Regulation of anterior neurectoderm specification and differentiation by BMP signaling in ascidians

Agnès Roure, Rafath Chowdhury and Sébastien Darras
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Reviewer 1

Evidence, reproducibility and clarity

Summary

In this article Roure et al address the role of BMP during formation of the ascidian palps, using Ciona intestinalis. Overexpression of BMP (specifically ADMP) from early stages of development results in complete suppression of palp formation, and early loss of the palp forming region (also called anterior neural border ANB). Using p-Smad1/5/8 antibody staining they show a marker of the ANB (FoxC) is expressed in a region negative for BMP signals. Inhibition of BMP signals is not sufficient to produce ectopic ANB. However, treatment with FGF protein from very early stages (8-cell stage) plus inhibition of BMP signaling (from 8-cell stage) increased FoxC expression. Looking at later stages of development the authors show that in a U-shaped expression domain of Foxg, Smad1/5/8 is active in the ventral-most part, which is expected to form the ventral-most palp. BMP2 treatment from gastrula stages results in loss of the ventral most palp expression of Isl and repression of ventral Foxg expression. Inhibition of BMP signaling from gastrula or neurula stages results in failure of a U-shaped pattern of Isl expression to resolve into the three palp expression domains, and by late tailbud stages, Sp6/7/8/9 (proposed as a repressor of Foxg in the inter-palp territory) expression is reduced and the numbers of specific cell-types making up the palps is increased. These cells are present in a single large palp of dorsal identity. Thus, inhibition of BMP from early gastrula stages results in a single palp made of more cells than the three palps of control larvae, presumably due to recruitment of cells usually present between the palps.

The authors then show a similar phenotype in another ascidian species Phallusia mammillata. Using their previous RNA-Seq data of embryos treated with BMP4, they looked for potential novel palp markers and identify a further eight novel markers of the palps. Looking further into this data and at a list of 68 genes expressed in palps (but not exclusively) they find that in whole embryo RNA-Seq data 70% were regulated by BMP signaling, mostly repressed, but some activated by BMP. 30 of these genes were regulated by Notch.

Apart from the confusion I explained in my comments below, the data seems to be carefully presented and interpreted. Overall, this manuscript presents a more detailed analysis of the role of BMP signaling during ascidian palp formation, but it remains to be precisely understood.

Major comments

1. I am a little confused about the timing of the protein treatments. In Figure 2, the authors show nicely that at the neurula stages, P-Smad1/5/8 staining abuts the FoxC ANB territory. Then at late neurula P-Smad1/5/8 is detected in the ventral-most part of the Foxg U-shaped part of the palp forming region, presumably the ventral most palp. However, the protein treatments with BMP (and FGF) are carried out from the 8-cell stage, which seems a bit drastic and embryos look difficult to orientate (e.g. Fig. 3D).
While BMP-treatment from early stages inhibits all palp gene expression and any sign of palp formation (Figure 1), treatment with BMP from the early gastrula stage, when Smad1/5/8 is detected only in mesendoderm cells and before it is detected in any ectoderm, is sufficient only to block ventral palp formation and cause a partial down-regulation of FoxC expression in the ANB. Thus, there seems to be a discrepancy between the roles proposed for BMP during ANB and palp formation as judged by P-Smad1/5/8 staining and the temporal evidence from BMP- and BMP-inhibitor treatment. Do the authors have some explanation for why they need to treat at least one hour before the BMP-mediated patterning mechanism (as indicated from the P-Smad1/5/8 staining) is taking place? For example, could the authors check how long it takes DMH1 to inhibit P-Smad1/5/8 positive staining? Or BMP to strongly induce P-Smad1/5/8? This seems to be a simple experiment and might go some way to explaining why they need to treat embryos much earlier than I would have thought necessary.
2. It does not make sense to me that BMP treatment from gastrula stage blocks only ventral palp formation (Figure 4) and ventral Foxg expression (Fig. 5G). In particular, it is the ventral palp region which is positive for P-Smad1/5/8 (Fig.2I,J) so I would not expect the ventral palp to be the most sensitive to BMP-treatment.

Minor comments

line 185 I see what the authors are trying to say but I don't agree that BMP limits the domain of FoxC expression as inhibition of BMP has no effect on FoxC. Rather BMP has to be kept out of the ANB in order to allow ANB formation.

The relationship between Foxg and Sp6/7/8/9 expression is not really clear and it would be better to do this with double ISH if the authors want to show mutually exclusive expression domains, or at least provide a summary figure.

Line 218, I do not see the data showing that Isl is expressed at a U-shape at st. 23, it seems to be expressed in three dots, unless embryos are treated with DMH1.

Figure 6B, G. It could be nice to show a close up of the palps to see elongated cells.

Figure 6K. It is better to use a statistical test to support the authors conclusions.

It could be nice to provide a timeline for Smad1/5/8 signaling and the role for BMP signals that are proposed in this manuscript as a summary diagram.

lines 66-74 is lacking references.

Significance

While it is still not clear how BMP signals are established (which ligands for example) and their precise role in palp formation, this manuscript adds more information to our current understanding of the role of BMP signaling during palp formation. In particular it shows that BMP signals need to be kept out of the ANB for its formation and that it is required to resolve the later forming palp territory into three discrete palp regions. However, there is some way to go before this is fully understood. This article will certainly be of interest to ascidian developmental biologists trying to understand the formation and patterning of the larval PNS. It may also be of some interest to evolutionary biologists trying to understand the relationship between the telencephalon territory of vertebrates and the palp forming territory of ascidians as some links have been proposed between these two developmental territories (e.g. line 78).

Reviewer 2

Evidence, reproducibility and clarity

Summary

The manuscript presents a detailed examination of how dynamic changes in BMP signaling during the development of the ascidian larval palps. Early in development BMP inhibition is responsible for the formation of a large field within the neuroectoderm that includes, among other fates, the presumptive palps. As development progresses, the territories of BMP activity/inhibition appear to be spatially refined within the palp-forming territory to specify palp versus interpalp fate. The experiments are presented with sufficient replication and statistical rigor.

Major Comments.

1. The researchers should look at otx expression in pFOG>Admp overexpressing embryos. It is difficult to assess from Figure 1, but it appears possible the the entire anterior sensory vesicle (not just the palps) are absent in the pFOG>Admp embryos (can the authors say briefly whether other ectodermal structures such as the atrial primordia or the oral siphon are still present?). Thus, is it possible that the entire a-lineage is disrupted? This would be an important distinction to make: are the defects attributed to experimental BMP activation specific to the palps, or are they more widespread in the anterior neuroectoderm? If the entire a-lineage is mis-fated, might this change the interpretation of the role of BMP inhibition? For example, might the formation of the palps depend on the proper development of the neighboring anterior neural plate? To address this concern, the authors should use a different driver to restrict Admp overexpression only to the palp forming region.
2. The authors hypothesize that papilla versus inter-papilla fate is controlled by differential BMP signaling. Is it possible to show differential P-Smad staining in papilla versus inter-papilla territories, as in Figure 2 for earlier gastrula-stage embryos? This data would make the authors hypothesis much more compelling. It appears that the authors have the necessary reagents.

Minor Comments

1. There is no mention of panels Figure 1 U and V in the text. In the figure legend they are misidentified as panels S and T.

2. Very small issue with English usage that occurs throughout the manuscript. The authors should check the use of "palps" versus "palp", particularly when expressions such as the following are used: "palps formation", "palps network", "palps lineage", "palps differentiation", "palps molecular markers", "palps neuronal markers", "palps phenotypes", etc . For example, the sentence, "Here, we show that BMP signaling regulates two phases of palps formation in Ciona intestinalis", should read instead "Here, we show that BMP signaling regulates two phases of palp formation in Ciona intestinalis".

3. It would be worth mentioning possible relationships between the tunicate palps and the adhesive glands for larval fish and amphibians. Are there common mechanisms? All of these are anterior ectoderm derivatives.

4. Please consider providing references in the Introduction for the sentences which end on the following lines of text: 36 (. . . is the sister group of vertebrates), 46 (. . . and sensory properties), 48 (. . . the secretion of adhesive materials), 57 (. . . on the nervous system in chordates), 68 (. . . also known as Ap2-like), 74 (. . . anterior neural territories)

5. To provide extra emphasis and to help the figures to stand alone with their respective legends, can you mention in the legend for Fig. 2 that D and E are controls? Also, can a brief legend be provided for S2 to give overall indication of staging, scale, orientation, etc.?

Significance

This study presents an advance in our understanding of the fine-structure regulation of BMP signaling in sculpting neuroectoderm derivatives. While this study is potentially of broad interest, the authors fail to fully discuss the comparative aspects of this study in the context of conserved chordate developmental mechanisms. This could be remedied without too much difficulty in the Discussion section.

Reviewer 3

Evidence, reproducibility and clarity

Summary:

This paper explores the role of BMP signaling for palp formation in ascidians using gain and loss of function approaches. The paper shows that while BMP at early (gastrula) stages prevents formation of the Foxc-positive palp ectoderm in Ciona, at later stages it appears to be essential for separation of the palps (possibly by promoting differentiation of interpapillary cells). The paper further shows that BMP plays similar roles in a different ascidian, Phallusia mammillata. Using previously published RNA-Seq results for the latter species after BMP up-regulation, the authors were able to identify additional BMP-responsive genes expressed in the palp region of ascidians.

Major comments:

However, while the effect of BMP overexpression at early stages has been confirmed by two independent strategies (electroporation of the BMP agonist ADMP and BMP2 treatment), the effects of late BMP activation as well as the effects of BMP inhibition at both early and late stages have been studied exclusively by pharmacological treatments with a single BMP signaling agonist (BMP2) and antagonist (DMH1). To substantiate these findings and rule out unspecific side effects, it would have been desirable to verify them with alternative strategies.

Therefore, while this study provides some new insights into the role of BMP in the specification of the palp forming region and subsequent palp development in ascidians, the evidence provided is relatively weak. Moreover, the scope of the study is quite limited. While identifying some BMP-responsive genes expressed in the palp region and describing the effects of BMP dysregulation on palp morphology, the study does not provide further insights into the underlying mechanisms how BMP patterns this region or affects subsequent palp formation.

Minor comments:

- 63: ...as the anterior...
- 68, 71, 74: references missing
- 73: better: anterior neural territories and placodes
- 76: palp territories also share molecular signature with anterior (eg. olfactory) placodes, not only telencephalon
- 106: awkward sentence
- 114: at what stage was ADMP electroporated?
- 134: to facilitate comparison between stages it would be useful to label cells in Fig. 2(eg. which are a-line and b-line cells? Where is the border between them?)
- 152: since Foxc and Foxg overlap with pSMAD1/5/8 at neurula but not gastrula stages, do you know whether this is due to a dorsal expansion of BMP activity or a ventral expansion of Foxc/Foxg expression? Again, labeling of the nuclei would help
- 174: the description is not clear here; what proportion of embryos did show reduction versus expansion of expression?. Why is the reduction shown in Fig.3 D asymmetrical?
- 198: ... of endogenous...
- 208: I suggest to highlight the regions of changes in Fig. with asterisks/arrows etc.
- 218: contrary to what is stated here, there is no depiction of u-shaped Isl1 expression in control embryos of Fig. 4
- 220: the cell shapes referred to here cannot be seen in Fig. 4 (too small)

- 271: the description here is confusing: first you talk about 53 genes and the mention palp expression of 12/26. Where does number 26 come from? And why was in situ done then for 27 additional genes? Also, while the comparison with previously published RNA-Seq data was valuable in uncovering additional BMP-sensitive palp markers, it does not provide any substantial new insights into how BMP patterns this territory.
- line 624: where
- Fig. 2: to facilitate comparison between stages it would be useful to label cells (eg. which are a-line and b-line cells? Where is the border between them?)
-Fig. 3: Why is the expression in D asymmetrical? In the main text you write that expression is expanded in some embryos but reduced in others - Please show examples also of the expanded phenotype and give numbers
- Fig. 6: small panels in I, L, N need to be explained (single channels), white signal needs to be explained (overlap ?)
- Fig. S2: legend is missing

Significance

Since the study does not provide substantial new insights into the mechanisms how BMP patterns the palp forming region or affects subsequent palp formation in ascidians, it will be of interest mostly for a specialized audience in the field of developmental biology.

Author response to reviewers' comments

1. General Statements [optional]

Our manuscript *Regulation of anterior neurectoderm specification and differentiation by BMP signaling in ascidians* describes the role of BMP in the formation of the ascidian palps, an adhesive and sensory organ located at the anterior-most region of the larva that bears similarities with vertebrate anterior neurectoderm derivatives. Our work has been evaluated by three reviewers. While some major points have been raised, the reviewers acknowledge the quality of the work. Reviewer 3 considers that our work is dedicated to an audience specialized in developmental biology, but the other two reviewers envision a broader audience.
We would like to clarify some possible misunderstanding that appeared to us by reading the comments from reviewer 3 but also from reviewer 1. We think that our study does not bring only 'some new insights' into the role of BMP signaling in palp formation. To our knowledge, the only previous data mentioning a possible role of BMP signaling during palp formation come from a study by Darras and Nishida (2001) in the ascidian *Halocynthia roretzi*. What is described are the morphological consequences of BMP2/4 (agonist) or Chordin (antagonist) overexpression by mRNA injection. We have observed the same phenotypes in a different ascidian species, *Ciona intestinalis* (around 400 My divergence time). But we have presented extensive additional data:
- by finely mapping BMP activity and modulating BMP signaling through time, we evidenced two main functions for BMP in palp formation (anterior neural border specification, and papilla/inter-papilla fate selection).
- BMP function in palp formation seems conserved in another ascidian species *Phallusia mammillata* (275 My divergence time). With the data obtained in *Halocynthia roretzi*, this suggests a broad conservation in ascidians; and this is not a trivial finding in ascidians, a highly divergent group of animals.
- we identified novel palp molecular markers, made a census of available palp genes and their possible regulation by BMP and Notch pathway.
We thus consider that our study provides significant new insights in understanding palp formation in ascidians. Our work also suggests a conserved role for BMP signaling pathway in chordates, in particular the fact early specification of the anterior neurectoderm needs to occur in a region free of BMP. This will be of interest for ascidian developmental biologists, but also to a broad readership of developmental biologists and scientists interested in the evolution of developmental mechanisms.

2. Description of the planned revisions

There are 3 points requiring additional experiments and analyses that we propose to address. While the first point will certainly yield a better understanding of BMP signaling function in palp formation and increase the quality of our manuscript, the other two points do not seem essential to us, and we let them at the appreciation of the editors and reviewers.

Point 1:
[Major comment from Reviewer #1]
2) It does not make sense to me that BMP treatment from gastrula stage blocks only ventral palp formation (Figure 4) and ventral Foxg expression (Fig. 5G). In particular, it is the ventral palp region which is positive for P-Smad1/5/8 (Fig.2I,J) so I would not expect the ventral palp to be the most sensitive to BMP-treatment.
[Response]
We were, like the reviewer, surprised by the phenotype. The time window to obtain this phenotype is quite narrow, and most likely deals with the full acquisition of the palp fate ('consolidation' of *Foxc* expression, onset of *Foxg*). This is actually a phenotype that we have not characterized in details. And such a characterization may help clarify the role of BMP: does BMP regulate papilla/inter-papilla fates only for the ventral palp or for all three palps? Does BMP 'only' regulate the dorso-ventral identities of the palps?
To better understand the role of BMP in palp formation, we propose to describe this specific phenotype: loss of ventral palp induced by BMP2 treatment at St. 10. We propose to test the following hypotheses. What is the fate of the ventral palp? Conversion into epidermis (more ventral fate)? Conversion into inter-papilla fate? What is the identity of the 2 remaining presumptive palps? Do they still have a dorsal identity? Are they converted into ventral palps?

Point 2:
[Major comment from Reviewer #1]
While BMP-treatment from early stages inhibits all palp gene expression and any sign of palp formation (Figure 1), treatment with BMP from the early gastrula stage, when Smad1/5/8 is detected only in mesendoderm cells and before it is detected in any ectoderm, is sufficient only to block ventral palp formation and cause a partial down-regulation of FoxC expression in the ANB. Thus, there seems to be a discrepancy between the roles proposed for BMP during ANB and palp formation as judged by P-Smad1/5/8 staining and the temporal evidence from BMP- and BMP-inhibitor treatment. Do the authors have some explanation for why they need to treat at least one hour before the BMP-mediated patterning mechanism (as indicated from the P-Smad1/5/8 staining) is taking place? For example, could the authors check how long it takes DMH1 to inhibit P-Smad1/5/8 positive staining? Or BMP to strongly induce P-Smad1/5/8? This seems to be a simple experiment and might go some way to explaining why they need to treat embryos much earlier than I would have thought necessary.
[Response]
We understand the reviewer's concerns, but we do not think that there are major discrepancies in the timing of events. The main rationale is to consider the onset of expression for the main genes of interest. We have examined their dynamics of expression in details, but we do not show them since our conclusions are in agreement with a previous report (Figure 1 from Liu and Satou, 2019). We have summarized the data in the modified Figure 2. *Foxc* can be detected from early gastrula stages (St. 10) when the palp precursors consist of a single row of 4 cells. This is the exact developmental time when the treatment with BMP2 has partial effects (Figure 4). Once the cells divide to make 2 rows of 4 cells robustly expressing *Foxc* (St. 12), BMP2 treatment has no effect on *Foxc*. Similarly, DMH1 treatment has no effect from late neurula stage (St. 16) (Figure 4) that corresponds to the onset of *Sp6/7/8/9* expression. We thus consider that modulating BMP pathway has no effect once key regulatory genes have acquired a robust expression in their normal domains. We have enhanced these points in the main text (lines 205-208, lines 228-229).
We think the above discussion should address the points raised by the reviewer. In the contrary, we are willing to perform the suggested experiments.

Point 3:
[Minor comment from Reviewer #1]
The relationship between Foxg and Sp6/7/8/9 expression is not really clear and it would be better to do this with double ISH if the authors want to show mutually exclusive expression

domains, or at least provide a summary figure.
[Response]
We have modified Figure 5 by adding schematic representations of our understanding of the expression patterns in relation to the different precursors of the palp lineage.
In case the reviewer does not find this clarification sufficient, we propose to perform the double fluorescent *in situ* hybridizations as part of the revision plan.

3. Description of the revisions that have already been incorporated in the transferred manuscript

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary
In this article Roure et al address the role of BMP during formation of the ascidian palps, using Ciona intestinalis. Overexpression of BMP (specifically ADMP) from early stages of development results in complete suppression of palp formation, and early loss of the palp forming region (also called anterior neural border ANB). Using p-Smad1/5/8 antibody staining they show a marker of the ANB (FoxC) is expressed in a region negative for BMP signals.
Inhibition of BMP signals is not sufficient to produce ectopic ANB. However, treatment with FGF protein from very early stages (8-cell stage) plus inhibition of BMP signaling (from 8-cell stage) increased FoxC expression. Looking at later stages of development the authors show that in a U-shaped expression domain of Foxg, Smad1/5/8 is active in the ventral-most part, which is expected to form the ventral-most palp. BMP2 treatment from gastrula stages results in loss of the ventral most palp expression of Isl and repression of ventral Foxg expression. Inhibition of BMP signaling from gastrula or neurula stages results in failure of a U-shaped pattern of Isl expression to resolve into the three palp expression domains, and by late tailbud stages, Sp6/7/8/9 (proposed as a repressor of Foxg in the inter-palp territory) expression is reduced and the numbers of specific cell-types making up the palps is increased. These cells are present in a single large palp of dorsal identity. Thus, inhibition of BMP from early gastrula stages results in a single palp made of more cells than the three palps of control larvae, presumably due to recruitment of cells usually present between the palps.
The authors then show a similar phenotype in another ascidian species Phallusia mammillata. Using their previous RNA-Seq data of embryos treated with BMP4, they looked for potential novel palp markers and identify a further eight novel markers of the palps. Looking further into this data and at a list of 68 genes expressed in palps (but not exclusively) they find that in whole embryo RNA-Seq data 70% were regulated by BMP signaling, mostly repressed, but some activated by BMP. 30 of these genes were regulated by Notch.
Apart from the confusion I explained in my comments below, the data seems to be carefully presented and interpreted. Overall, this manuscript presents a more detailed analysis of the role of BMP signaling during ascidian palp formation, but it remains to be precisely understood.
[Response]
We thank the reviewer for the evaluation of our work.

Major comments

1) I am a little confused about the timing of the protein treatments. In Figure 2, the authors show nicely that at the neurula stages, P-Smad1/5/8 staining abuts the FoxC ANB territory. Then at late neurula P-Smad1/5/8 is detected in the ventral-most part of the Foxg U-shaped part of the palp forming region, presumably the ventral most palp. However, the protein treatments with BMP (and FGF) are carried out from the 8-cell stage, which seems a bit drastic and embryos look difficult to orientate (e.g. Fig. 3D).
[Response]
We first would like to clarify the issue raised from Figure 3. Actually, Figure 3D was the only case where the embryo was shown from the side (the description as a lateral view was inadvertently omitted in the legend). We have now modified Figure 3 by properly showing only dorsal (neural plate) views and lateral views in insets when necessary. In addition, we have added schemes of embryos depicting the main tissues we have examined (palps, CNS and epidermis) and their localization depending on the treatments.
Regarding the timing of treatments, we performed them at the 8-cell stage to make them manageable to perform. At the latest, bFGF treatment should be performed at the 16-cell stage (before neural induction at the 32-cell stage), while BMP2 treatment should be performed at the

64-cell stage (before the onset of *Foxc*/partial effect at early gastrula (St. 10)). In principle, sequential treatment (first bFGF, then BMP2) could thus be performed. Since earlier treatments, produce the same effects, we reasoned that combined treatments from the 8-cell stage should be equivalent and would avoid fastidious repeated manipulation of the embryos that could negatively impact their development. We are convinced that the way we performed the treatment has no impact on our results (except for the treatment by bFGF alone on *Foxc* as already discussed in the text) and conclusions.

While BMP-treatment from early stages inhibits all palp gene expression and any sign of palp formation (Figure 1), treatment with BMP from the early gastrula stage, when Smad1/5/8 is detected only in mesendoderm cells and before it is detected in any ectoderm, is sufficient only to block ventral palp formation and cause a partial down-regulation of FoxC expression in the ANB. Thus, there seems to be a discrepancy between the roles proposed for BMP during ANB and palp formation as judged by P-Smad1/5/8 staining and the temporal evidence from BMP- and BMP-inhibitor treatment. Do the authors have some explanation for why they need to treat at least one hour before the BMP-mediated patterning mechanism (as indicated from the P-Smad1/5/8 staining) is taking place? For example, could the authors check how long it takes DMH1 to inhibit P-Smad1/5/8 positive staining? Or BMP to strongly induce P-Smad1/5/8? This seems to be a simple experiment and might go some way to explaining why they need to treat embryos much earlier than I would have thought necessary.

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[Response]

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Minor comments

line 185 I see what the authors are trying to say but I don't agree that BMP limits the domain of FoxC expression as inhibition of BMP has no effect on FoxC. Rather BMP has to be kept out of the ANB in order to allow ANB formation.

[Response]

We have modified the sentence (lines 195-196).

The relationship between Foxg and Sp6/7/8/9 expression is not really clear and it would be better to do this with double ISH if the authors want to show mutually exclusive expression domains, or at least provide a summary figure.
[Response]
We have modified Figure 5 by adding schematic representations of our understanding of the expression patterns in relation to the different precursors of the palp lineage.
In case the reviewer does not find this clarification sufficient, we propose to perform the double fluorescent *in situ* hybridizations as part of the revision plan (see dedicated file).

Line 218, I do not see the data showing that Isl is expressed at a U-shape at st. 23, it seems to be expressed in three dots, unless embryos are treated with DMH1.
[Response]
We apologize for the misunderstanding since the sentence was not clear. We referred to the U-shaped *Isl* expression under BMP inhibition. Indeed, *Isl* starts to be expressed in 3 separate domains in the palp forming region, and not following a U-shape as its upstream regulator *Foxg* (Liu and Satou, 2019). We amended the sentence (lines 234-235).

Figure 6B, G. It could be nice to show a close up of the palps to see elongated cells.
[Response]
The close-up pictures have now been added in the modified Figure 6.

Figure 6K. It is better to use a statistical test to support the authors conclusions.
[Response]
As suggested, we have performed a statistical evaluation (Mann-Whitney U test) of the cell counts. The p-values are presented in Figure 6Q. The slight increase of *Celf3/4/5/6* is not statistically significant, but it does not impact our conclusion that the number of papilla cells increases following BMP inhibition.

It could be nice to provide a timeline for Smad1/5/8 signaling and the role for BMP signals that are proposed in this manuscript as a summary diagram.
[Response]
Following the suggestion, we have added summary diagrams in Figure 2 for BMP signaling in relation to lineages and gene expression.

lines 66-74 is lacking references.
[Response]
This is now corrected (lines 70-80).

Reviewer #1 (Significance (Required)):

Significance
While it is still not clear how BMP signals are established (which ligands for example) and their precise role in palp formation, this manuscript adds more information to our current understanding of the role of BMP signaling during palp formation. In particular it shows that BMP signals need to be kept out of the ANB for its formation and that it is required to resolve the later forming palp territory into three discrete palp regions. However, there is some way to go before this is fully understood. This article will certainly be of interest to ascidian developmental biologists trying to understand the formation and patterning of the larval PNS. It may also be of some interest to evolutionary biologists trying to understand the relationship between the telencephalon territory of vertebrates and the palp forming territory of ascidians as some links have been proposed between these two developmental territories (e.g. line 78).

[Reviewer's comments]
Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary.
The manuscript presents a detailed examination of how dynamic changes in BMP signaling during the development of the ascidian larval palps. Early in development BMP inhibition is responsible for the formation of a large field within the neuroectoderm that includes, among other fates, the presumptive palps. As development progresses, the territories of BMP activity/inhibition appear

to be spatially refined within the palp-forming territory to specify palp versus interpalp fate. The experiments are presented with sufficient replication and statistical rigor.
[Response]
We thank the reviewer for the evaluation of our work.

Major Comments.

1. The researchers should look at otx expression in pFOG>Admp overexpressing embryos. It is difficult to assess from Figure 1, but it appears possible the the entire anterior sensory vesicle (not just the palps) are absent in the pFOG>Admp embryos (can the authors say briefly whether other ectodermal structures such as the atrial primordia or the oral siphon are still present?). Thus, is it possible that the entire a-lineage is disrupted? This would be an important distinction to make: are the defects attributed to experimental BMP activation specific to the palps, or are they more widespread in the anterior neuroectoderm? If the entire a-lineage is mis-fated, might this change the interpretation of the role of BMP inhibition? For example, might the formation of the palps depend on the proper development of the neighboring anterior neural plate? To address this concern, the authors should use a different driver to restrict Admp overexpression only to the palp forming region.
[Response]
In Figure 1, we show that *Celf3/4/5/6*, a general neural marker was still expressed in pFog>Admp embryos. We explain, in the Figure 1 legend, that this most likely corresponds to the CNS. It does not demonstrate that the anterior sensory vesicle (a-line induced CNS lineage) is still present. Unfortunately, *Otx* cannot be used as a suitable marker since it is also expressed in the posterior sensory vesicle (A-line lineage) (Hudson et al., 2003). Other a-line markers do exist. However, determining their expression at tailbud stages may not be conclusive since it is most likely that the patterning of the sensory vesicle (hence the expression of these markers) is modified after BMP activation. We have presented in former Figure 3 and Figure S1, strong evidence that the a-line neural lineage is intact at the neural plate stage. To better communicate these data, we have combined then in a modified Figure 3 that includes all markers examined and interpretative embryonic schemes. We show that, following BMP2 treatment, *Otx* and *Celf3/4/5/6* were downregulated in the palp lineage but otherwise normal. Consequently, the a- line CNS lineage is most likely not affected by BMP pathway activation. This does not mean that its later derivatives form normally, but this is an issue that we have not addressed. A previous report indicates that BMP activation leads to *Six1/2* repression and, possibly, the absence of oral siphon primordium (based on the images, no description in this paper) (Figure 1 from Abitua et al., 2015).
We think that we have addressed the concern of the reviewer, but would like to comment on the suggested experiment. It is very difficult to find a driver that would allow BMP activation only in the palp lineage (by overexpressing a constitutive active BMP receptor for example). a-line neural linage and palp lineage are intimately linked and separate at gastrula stages (St. 10).
The regulatory sequences of *Foxc*, the first palp specific gene that we know, would thus be interesting. But it is most likely too late according to our whole embryo protein treatments (Figure 4). In agreement with this assumption, overexpressing Bmp2/4 (another BMP ligand) using the regulatory sequences of *Dmrt* (a master regulator of the palp+a-line CNS lineage expressed just before *Foxc*) does not apparently abolish palp formation (Extended Data Figure 5 from Abitua et al., 2015).

2. The authors hypothesize that papilla versus inter-papilla fate is controlled by differential BMP signaling. Is it possible to show differential P-Smad staining in papilla versus inter-papilla territories, as in Figure 2 for earlier gastrula-stage embryos? This data would make the authors hypothesis much more compelling. It appears that the authors have the necessary reagents.
[Response]
The actual lineage and fate segregation of papilla and inter-papilla lineage has not been determined as far as we know. Our current understanding comes from indirect evidence from gene expression and gene function, in particular from the study of *Foxg* and *Sp6/7/8/9* by Liu and Satou (2009). Papillae originate from the 3 *Foxg*/*Isl* positive spots that are visible at very early tailbud stages. At earlier stages, *Isl* is not expressed and *Foxg* is expressed with a U- shape (Figure 5). Within this U, it is most likely that the segregation of papilla and inter-papilla fates takes place when *Sp6/7/8/9* starts being expressed at late neurula stages. It is thought that *Sp6/7/8/9*$^{+}$/*Foxg*$^{+}$ cells will become inter-papilla cells while *Sp6/7/8/9*$^{-}$/*Foxg*$^{+}$ will become papilla. Our data indicate that BMP signaling is active in the future ventral papilla. We have

mapped these data on schematics in the modified Figure 2.

[Reviewer's comments]
Minor Comments.

1. There is no mention of panels Figure 1 U and V in the text. In the figure legend they are misidentified as panels S and T.
[Response]
This has been corrected.

2. Very small issue with English usage that occurs throughout the manuscript. The authors should check the use of "palps" versus "palp", particularly when expressions such as the following are used: "palps formation", "palps network", "palps lineage", "palps differentiation", "palps molecular markers", "palps neuronal markers", "palps phenotypes", etc . For example, the sentence, "Here, we show that BMP signaling regulates two phases of palps formation in Ciona intestinalis", should read instead "Here, we show that BMP signaling regulates two phases of palp formation in Ciona intestinalis".
[Response]
Thank you, we have corrected these mistakes.

3. It would be worth mentioning possible relationships between the tunicate palps and the adhesive glands for larval fish and amphibians. Are there common mechanisms? All of these are anterior ectoderm derivatives.
[Response]
Thank you for the suggestion. We have added a section on that topic in the discussion (line 358).

4. Please consider providing references in the Introduction for the sentences which end on the following lines of text:
36 (. . . is the sister group of vertebrates), 46 (. . . and sensory properties), 48 (. . . the secretion of adhesive materials), 57 (. . . on the nervous system in chordates), 68 (. . . also known as Ap2-like), 74 (. . . anterior neural territories)
[Response]
References have now been added.

5. To provide extra emphasis and to help the figures to stand alone with their respective legends, can you mention in the legend for Fig. 2 that D and E are controls? Also, can a brief legend be provided for S2 to give overall indication of staging, scale, orientation, etc.?
[Response]
Actually, the original Fig 2D and 2E correspond to treated embryos as explained in the legend. For clarity, these embryos have been separated from control embryos in the modified Figure 2. Figure S2 has modified and a legend has been added.

Reviewer #2 (Significance (Required)):

Significance.

This study presents an advance in our understanding of the fine-structure regulation of BMP signaling in sculpting neuroectoderm derivatives. While this study is potentially of broad interest, the authors fail to fully discuss the comparative aspects of this study in the context of conserved chordate developmental mechanisms. This could be remedied without too much difficulty in the Discussion section.

[Reviewer's comments]
Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:
This paper explores the role of BMP signaling for palp formation in ascidians using gain and loss of function approaches. The paper shows that while BMP at early (gastrula) stages prevents formation of the Foxc-positive palp ectoderm in Ciona, at later stages it appears to be essential for separation of the palps (possibly by promoting differentiation of interpapillary cells). The

paper further shows that BMP plays similar roles in a different ascidian, Phallusia mammillata. Using previously published RNA-Seq results for the latter species after BMP up- regulation, the authors were able to identify additional BMP-responsive genes expressed in the palp region of ascidians.
[Response]
We thank the reviewer for the evaluation of our work.

Major comments:
However, while the effect of BMP overexpression at early stages has been confirmed by two independent strategies (electroporation of the BMP agonist ADMP and BMP2 treatment), the effects of late BMP activation as well as the effects of BMP inhibition at both early and late stages have been studied exclusively by pharmacological treatments with a single BMP signaling agonist (BMP2) and antagonist (DMH1). To substantiate these findings and rule out unspecific side effects, it would have been desirable to verify them with alternative strategies.
[Response]
The reviewer may have missed some of our data. We have shown that BMP inhibition through overexpression of the secreted antagonist Noggin *via* electroporation using the early ectodermal driver pFog gives the same phenotypes as DMH1 treatment. The effects on *Foxc* were presented in Figure S1, and are now presented in the modified Figure 3 (line 170). We also showed that the morphological *Cyrano* phenotype was observed with Noggin overexpression (modified Figure 6H). We now present a novel Figure S1 with expression of *Isl* and *Celf3/4/5/6* following Noggin overexpression, and stress the use of this independent way of inhibiting BMP (lines 260-264). Given that early or late BMP inhibition led to the same phenotype, we do not consider that overexpressing Noggin at gastrula stages is necessary.
Regarding BMP activation from gastrula stages, we have only used BMP2 treatment. It may be possible to overexpress Admp using promoters active in the palp lineage such as the ones of *Dmrt*, *Foxc* or *Foxg*. However, it may be difficult to phenocopy the phenotype obtained using BMP2 protein (loss of ventral palp), for two reasons. First, the precise timing to reach high BMP activation is not tightly controlled using such a method. Hence, all drivers should be tested. Second, the different promoters are active progressively later in development and in more and more restricted regions. Consequently, we consider that this requires a huge effort to validate a method (BMP protein treatment) that we already validated for the early effects and that has been used in several publications.

Therefore, while this study provides some new insights into the role of BMP in the specification of the palp forming region and subsequent palp development in ascidians, the evidence provided is relatively weak. Moreover, the scope of the study is quite limited. While identifying some BMP-responsive genes expressed in the palp region and describing the effects of BMP dysregulation on palp morphology, the study does not provide further insights into the underlying mechanisms how BMP patterns this region or affects subsequent palp formation.
[Response]
We are surprised by the appreciation of the reviewer describing our work as 'some new insights'. To our knowledge, this is the first report addressing the role of BMP signaling in palp formation at the molecular level. The only previous report by Darras and Nishida (2001) describes solely the morphology of the palps following overexpression of Bmp2/4 and Chordin overexpression by mRNA injection. We have brought significant novel findings 1) two important steps in palp formation with a precise description of the cellular and molecular actors, and a proposed function for BMP at each step, 2) evidence for conservation of this process in different ascidian species and 3) significant enrichment in the molecular description of this process.
Moreover, the reviewer does not ask for specific items, we thus feel in the impossibility to offer satisfaction.

Minor comments:

- 63: ...as the anterior...

[Response]
Corrected.

- 68, 71, 74: references missing

Response]

References have now been added.

- 73: better: anterior neural territories and placodes
[Response]
Corrected.

- 76: palp territories also share molecular signature with anterior (eg. olfactory) placodes, not only telencephalon
[Response]
Corrected.

- 106: awkward sentence
- [Response]
Corrected.

- 114: at what stage was ADMP electroporated?
[Response]
Electroporation of plasmid DNA is performed in the fertilized egg. Transcription of the transgene is controlled by the driver. In this case, with pFog, it occurs from the 16-cell stage. This precision has been added in line 121.

- 134: to facilitate comparison between stages it would be useful to label cells in Fig. 2(eg. which are a-line and b-line cells? Where is the border between them?)
[Response]
As suggested by the reviewer, we have modified Figure 2 with embryo outlines and schemes to better appreciate where BMP signaling is active.

- 152: since Foxc and Foxg overlap with pSMAD1/5/8 at neurula but not gastrula stages, do you know whether this is due to a dorsal expansion of BMP activity or a ventral expansion of Foxc/Foxg expression? Again, labeling of the nuclei would help
[Response]
The change corresponds to a dorsal expansion of P-Smad1/5/8. Our conclusion comes from combining nuclear staining (not shown for simplicity) and available fate maps. The results are presented in schematic diagrams of embryos in frontal views in the modified Figure 2.

- 174: the description is not clear here; what proportion of embryos did show reduction versus expansion of expression?. Why is the reduction shown in Fig.3 D asymmetrical?
[Response]
The proportions are now indicated in line 184.
We apologize for the impression led by Fig 3D. Actually, it was the only case where the embryo was shown from the side (the description as a lateral view was inadvertently omitted in the legend). It did not show an asymmetric repression but an ectopic expression. We have now modified Figure 3 by properly showing only dorsal (neural plate) views and lateral views in insets when necessary. In addition, we have added schemes of embryos depicting the main tissues we have examined (palps, CNS and epidermis) and their localization depending on the treatments. We hope that the results are now clearly presented.

- 198: ... of endogenous...
- [Response]
Corrected (line 213).

- 208: I suggest to highlight the regions of changes in Fig. with asterisks/arrows etc.
[Response]
We have added schematic embryos to highlight expression changes in the modified Figure 5.

- 218: contrary to what is stated here, there is no depiction of u-shaped Isl1 expression in control embryos of Fig. 4
[Response]
As also pointed by reviewer 1, we apologize for the misunderstanding since the sentence was not clear. We referred to the U-shaped *Isl* expression under BMP inhibition. Indeed, *Isl* starts to be

expressed in 3 separate domains in the palp forming region, and not following a U-shape as its upstream regulator *Foxg* (Liu and Satou, 2019). We amended the sentence (lines 234-235).

- 220: the cell shapes referred to here cannot be seen in Fig. 4 (too small)

[Response]
We have modified Figure 6 to include close up of the palps.

- 271: the description here is confusing: first you talk about 53 genes and the mention palp expression of 12/26. Where does number 26 come from? And why was in situ done then for 27 additional genes? Also, while the comparison with previously published RNA-Seq data was valuable in uncovering additional BMP-sensitive palp markers, it does not provide any substantial new insights into how BMP patterns this territory.

[Response]
We have amended the sentence to make it clearer (lines 291-295).

- line 624: where

[Response]
Thank you. Corrected line 731.

- Fig. 2: to facilitate comparison between stages it would be useful to label cells (eg. which are a-line and b-line cells? Where is the border between them?)

[Response]
Already responded above.

- Fig. 3: Why is the expression in D asymmetrical? In the main text you write that expression is expanded in some embryos but reduced in others - Please show examples also of the expanded phenotype and give numbers

[Response]
Already responded above.

- Fig. 6: small panels in I, L, N need to be explained (single channels), white signal needs to be explained (overlap ?)

[Response]
We used white for better display of separate single channels. Given the confusion and the good quality of the 2-color fluorescent *in situ* images, we removed these panels in the modified Figure 6.
White in K and L correspond to overlap (explained in the legend).

- Fig. S2: legend is missing [Response]

This has been amended.

Reviewer #3 (Significance (Required)):

Since the study does not provide substantial new insights into the mechanisms how BMP patterns the palp forming region or affects subsequent palp formation in ascidians, it will be of interest mostly for a specialized audience in the field of developmental biology.

[Response]
We do not agree with the reviewer as discussed above. The description of the role of BMP signaling in the specification of the ANB and its subsequent patterning in ascidians has interesting evolutionary implications and should be of interest for a broader audience.

4. Description of analyses that authors prefer not to carry out

[Major comment from Reviewer #3]
However, while the effect of BMP overexpression at early stages has been confirmed by two independent strategies (electroporation of the BMP agonist ADMP and BMP2 treatment), the effects of late BMP activation as well as the effects of BMP inhibition at both early and late stages have been studied exclusively by pharmacological treatments with a single BMP signaling agonist (BMP2) and antagonist (DMH1). To substantiate these findings and rule out unspecific side effects, it would have been desirable to verify them with alternative strategies.

[Response]
The reviewer may have missed some of our data. We have shown that BMP inhibition through overexpression of the secreted antagonist Noggin *via* electroporation using the early ectodermal driver pFog gives the same phenotypes as DMH1 treatment. The effects on *Foxc* were presented in Figure S1, and are now presented in the modified Figure 3 (line 170). We also showed that the morphological *Cyrano* phenotype was observed with Noggin overexpression (modified Figure 6H). We now present a novel Figure S1 with expression of *Isl* and *Celf3/4/5/6* following Noggin overexpression, and stress the use of this independent way of inhibiting BMP (lines 260-264). Given that early or late BMP inhibition led to the same phenotype, we do not consider that overexpressing Noggin at gastrula stages is necessary.
Regarding BMP activation from gastrula stages, we have only used BMP2 treatment. It may be possible to overexpress Admp using promoters active in the palp lineage such as the ones of *Dmrt*, *Foxc* or *Foxg*. However, it may be difficult to phenocopy the phenotype obtained using BMP2 protein (loss of ventral palp), for two reasons. First, the precise timing to reach high BMP activation is not tightly controlled using such a method. Hence, all drivers should be tested. Second, the different promoters are active progressively later in development and in more and more restricted regions. Consequently, we consider that this requires a huge effort to validate a method (BMP protein treatment) that we already validated for the early effects and that has been used in several publications.

Original submission

First decision letter

MS ID#: DEVELOP/2022/201575

MS TITLE: Regulation of anterior neurectoderm specification and differentiation by BMP signaling in ascidians

AUTHORS: Agnès Roure, Rafath Chowdhury and Sèbastien Darras

Thank you for transferring your manuscript to Development. I have read it along with the referees' reports and your revision plan, and have reached a decision.

The referees express considerable interest in your work, but have some significant criticisms that require a substantial revision of your manuscript before we can consider publication. I will be happy to receive a revised version of the manuscript along the lines indicated in your revision plan. Please resubmit the fully revised manuscript via our online system. 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.

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.

First revision

Author response to reviewers' comments

[Reviewer's comments]
Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary
In this article Roure et al address the role of BMP during formation of the ascidian palps, using Ciona intestinalis. Overexpression of BMP (specifically ADMP) from early stages of development results in complete suppression of palp formation, and early loss of the palp forming region (also called anterior neural border ANB). Using p-Smad1/5/8 antibody staining they show a marker of the ANB (FoxC) is expressed in a region negative for BMP signals.
Inhibition of BMP signals is not sufficient to produce ectopic ANB. However, treatment with FGF protein from very early stages (8-cell stage) plus inhibition of BMP signaling (from 8-cell stage) increased FoxC expression. Looking at later stages of development the authors show that in a U-shaped expression domain of Foxg, Smad1/5/8 is active in the ventral-most part, which is expected to form the ventral-most palp. BMP2 treatment from gastrula stages results in loss of the ventral most palp expression of Isl and repression of ventral Foxg expression. Inhibition of BMP signaling from gastrula or neurula stages results in failure of a U-shaped pattern of Isl expression to resolve into the three palp expression domains, and by late tailbud stages, Sp6/7/8/9 (proposed as a repressor of Foxg in the inter-palp territory) expression is reduced and the numbers of specific cell-types making up the palps is increased. These cells are present in a single large palp of dorsal identity. Thus, inhibition of BMP from early gastrula stages results in a single palp made of more cells than the three palps of control larvae, presumably due to recruitment of cells usually present between the palps.
The authors then show a similar phenotype in another ascidian species Phallusia mammillata. Using their previous RNA-Seq data of embryos treated with BMP4, they looked for potential novel palp markers and identify a further eight novel markers of the palps.
Looking further into this data and at a list of 68 genes expressed in palps (but not exclusively) they find that in whole embryo RNA-Seq data 70% were regulated by BMP signaling, mostly repressed, but some activated by BMP. 30 of these genes were regulated by Notch.
Apart from the confusion I explained in my comments below, the data seems to be carefully presented and interpreted. Overall, this manuscript presents a more detailed analysis of the role of BMP signaling during ascidian palp formation, but it remains to be precisely understood.
[Response]
We thank the reviewer for the evaluation of our work.

Major comments

1) I am a little confused about the timing of the protein treatments. In Figure 2, the authors show nicely that at the neurula stages, P-Smad1/5/8 staining abuts the FoxC ANB territory. Then at late neurula P-Smad1/5/8 is detected in the ventral-most part of the Foxg U-shaped part of the palp forming region, presumably the ventral most palp. However, the protein treatments with BMP (and FGF) are carried out from the 8-cell stage, which seems a bit drastic and embryos look difficult to orientate (e.g. Fig. 3D).
[Response]
We first would like to clarify the issue raised from Figure 3. Actually, Figure 3D was the only case where the embryo was shown from the side (the description as a lateral view was inadvertently omitted in the legend). We have now modified Figure 3 by properly showing only dorsal (neural plate) views and lateral views in insets when necessary. In addition, we have added schemes of embryos depicting the main tissues we have examined (palps, CNS and epidermis) and their localization depending on the treatments.
Regarding the timing of treatments, we performed them at the 8-cell stage to make them manageable to perform. At the latest, bFGF treatment should be performed at the 16-cell stage (before neural induction at the 32-cell stage), while BMP2 treatment should be performed at the 64-cell stage (before the onset of *Foxc*/partial effect at early gastrula (St. 10)). In principle, sequential treatment (first bFGF, then BMP2) could thus be performed. Since earlier treatments, produce the same effects, we reasoned that combined treatments from the 8-cell stage should be equivalent and would avoid fastidious repeated manipulation of the embryos that could negatively impact their development. We are convinced that the way we performed the treatment has no impact on our results (except for the treatment by bFGF alone on *Foxc* as already discussed in the text) and conclusions.

While BMP-treatment from early stages inhibits all palp gene expression and any sign of palp formation (Figure 1), treatment with BMP from the early gastrula stage, when Smad1/5/8 is detected only in mesendoderm cells and before it is detected in any ectoderm, is sufficient only

to block ventral palp formation and cause a partial down-regulation of FoxC expression in the ANB. Thus, there seems to be a discrepancy between the roles proposed for BMP during ANB and palp formation as judged by P-Smad1/5/8 staining and the temporal evidence from BMP- and BMP-inhibitor treatment. Do the authors have some explanation for why they need to treat at least one hour before the BMP-mediated patterning mechanism (as indicated from the P-Smad1/5/8 staining) is taking place? For example, could the authors check how long it takes DMH1 to inhibit P-Smad1/5/8 positive staining? Or BMP to strongly induce P-Smad1/5/8? This seems to be a simple experiment and might go some way to explaining why they need to treat embryos much earlier than I would have thought necessary.
[Response]
We understand the reviewer's concerns, and performed the suggested experiments (new Fig S1). Treatments as short as 30 min with BMP2 were sufficient to induce robust P-Smad1/5/8 ectopic staining. For DMH1, treatments for at least 1 hr were needed to dramatically reduce (but not abolish) P-Smad1/5/8 staining. The timing of treatments that we performed are thus quite close indicators for the actual timing of the process they interfere with.
We do not think that there are major discrepancies in the timing of events, once we consider the onset of expression for the main genes of interest. We have examined their dynamics of expression in details, and our conclusions are in agreement with a previous report (Figure 1 from Liu and Satou, 2019). We have summarized the data in the modified Figure 2 and added some details for *Foxg* and *Sp6/7/8/9* in the new Figure 7. *Foxc* can be detected from early gastrula stages (St. 10) when the palp precursors consist of a single row of 4 cells. This is the exact developmental time when the treatment with BMP2 has partial effects (Figure 4). Once the cells divide to make 2 rows of 4 cells robustly expressing *Foxc* (St. 12), BMP2 treatment has no effect on *Foxc*. Similarly, DMH1 treatment has little effect from late neurula stage (St. 16) (Figure 4) that corresponds to the onset of *Sp6/7/8/9* expression. We thus consider that modulating BMP pathway has no effect once key regulatory genes have acquired a robust expression in their normal domains. We have enhanced these points in the main text (lines 212-215, lines 298-300).

2) It does not make sense to me that BMP treatment from gastrula stage blocks only ventral palp formation (Figure 4) and ventral Foxg expression (Fig. 5G). In particular, it is the ventral palp region which is positive for P-Smad1/5/8 (Fig.2I,J) so I would not expect the ventral palp to be the most sensitive to BMP-treatment.
[Response]
Given that BMP is active in the future ventral palp and that ventral palp is missing when BMP is inhibited, we concluded that BMP specifies ventral palp fate. We were, like the reviewer, surprised by the phenotype since we predicted ectopic ventral palp formation for BMP treatment from gastrula stages. In this revised version, we provide a novel Fig 6 that characterizes the phenotype of this late BMP treatment. In agreement with the loss of the ventral spot of *Isl*, we observed the loss of *Msx* expression and a lack of ventral papilla protrusion. However, ventral palp fate was not fully repressed since we observed partial expression of *Pou4* and *Sp6/7/8/9*. Also, we did not detect conversion into epidermis. Dorsal palps were not ventralized and developed normally as far as we can tell. We concluded that too high, or rather too ealy, BMP levels prevent ventral palp formation.

Minor comments
line 185 I see what the authors are trying to say but I don't agree that BMP limits the domain of FoxC expression as inhibition of BMP has no effect on FoxC. Rather BMP has to be kept out of the ANB in order to allow ANB formation.
[Response]
We have modified the sentence (lines 193-194).

The relationship between Foxg and Sp6/7/8/9 expression is not really clear and it would be better to do this with double ISH if the authors want to show mutually exclusive expression domains, or at least provide a summary figure.
[Response]
We have tried very hard to perform these double fluorescent *in situ* hybridizations. However, the developmental time window when the two genes are co-expressed at a level sufficient for a robust detection is very transient, as previously described (Liu and Satou, 2019). We thus did not manage results that allowed simultaneous detection of the two genes and turned to colorimetric *in situs* at different developmental stages. We have detailed that in the main text (lines 286-292). We

have modified Figure 5 into a new Figure 7 by adding schematic representations of our understanding of the expression patterns in relation to the different precursors of the palp lineage. We have also added a model for the role of BMP in the transition (U-shape to 3 spots) of *Foxg* expression.

Line 218, I do not see the data showing that Isl is expressed at a U-shape at st. 23, it seems to be expressed in three dots, unless embryos are treated with DMH1.
[Response]
We apologize for the misunderstanding since the sentence was not clear. We referred to the U-shaped *Isl* expression under BMP inhibition. Indeed, *Isl* starts to be expressed in 3 separate domains in the palp forming region, and not following a U-shape as its upstream regulator *Foxg* (Liu and Satou, 2019). We amended the sentence (lines 227-228).

Figure 6B, G. It could be nice to show a close up of the palps to see elongated cells.
[Response]
The close up pictures have now been added in the new Figure 5.

Figure 6K. It is better to use a statistical test to support the authors conclusions.
[Response]
As suggested, we have performed a statistical evaluation (Mann-Whitney U test) of the cell counts. The p-values are presented in the new Figure 5Q. The slight increase of *Celf3/4/5/6* is not statistically significant, but it does not impact our conclusion that the number of papilla cells increases following BMP inhibition.

It could be nice to provide a timeline for Smad1/5/8 signaling and the role for BMP signals that are proposed in this manuscript as a summary diagram.
[Response]
Following the suggestion, we have added summary diagrams in Figure 2 for BMP signaling in relation to lineages and gene expression.

lines 66-74 is lacking references.
[Response]
This is now corrected (lines 72-82).

Reviewer #1 (Significance (Required)):

Significance
While it is still not clear how BMP signals are established (which ligands for example) and their precise role in palp formation, this manuscript adds more information to our current understanding of the role of BMP signaling during palp formation. In particular it shows that BMP signals need to be kept out of the ANB for its formation and that it is required to resolve the later forming palp territory into three discrete palp regions. However, there is some way to go before this is fully understood. This article will certainly be of interest to ascidian developmental biologists trying to understand the formation and patterning of the larval PNS. It may also be of some interest to evolutionary biologists trying to understand the relationship between the telencephalon territory of vertebrates and the palp forming territory of ascidians as some links have been proposed between these two developmental territories (e.g. line 78).

[Reviewer's comments]
Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary.
The manuscript presents a detailed examination of how dynamic changes in BMP signaling during the development of the ascidian larval palps. Early in development BMP inhibition is responsible for the formation of a large field within the neuroectoderm that includes, among other fates, the presumptive palps. As development progresses, the territories of BMP activity/inhibition appear to be spatially refined within the palp-forming territory to specify palp versus interpalp fate. The experiments are presented with sufficient replication and statistical rigor.
[Response]
We thank the reviewer for the evaluation of our work.

Major Comments.

1. The researchers should look at otx expression in pFOG>Admp overexpressing embryos. It is difficult to assess from Figure 1, but it appears possible the the entire anterior sensory vesicle (not just the palps) are absent in the pFOG>Admp embryos (can the authors say briefly whether other ectodermal structures such as the atrial primordia or the oral siphon are still present?). Thus, is it possible that the entire a-lineage is disrupted? This would be an important distinction to make: are the defects attributed to experimental BMP activation specific to the palps, or are they more widespread in the anterior neuroectoderm? If the entire a-lineage is mis-fated, might this change the interpretation of the role of BMP inhibition? For example, might the formation of the palps depend on the proper development of the neighboring anterior neural plate? To address this concern, the authors should use a different driver to restrict Admp overexpression only to the palp forming region.

[Response]

In Figure 1, we show that *Celf3/4/5/6*, a general neural marker was still expressed in pFog>Admp embryos. We explain, in the Figure 1 legend, that this most likely corresponds to the CNS. It does not demonstrate that the anterior sensory vesicle (a-line induced CNS lineage) is still present. Unfortunately, *Otx* cannot be used as a suitable marker since it is also expressed in the posterior sensory vesicle (A-line lineage) (Hudson et al., 2003). Other a-line markers do exist. However, determining their expression at tailbud stages may not be conclusive since it is most likely that the patterning of the sensory vesicle (hence the expression of these markers) is modified after BMP activation. We have presented in former Figure 3 and Figure S1, strong evidence that the a-line neural lineage is intact at the neural plate stage. To better communicate these data, we have combined then in a modified Figure 3 that includes all markers examined and interpretative embryonic schemes. We show that, following BMP2 treatment, *Otx* and *Celf3/4/5/6* were specifically downregulated in the palp lineage but expressed normally in the CNS including a-line lineage. Consequently, the a-line CNS lineage is most likely not affected by BMP pathway activation. This does not mean that its later derivatives form normally, but this is an issue that we have not addressed. A previous report indicates that BMP activation leads to *Six1/2* repression and, possibly, the absence of oral siphon primordium (based on the images, no description in this paper) (Figure 1 from Abitua et al., 2015).

We think that we have addressed the concern of the reviewer, but would like to comment on the suggested experiment. It is very difficult to find a driver that would allow BMP activation only in the palp lineage (by overexpressing a constitutive active BMP receptor for example). a-line neural linage and palp lineage are intimately linked and separate at gastrula stages (St. 10). The regulatory sequences of *Foxc*, the first palp specific gene that we know, would thus be interesting. But it is most likely too late according to our whole embryo protein treatments (Figure 4). In agreement with this assumption, overexpressing Bmp2/4 (another BMP ligand) using the regulatory sequences of *Dmrt* (a master regulator of the palp+a-line CNS lineage expressed just before *Foxc*) does not apparently abolish palp formation (Extended Data Figure 5 from Abitua et al., 2015).

2. The authors hypothesize that papilla versus inter-papilla fate is controlled by differential BMP signaling. Is it possible to show differential P-Smad staining in papilla versus inter- papilla territories, as in Figure 2 for earlier gastrula-stage embryos? This data would make the authors hypothesis much more compelling. It appears that the authors have the necessary reagents.

[Response]

The actual lineage and fate segregation of papilla and inter-papilla lineage has not been determined as far as we know. Our current understanding comes from indirect evidence based on gene expression and gene function, in particular from the study of *Foxg* and *Sp6/7/8/9* by Liu and Satou (2009). Papillae originate from the 3 *Foxg/Isl* positive spots that are visible at very early tailbud stages. At earlier stages, *Isl* is not expressed and *Foxg* is expressed with a U-shape (new Figure 7). Within this U, it is most likely that the segregation of papilla and inter-papilla fates takes place when *Sp6/7/8/9* starts being expressed at late neurula stages. It is thought that *Sp6/7/8/9*$^{+}$/*Foxg*$^{+}$ cells will become inter-papilla cells while *Sp6/7/8/9*$^{-}$/*Foxg*$^{+}$ will become papilla. Our data indicate that BMP signaling is active in the future ventral papilla. We have mapped these data on schematics in the modified Figure 2. In addition, we are now proposing a more explicit model on the action of BMP in this process that is depicted in the new Figure 7.

[Reviewer's comments]

Minor Comments.

1. There is no mention of panels Figure 1 U and V in the text. In the figure legend they are misidentified as panels S and T.
[Response]
This has been corrected.

2. Very small issue with English usage that occurs throughout the manuscript. The authors should check the use of "palps" versus "palp", particularly when expressions such as the following are used: "palps formation", "palps network", "palps lineage", "palps differentiation", "palps molecular markers", "palps neuronal markers", "palps phenotypes", etc . For example, the sentence, "Here, we show that BMP signaling regulates two phases of palps formation in Ciona intestinalis", should read instead "Here, we show that BMP signaling regulates two phases of palp formation in Ciona intestinalis".
[Response]
Thank you, we have corrected these mistakes.

3. It would be worth mentioning possible relationships between the tunicate palps and the adhesive glands for larval fish and amphibians. Are there common mechanisms? All of these are anterior ectoderm derivatives.
[Response]
Thank you for the suggestion. We have added a section on that topic in the discussion (lines 410-426).

4. Please consider providing references in the Introduction for the sentences which end on the following lines of text:
36 (. . . is the sister group of vertebrates), 46 (. . . and sensory properties), 48 (. . . the secretion of adhesive materials), 57 (. . . on the nervous system in chordates), 68 (. . . also known as Ap2-like), 74 (. . . anterior neural territories)
[Response]
References have now been added.

5. To provide extra emphasis and to help the figures to stand alone with their respective legends, can you mention in the legend for Fig. 2 that D and E are controls? Also, can a brief legend be provided for S2 to give overall indication of staging, scale, orientation, etc.?
[Response]
Actually, the original Fig 2D and 2E correspond to treated embryos as explained in the legend. These embryos are now presented with additional treatments suggested by reviewer 1 in a novel Figure S1.
We have modified and added a legend to Figure S2 that is now Figure S4.

Reviewer #2 (Significance (Required)):

Significance.

This study presents an advance in our understanding of the fine-structure regulation of BMP signaling in sculpting neuroectoderm derivatives. While this study is potentially of broad interest, the authors fail to fully discuss the comparative aspects of this study in the context of conserved chordate developmental mechanisms. This could be remedied without too much difficulty in the Discussion section.

[Reviewer's comments]
Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:
This paper explores the role of BMP signaling for palp formation in ascidians using gain and loss of function approaches. The paper shows that while BMP at early (gastrula) stages prevents formation of the Foxc-positive palp ectoderm in Ciona, at later stages it appears to be essential for separation of the palps (possibly by promoting differentiation of interpapillary cells). The paper further shows that BMP plays similar roles in a different ascidian, Phallusia mammillata. Using previously published RNA-Seq results for the latter species after BMP up- regulation, the authors were able to identify additional BMP-responsive genes expressed in the palp region of

ascidians.
[Response]
We thank the reviewer for the evaluation of our work.

Major comments:
However, while the effect of BMP overexpression at early stages has been confirmed by two independent strategies (electroporation of the BMP agonist ADMP and BMP2 treatment), the effects of late BMP activation as well as the effects of BMP inhibition at both early and late stages have been studied exclusively by pharmacological treatments with a single BMP signaling agonist (BMP2) and antagonist (DMH1). To substantiate these findings and rule out unspecific side effects, it would have been desirable to verify them with alternative strategies.
[Response]
The reviewer may have missed some of our data. We have shown that BMP inhibition through overexpression of the secreted antagonist Noggin *via* electroporation using the early ectodermal driver pFog gives the same phenotypes as DMH1 treatment. The effects on *Foxc* were presented in the original Figure S1, and are now presented in the modified Figure 3 (line 173). We also showed that the morphological *Cyrano* phenotype was observed with Noggin overexpression (novel Figure 5F). We now present a novel Figure S3 with expression of *Isl* and *Celf3/4/5/6* following Noggin overexpression, and stress the use of this independent way of inhibiting BMP (lines 251-255). Given that early or late BMP inhibition lead to the same phenotype, we do not consider that overexpressing Noggin at gastrula stages is necessary.
Regarding BMP activation from gastrula stages, we have only used BMP2 treatment. It may be possible to overexpress Admp using promoters active in the palp lineage such as the ones of *Dmrt*, *Foxc* or *Foxg*. However, it may be difficult to phenocopy the phenotype obtained using BMP2 protein (loss of ventral palp), for two reasons. First, the precise timing to reach high BMP activation is not tightly controlled using such a method. Hence, all drivers should be tested. Second, the different promoters are active progressively later in development and in more and more restricted regions. Consequently, we consider that this requires a huge effort to validate a method (BMP protein treatment) that we already validated for the early effects and that has been used in several publications.

Therefore, while this study provides some new insights into the role of BMP in the specification of the palp forming region and subsequent palp development in ascidians, the evidence provided is relatively weak. Moreover, the scope of the study is quite limited. While identifying some BMP-responsive genes expressed in the palp region and describing the effects of BMP dysregulation on palp morphology, the study does not provide further insights into the underlying mechanisms how BMP patterns this region or affects subsequent palp formation.
[Response]
We are surprised by the appreciation of the reviewer describing our work as 'some new insights'. To our knowledge, this is the first report addressing the role of BMP signaling in palp formation at the molecular level. The only previous report by Darras and Nishida (2001) describes solely the morphology of the palps following overexpression of Bmp2/4 and Chordin overexpression by mRNA injection. We have brought significant novel findings 1) two important steps in palp formation with a precise description of the cellular and molecular actors, and a proposed function for BMP at each step, 2) evidence for conservation of this process in different ascidian species and 3) significant enrichment in the molecular description of this process. Moreover, the reviewer does not ask for specific items, we thus feel in the impossibility to offer satisfaction.

Minor comments:

- 63: ...as the anterior...

[Response]
Corrected.

- 68, 71, 74: references missing
- [Response]

References have now been added.

- 73: better: anterior neural territories and placodes
[Response]
Corrected.

- 76: palp territories also share molecular signature with anterior (eg. olfactory) placodes, not only telencephalon
[Response]
Corrected.

- 106: awkward sentence
[Response]
Corrected.

- 114: at what stage was ADMP electroporated?
[Response]
Electroporation of plasmid DNA is performed in the fertilized egg. Transcription of the transgene is controlled by the driver. In this case, with pFog, it occurs from the 16-cell stage. This precision has been added in line 122.

- 134: to facilitate comparison between stages it would be useful to label cells in Fig. 2(eg. which are a-line and b-line cells? Where is the border between them?)
[Response]
As suggested by the reviewer, we have modified Figure 2 with embryo outlines and schemes to better appreciate where BMP signaling is active.

- 152: since Foxc and Foxg overlap with pSMAD1/5/8 at neurula but not gastrula stages, do you know whether this is due to a dorsal expansion of BMP activity or a ventral expansion of Foxc/Foxg expression? Again, labeling of the nuclei would help
[Response]
The change corresponds to a dorsal expansion of P-Smad1/5/8. Our conclusion comes from combining nuclear staining (not shown for simplicity) and available fate maps. The results are presented in schematic diagrams of embryos in frontal views in the modified Figure 2.

- 174: the description is not clear here; what proportion of embryos did show reduction versus expansion of expression?. Why is the reduction shown in Fig.3 D asymmetrical?
[Response]
The proportions are now indicated in lines 187-188.
We apologize for the impression led by Fig 3D. Actually, it was the only case where the embryo was shown from the side (the description as a lateral view was inadvertently omitted in the legend). It did not show an asymmetric repression but an ectopic expression. We have now modified Figure 3 by properly showing only dorsal (neural plate) views and lateral views in insets when necessary. In addition, we have added schemes of embryos depicting the main tissues we have examined (palps, CNS and epidermis) and their localization depending on the treatments. We hope that the results are now clearly presented.

- 198: ... of endogenous...
[Response]
Corrected (line 276).

- 208: I suggest to highlight the regions of changes in Fig. with asterisks/arrows etc.
[Response]
We have added schematic embryos to highlight expression changes in the modified Figure 5 that is now Figure 7.

- 218: contrary to what is stated here, there is no depiction of u-shaped Isl1 expression in control embryos of Fig. 4
[Response]
As also pointed by reviewer 1, we apologize for the misunderstanding since the sentence was not clear. We referred to the U-shaped *Isl* expression under BMP inhibition. Indeed, *Isl* starts to be expressed in 3 separate domains in the palp forming region, and not following a U-shape as its

upstream regulator *Foxg* (Liu and Satou, 2019). We amended the sentence (lines 227-228).

- 220: the cell shapes referred to here cannot be seen in Fig. 4 (too small)
[Response]
We have modified Figure 6 (now Figure 5) to include close up of the palps.

- 271: the description here is confusing: first you talk about 53 genes and the mention palp expression of 12/26. Where does number 26 come from? And why was in situ done then for 27 additional genes? Also, while the comparison with previously published RNA-Seq data was valuable in uncovering additional BMP-sensitive palp markers, it does not provide any substantial new insights into how BMP patterns this territory.
[Response]
We have amended the sentence to make it clearer (lines 334-341).

- line 624: where
[Response]
Thank you. Modified line 787.

- Fig. 2: to facilitate comparison between stages it would be useful to label cells (eg. which are a-line and b-line cells? Where is the border between them?)
[Response]
Already responded above.

- -Fig. 3: Why is the expression in D asymmetrical? In the main text you write that expression is expanded in some embryos but reduced in others - Please show examples also of the expanded phenotype and give numbers
[Response]
Already responded above.

- Fig. 6: small panels in I, L, N need to be explained (single channels), white signal needs to be explained (overlap ?)
[Response]
We used white for better display of separate single channels. Given the confusion and the good quality of the 2 color fluorescent *in situ* images, we removed these panels in the modified Figure 6 that is now Figure 5.
White in K and L correspond to overlap (explained in the legend).

- Fig. S2: legend is missing
[Response]
This has been amended (now Figure S4).

Reviewer #3 (Significance (Required)):

Since the study does not provide substantial new insights into the mechanisms how BMP patterns the palp forming region or affects subsequent palp formation in ascidians, it will be of interest mostly for a specialized audience in the field of developmental biology.

Second decision letter

MS ID#: DEVELOP/2022/201575

MS TITLE: Regulation of anterior neurectoderm specification and differentiation by BMP signaling in ascidians

AUTHORS: Agnès Roure, Rafath Chowdhury and Sèbastien Darras
ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Development through Review Commons

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 study provides new insights into the role of BMP in the specification of the palp forming region and subsequent palp development in ascidians.

Comments for the author

The reviewers have successfully addressed my previous comments and those of other reviewers.

Reviewer 2

Advance summary and potential significance to field

In the article by Roure et al, the experiments seem to be carefully conducted and the presentation is much improved in this revised version. With this data, readers will understand somewhat better the role of BMP in palp formation. However, as mentioned in my previous report, this is still far from being fully understood. For example, while BMP signalling is not compatible with ANB formation, it is not clear which BMP ligands might be expressed near this territory or whether there is any specific mechanism present, or required, to protect the ANB from BMP signalling. In other words, it is difficult to access the biological significance of this part of the study.
Later in development, Smad1/5/8 becomes active in the ventral-most palp forming territory. BMP signals appear to be required for two aspects of palp patterning during these later stages. When BMP signals are inhibited with a pharmacological inhibitor from st.10, ventral palp identity seems to be missing (loss of Msx, changes in Pou4 expression) and at the same time the palp-inter-palp fate choice seems to be disrupted (more palp cells less inter-palp cells, Fig.5). On the other hand, BMP treatment resulted in partial loss of the ventral palp (Isl and Msx are lost, but some Pou4 cells remain, Fig. 6). Thus, both loss and gain of BMP signals result in loss of ventral palp. The authors offer a model for the papilla vs inter-papilla fate choice by proposing the presence of a second, unknown signal to reconcile the observations that both loss and gain of BMP signals result in loss of ventral palp (Fig. 7U-X). It seems to be clear that BMP signalling does not impact dorsal palp formation.
Thus, overall, while the study brings new insights into the potential roles of BMP in various aspects of ascidian papilla formation, a detailed molecular understanding is still lacking.

Comments for the author

Specific additional point:
It is not clear why inhibition of BMP results in a reduction in the number of Isl positive cells at stage 19 (Fig. 5KLOP, 12 green dots vs 8) and an increase in the number of Isl positive cells at stage 23 (Fig. 5IJNO, 12 green dots vs 15).