we looking at here: seconds, minutes, more? The explanation of "relaxation" in panels B and D leaves me in the fog. In the absence of critical experimental and image analysis details, this work will be very difficult to reproduce or even understand.

ANS#40) Detailed explanations are now provided in all figures. Descriptions of panel photos, how they were taken, time/scales, and heat maps (looking at the time) were noted. We have added an explanation of relaxation, etc.

We have re-wrote the figure legend for Figure 3 as follows (in line 395). “UV-laser cutting experiments were performed on tailbud *Ciona* embryos. An inverted Axio Observer Z1 (Zeiss) microscope equipped with a confocal spinning disk (Andor Revolution Imaging System, Yokogawa CSU-X1), a Q-switched solid-state 355 nm UV-A laser (Powerchip, Teem Photonics), a C-APOCHROMAT 63x/1.2 W Korr UV-VIS-IR water immersion objective (Behrndt et al., 2012), and a home-made cooling stage was used. The membrane of tail epidermal cells of tailbud embryos was labeled with FM-64 (ThermoFisher). Each ventral midline epidermal cell was cut along the apico-basal axis (5 to 10 µm lines each) by applying 25 UV pulses at 0.7 kHz. The embryos were imaged every 0.2 s frame rate with an exposure time of 150 ms. Single fluorescent images were used to measure tail relaxation 3 s post-ablation, and the percentage of relaxation was calculated as the area of movement of the tail region 3 s after laser cutting.”

The same comment applies to supplementary figures. For instance, the DV axis should be shown in Fig S2A. How the ratio in S2B is precisely calculated should be indicated (is this the peak intensity value? The intensity value integrated on several pixels flanking the peak value? The two measures may give different results as the dorsal and ventral intensity peaks have different shapes), etc.

ANS#41) The DV axis is shown in Figure S2A. The ratio of Suppl. Fig. 2B is calculated from the data of the peak intensity values in Suppl. Fig. 2A. The average of this ratio is shown in each graph.

2) I find the term “triangular cells” very confusing, as cells are 3D objects while a triangle is a 2D shape. If the 3D shapes shown in S5C and D represent a “triangular cell”, then such a cell will not appear as triangular on all 2D optical sections.

Many non-triangular cells in a 2D section may turn up to be triangular in another and the 2D approach may be unsuitable. The lack of proper description of what the authors are showing makes me wonder whether my overall confusion is due to a fundamental flaw in the work presented of to its very poor presentation!

I suggest to change the presentation of these cells, possibly by first describing their 3D shape and orientation in the context of the embryo and then explain how this translates on the 2D sections shown throughout the manuscript.

ANS#42) Thank you for your advice. We changed the manuscript structure and first described the 3D shape and orientation of these cells in the new Figure 3. The term triangular cell is not used in the new manuscript. Instead, we use the term boat cells when referring to 3D structures, which display a triangular shape in 2D sections.

3) I understand that Figure S5C may describe the 3D shapes of a “triangular cell” (blue? Please confirm) and of a non-triangular one (mauve? Please confirm). But what is the orientation of these cells with respect to the embryo? Is anterior to the top and posterior to the bottom?

How does this relate to intercalation?

ANS#43) We added the AP orientation to the new Figure 3. All boat cells are under intercalation. To facilitate the understanding of how the boat cells intercalate and show ventroflexion at the tissue level, we have prepared a new figure (Suppl. Fig. 5).

4) I am puzzled by the relationships between apical myosin accumulation in the “triangular cells”, intercalation of ventral epidermal cells and ventral tail bending.

Cell intercalation, in convergent extension movements for instance, is generally a mechanism driving tissue elongation. The authors say that ventral epidermis midline cells fail to undergo intercalation when ADMP signalling is blocked. This sounds counter-intuitive to me, as this should favor tail bending! Indeed Figures 4C and S6 do not really show a defect in cell intercalation (there are 3 rows of cells with or without ADMP signalling) but rather a defect in the organisation of these rows, which become irregular in the absence of ADMP. This should be clarified.

ANS#44) Although cell intercalation is certainly a mechanism that promotes tissue elongation in general, we have shown that, contrary to previous expectations, during the period of ventroflexion in WT (st.19–st.22), all cells in the ventral epidermis are under intercalation. Yet despite this (Suppl. Fig. 3), we found that the length of the ventral epidermis in the AP direction did not change (Fig. 2B). This suggests that intercalation of the ventral epidermis does not promote elongation in the AP direction at least during the early period. We called this early intercalation.

See also ANS#14. All midline epidermal cells undergoing the early intercalation extend left and right and form boat-shaped cells. Figure 2B indicates that this process does not extend in the AP axial direction at the tissue level. In line 299 of the discussion, “we hypothesized that the myosin accumulation on the apical side of these boat-shaped cells resists the force of AP notochord elongation in early intercalation and causes no ventral epidermis elongation (Fig. 6B). Therefore, ADMP-MO, in which ventral epidermal midline cells disrupt early intercalation, inhibits the temporary stops of the AP elongation, and thus tail bending does not occur.

There is no difference between the width along the L/R axis of WT and Admp MO embryo at st. 24 (data not shown). We believe this is because intercalation of the other five rows (other than the ventral epidermis) occurs normally (Fig. 2D), and the notochord AP elongation force is sufficient to narrow the Admp MO tail.”

5) This may not strictly be in line with the authors argumentation, but if intercalation during convergent extension is indeed a player during tail bending, I would expect: 1) that ventral cell intercalation is delayed compared with dorsal intercalation (this is indeed shown on Figure S7!);

ANS#45) Thank you for your suggestion.

As we previously mentioned, the halt of the AP elongation during early intercalation is required for ventral bending, and ADMP is thought to regulate this specialized cell intercalation in the ventral midline epidermal cells. This is because ADMP MO completely prevents intercalation of ventral midline epidermal cells (Fig. 2; Suppl. Fig. 4). Thus, the role of Admp is not to delay intercalation but to cause intercalation specific to the ventral midline epidermis.

6) The systematic use of the Rock-inhibitor Y27632 may not be informative concerning the role of myosin or ADMP in the epidermis, as this treatment inhibits Rock and myosin regulatory light chain activation in both epidermis and notochord. Using a more targeted approach, Lu et al. (2020) showed that myosin regulatory light chain activity in the notochord is needed for tail elongation. How do the authors differentiate the epidermal and notochord role of myosin in these experiments? I suggest either removing these experiments (Fig 3C) or replacing them with dorsomorphin-treated embryos (Fig S4).

ANS#47) Thank you for your suggestion. We deleted the Y27632 experiment of Figure 3C. We further showed that ventroflexion did not occur in dorsomorphin- treated embryos but remained asymmetrical notochord actin (Suppl. Fig. 2). Ectopic expression of the Bmp2/4 experiment (Fig. 4C) indicates that Admp/BMP controls pMLC translocation from the basal to the apical side.

7) Is ADMP only affecting the epidermis? Although the legend of Figure 1 is unclear on this point, I imaging that the P-smad staining only shows the epidermis. Are the authors sure that ADMP does not also signal to the notochord? Figure 2A reveals that the asymmetric distribution of actin and activated myosin in the notochord is affected by the inhibition of ADMP signalling...

What happens at earlier stages?

ANS#48) pSmad was detected only in the ventral epidermis in WT and not in the notochord. We mentioned this in the legend of Figure 1E. Regarding actomyosin in the notochord at early stages in AdmpMO embryos, please see ANS#6. We observed the Admp MO and Dorsomorphin-treated embryo at st 18 to 22, and they retain the asymmetrical accumulation of actomyosin in the notochord, similar to WT (Suppl. Fig. 2). From this result, it was suggested that the DV difference of actin of notochord is not regulated by Admp and that the difference is not critical for ventroflexion.

8) Minor issues:

a. The English syntax should be checked throughout the manuscript. Some sections, presumably coming from one of the two labs involved, are very well written. This suggests that the authors should better coordinate to provide a uniformly satisfying text.

ANS#49) The English in the revised manuscript was checked by all authors and a professional English editing service.

b. The author may want to comment why the effect of the laser cutting is much more subtle than the ADMP morpholino.

ANS#50) We discussed line 317 as follows.“ The cells and tissues of the developing embryos are changing their shape and arrangement from moment to moment, and we propose that those changes affect the mechanical state of other tissues where Admp was not originally involved. At this time, the newly appeared cells on the dorsal midline may irreversibly stabilize the whole-embryo shape, so the laser-cut experiment could not completely straighten the tail bending (Fig. 5A). Compared with Admp MO, the cell number of the ventral midline epidermis in WT at st. 22 did not change, but the cell number of the dorsal midline epidermis increased, even if Admp/Bmp signal was only exerted in the ventral epidermis (Suppl. Fig. 7). We propose that, in the WT embryo, ventroflexion has a mechanical effect on dorsal tissue, and because of promoting cell intercalation (Caussinus et al., 2008) and/or cell division (Campinho et al., 2013), the number of dorsal midline epidermal cells is increased (Suppl. Fig. 7).”

c. Line 80: scientists do not aim at “confirming their hypothesis” but at testing it rigorously and without preconception of the results, as formalised by Karl Popper.

ANS#51) We have now removed the term “confirming their hypothesis” and instead raised a new hypothesis based on our results.

d. I am not convinced that there are enough data in vertebrate systems to claim (in the abstract and in the discussion) that the identified role of ADMP may be conserved in bilaterians.

ANS#52) We have now deleted this sentence. In the new version of the manuscript, on line 341, we mentioned, “In invertebrate chordate animals, such as sea urchins and hemichordates, Admp is expressed at the ectoderm (Chang et al., 2016; Lowe et al., 2006) of embryos. It would be interesting to investigate whether the MLC regulation by Admp is conserved among primitive chordate embryogenesis and causes the body shape change in these animals. Since the apical surface is ventral to the cells of the ventral ectoderm in such small primitive chordates, the regulation of AP polarity at the cellular level by Admp/Bmp might directly influence the regulation of the DV polarity in the whole body.”

Second decision letter

MS ID#: DEVELOP/2021/200215

MS TITLE:*Admp* regulates tail bending by controlling ventral epidermal cell polarity via phosphorylated myosin translocation

AUTHORS: Yuki S. Kogure, Hiromochi Muraoka, Wataru C. Koizumi, Raphael Gelin-alessi, Benoit Godard, Carl-Philipp Heisenberg, Kotaro Oka, and Kohji Hotta

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

The overall evaluation is very positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. No further experiment nor analysis is needed but a text edit is essential to improve the clarity of the manuscript. Reviewers make suggestions to improve the text. Please attend to the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

This manuscript shows that the BMP family ligand Admp is required for proper morphogenesis of the ventral epidermal midline in the Ciona embryo and that this plays a major role in the transient but characteristic ventral curvature of the elongating tail. The paper provides compelling data that the morphogenesis and tissue mechanics of the ventral epidermal midline are of great importance in tail curvature and provides an important counterpoint to earlier work proposing that tail curvature is controlled by DV biased actomyosin asymmetries in just the notochord.

Comments for the author

This revised manuscript is much improved over the original submission. There is now much better data and greater clarity of presentation to help the reader assess the data here in the context of the prior work of Lu et al. The ‘boat-shaped’ cell shape description is effective and the new clarity that triangular and trapezoidal cell cross sections represent different cutting planes through these boat-shaped cells is very helpful. I am confident that a major contribution has been made to understanding ventral tail bending in the Ciona embryo and I don’t think that any new experiments are required for this to be a Development paper. There are still some areas where conclusions have been overstated or problematic language has been used but I feel they can be handled with changes to the text or reanalysis of existing data. The writing in the revised manuscript is greatly improved but it would still benefit from extensive copy-editing.

Larger concerns:

Fig 2D It is clear that there is a major and specific defect in the morphogenesis of the ventral and ventrolateral midline epidermal cells in the ADMP morphant, but the nature of that defect is still not clear to me. Can the ventral vs ventrolateral cells actually be distinguished in the morphant? If the mixed orange/red color scheme in the ventral ADMP MO panel is being used to indicate that those cells cannot be distinguished, then that should be stated explicitly in the legend and/or the text. If that is the case though, it is not clear to me that this is actually an intercalation defect. If you include the ventralmost row and the two ventrolateral rows, then the wildtype ventral midline is 3 cells wide. If there is an intercalation defect in the morphant, then the comparable territory should be >3 cells wide and that does not appear to be the case… The same is seen for the

dorsomorphin treatment in Supp Fig 4. The three very stereotyped single file rows of ventral epidermis are not evident, but the ventral epidermal territory is still roughly 3 cells wide. It seems possible that this could be a defect in cell/tissue shape independent of intercalation per se. I would note for example that the wildtype ventral cells in Fig 3D seem much longer in the AP dimension than their morphant counterparts. The overall convergence and extension of a tissue can involve both cell neighbor exchanges (intercalation) and also changes in cell size/shape/aspect ratio. Those have not really been teased apart here. Unless you have other timelapse or clonal analysis data clearly showing a defect in cells intercalating between one another, it would be best to soften the conclusion that this is fundamentally an intercalation defect. I suspect this is something complicated where the ventral midline intercalates actively and autonomously in the wildtype condition and more passively/non-autonomously in the Admp morphant, but that is not clearly demonstrated here.

The term ‘translocation’ is problematic throughout the paper. ‘Translocation’ implies that already phosphorylated myosin is being moved from one place to another. There is no evidence that pMLC is being moved from the basal side to the apical side, just that pMLC levels are higher apically than basally in some cell sections. It seems more appropriate to talk about apically enriched phosphomyosin ‘localization’ rather than ‘translocation’. Note my comments later though with respect to potential nuance about whether this really an apical enrichment vs an ML enrichment. 210-212 If there is convincing publication-quality data about differential effects from AP vs ML oriented cuts that data should be shown. Otherwise this should be omitted. That would be much too important of a finding to leave as ‘data not shown’.

Mid-sized concerns:

163-180. In the discussion it becomes clear that you think all of the ventral epidermal cells are boat-shaped but that needs to be made clear here where you first introduce this topic. In the morphants, is it that the ventral epidermal cells are quantitatively less boat-shaped or that some cells are still boat-shaped and others are not? Or are they still boat-shaped but packed together in a more chaotic way? This can’t be distinguished from single midline sections as in Fig 3D,E, but should be evident if you’ve imaged these with 3D confocal stacks… I’m not asking for additional experiments, but this seems like an important point and it should be addressed if you have data that speaks to it or at least discussed if you do not have such data. Note that the abstract claims that there are fewer boat cells in the morphant but no data to that effect are shown. The observation at lines 178-180 that there are fewer triangular-shaped cell sections along the midline plane doesn’t seem adequate to conclude that there are fewer boat cells without at least a discussion of what these cells look like in 3D.

It would be helpful to use a cartoon diagram similar to 3B to highlight where the pMLC staining is strongest. If I am understanding things correctly, it is on the prow/stern of each boat shaped cell (the medial and lateral ridges connecting the apical and basal sides). If so, it’s possible that the Admp MO defect is a mediolateral planar polarity defect as much as an apicobasal polarity defect. This possibility should be discussed. Is there actually a difference in pMLC intensity between the major apical and basal surfaces (colored orange vs red in Fig 3B..) or is it all on the medial/lateral vertices connecting those apical and basal surfaces? Do those vertices remain on the surface of the embryo for their full lengths or do they end up buried between other cells on their more basal ends?

supp fig 2 needs more clarity on the stats. Are they showing that the various ratios are significantly different than 1 or are they showing that the ADMP Mo or dorsomorphin ratios are different from the control ratios?

supp fig 3 how are the putative epidermal midline cells identified to be highlighted in yellow? Are these really comparable groups of cells? In supp fig 3B for example the ventral view seems to include ventral epidermal cells in the trunk whereas in the later panels it is just the tail. Does the ventral epidermal midline really become shorter in the AP dimension as shown in in panel C, or is that just an artifact of the angle of the embryo and the bend in the tail? The yellow ventral tracings seem to all be based on single confocal sections through the curved tail, so they are not super useful in terms of showing the overall convergence, extension and intercalation of the ventral epidermal midline.

Figure 2B is very helpful but it would be useful to show a comparable plot if possible for ADMP MO embryos.

Fig 4B. I don't find the proposed apical-basal differences in pMLC intensity in the TSCC areas to be particularly compelling. The sample sizes are very small and line trace measurements on single confocal sections are very noisy. The figure legend should make it clear whether the 'n's reported are for the number of cells quantified or the number of different embryos quantified. Ideally both those values should be provided. It's very hard to analyze this sort of phenomenon without a 3D analysis of all the cells in the entire ventral midline. That might not be feasible here, but I think it's important not to overinterpret this sort of data where the sample size is very low and the data itself is a 2d simplification of a fundamentally 3D issue. This doesn't affect the seemingly robust conclusion that there are fewer of the high pMLC regions that are triangular in section in the morphants.
~191 The BMP overexpression experiments need to be properly introduced. They are introduced very abruptly here and it is not clear from the text that this is a new experimental perturbation.
203-204 I find the laser cutter experiments convincing that the mechanical properties of the ventral epidermal midline are very important for ventral tail curvature, ,but I disagree with the inference here that this is necessarily downstream of apical pMLC accumulation. That is probably correct, but the cuts made aren't targeted specifically to triangular cell cross sections so these experiments are showing that it is the mechanical integrity of the ventral epidermal midline that is important for keeping the tail bent but don't directly speak to the importance of polarized pMLC. This should be reworded to distinguish better between the direct conclusion of the cutting experiments and the broader inference about polarized pMLC.
The enlarged ventral view in Supp Fig 6 are very interesting but hard to make sense of. In Db' and Eb' for example there are some very dramatic plane polarized pMLC accumulations but it is not clear if they are on AP cell surfaces or on protrusions between interdigitating cells. Is there some sort of transition between having most of the pMLC on the flat A and P cell surfaces to the wedge-shaped M and L sides? A bit more annotation and description here would be really useful, especially given that the functional relationship between intercalation and bending remains somewhat murky.
316-328 It seems odd to be bringing up new data from Supp Figure 7 this late in the discussion, and the data itself is quite cursory. If there was actually more proliferation in the dorsal midline epidermis because of mechanical effects from the ventral midline epidermis, that would be incredibly interesting. If it's just that epidermal cells intercalate slightly differently in the morphant so that a different number get counted as being part of the dorsal midline, then that seems less interesting. In either case though, it doesn't seem like this is particularly germane to the broader question of why the tail bends ventrally. Although ventral tail bending does not require physical constraint from the chorion, isn't it enough just to speculate that it may have evolved as a response to that constraint?

wording issues and small things (this is not meant to be an exhaustive list- the ms still needs quite a bit of copyediting):

26 better to read 'Morpholino knockdown of ADMP completely inhibited ventral tail bending'
27 better to read 'specific inhibition of cell-cell intercalation in the ventral epidermis…'
28 better to read ' in the early stages of intercalation…'
30 better to read 'the number of boat cells was reduced and pMLC was observed at the basal side.'
32 has the relaxation really been shown to be pMLC dependent?
45-46 better to read 'most tailbud embryos become curved in shape with a ventrally bending tail.'
47 -51 awkward paragraph that needs copyediting 52-52 should this be part of the previous paragraph?
54-70 'AP' would be clearer here than 'longitudinal'
71 better to say 'To identify upstream regulators of tail bending…'
82 get rid of the 'some' unless there are actually contradictory results in earlier ADMP knockdown studies. If you want to qualify this, make it clear that these previous studies weren't focused on tail bending but the phenotype is apparent in their images.
95-97 'This indicates that Admp/BMP signaling specifically regulates the ventroflexion of ascidian tailbud embryos'
98 is 'shown' the best verb here? maybe 'proposed'?
110 'independently' would be more apt than 'apart'

116 -117 Does the 2012 Imai paper really show that Admp is a morphogen, in the sense of forming a concentration gradient and having distinct effects at different concentrations? I'm not convinced… Aren't short range interactions between touching cells sufficient to explain its effects here, especially given they may be relayed via BMP4? Does the issue even need to be discussed given ADMP isn't being proposed to be a directly polarizing cue?
127-128 rephrase- these embryos were not injected with MO at stage 16…
139 better to read 'cells started at st.19 and was completed at st. 24'
142-145 very confusing sentence…
149 'physiological factors' is unclear. 'mechanical properties' perhaps?
165-166 very confusing sentence! Maybe rephrase to 'We suspected that the inhibition of ventroflexion in ADMP morphants involves abnormal cell intercalation of the ventral epidermis' As discussed earlier though it's not clear if this is fundamentally an intercalation defect.
171,184 The word 'unique' to describe the boat shaped cells seems inappropriate. This could well be a common feature of compound-curved epithelia that just hasn't been described before. 'Distinctive' or the like would be more suitable 174 not clear what is meant by 'compiling them alternately'
183 this paragraph should introduce the topic of myosin phosphorylation and make it clear that the pMLC antibody is being used here to infer sites of high actomyosin contractility.
194, 196 the 'lateral side of epidermal cells' language here is deeply ambiguous. It's not the lateral sides of ventral epidermal cells that are being discussed- it's the epidermal cells on the lateral sides of the tail that aren't normally triangular in cross section…
253-254 the conclusion about intercalation is problematic as discussed earlier 264 'generate resistance temporally' is not clear Fig3A,B would benefit from more annotation to make it clear that the long axis of the boat shaped cells runs mediolaterally in the embryo and the short axis runs AP.
270-271, 294-296 data not shown here should either be shown or the discussion should be omitted.
291 'in which pMLC their translocate'?
315 ventroflexion not ventrofrexion Fig 5A The temporal colormap needs an indication of the actual time scale. I think (from the methods) that it shows from 0 to 3 seconds post cut but that should be indicated on the color bars.

Reviewer 2

Advance summary and potential significance to field

This paper reports about functional importance of two developmentally regulated growth factors ADMP and BMP and demonstrated that they contribute to the cell shape change in notochord bending through the sophisticated 3D modeling of the cells, which would provide novel information to the community and thus merits publication in Development.

Comments for the author

Most of the points which I raised have been adequately addressed by adding new experiments or well explained by the authors, which is satisfactory to this reviewer.

Reviewer 3

Advance summary and potential significance to field

The revision carried out by the authors has significantly strengthened the case for a major role of ADMP in ventral tail bending from the earliest stages of the process. I fully agree with Referee 1 that the authors results strongly suggest the Dong paper is wrong in its interpretation: while notochord contractility powers tail elongation, ADMP probably controls ventral binding.

Comments for the author

I therefore strongly supports publication of this study in Development. My remaining concerns can be dealt with by (major) text editions.

Last section of the discussion, lines 340 to 347: Urchins and hemichordates are invertebrate non-chordate deuterostomes. They are NOT chordates. This error should absolutely be corrected.
Line 47, "protochordata" should be replaced by "tunicate": ascidians are not the most basal chordates and there is considerable evidence that they are highly derived.
The manuscript remains overall very difficult to read because of its poor English quality. The work is very nice and I strongly urge the authors to have their manuscript edited by one or several native English speakers with some understanding of biological sciences. The "professional English editing service" they consulted should be avoided.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
This manuscript shows that the BMP family ligand Admp is required for proper morphogenesis of the ventral epidermal midline in the Ciona embryo and that this plays a major role in the transient but characteristic ventral curvature of the elongating tail. The paper provides compelling data that the morphogenesis and tissue mechanics of the ventral epidermal midline are of great importance in tail curvature and provides an important counterpoint to earlier work proposing that tail curvature is controlled by DV biased actomyosin asymmetries in just the notochord.

Reviewer 1 Comments for the Author:
This revised manuscript is much improved over the original submission. There is now much better data and greater clarity of presentation to help the reader assess the data here in the context of the prior work of Lu et al. The ‘boat-shaped’ cell shape description is effective, and the new clarity that triangular and trapezoidal cell cross sections represent different cutting planes through these boat-shaped cells is very helpful. I am confident that a major contribution has been made to understanding ventral tail bending in the Ciona embryo and I don’t think that any new experiments are required for this to be a Development paper. There are still some areas where conclusions have been overstated or problematic language has been used but I feel they can be handled with changes to the text or reanalysis of existing data. The writing in the revised manuscript is greatly improved but it would still benefit from extensive copy-editing.

Larger concerns:

Fig 2D It is clear that there is a major and specific defect in the morphogenesis of the ventral and ventrolateral midline epidermal cells in the ADMP morphant, but the nature of that defect is still not clear to me. Can the ventral vs ventrolateral cells actually be distinguished in the morphant? If the mixed orange/red color scheme in the ventral ADMP MO panel is being used to indicate that those cells cannot be distinguished, then that should be stated explicitly in the legend and/or the text.
ANS#1
The mixed orange/red color scheme in the ventral ADMP MO panel in Fig.2D is being used to indicate that these cells cannot be distinguished, something we now explicitly stated in the revised manuscript.

If that is the case though, it is not clear to me that this is actually an intercalation defect. If you include the ventralmost row and the two ventrolateral rows, then the wildtype ventral midline is 3 cells wide. If there is an intercalation defect in the morphant, then the comparable territory should be >3 cells wide and that does not appear to be the case…
The same is seen for the dorsomorphin treatment in Supp Fig 4. The three very stereotyped single file rows of ventral epidermis are not evident, but the ventral epidermal territory is still roughly 3 cells wide. It seems possible that this could be a defect in cell/tissue shape independent of intercalation per se. I would note for example that the wildtype ventral cells in Fig 3D seem much longer in the AP dimension than their morphant counterparts. The overall convergence and extension of a tissue can involve both cell neighbor exchanges (intercalation) and also changes in

cell size/shape/aspect ratio. Those have not really been teased apart here. Unless you have other timelapse or clonal analysis data clearly showing a defect in cells intercalating between one another, it would be best to soften the conclusion that this is fundamentally an intercalation defect.

ANS#2

We agree with the referee that neighbor exchanges still occur in Admp morphants. Thus, we change the conclusion to Admp interference leading to "disordered intercalation."

We added as follows in line 167.

"In contrast, the ventral three-rows in Admp morphant embryos were disorganized into one or two rows, making it difficult to clearly distinguish between midline and medio-lateral cells (Fig. 2F, Admp MO; mixed orange/red color). Dorsomorphin- treated embryos showed a similar disordered ventral midline intercalation phenotype (Suppl. Fig. 5), further supporting the notion that Admp regulates ordered ventral epidermal cell-cell intercalation. "And also added the explanation about cell shape change and aspect ratio as follows in line 154, "During st.20 to 22 the ventral epidermis in WT embryos showed a preferential accumulation of junctional F-actin in the medio-lateral direction (ML accumulation) (Fig. 2C). Antibody staining of pMLC also showed such ML accumulation, especially at st. 19 to 22 (Suppl. Fig. 4). In contrast, no such ML accumulation was observed in Admp morphant embryos during st. 20 to 22 (Fig. 2C). In addition, while the AP/ML aspect ratio of ventral epidermal cells decreased in WT embryos during st. 18 to 22, no such decrease was found in Admp morphants (Fig. 2D). This suggests that Admp is required for proper asymmetric junctional actin accumulation and ML elongation of ventral epidermal cells during early intercalation.", and in line 276, "When ventral epidermal cell intercalation is completed (st. 24~), ventral epidermal cells drastically change their polarity into the AP direction in WT embryos. This does not occur to the same extent in Admp morphants (Fig. 2D), suggesting that Admp is also required for this later shift in cell polarity."

I suspect this is something complicated where the ventral midline intercalates actively and autonomously in the wildtype condition and more passively/non- autonomously in the Admp morphant, but that is not clearly demonstrated here.

ANS#3

We agree that the changes in intercalation behavior observed in Admp morphant embryos is because the ventral midline intercalates actively and autonomously in the wildtype condition and more passively/non-autonomously in the Admp morphant condition. To clearly mention this, we revised the discussion in line 280, "Ventral tail epidermal cells in WT, but not Admp morphant embryos, arrange into three ordered rows at st. 24. This suggests that Admp may be required for the cell autonomous ML intercalation of ventral epidermal cells by controlling ML cell polarization and protrusion formation. Notably, in Admp morphants, the ventral epidermis was disordered but kept a three-cell width, suggesting that some intercalation of ventral epidermal cells might still occur in the absence of Admp."

The term 'translocation' is problematic throughout the paper. 'Translocation' implies that already phosphorylated myosin is being moved from one place to another. There is no evidence that pMLC is being moved from the basal side to the apical side, just that pMLC levels are higher apically than basally in some cell sections. It seems more appropriate to talk about apically enriched phosphomyosin 'localization' rather than 'translocation'. Note my comments later though with respect to potential nuance about whether this really an apical enrichment vs an ML enrichment.

ANS#4

We have now changed the term "Translocation" to "Localization".

210-212 If there is convincing publication-quality data about differential effects from AP vs ML oriented cuts, that data should be shown. Otherwise this should be omitted. That would be much too important of a finding to leave as 'data not shown'.

ANS#5

In the revised manuscript, we have now added Suppl. Mov. 4 showing exemplary cases for AP vs ML oriented cuts.

Mid-sized concerns:
1
163-180. In the discussion it becomes clear that you think all of the ventral epidermal cells are boat-shaped, but that needs to be made clear here where you first introduce this topic.
ANS#6
To clarify this, we added the following sentence to the discussion (line 182):
" Almost all anterior ventral epidermal cells showed this shape (Suppl. Mov. 2), consistent with previous reports that tail bending only occurs in the anterior tail of Ciona (Lu et al., 2020)."

In the morphants, is it that the ventral epidermal cells are quantitatively less boat- shaped or that some cells are still boat-shaped and others are not? Or are they still boat-shaped but packed together in a more chaotic way? This can't be distinguished from single midline sections as in Fig 3D,E, but should be evident if you've imaged these with 3D confocal stacks… I'm not asking for additional experiments, but this seems like an important point and it should be addressed if you have data that speaks to it or at least discussed if you do not have such data. Note that the abstract claims that there are fewer boat cells in the morphant but no data to that effect are shown. The observation at lines 178-180 that there are fewer triangular-shaped cell sections along the midline plane doesn't seem adequate to conclude that there are fewer boat cells without at least a discussion of what these cells look like in 3D.
ANS#7
In the morphants, there are less ventral epidermal cells displaying a clear boat- shape. In fact, most ventral epidermal cells are not boat-shaped, as shown in Supple. Mov. 2.
To more explicitly mention this, we added the following sentence to the Result section of the revised manuscript (line 188):
" In Admp morphant embryos at st. 22, the number of TSBCs in ventral epidermal cells was strongly reduced (Fig. 3D and 3E; Admp MO, N= 12, WT, N = 7, p = 0.05×10^-5), while the number of non-TSBCs was increased (the section of non- boat cell) indicative of a reduced number of boat-cells in all ventral epidermis sections of morphant embryos (Suppl. Mov. 2)."

It would be helpful to use a cartoon diagram similar to 3B to highlight where the pMLC staining is strongest. If I am understanding things correctly, it is on the prow/stern of each boat shaped cell (the medial and lateral ridges connecting the apical and basal sides). If so, it's possible that the Admp MO defect is a mediolateral planar polarity defect as much as an apicobasal polarity defect. This possibility should be discussed. Is there actually a difference in pMLC intensity between the major apical and basal surfaces (colored orange vs red in Fig 3B..) or is it all on the medial/lateral vertices connecting those apical and basal surfaces? Do those vertices remain on the surface of the embryo for their full lengths or do they end up buried between other cells on their more basal ends?
ANS#8
The pMLC localization in the WT case is shown by the red color in Fig. 3C. To better illustrate this, we have added a schematic diagram of the morphological changes of boat-cells and accompanying changes in the localization of pMLC (Fig. 6B).
We have also mentioned the role of Admp in apicobasal pMLC localization in the discussion section of the revised manuscript (line 301):
" The preferential accumulation of pMLC in ventral epidermal cells along ML junctions (Suppl. Fig. 4Db', Eb') is found at the apical side of cell boundaries of TSBC and/or SSBC and might correspond to protrusion-like extensions formed between interdigitating boat cells (Fig. 6B). The lack of such polarized distribution of pMLC suggests that Admp might be required for both planar and apicobasal polarization of these cells."

supp fig 2 needs more clarity on the stats. Are they showing that the various ratios are significantly different than 1 or are they showing that the ADMP Mo or dorsomorphin ratios are different from the control ratios?
ANS#9
The ratio of the F-actin intensity of ventral /dorsal at notochord in the Admp morphants are bigger than 1 throughout all stages, as well as WT. This suggests that the asymmetrical localization of actomyosin along the dorsoventral axis of notochordal cells remains unchanged between the WT and the Admp morphant embryos.
We have now more clearly mentioned this in the results section of the revised manuscript (line 132):

" To test whether Admp functions in ventroflexion by affecting asymmetric actomyosin contraction within notochord cells, we analyzed Actin localization in Admp signaling defective embryos. We found that in both dorsomorphin-treated and Admp morphant embryos, asymmetric actin localization in notochord cells remained unchanged (Suppl.Fig.2). This indicates that Admp/BMP signaling affects ventroflexion independently from the proposed function of asymmetric actomyosin contraction in notochord cells. "

supp fig 3 how are the putative epidermal midline cells identified to be highlighted in yellow? Are these really comparable groups of cells? In supp fig 3B for example the ventral view seems to include ventral epidermal cells in the trunk whereas in the later panels it is just the tail. Does the ventral epidermal midline really become shorter in the AP dimension as shown in panel C, or is that just an artifact of the angle of the embryo and the bend in the tail? The yellow ventral tracings seem to all be based on single confocal sections through the curved tail, so they are not super useful in terms of showing the overall convergence, extension and intercalation of the ventral epidermal midline.

ANS#10

We removed the color-labelling of putative ventral epidermal midline cells, which can be identified by F-actin/Phalloidin staining. As ventral epidermal cells show enhanced accumulation of F-actin at their ML boundaries, we could use this feature to distinguish midline form non-midline cells. We also re-checked the elongation of midline cells along the AP axis within a small region of the ventral tail epidermis (Fig. 2B) to avoid any artefacts caused by large-scale tail bending.

Figure 2B is very helpful but it would be useful to show a comparable plot if possible for ADMP MO embryos.

ANS#11

It is very difficult to reliably measure the length of the midline epidermis in Admp morphant embryos from time-lapse movies taken at a stereo-microscope, since their tail is straight and thus it's nearly impossible to stably mount them along their dorsoventral axis.

*Fig 4B. I don't find the proposed apical-basal differences in pMLC intensity in the TSCC areas to be particularly compelling. The sample sizes are very small and line trace measurements on single confocal sections are very noisy. The figure legend should make it clear whether the 'n's reported are for the number of cells quantified or the number of different embryos quantified. Ideally both those values should be provided. It's very hard to analyze this sort of phenomenon without a 3D analysis of all the cells in the entire ventral midline. That might not be feasible here, but I think it's important not to overinterpret this sort of data where the sample size is very low and the data itself is a 2d simplification of a fundamentally 3D issue. This doesn't affect the seemingly robust conclusion that there are fewer of the high pMLC regions that are triangular in section in the morphants.

ANS#12

The number of TSBC is N=7; n=7 in the Admp morphants, and N=10, n=10 in the WT. We add this to the figure legend. We also changed the term "Translocation" to "Localization".

191~ The BMP overexpression experiments need to be properly introduced. They are introduced very abruptly here and it is not clear from the text that this is a new experimental perturbation.

ANS#13

We have revised our description of the experiments in the results section of the revised manuscript (line 203):

"To test whether Admp/BMP signaling can ectopically affect the localization of pMLC and thereby generate TSBC (Fig. 4D), we performed ectopic BMP-expression experiments. In WT embryo, both apical pMLC accumulation and TSBCs were not observed in epidermal cells except ventral epidermal cells, where also pSmad signal was detected (Fig. 4D, a frontal section of WT). In contrast, in embryos ectopically expressing BMP, pSmad signal was detected in all epidermal cells, accompanied by apical pMLC accumulation and TSBC formation not only in ventral tail epidermal cells but also within the remainder of the tail epidermis (Fig. 4D)."

203-204 I find the laser cutter experiments convincing that the mechanical properties of the ventral epidermal midline are very important for ventral tail curvature, ,but I disagree with the inference here that this is necessarily downstream of apical pMLC accumulation. That is probably

correct, but the cuts made aren't targeted specifically to triangular cell cross sections so these experiments are showing that it is the mechanical integrity of the ventral epidermal midline that is important for keeping the tail bent but don't directly speak to the importance of polarized pMLC. This should be reworded to distinguish better between the direct conclusion of the cutting experiments and the broader inference about polarized pMLC.

ANS#14

We have changed the description of the laser-cutting experiment in the result section of the revised manuscript (line 218):

" To investigate whether the ventral epidermis indeed locally resists tail elongation, eventually leading to ventroflexion, we cut either ventral or dorsal epidermal cells at their apex along the AP axis using an ultraviolet (UV)-laser cutter (Fig. 5, yellow lines)."

The enlarged ventral view in Supp Fig 6 are very interesting but hard to make sense of. In Db' and Eb' for example there are some very dramatic plane polarized pMLC accumulations but it is not clear if they are on AP cell surfaces or on protrusions between interdigitating cells. Is there some sort of transition between having most of the pMLC on the flat A and P cell surfaces to the wedge-shaped M and L sides? A bit more annotation and description here would be really useful, especially given that the functional relationship between intercalation and bending remains somewhat murky.

ANS#15

We have changed the description of the pMLC localization in the results section of the revised manuscript (line 301):

" Ventral epidermal cells take a distinct boat-cell shape, which likely contributes the ventroflexion (Fig. 4). The preferential accumulation of pMLC in ventral epidermal cells along ML junctions (Suppl. Fig. 4Db', Eb') is found at the apical side of cell boundaries of TSBC and/or SSBC and might correspond to protrusion-like extensions formed between interdigitating boat cells (Fig. 6B)."

316-328 It seems odd to be bringing up new data from Supp Figure 7 this late in the discussion, and the data itself is quite cursory. If there was actually more proliferation in the dorsal midline epidermis because of mechanical effects from the ventral midline epidermis, that would be incredibly interesting. If it's just that epidermal cells intercalate slightly differently in the morphant so that a different number get counted as being part of the dorsal midline, then that seems less interesting. In either case though, it doesn't seem like this is particularly germane to the broader question of why the tail bends ventrally. Although ventral tail bending does not require physical constraint from the chorion, isn't it enough just to speculate that it may have evolved as a response to that constraint?

ANS#16

We have removed this part of the discussion.

wording issues and small things (this is not meant to be an exhaustive list- the ms still needs quite a bit of copyediting):

26 better to read 'Morpholino knockdown of ADMP completely inhibited ventral tail bending'
27 better to read 'specific inhibition of cell-cell intercalation in the ventral epidermis…'
28 better to read ' in the early stages of intercalation…'
30 better to read 'the number of boat cells was reduced and pMLC was observed at the basal side.'
32 has the relaxation really been shown to be pMLC dependent?
45-46 better to read 'most tailbud embryos become curved in shape with a ventrally bending tail.'
47 -51 awkward paragraph that needs copyediting 52-52 should this be part of the previous paragraph?
54-70 'AP' would be clearer here than 'longitudinal'
71 better to say 'To identify upstream regulators of tail bending…'
82 get rid of the 'some' unless there are actually contradictory results in earlier ADMP knockdown studies. If you want to qualify this, make it clear that these previous studies weren't focused on tail bending but the phenotype is apparent in their images.
95-97 'This indicates that Admp/BMP signaling specifically regulates the ventroflexion of ascidian tailbud embryos'
98 is 'shown' the best verb here? maybe 'proposed'? 110

‘independently’ would be more apt than ‘apart’
ANS#17
We changed the text along the lines suggested by the referee.

116 -117 Does the 2012 Imai paper really show that Admp is a morphogen, in the sense of forming a concentration gradient and having distinct effects at different concentrations? I’m not convinced… Aren’t short range interactions between touching cells sufficient to explain its effects here, especially given they may be relayed via BMP4? Does the issue even need to be discussed given ADMP isn’t being proposed to be a directly polarizing cue?
ANS#18
We have removed this sentence.

127-128 rephrase- these embryos were not injected with MO at stage 16… 139 better to read ’cells started at st.19 and was completed at st. 24’
142-145 very confusing sentence…
149 ‘physiological factors’ is unclear. ‘mechanical properties’ perhaps?
165-166 very confusing sentence! Maybe rephrase to ‘We suspected that the inhibition of ventroflexion in ADMP morphants involves abnormal cell intercalation of the ventral epidermis’ As discussed earlier though, it’s not clear if this is fundamentally an intercalation defect.
171,184 The word ‘unique’ to describe the boat shaped cells seems inappropriate. This could well be a common feature of compound-curved epithelia that just hasn’t been described before. ‘Distinctive’ or the like would be more suitable
ANS#19
We rephrased the text along the lines suggested by the referee.

174 not clear what is meant by ‘compiling them alternately
ANS#20
We have revised this phrasing.

183 this paragraph should introduce the topic of myosin phosphorylation and make it clear that the pMLC antibody is being used here to infer sites of high actomyosin contractility.
ANS#21
We have more clearly mentioned by adding the following sentence to the results sec tion of the revised manuscript (line 214):
“pMLC levels at the apex increase actomyosin contractility”

194, 196 the ‘lateral side of epidermal cells’ language here is deeply ambiguous. It’s not the lateral sides of ventral epidermal cells that are being discussed- it’s the epidermal cells on the lateral sides of the tail that aren’t normally triangular in cross section…
ANS#22
We have revised the sentence in line 203 as follows:
“To test whether Admp/BMP signaling can ectopically affect the localization of pMLC and thereby generate TSBC (Fig. 4D), we performed ectopic BMP-expression experiments. In WT embryo, both apical pMLC accumulation and TSBCs were not observed in epidermal cells except ventral epidermal cells, where also pSmad signal was detected (Fig. 4D, a frontal section of WT). In contrast, in embryos ectopically expressing BMP, pSmad signal was detected in all epidermal cells, accompanied by apical pMLC accumulation and TSBC formation not only in ventral tail epidermal cells but also within the remainder of the tail epidermis (Fig. 4D).”

253-254 the conclusion about intercalation is problematic as discussed earlier
ANS#23
We have revised the sconcluding entence in line 259 as follows:
" Admp controls ventral, but not dorsal tail bending by determining early ventral epidermal cell intercalation (Fig. 2), and the shape of ventral epidermal midline cells (Fig. 3) through the localization of pMLC (Fig. 4)."

264 ‘generate resistance temporally’ is not clear
ANS#24
We changed the term ’temporally’ to ‘transiently’.

Fig3A,B would benefit from more annotation to make it clear that the long axis of the boat shaped cells runs mediolaterally in the embryo and the short axis runs AP.
ANS#25
We clarified that the long axis of the boat-cells runs mediolaterally in the embryo and the short axis runs along the AP axis in the revised manuscript.

270-271, 294-296 data not shown here should either be shown or the discussion should be omitted.
ANS#26
We have removed this sentence.

291 'in which pMLC their translocate'?
ANS#27
We have revised this phrase.

315 ventroflexion not ventrofrexion Fig 5A The temporal colormap needs an indication of the actual time scale. I think (from the methods) that it shows from 0 to 3 seconds post cut but that should be indicated on the color bars.
ANS#28
We have revised the figure legend of Fig. 5A as follows:
"The pictures of the tail region stained by FM4-64 of the tailbud embryos. Color bar indicates the time after laser cut 0 (before) to 30 (after) seconds post cut."

Reviewer 2 Advance Summary and Potential Significance to Field:
This paper reports about functional importance of two developmentally regulated growth factors ADMP and BMP and demonstrated that they contribute to the cell shape change in notochord bending through the sophisticated 3D modeling of the cells, which would provide novel information to the community and thus merits publication in Development.

Reviewer 2 Comments for the Author:
Most of the points which I raised have been adequately addressed by adding new experiments or well explained by the authors, which is satisfactory to this reviewer.
ANS#29
We thank the referee for his/her encouraging remarks.

Reviewer 3 Advance Summary and Potential Significance to Field:
The revision carried out by the authors has significantly strengthened the case for a major role of ADMP in ventral tail bending from the earliest stages of the process. I fully agree with Referee 1 that the authors results strongly suggest the Dong paper is wrong in its interpretation: while notochord contractility powers tail elongation, ADMP probably controls ventral binding.

Reviewer 3 Comments for the Author:
I therefore strongly supports publication of this study in Development. My remaining concerns can be dealt with by (major) text editions.

Last section of the discussion, lines 340 to 347: Urchins and hemichordates are invertebrate non-chordate deuterostomes. They are NOT chordates. This error should absolutely be corrected.
ANS#30
We changed this as advised.

Line 47, "protochordata" should be replaced by "tunicate": ascidians are not the most basal chordates and there is considerable evidence that they are highly derived.
ANS#31
We changed this as advised.

The manuscript remains overall very difficult to read because of its poor English quality. The work is very nice and I strongly urge the authors to have their manuscript edited by one or several native English speakers with some understanding of biological sciences. The "professional English editing service" they consulted should be avoided.

ANS#32
We have revised the text of the entire manuscript.

Third decision letter

MS ID#: DEVELOP/2021/200215

MS TITLE: *Admp* regulates tail bending by controlling ventral epidermal cell polarity via phosphorylated myosin localization

AUTHORS: Yuki S. Kogure, Hiromochi Muraoka, Wataru C. Koizumi, Raphael Gelin-alessi, Benoit Godard, Kotaro Oka, Carl-Philipp Heisenberg, and Kohji Hotta
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.

Reviewer 1

Advance summary and potential significance to field

This manuscript provides strong evidence that ADMP is a major regulator of ventral tail bending in the Ciona embryo via its effects on cell behaviors in the ventral epidermal midline. The manuscript provides an important counterpoint to an earlier paper proposing that ventral tail bending is controlled by asymmetric contractility in the notochord.

Comments for the author

I am satisfied with the changes that have been made and enthusiastically support publication of this manuscript in Development.