Deciphering and modelling the TGF-β signalling interplays specifying the dorsal-ventral axis of the sea urchin embryo.

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MS TITLE: Deciphering and modelling the TGF-&[beta] signalling interplays specifying the dorsal-ventral axis of the sea urchin embryo.

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend various revisions to your manuscript. 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 currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

In this manuscript, Flochlay et al. model the interplay between Nodal and BMP signaling during dorsal-ventral patterning in sea urchin embryos. The models build on experimental data from sea urchins and other organisms and increase in complexity to describe the initial partitioning of the ventral Nodal expression domain, the interplay between Nodal and BMP signaling without explicitly considering ligand diffusion, and finally spatiotemporal changes in cell ensembles. The models nicely recapitulate the abstracted wild type patterns as well as various signaling perturbations. However, although the summary statement states the proposal of a predictive model, the authors did not use the models to generate or experimentally test new predictions. The models were also not used to address several of the questions set up in the introduction, e.g. regarding the mechanism underlying the antagonism of BMP and Nodal signaling or the mechanism why Nodal and BMP signaling are located at opposite poles despite co-expression on the ventral side. Nevertheless, the current models can serve as the basis for more detailed simulations in the future.

Comments for the author

MAJOR POINTS

1) Model building and details a) The iterative process of modeling is currently only vaguely described, but the paper would benefit from a dedicated section about the challenges that were encountered. This might also expose sensitive parameters that are crucial for the model to work.
b) A major shortcoming of the models is the lack of feedback, i.e. there is no coupling of the output to the input, and Lefty, Chordin and Bmp2/4 are purely readout nodes. Furthermore, positive feedback by Glypican5 is mentioned in the introduction but not included in the model. Since feedback is crucial in this system, it should be included in the model.
c) The rules in Table 2 imply that Chordin diffuses less than BMP and that Lefty diffuses less than Nodal, which is inconsistent with other studies in sea anemones, flies, fish and frogs (e.g. PMIDs 25772352, 15935780, 28857744, 22499809, 24284174). Given that the diffusion coefficients in sea urchins are unknown, the authors should also try alternative models that take the data from other systems into account.
d) Smad6 is well known to predominantly affect BMP signaling (PMID 10733523). Since the antagonistic effect of Smad6 on Smad2/3 is modeled, it should also be included for the more important inhibition of Smad1/5/8. Alternatively, I suggest to remove Smad6 from the model.
e) Table 1B lists an entry “Admp2_Trans”, which is probably a carryover from the nomenclature in the GINsim model file. In contrast to the role of Admp2_In in Fig. 3, the model file says that the corresponding Admp2_Trans is not an input - please clarify.
f) Admp1 is missing from Table 1 - please add this parameter. Please also clarify whether the 0 input for Panda listed in Table 1 is correct.

2) Model predictions The models currently only recapitulate known phenotypes and can be considered “overfitted” given the model building procedure. To validate the model, it will therefore be crucial to generate and test new predictions as detailed below.
a) The model predicts that Onecut should be expressed transiently in the ventral region (see Fig.7). Can this also be observed experimentally?
b) The model in Fig. S1 provides a strong new prediction that must be experimentally tested to assess the validity of the model. Are ciliary and dorsal fates indeed switched on transiently in Lefty morphant embryos?
c) The dynamics are very important and decisive for a model’s utility to describe a biological process. It would be good to see the time course for the wild type data shown in Fig. 8. The dynamics should then be corroborated experimentally using in situ stainings at early embryonic stages. This would also help to more directly relate the 1D simulations with the experimental data and embryo geometry.

d) In Fig. 8D, the field comprises many more cells than in normal embryos, which is not in agreement with the experimental conditions. Therefore, the authors should repeat the simulations with identical field sizes and cell numbers.

3) BMP signaling dominance and the role of Smad4
a) In Fig. 3, BMP signaling has 4 activating inputs and only 1 inhibitory input, whereas Nodal signaling has only 2 activating but 2 inhibitory inputs. This easily explains the dominance of BMP signaling in the simulations. However, the real situation is more complicated since BMP signaling receives additional inhibitory inputs that could possibly lead to a balance of Nodal and BMP signaling in the model. For example, Noggin and Follistatin are well known inhibitors of BMP signaling that are currently not included in the model. Furthermore, Tolloid should not be a direct positive input in the model since it works indirectly and does not activate BMP signaling in the absence of Chordin. The authors should therefore assess whether their model outcomes still hold with these necessary amendments.
b) The paper states that there is a competition for Smad4 between BMP and Nodal signaling, e.g. “In other words, the dorsal pathway is more likely to win the competition for Smad4”, but this is not clear from the modeling or the experiments. First, as far as I know, the phenotype of Smad4 loss-of-function in sea urchin embryos is not known (but this system could be used to test whether Smad4 is truly the key node for competition). Second, the competition for Smad4 is not explicitly implemented in the model although it is considered to be the key integrator. Third, other mechanisms of antagonism that do not rely on competition for Smad4 are possible, e.g. at the extracellular level or downstream of Smad4. Statements such as those in lines 315-318 of the results section can therefore only be considered as speculation. Such statements would be bolstered by explicit modeling of Smad4 or by experimentally testing the dominance of BMP over Nodal using different input doses with simultaneous injections of recombinant BMP and Nodal. Alternatively, the authors should tone down their conclusions.

4) Chordin loss-of-function phenotypes Chordin morphants display “weak dorsal patterns”, which the authors argue presumably result “from the co-activation of the two antagonistic pathways”. However, alternatively it is also possible that the phenotype is not fully penetrant due to an incomplete block of Chordin by the morpholino. The authors could address this concern by injecting higher concentrations of the morpholino or by corroborating the results with a second morpholino (also compare to findings in PMID 19389361). Has the morpholino been validated in rescue experiments (I could not find the relevant data in the references listed in line 498)?

MINOR POINTS

1) Materials and methods
In the Materials and methods section, please provide details, exactly which recombinant proteins were used (e.g. a mix of BMP2 and BMP4?). Please cite the papers where the constructs for overexpression were originally published and what volume of the mRNA stocks was injected, so that the amount per embryo can be calculated.

2) Model details
a) The logical rule for Univin in Table 1B should be !Smad1_4_5_8 according to Fig. 3, not !Smad2_3_4. Please clarify.
b) It would be useful to show the database links also in Fig. 3 (similar to the GINsim model file) for permanent documentation and for readers who do not want to install the software. Furthermore, it would be helpful to provide references for each of the statements in Table 1. The model files should be published with the paper for a permanent record.
c) The model assumes that the domains are exclusive and do not overlap, and it would be useful to assess this assumption using multiplexed in situ stainings if technically feasible.
d) The division between early and late patterns seems artificial and is not molecularly justified. If the authors cannot simulate the entire process due to technical difficulties, they should at least provide a justification. Furthermore, the transition from the upper to the lower panel in Fig. 5B is unclear. Was “diffusion/shuttling” manually applied? If this is the case, the model apparently has little predictive power.
e) In the current model, Tolloid is only present dorsally. This is different from a previous modeling study (PMID 25167787) and should be discussed.

f) Admp1/2 are expressed in different poles in sea urchin embryos (PMID 26423516). Please clarify why this aspect was removed from the model.

3) Manuscript organization
a) The results mentioned in Fig. S2 and Fig. S3 currently only appear in the Discussion section and should be moved to the Results section.
b) In Fig. 1F, please also show the ventral view for completeness.
c) Please consistently add slashes for BMP2/4 and pSMAD1/5/8 in the figures.
d) The introduction section should include more references to the primary literature (e.g. for statements such as “Glypican5 is expressed downstream of BMP2/4 signalling […]” etc.).
e) Please clarify why Univin is listed as “Ciliary” in Fig. 2 but as “Ventral” in Fig. 3.
f) Line 172 states “eight nodes shown in green”, but there are 9 green nodes in Fig. 3. Please clarify.
g) Please clarify how “intermediate” levels of BMP and Nodal are defined.
h) Please provide statistics for all experimental phenotypes shown in the figures.
i) Are the units shown in Fig. 4 correct (panel A: mg/ml; panel B: ug/ml)?
j) The paragraph in lines 298-304 seems disconnected from the model, since the feedback on BMP activation is not included in the model (also see Major Point 1b above).

Reviewer 2

Advance summary and potential significance to field

This manuscript describes the formulation of a Boolean logic model for the gene regulatory network controlling the dorsal/ventral axis in sea urchin embryos. There is a lot of experimental evidence available in this system including results from the authors’ lab, and the goal to incorporate this into a dynamic model is important and timely. The GRN model the authors present here consists mostly of components of the nodal and bmp signaling pathways and their cross-interactions as well as their target genes. This model reproduces the early patterning events that subdivide the dorsal/ventral axis into three distinct cell fate domains, ventral, dorsal and ciliary band ectoderm and has therefore the potential to illuminate the mechanisms underlying this process. However, in the present form, I am not sure what to learn from this model and this is due in part to the way the model is built and in part to the way it is described in the manuscript.

Comments for the author

The logic model for the dorso/ventral axis has been built using an “iterative process” starting with experimental evidence and then using computational simulation until the model reproduces the experimental evidence derived from wildtype and experimentally perturbed embryos. In the following, the authors show that the model reproduces the observed expression pattern in wildtype and perturbed embryos, but since the model has been trained to do so, this is a circular argument and does not provide evidence that the model is correct. These results are expected based on the way the model has been generated. So besides showing that the model reproduces wildtype and mutant stable states, what have the authors learned about the GRN or the dynamic process of axis specification from generating this model? What are the nodes or mechanisms or interactions that are predicted by the model to be critical for the specification of the dorsal/ventral axis beyond what was already known based on experiments?

In order to use the computational model of the dorsal/ventral axis GRN to either assess the sufficiency of current understanding or to generate predictions, it would be important to distinguish between observed and predicted/assumed regulatory interactions. Which part of the model is supported by evidence, and which part has been inferred in order to reproduce the correct outcome? Is there any evidence supporting the inferred regulatory interactions? The GRN model in Fig. 3 does not show which interactions are supported by experimental evidence, and clarifications in the text, in the GRN model in Fig. 3, and/or in a table summarizing experimentally observed regulatory interactions would be extremely important and useful.

In this system, there is experimental evidence available that supports some regulatory interactions in the dorsal/ventral GRN. Were these known interactions used at all to constrain the

computational model in any way? In the method section you mention that the logical rules were refined, but were all interactions subjected to being refined and modified during the model building process or were some interactions maintained as they were supported by experimental data?

The model in Fig. 3 shows one GRN, however, depending on the inputs, there are different parts of the overall GRN that are active in the three domains along the D/V axis. According to the model, what are the nodes or mechanisms that are critical for generating the three cell fates in three distinct domains? What have you learned from the model about the mechanisms operating in the three domains? Does it confirm previously assumed mechanisms or predict new ones? Again, the fact that three states are produced is not very surprising given the way the model was built, but the model could be used to asses for example different possible input states and their ability to compute the correct output states or to assess critical nodes in the GRN.

The authors might have done this but the manuscript should state such results more clearly.
The model relies mainly on the mutual repression between Smad2/3/4 and Smad 1/4/5/8. Is there any cis-regulatory evidence for these interactions? Is there evidence that the smad genes are repressed in dorsal or ventral domains? How do you explain that nodal and bmp are co-expressed in the same ventral domain and yet nodal signaling is dominant? Does the model make any predictions about why this occurs?

The experiments with bmp and nodal show that both can induce one fate and suppress the alternative fate. However, in the bmp experiment, the effect is much stronger if embryos are exposed to bmp right after fertilization than if the protein is applied later in development. Based on this evidence and on the signaling that occurs during wildtype development I am not convinced that the signaling effects are independent of the relative timing of nodal and bmp signaling. One explanation for nodal signaling being dominant in ventral ectoderm could be that it is expressed earlier. Please clarify.

Furthermore, the repression circuit between the smads explains the formation of two alternative states, ventral and dorsal. But what about the ciliary fate? The model shows two genes, fgfa and onecut, as representing the ciliary state, but there is no interaction activating the two genes. Similarly, there is no activation of brachyury and foxa. How does this model produce three alternative states without assuming a mechanism to activate the genes representing all three states?

The modeling outcome is shown in form of the three cell fates, dorsal ciliary, and ventral, yet the GRN model is based on genes and their interactions. How do the authors define the three cell states in terms of expression states? Is it just based on individual representatives or are all blue genes required to be ON in the ventral state? It is also not clear whether 0, 1, and 2 values in the model correspond to signaling function or to gene expression state. This would need clarification.

In order to capture the spatio-temporal aspect of this patterning mechanism diffusion rates and signaling regulatory interactions are captured in a combined model. You mention that simple assumptions are sufficient to reproduce the patterning of the dorsal/ventral axis, but what are the critical assumptions in order for this to work? Does the model assume different diffusion rates for nodal, bmp, or some if their inhibitors? Is diffusion rate critical at all or are the regulatory interactions in Fig. 3 providing sufficient information for the three cell fates? Is it critical that nodal is expressed first? Why is bmp expressed in the cells on the right side in Fig. 8C? As far as I understand, the model only requires input values for nodal and univin, and the rest of the model operates correctly.
This would be an interesting result which might be most similar to the sea urchin embryo and would deserve being discussed together with the assumptions that have to be made in the model.

In Fig. 4 there are issues with concentrations shown as mg/ml and microg/ml in A and B.

In Fig. 5 A, the modeling inputs are shown as ubiquitous nodal and localized panda. But this is not consistent with the data provided by the author’s lab and others showing that nodal is localized at 32 cell stage. Similarly nodal is shown as expressed at low levels ubiquitously in Fig. 5B, but this

does not seem to be consistent with results. Why does the model need to be split into before and after 32 cell stage? Wouldn't it run to the same stable state with just the initial state in Fig. 5A?

In the stochastic model, the first fate to be induced is the ciliary band fate, as shown in Fig. 7. But is there any evidence that this occurs in the embryo? Why is the ciliary band assumed to be the default state in this model?

Reviewer 3

Advance summary and potential significance to field

In the paper by Floc'hlay et al, they authors develop a multi-level Boolean model to simulate the dorsal/ventral axis formation by BMPs and Nodals in the sea urchin embryo. This is an important and timely paper that couples a combined experimental and modeling of Nodal and BMP2/4 GRN. The primary outcomes of the paper support the mutual antagonism hypothesis. The paper also touches on the order and dominance of the antagonism and offers a confirmatory outcome that will be of interest to the developmental biology community.

Comments for the author

While the authors do a good job of developing and presenting the simulation, some of the concepts of the model were not clearly explained. In addition, there are areas of interpretation and figures that could increase clarity and specific examples are listed below.

1) In figure 1 E and 1F, the morphological phenotypes between BMP2/3 inhibition, and both BMP2/4 and Panda inhibition are compared and it is claimed that panda is sufficient to break the radial symmetry of the embryo and necessary to specify the D/V axis. However, this conclusion is based on the fact that in both cases BMP2/4 is inhibited. Clarity on the epistasis of BMP2/4 and Panda will need to be provided.

2) I think a lot more explanation of the model is needed. In the single-cell level simulation, the author claimed that the model was revised based on the experimental results. However, how the revision process was done was not clear. More details and examples for the model or figures could be included to increase clarity.

3) The single-cell logical model using the Boolean case cannot represent the interaction between neighbor cells. The spatial information between the cells depends on the input level of specific species this is not sufficient to represent the diffusion and reactions between different species. It is still a little bit unclear wreathe the single-cell model was included in the multicellular model and how they match each other both in single-cell and multicellular level experimental evidence.

4) The interactions between different protein ligands included in the model, this could be insufficient to the model. For example, as it was well known, the BMP2/4 ligands can react with Chordin ligand and formed the BMP-Chordin compound which is inhibited the signaling ability of BMP2/4. In the model shown in Figure 3, this mechanism was represented by Chordin inhibit BMP receptor Alk1_2_3_6. This may be sufficient in the circumstance that Chordin inhibits the BMP singling by preventing BMP bound it receptor this is equal to the fact that it reduces the receptor level itself. If this is the assumption made by the author, this needs to be clarified.

5) The embryo shape was not considered in the study. As shown in Figure 1, at the MB stage the embryo is a ring-like shape. However, in the multicellular simulation shown in figure 8, the author illustrates the layers of hexagons to represent the cell, and based on the description, the diffusion of the species is relayed on the cell by cell transport. If so, the cell number and the relative distance between the cell should match with the embryo as close as possible to represent the real spatial distribution of the cells. How does the ring- like aspect get incorporated- throught the boundary conditions of cells at the ends?

6) The "diffusion" mechanism represented in this study is based on the logical rule of passing the species between the 'cells' directly. The author has not shown enough evidence of how the logical rule of diffusion was constructed. This also coincides with the multiple integer level of some

components used in the Boolean model. Do these extend to the diffusion as well? How is range determined?

First revision

Author response to reviewers' comments

We warmly thank the editor for selecting our manuscript for reviewing and the reviewers for their time and valuable comments. Please find below our answer to the points raised by the reviewers.

In order to ease the modification tracking, we have highlighted in the revised manuscript the sections added and/or modified according to the reviewer suggestions and referred to their location in the answer by underlined text.

Reviewer comments are numbered and formatted in black, answers are formatted in grey and start with a bullet point.

I) Answer to reviewer 1

A) Major points

Comments regarding model building:

1 - The iterative process of modeling is currently only vaguely described, but the paper would benefit from a dedicated section about the challenges that were encountered. This might also expose sensitive parameters that are crucial for the model to work.

- We thank the reviewer for pointing this need of clarification, also stressed by the two other reviewers. We have expanded our explanation of the different steps and challenges encountered to set up the model in the result section (page 6-7) and adapted the Fig.2.

2 - A major shortcoming of the models is the lack of feedback, i.e. there is no coupling of the output to the input, and Lefty, Chordin and Bmp2/4 are purely readout nodes. Furthermore, positive feedback by Glypican5 is mentioned in the introduction but not included in the model. Since feedback is crucial in this system, it should be included in the model.

- The unicellular model does indeed include only a few feedback mechanisms explicitly (FoxA auto-regulation and FoxA - Brachyury positive regulatory circuits). This can be explained by the fact that a large fraction of the regulatory mechanisms involved in the dorsal-ventral axis specification are based on intercellular signalling and diffusion, which cannot be fully considered in our unicellular model. In order to tackle this problem, we have integrated our unicellular model into a multicellular framework, which explicitly accounts for intercellular signalling, in terms of diffusion rules assigned to each input node, which integrate signalling events coming from neighbouring cells. In this framework, the links between inputs and output nodes corresponding to Nodal, BMP2/4, Lefty and Chordin implement various autocrine and paracrine signalling circuits.

- The reviewer is right to stress the importance of Glypican5 in the system. According to Lapraz *et al*. 2009, Glypican5 has been shown to reinforce the BMP2/4 dorsal signalling following the initial activation of the BMP2/4 pathway. However, it does not seem to be sufficient to activate the dorsal cascade by itself. We believe that Glypican5 is indeed a crucial actor to regulate the kinetic of the dorsal cascade, but is not necessary for the primary BMP2/4 cascade activation. As this study focuses on the primary activation of the dorsal cascade, we chose to leave out the positive feedback exerted by Glypican5 at this stage. However, it would be interesting to integrate this positive feedback in a refined version of the model covering later developmental stages, although this might require the use of a more quantitative modelling framework (e.g.

ODEs).

3 - The rules in Table 2 imply that Chordin diffuses less than BMP and that Lefty diffuses less than Nodal, which is inconsistent with other studies in sea anemones, flies, fish and frogs (e.g. PMIDs 25772352, 15935780, 28857744, 22499809, 24284174). Given that the diffusion coefficients in sea urchins are unknown, the authors should also try alternative models that take the data from other systems into account.

- The point raised by the reviewer is very interesting. It led us to adapt our multicellular model to check the impact of using similar diffusion rules for Nodal, Chordin, BMP2/4 and Lefty (i.e. they all take the value 1 as input node if any neighbour cells on the epithelium express them as output node at minimum level 1, cf. revised Table 2). Interestingly, using this revised model, we could now address two other points raised by the reviewer, namely that dealing with the ubiquitous expression of Tolloid, and that dealing with the transient dorsal specification in the Lefty morphants (see below).

4 - Smad6 is well known to predominantly affect BMP signaling (PMID 10733523). Since the antagonistic effect of Smad6 on Smad2/3 is modeled, it should also be included for the more important inhibition of Smad1/5/8. Alternatively, I suggest to remove Smad6 from the model.

- Following the suggestion from the reviewer, we chose to remove Smad6 from our model (cf. Fig. 3). Although Smad6 is a crucial actor in the dorsal-ventral axis specification, its action mainly concerns the maintenance of the territory specification, rather than its initial activation. Indeed, the negative regulation of Smad6 by BMP2/4 presumably contributes to stabilise BMP2/4 activation in the dorsal territory, but would not be necessary for its initial activation.

5 - Table 1B lists an entry “Admp2_Trans”, which is probably a carryover from the nomenclature in the GINsim model file. In contrast to the role of Admp2_In in Fig. 3, the model file says that the corresponding Admp2_Trans is not an input - please clarify.

- We thank the reviewer for pointing this mistake, which has been corrected in the Table 1 of this revised manuscript.

6 - Admp1 is missing from Table 1 - please add this parameter. Please also clarify whether the 0 input for Panda listed in Table 1 is correct.

- We thank the reviewer for pointing the omission of Admp1, which has been corrected in the Table 1 of the revised manuscript. The 0 input for Panda is indeed correct, as Table 1 refers to the unicellular late stage simulation, where the maternal input is not present anymore. However, Panda input node is set to the level 1 for the early stage simulation, as depicted in the new version of Fig.5.

Comments regarding model prediction:

7 - The models currently only recapitulate known phenotypes and can be considered “overfitted” given the model building procedure. To validate the model, it will therefore be crucial to generate and test new predictions as detailed below.

- A new section has been added to the manuscript to clarify the main modelling workflow steps, especially the interplay between the iterative model construction and experimental hypothesis testing (page 6-7).

8 - The model predicts that Onecut should be expressed transiently in the ventral region (see Fig.7). Can this also be observed experimentally?

- The finding displayed in Fig.7 pinpointed by the reviewer has indeed been observed experimentally: evidence for a transient activation of the ciliary marker gene Onecut can be found in the Fig. 1E of Saudemont et al. (2010). This information has been added to the revised manuscript (page 11).

9 - The model in Fig. S1 provides a strong new prediction that must be experimentally tested to assess the validity of the model. Are ciliary and dorsal fates indeed switched on transiently in Lefty morphant embryos?

● The results presented in Molina *et al.* (2018) suggest that there is no transient expression of ciliary or dorsal fate markers in Lefty morphants. Indeed, in such conditions, the embryos express ectopic nodal in the whole ectoderm from early stages of development, at 128 cells (Molina et al 2018), when dorsal or ciliary band marker are still not expressed, even in control embryos. At later stages of development, when control embryos do express dorsal and ciliary band markers, lefty morphants continue to express ectopic nodal in the whole ectoderm (Duboc et al 2007). The ectopic expression of nodal observed in Lefty morphants correlates with an expanded expression of the nodal target genes bmp2/4 and its inhibitor chordin, among others. Activation of BMP2/4 signalling in the presence of its inhibitor chordin does not result in the expression of dorsal fate markers but, interestingly, it is sufficient to impair the expression of some nodal target genes, such as admp1 (Lapraz et al., 2015). Thus, weak activation of BMP2/4 signalling occurs in ventralised embryos that ectopically expressed nodal, although it is not sufficient to switch on dorsal or ciliary band fates.

● Following the suggestion of the reviewer concerning the adaptation of diffusion rules in the multicellular model (see above), the refined model does not predict a transient dorsal or ciliary activation anymore. On the contrary, our model now predicts an ectopic ventral activation in the whole embryo, starting very early on, thus preventing the dorsal cascade activation. The modification of the diffusion rules in our model (cf. revised Table 2) thus better match current experimental results for Lefty morphants.

10 - The dynamics are very important and decisive for a model's utility to describe a biological process. It would be good to see the time course for the wild type data shown in Fig. 8. The dynamics should then be corroborated experimentally using in situ stainings at early embryonic stages. This would also help to more directly relate the 1D simulations with the experimental data and embryo geometry.

● We are now including a supplementary figure (Sup. Fig. 1) representing the time course of the activation of key input and output nodes for the multicellular model.

11 - In Fig. 8D, the field comprises many more cells than in normal embryos, which is not in agreement with the experimental conditions. Therefore, the authors should repeat the simulations with identical field sizes and cell numbers.

● One current limitation of the EpiLog tool is its fixed epithelium size over the simulation time course. From the 32-cell stage up to the early blastula stage, the sea urchin embryo increases its number of cells through several rounds of cell division. This increase in cell number cannot be formally implemented within our multicellular epithelium. To circumvent this problem, we simulated the siamese experiment with a fixed size epithelium, representing the initial opposite *nodal* mRNA cell injections by setting Nodal output node as active in the two extreme opposite columns of cells in our epithelium (which are seen as circles of cells, as the top and bottom rows of the epithelium are linked in order to create a cylinder in the simulation, cf. Fig.8D). Although this result matches the experimental observation, it is still not perfect as it does not reflect the cell number increase. Hopefully, this limitation could be resolved in the future with the release of a novel version of EpiLog enabling the consideration of cell proliferation. All simulations have been repeated with identical field size and cell number in the revised manuscript (Fig. 8 and method p.21).

Comments regarding BMP dominance and the role of Smad4:

12 - In Fig. 3, BMP signaling has 4 activating inputs and only 1 inhibitory input, whereas Nodal signaling has only 2 activating but 2 inhibitory inputs. This easily explains the dominance of BMP signaling in the simulations. However, the real situation is more complicated since BMP signaling receives additional inhibitory inputs that could possibly lead to a balance of Nodal and BMP signaling in the model. For example, Noggin and Follistatin are well known inhibitors of BMP signaling that are currently not included in the model. Furthermore, Tolloid should not be a direct

positive input in the model since it works indirectly and does not activate BMP signaling in the absence of Chordin. The authors should therefore assess whether their model outcomes still hold with these necessary amendments.

● We understand the point brought by the reviewer concerning the potential impact of the different numbers of activatory and inhibitory inputs considered. However, in the context of logical model, the relative importance of each repression and activation interaction is modulated by the logical rule. For example, for a node A having two activators B and C and one repressor D, all active, we can represent the following alternative situations:
The repression is dominant with the logical rule D -> 1 : (B | C) & !D; The activations are dominant with the logical rule D -> 1 : (B|C) | !D.
Presumably, the stochastic simulations with MaBoSS do address the point raised by the reviewer. Indeed, using equal rates of activation, we observe a dorsal dominance explained by the structure of the model and taking into account the weight given to the interaction by the regulatory rules.

● Concerning the consideration of Noggin and Follistatin, although the corresponding genes have been identified in sea urchin genome, data regarding the regulation of their expression is still lacking and we thus decided to leave them aside at this stage. Regarding Tolloid, the reviewer points correctly that this protein does not directly act on the Alk receptors. To take into account the indirect effect of Tolloid, we build the regulatory rule driving Alk activity so that Tolloid alone is not sufficient to drive the activation but is only necessary for the repression of chordin (cf. Table 1).

13 - The paper states that there is a competition for Smad4 between BMP and Nodal signaling, e.g. “In other words, the dorsal pathway is more likely to win the competition for Smad4”, but this is not clear from the modeling or the experiments. First, as far as I know, the phenotype of Smad4 loss-of- function in sea urchin embryos is not known (but this system could be used to test whether Smad4 is truly the key node for competition). Second, the competition for Smad4 is not explicitly implemented in the model although it is considered to be the key integrator. Third, other mechanisms of antagonism that do not rely on competition for Smad4 are possible, e.g. at the extracellular level or downstream of Smad4. Statements such as those in lines 315-318 of the results section can therefore only be considered as speculation. Such statements would be bolstered by explicit modeling of Smad4 or by experimentally testing the dominance of BMP over Nodal using different input doses with simultaneous injections of recombinant BMP and Nodal. Alternatively, the authors should tone down their conclusions.

● We agree with the reviewer that we have not demonstrated experimentally that there is a competition between the Nodal and BMP pathways at the level of Smad4. The reviewer is also correct in pointing that the phenotype caused by the injection of a morpholino targeting Smad4 has not been described so far. Unfortunately, due to the Covid-19 pandemics, which led to the closing of the University during three months, we have not been able to perform any additional experiments, and we were furthermore impeded to renew our stock of sea urchins before the end of the sea urchin season. Therefore, to date, we cannot provide additional data to support the idea of the competition between the two pathways. However, we would like to mention preliminary results from experiments initiated before the sanitary crisis on the Smad4 gain- and loss-of-function, since we think these support the idea that Smad4 is a limiting factor for both Nodal and BMP2/4 pathways, and that Smad4 plays a central role in the competition between the two pathways.

● The first observation that points to Smad4 being the object of a competition between the Nodal and BMP pathways is that embryos injected with a Smad4 morpholino seem to display a phenotype very similar to the BMP2/4 loss of function, i.e. loss of dorsal tissues, which are replaced by ciliary tissue. At the molecular level, this is accompanied by a reduction of ventral gene expression (chordin) and by an almost complete loss of dorsal gene expression (tbx2/3). So maternal Smad4 protein is limiting in the embryo and zygotic Smad4 protein appears necessary to sustain BMP2/4 signalling.
A first attempt to overexpress Smad4 mRNA also gave a clear phenotype: adding Smad4 mRNA ventralises the embryo, expanding chordin and suppressing tbx2/3 expression. So, despite the fact that Smad4 is a common Smad and is not supposed to be limiting for Nodal, it appears to be limiting as adding more Smad4 allows endogenous Nodal to ventralise.

We do not intend to include these experiments in the paper because they need to be repeated and the results confirmed, together with proper control experiments for the morpholinos.
Hence, a definitive demonstration of the role of Smad4 will require additional experiments postponed to the next sea urchin season.

- We have expanded the discussion of this hypothesis of a competition for Smad4, mentioning the need for further experimental validation in the revised manuscript discussion (p. 13-14).

Comments regarding Chordin loss-of-function phenotypes:

14 - Chordin morphants display "weak dorsal patterns", which the authors argue presumably result "from the co-activation of the two antagonistic pathways". However, alternatively it is also possible that the phenotype is not fully penetrant due to an incomplete block of Chordin by the morpholino. The authors could address this concern by injecting higher concentrations of the morpholino or by corroborating the results with a second morpholino (also compare to findings in PMID 19389361). Has the morpholino been validated in rescue experiments (I could not find the relevant data in the references listed in line 498)?

- Several concentrations of a second morpholino have been tested (cf. figure below). As previously reported, chordin morphants mainly develop into pluteus larva that grow parallel spicules, show ectopic PSmad1/5/8 staining and are partially dorsalised (Lapraz et al., 2009). When increasing the concentration of Chordin splice morpholino, some embryos differentiated an ectopic ciliary band along the dorsal ectoderm and resemble bmp2/4 morphants. In a few cases, the whole ectoderm differentiated into ciliary band and the embryos appeared radialized, resembling nodal morphants. In the sea urchin, bmp2/4 expression is fully dependent on Nodal signalling (Duboc et al., 2004). Hence, these results suggest that the activation of BMP2/4 signalling observed in chordin morphants blocks Nodal signalling and the source of bmp2/4 gene, giving rise to radialized embryos, which eventually lose both dorsal and ventral fates.

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

B) minor points

Comments regarding Material and methods:

1 - In the Materials and methods section, please provide details, exactly which recombinant proteins were used (e.g. a mix of BMP2 and BMP4?). Please cite the papers where the constructs for overexpression were originally published and what volume of the mRNA stocks was injected, so that the amount per embryo can be calculated.

- This information has been added to the revised manuscript. (cf. subsections "Animal, embryos and treatments" and "Overexpression of mRNAs and morpholino injections" of the Material and methods section, p. 16-17).

Comments regarding model details:

2 - The logical rule for Univin in Table 1B should be !Smad1_4_5_8 according to Fig. 3, not ! Smad2_3_4. Please clarify.

- We thank the reviewer for pointing this error, which has been corrected it the Table 1 of our revised manuscript.

3 - It would be useful to show the database links also in Fig. 3 (similar to the GINsim model file) for permanent documentation and for readers who do not want to install the software. Furthermore, it would be helpful to provide references for each of the statements in Table 1. The model files should be published with the paper for a permanent record.

● We have exported the documentation from the GINsim model file as a standalone supplementary material accessible from the corresponding entry in the GINsim repository page (http://ginsim.org/node/236).

4 - The model assumes that the domains are exclusive and do not overlap, and it would be useful to assess this assumption using multiplexed in situ stainings if technically feasible.

● Several double in situs have been realised for several pairs of dorsal and ventral markers in Lapraz et al., 2009 (Fig.6) and Saudemont et al., 2011. (Fig.1). The results showed no overlapping pattern of expression.

5 - The division between early and late patterns seems artificial and is not molecularly justified. If the authors cannot simulate the entire process due to technical difficulties, they should at least provide a justification. Furthermore, the transition from the upper to the lower panel in Fig. 5B is unclear. Was “diffusion/shuttling” manually applied? If this is the case, the model apparently has little predictive power.

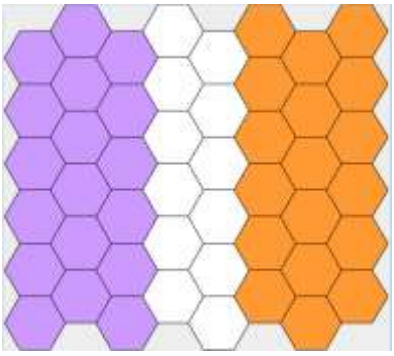
● The link between early and late patterning relies on diffusion, which could not be taken into account explicitly with the unicellular model. Hence, the initiation of the blastula stage simulation (input and initial state) for the unicellular model was derived from the outputs of the simulation of the earlier stage (32-cell) and manually specified (cf. revised Fig.5).
However, in the case of the multicellular model, signal diffusion can be properly considered. In the revised version of the multicellular model, we are now able to recapitulate the two previous stages of axis specification into a single simulation (cf. revised Fig.8 and results p. 12), starting from the early Panda + Nodal embryo patterning and resulting in the Ventral- Ciliary-Dorsal pattern expected at the blastula stage. In our revised manuscript, we now clarify how the inputs for the blastula stage were derived from the simulation of the previous 32-cell stage (cf. revised Fig.5).

6 - In the current model, Tolloid is only present dorsally. This is different from a previous modeling study (PMID 25167787) and should be discussed.

● In our initial study, we considered that Tolloid was active only dorsally. However, as pointed by the reviewer, Tolloid is expressed more broadly. In the revised version of our multicellular model, we now consider that Tolloid is ubiquitously present and active in the full ectoderm (cf. revised Table 1), but that this activity is contextualised by other inputs (through the logical rules). These simulations are thus now consistent with the previous modelling study mentioned by the reviewer (van Heijster et al, 2014 - PMID 25167787 : "we use the whole embryo circumference as the production domain for Tld").

7 - Admp1/2 are expressed in different poles in sea urchin embryos (PMID 26423516). Please clarify why this aspect was removed from the model.

● As noted by the reviewer and the mentioned study (Lapraz et al, 2015 - PMID 26423516), Admp1 and Admp2 are indeed expressed in two opposite poles of the sea urchin, namely the ventral (Admp1) and dorsal (Admp2) poles. This observation coincides with our wild-type simulation results (cf. figure below). Indeed, the output nodes of the multicellular model representing Admp1 (purple) and Admp2 (orange) expression are respectively active in the ventral and dorsal region of the sea urchin embryo.



Comments regarding manuscript organisation :

8 - The results mentioned in Fig. S2 and Fig. S3 currently only appear in the Discussion section and should be moved to the Results section.

- In our revised manuscript, we now refer to Fig. S2 in the result section (now indexed as Fig.S1, p.12). However, we prefer to keep the reference to Fig. S3 in the discussion, as it is related to the discussion of potential model extensions (now indexed as Fig.S2, p.14).

9 - In Fig. 1F, please also show the ventral view for completeness.

- The figure has been adapted according to the reviewer suggestions (cf. revised Fig.1).

10 - Please consistently add slashes for BMP2/4 and pSMAD1/5/8 in the figures.

- The figures and text have been adapted according to the reviewer suggestions.

11 - The introduction section should include more references to the primary literature (e.g. for statements such as “Glypican5 is expressed downstream of BMP2/4 signalling […]” etc.).

- Additional reference has been added to the manuscript according to the reviewer suggestions (p. 4).

12 - Please clarify why Univin is listed as “Ciliary” in Fig. 2 but as “Ventral” in Fig. 3.

- We thank the reviewer for pointing this error, which is now corrected in the revised manuscript (Fig. 2 and Fig.3).

13 - Line 172 states “eight nodes shown in green”, but there are 9 green nodes in Fig. 3. Please clarify.

- We thank the reviewer for pointing this error, now corrected in our revised manuscript (p. 7, note that, as Smad6 node was removed from the model, the number remains unchanged).

14 - Please clarify how “intermediate” levels of BMP and Nodal are defined.

- In our model, we try to minimise as much as possible the use of multilevel nodes in order to restrict the complexity of the model. The higher expression levels of multicellular nodes therefore distinguish the different regulatory states of the same species in wild type conditions (e.g. Nodal 1 < Lefty inhibition and Nodal 2 > Lefty inhibition) as well as mutant conditions (e.g. Nodal 3 (mRNA injection) > Nodal 1 & 2 wild type expression). We have expanded our explanation of the multilevel nodes in our revised manuscript (p. 8).

15 - Please provide statistics for all experimental phenotypes shown in the figures.

- The following sentence has been add to the subsection “Animals, embryos and

treatments" of the Material and methods section (p. 16):
"All the experiments described in this study have been repeated two or three times. At least 200 wild-type and 50 injected embryos were analysed for each condition or experiment. In the case of treatments with recombinant proteins, more than 500 embryos were scored for morphological phenotypes for each condition and about 250 embryos were used for in situ hybridisation. Only phenotypes observed in more than 90% of the embryos are shown."

16 - Are the units shown in Fig. 4 correct (panel A: mg/ml; panel B: ug/ml)?

- We thanks the reviewer for pointing this error, now corrected in the Fig.4 of our revised manuscript.

17 - The paragraph in lines 298-304 seems disconnected from the model, since the feedback on BMP activation is not included in the model (also see Major Point 1b above).

- We agree with the reviewer that the feedback on BMP activation is not formally included in the unicellular model. This feedback is considered by manually adjusting the inputs based on the output of the simulation of earlier stages (see Fig. 5). However, this feedback is explicitly taken into account in the multicellular model, via the definition of logical diffusion rules.
The paragraph pointed by the reviewer indeed summarises experimental observations that are not fully recapitulated by the model, as it relates to the conditions where discrepancies were observed between the model and the experimental observation (see Fig. 2). In this respect, to better understand the regulatory mechanisms driving the patterning of the embryo in chordin Mo conditions, we used stochastic simulations (Fig. 7).

II) Answer to reviewer 2

1- The logic model for the dorso/ventral axis has been built using an "iterative process" starting with experimental evidence and then using computational simulation until the model reproduces the experimental evidence derived from wildtype and experimentally perturbed embryos. In the following, the authors show that the model reproduces the observed expression pattern in wildtype and perturbed embryos, but since the model has been trained to do so, this is a circular argument and does not provide evidence that the model is correct. These results are expected based on the way the model has been generated. So besides showing that the model reproduces wildtype and mutant stable states, what have the authors learned about the GRN or the dynamic process of axis specification from generating this model? What are the nodes or mechanisms or interactions that are predicted by the model to be critical for the specification of the dorsal/ventral axis beyond what was already known based on experiments?

- The main aim of the iterative modelling process is to integrate the different pieces of data documented in the scientific literature into a common framework (regulatory graph), and then assess whether a set of consistent regulatory rules can enable the wild-type and mutant gene expression patterns observed. In order words, the logical simulations demonstrate that a given network is able to generate the observed patterns. The logical rules themselves are constrained by experimental data (e.g. the formation of an active complex implies the use of an AND operator in the rules driving the dynamics of the targets of this complex).

- Model simulations further enabled us to explore alternative mechanisms for dorsal-ventral pattern specification. For example, our first model simulations of Chordin KO conditions suggested that both ventral and dorsal territory could be specified in the presumptive ventral territory, whereas only dorsal specification is observed experimentally. This led us to distinguish two alternative mechanisms for the activation onset of the two TGF-beta pathways, the first based on some differential activation timing, the second based on protein dosage. This led us to perform some additional experiments, whose results favoured the dosage mechanism, which in turn led us to refine the logical rules of the model accordingly.

2 - In order to use the computational model of the dorsal/ventral axis GRN to either assess the sufficiency of current understanding or to generate predictions, it would be important to distinguish between observed and predicted/assumed regulatory interactions. Which part of the

model is supported by evidence, and which part has been inferred in order to reproduce the correct outcome? Is there any evidence supporting the inferred regulatory interactions? The GRN model in Fig. 3 does not show which interactions are supported by experimental evidence, and clarifications in the text, in the GRN model in Fig. 3, and/or in a table summarizing experimentally observed regulatory interactions would be extremely important and useful.

● All the data supporting our model network and associated regulatory rules are mentioned in the annotations included in the model file, and are now further included as a standalone supplementary material accessible from the corresponding entry in the GINsim repository page (http://ginsim.org/node/236).

3 - In this system, there is experimental evidence available that supports some regulatory interactions in the dorsal/ventral GRN. Were these known interactions used at all to constrain the computational model in any way? In the method section you mention that the logical rules were refined, but were all interactions subjected to being refined and modified during the model building process or were some interactions maintained as they were supported by experimental data?

● We thank the reviewer for pointing this need of clarification, also stressed by the two other reviewers. In order to better clarify the distinction between the initial iterative process of model construction from the rule refinement based on experiments, we have revisited the Fig. 2 and expanded our explanation of the different steps and challenges encountered to set up the model in the result section (page 6-7).

4 - The model in Fig. 3 shows one GRN, however, depending on the inputs, there are different parts of the overall GRN that are active in the three domains along the D/V axis. According to the model, what are the nodes or mechanisms that are critical for generating the three cell fates in three distinct domains? What have you learned from the model about the mechanisms operating in the three domains? Does it confirm previously assumed mechanisms or predict new ones? Again, the fact that three states are produced is not very surprising given the way the model was built, but the model could be used to assess for example different possible input states and their ability to compute the correct output states or to assess critical nodes in the GRN. The authors might have done this but the manuscript should state such results more clearly.

● Indeed, the outcome of model simulations critically depends on the states of the input nodes. For any set of active input node, the model leads to the activation of a gene expression pattern corresponding to one of the three know ectodermal territories (ciliary, ventral, dorsal). As expected, the wild-type input conditions correctly recapitulate the wild-type pattern as defined by the main marker genes considered. Depending on the region considered, a subset of the network components are active, although the regulatory network is the same throughout the embryo. In the supplementary Jupyter notebook accessible from the corresponding entry in the GINsim repository page (http://ginsim.org/node/236), we are now including a few additional command lines enabling the visualisation of the three reference (wild-type) patterns on the GRN itself.

● Our analysis of the impact of different input combinations for different perturbations (i.e. restricting a node activity to either active or inactive to reflect Mo-mRNA injection) led to three main insights :

- The positive feedback of Nodal on its own regulatory cascade is critical to maintain territory specification active, even outside of the ventral region (i.e. Nodal KO prevents the dorsal BMP2/4 cascade to activate).

- The mechanisms establishing the boundaries of the territories rely on diffusion. More specifically, chordin and lefty morphogen gradients are critical to establish the proper location of boundaries between the three territories (i.e. Chordin KO and Lefty KO expand the dorsal and ventral territories, respectively).
- The competition between ventral and dorsal pathways for Smad4 is critical to maintain the mutual restriction between territories and lock cell fate by preventing the opposite pathway to be active (i.e. no overlapping markers from different territories in the multicellular simulation in any conditions).

● In our revised manuscript, we have adapted the Fig.5 to clarify the specification of inputs levels for the unicellular model.

5 - The model relies mainly on the mutual repression between Smad2/3/4 and Smad 1/4/5/8. Is there any cis-regulatory evidence for these interactions? Is there evidence that the smad genes are repressed in dorsal or ventral domains? How do you explain that nodal and bmp are co-expressed in the same ventral domain and yet nodal signaling is dominant? Does the model make any predictions about why this occurs?

● The reviewer is raising a very interesting point. Indeed, evidence for binding site of Smad complexes on the *cis*-regulatory regions of the target genes of the BMP2/4 and Nodal pathways has been shown (Hill, 2016 - PMID 27449814). This evidence support the regulatory role of Smad complexes in the activation of the BMP2/4 and Nodal TGF-beta pathways. We have added this point to the revised manuscript (p.13-14).

● Concerning the mutual repression of the two Smad complexes, there are no additional published evidence supporting this hypothesis to our knowledge. Please refer to our answer to the first reviewer about "BMP dominance and the role of Smad4" for a more detailed discussion on this point.

● Concerning the co-expression of nodal and bmp in the ventral territory, the model has enabled us to explore the regulatory dynamics occurring in this presumptive territory. In the ventral territory, although both nodal and bmp are present, the expression of chordin, an inhibitor of the BMP cascade, prevents bmp from activating its Alk receptor, giving an advantage to the nodal cascade activation. This point is further supported by experiments reported in Saudemont at al. 2010 (Fig. 9B), which show that chordin Mo embryos present active dorsal marker genes in the ventral territory. Hence, according to our model analysis the bmp cascade would presumably be active in the ventral territory in chordin Mo conditions (Fig. 6).

6 - The experiments with bmp and nodal show that both can induce one fate and suppress the alternative fate. However, in the bmp experiment, the effect is much stronger if embryos are exposed to bmp right after fertilization than if the protein is applied later in development. Based on this evidence and on the signaling that occurs during wildtype development I am not convinced that the signaling effects are independent of the relative timing of nodal and bmp signaling. One explanation for nodal signaling being dominant in ventral ectoderm could be that it is expressed earlier. Please clarify.

● The reviewer is right. We indeed believe that the relative timing of nodal and bmp signalling is crucial to properly pattern the sea urchin embryo. Specifically, the earlier activation of Nodal is crucial to properly set up the ventral organiser, which is then necessary to trigger and maintain bmp activation in the dorsal territory.
However, we suggest that the dosage of activation is also critical for proper embryo patterning. Indeed, the experiments reported in Fig. 4B suggest that increasing NODAL or

BMP protein levels at a later developmental stage, where both cascades are already active in their respective territory, is sufficient to reverse the cellular fate. For example, late NODAL protein treatment fully ventralises the embryo, even in the dorsal territory, where the dorsal marker tbx2/3 is already active in wild-type conditions.
We therefore suggest that relative timing of the two TGF-beta cascades is important for the initiation of the D-V axis patterning, but we further suggest that dosage is also critical to maintain the cascade active, as a higher concentration of the competing TGF-beta is sufficient to revert the cell specification.

7 - Furthermore, the repression circuit between the smads explains the formation of two alternative states, ventral and dorsal. But what about the ciliary fate? The model shows two genes, fgfa and onecut, as representing the ciliary state, but there is no interaction activating the two genes. Similarly, there is no activation of brachyury and foxa. How does this model produce three alternative states without assuming a mechanism to activate the genes representing all three states?

● Indeed, the regulatory rules assigned to FGFA, Onecut, FoxA and Brachyury do not require any activator to trigger their activation. As a result, these nodes are active in the absence of the corresponding inhibitors (e.g. Repressor R1 for FoxA and Brachyury). The factors necessary for their expression are implicit in the model. For example, SoxB1 is a transcription factor necessary to activate ciliary genes such as onecut and FGFA (see Saudemont et al. 2010, Fig.1.F). As SoxB1 is broadly distributed in the embryo, its presence is implicitly considered by allowing the ciliary marker to be expressed when no inhibitor is present. SoxB1 (and its potential repressors, e.g. irxA or goosecoid) could be considered for inclusion in future extensions of this model.

● In the case of FoxA and brachyury, proper evidence regarding activators is still lacking. Some evidence suggest that activation of these two genes can be triggered by the activation of goosecoid, via the inhibition of repression mechanism (Saudemont et al 2010). At this point, in our model, we set the active state as a default state, as for onecut and FgfA. Thus, these gene will be expressed in the absence of their repressor Repressor R1.
As all ciliary gene present in the model have an active default state, their expression will be triggered in the absence of repressor without the need for an implicit activator. As they are repressed by ventral and dorsal genes, they are activated solely in the absence of any of the two TGF-beta cascades. Consequently, the ciliary fate is obtained in the absence of active input, as it behave as a default active state.

8 - The modeling outcome is shown in form of the three cell fates, dorsal, ciliary, and ventral, yet the GRN model is based on genes and their interactions. How do the authors define the three cell states in terms of expression states? Is it just based on individual representatives or are all blue genes required to be ON in the ventral state? It is also not clear whether 0, 1, and 2 values in the model correspond to signaling function or to gene expression state. This would need clarification.

● The assignment of our simulation results to dorsal, ciliary or ventrally fates is based on the levels of the key marker genes downstream of the Smad complexes: Goosecoid, FoxA, Brachyury, FGFA, Onecut, Tbx2/3, IrxA, ... (cf. Jupyter notebook)
These correspond to gene expression state, where 0 and 1 correspond to absence and presence of expression, respectively. In the case of multilevel nodes (denoted by rectangular nodes in the regulatory graph), the values higher than 0 denote increasing gene expression or activity levels (activity level 1 < activity level 2). Such multilevel variables enable us to discriminate between wild-type and ectopic expression level (e.g. case of Nodal). We have clarified the text related to the multilevel specification in the revised manuscript (p. 8).

9 - In order to capture the spatio-temporal aspect of this patterning mechanism, diffusion rates and signaling regulatory interactions are captured in a combined model. You mention that simple assumptions are sufficient to reproduce the patterning of the dorsal/ventral axis, but what are the critical assumptions in order for this to work? Does the model assume different diffusion rates for nodal, bmp, or some if their inhibitors? Is diffusion rate critical at all or are the regulatory interactions in Fig. 3 providing sufficient information for the three cell fates? Is it critical that nodal is expressed first? Why is bmp expressed in the cells on the right side in Fig. 8C? As far as I understand, the model only requires input values for nodal and univin, and the rest of the model operates correctly. This would be an interesting result which might be most similar to the sea urchin embryo and would deserve being discussed together with the assumptions that have to be made in the model.

● In response to a point raised by the first reviewer, we have further revised the diffusion rules for the multicellular model and we now explicitly consider an ubiquitous expression of tolloid at the initial state (cf. Fig. 5 and Table 2). As a result, our multicellular model is now able to fully simulate the DV patterning of the embryo starting from initial conditions corresponding to a broad Nodal expression and an asymmetrical activation of Panda mirroring its asymmetric maternal deposition. The Fig.8 has been adapted accordingly. Our wild-type and morphan simulations suggest that the diffusion of the signalling factors across the embryo is critical for the proper patterning of the embryo. Indeed, BMP diffusion is required for dorsal cascade activation, Nodal diffusion is required to maintain its own ventral expression, and Lefty and Chordin diffusion are necessary to specify the territory boundaries,by restricting the ventral and dorsal fates, respectively, in the ciliary band. In our revised model, the diffusion distances are now specified

similarly in the diffusion rules corresponding to each signalling molecule. As mentioned, our multicellular simulation is initiated with Nodal being expressed at level 1, as observed experimentally. This first activation prior to that of bmp is crucial for the proper initiation of the ventral organiser. Indeed, if bmp is also express at this early stage, it is able to abolish the ventral signalling via Smad repression.

- Concerning the Fig. 8C, the green labelling of the dorsal territory corresponds to the presence of active dorsal marker genes (e.g. irxA) solely, and does not represent the expression of bmp. In the new supplementary Figure S1, the territories of expression of bmp input and output nodes are more extensively depicted.

10 - In Fig. 4 there are issues with concentrations shown as mg/ml and microg/ml in A and B.

- We thanks the reviewer for pointing this error, now corrected in our revised manuscript.

11 - In Fig. 5 A, the modeling inputs are shown as ubiquitous nodal and localized panda. But this is not consistent with the data provided by the author's lab and others showing that nodal is localized at 32 cell stage. Similarly, nodal is shown as expressed at low levels ubiquitously in Fig. 5B, but this does not seem to be consistent with results. Why does the model need to be split into before and after 32 cell stage? Wouldn't it run to the same stable state with just the initial state in Fig. 5A?

- The point raised by the reviewer is very interesting. In the DV patterning, diffusion of signalling molecules across the ectoderm is crucial to trigger the specification of the dorsal territory from ventrally expressed genes (e.g. bmp). As diffusion could not be integrated into a unicellular model, we manually defined the input node states for late stage simulation based on early stage simulation results. In order to clarify this point, we have modified the Fig.5, now highlighting the link between early and late unicellular simulations.
Furthermore, in our revised multicellular model, the integration of diffusion rules now enables to simulate the DV patterning of the full ectoderm at both early and late stages (cf. Fig. 8).
This simulation start with a broad nodal expression and an asymmetric panda expression, as observed experimentally (Haillot et al., 2015). The results of the simulations of the new multicellular model are reported in Fig.8 of our revised manuscript.

12 - In the stochastic model, the first fate to be induced is the ciliary band fate, as shown in Fig. 7. But is there any evidence that this occurs in the embryo? Why is the ciliary band assumed to be the default state in this model?

- Such a transient expression of ciliary band markers has indeed be observed experimentally, at least for Onecut, as shown in the Fig. 1E of Saudemont et al. (2010). This information has been added to the revised manuscript (page 11)

III) Answer to reviewer 3

1 - In figure 1 E and 1F, the morphological phenotypes between BMP2/4 inhibition, and both BMP2/4 and Panda inhibition are compared and it is claimed that panda is sufficient to break the radial symmetry of the embryo and necessary to specify the D/V axis. However, this conclusion is based on the fact that in both cases BMP2/4 is inhibited. Clarity on the epistasis of BMP2/4 and Panda will need to be provided.

- Our claim is based on the results from Haillot et al. 2015. In this article (Fig.6B), the authors have performed panda Mo injection and observed that this perturbation was sufficient to break the radial symmetry, even in the absence of bmp Mo coupled injection.

2 - I think a lot more explanation of the model is needed. In the single-cell level simulation, the author claimed that the model was revised based on the experimental results. However, how the revision process was done was not clear. More details and examples for the model or figures could be included to increase clarity.

● We thank the reviewer for pointing this need of clarification, also stressed by the two other reviewers. A novel subsection now explains the different steps of this iterative modelling process (p. 6-7), including a revised figure (Fig. 2).

3 - The single-cell logical model using the Boolean case cannot represent the interaction between neighbor cells. The spatial information between the cells depends on the input level of specific species this is not sufficient to represent the diffusion and reactions between different species. It is still a little bit unclear wreathe the single-cell model was included in the multicellular model and how they match each other both in single-cell and multicellular level experimental evidence.

● The multicellular model is constructed on the basis of the unicellular model. Each cell (one hexagon) encompass one copy of the unicellular model. In the course of the simulations, the inputs of each of the cells are updated based on logical diffusion rules. These diffusion rules take into account the values of the output nodes corresponding to the same species and present in neighbouring cells. Once the input values are updated, the unicellular model present within each of the cell is also updated, in order to reflect the regulatory mechanisms triggered by the inputs active in this cell. We have clarified the method section related to the multicellular model (p. 20). For a more detailed explanation on EpiLog, please refer to the publication of Varela et al. (2019) (https://doi.org/10.12688/f1000research.15613.2).

4 - The interactions between different protein ligands included in the model, this could be insufficient to the model. For example, as it was well known, the BMP2/4 ligands can react with Chordin ligand and formed the BMP-Chordin compound which is inhibited the signaling ability of BMP2/4. In the model shown in Figure 3, this mechanism was represented by Chordin inhibit BMP receptor Alk1_2_3_6. This may be sufficient in the circumstance that Chordin inhibits the BMP singling by preventing BMP bound it receptor this is equal to the fact that it reduces the receptor level itself. If this is the assumption made by the author, this needs to be clarified.

● Indeed, the reviewer is right to mention that the BMP/chordin complex formation is not explicitly considered in the model, although it is actually forming in this context. We chose not to represent explicitly this complex formation for sake of simplicity. However, this mechanism is implicitly implemented in the regulatory rule defined for the Alk receptor complex, which restrict the activation of the BMP cascade when chordin is present.

5 - The embryo shape was not considered in the study. As shown in Figure 1, at the MB stage the embryo is a ring-like shape. However, in the multicellular simulation shown in figure 8, the author illustrates the layers of hexagons to represent the cell, and based on the description, the diffusion of the species is relayed on the cell by cell transport. If so, the cell number and the relative distance between the cell should match with the embryo as close as possible to represent the real spatial distribution of the cells. How does the ring- like aspect get incorporated- throught the boundary conditions of cells at the ends?

● The multicellular model was built using EpiLog, which does not currently enable the simulation of 3D epithelia or dynamic changes in cell numbers or topology.
However, to match as closely as possible the ring shape of the embryo, we used boundary conditions such that cells on the upper row are in direct contact with the cell from the lower row, thereby wrapping the epithelium into a cylinder of cells.

6 - The "diffusion" mechanism represented in this study is based on the logical rule of passing the species between the 'cells' directly. The author has not shown enough evidence of how the logical rule of diffusion was constructed. This also coincides with the multiple integer level of some components used in the Boolean model. Do these extend to the diffusion as well? How is range determined?

● We thank the reviewer for pointing this need for clarification. We have further developed the section explaining the construction of the regulatory and diffusion rules in our revised manuscript (p. 6-7 and p. 20).

● Concerning the propagation of the values of the multilevel nodes, the diffusion rules take into account the different levels of activity for the specification of the input activity levels. In our

case, we specified for all the diffusing element that they could reach an input level of 2 if and only if they were already expressing this species (as output) at a level 2 (autocrine signalling). Of note, in our model, we tried to minimise as much as possible the use of multilevel nodes in order to contain the complexity of the model. The higher expression levels of multicellular nodes have been introduced to enable us to distinguish the levels of activity reached in wild type conditions (e.g. Nodal 1 < Lefty and Nodal 2 > Lefty) versus ectopic expression conditions (e.g. Nodal 3 (mRNA injection) > Nodal 1 & 2 wild type expression). We have further clarified the choice for multilevel nodes in the revised manuscript (p.8).

Second decision letter

MS ID#: DEVELOP/2020/189944

MS TITLE: Deciphering and modelling the TGF-&[beta] signalling interplays specifying the dorsal-ventral axis of the sea urchin embryo.

AUTHORS: Swann Floc'hlay, Maria Dolores Molina Jimenez, Celine Hernandez, Emmanuel Haillot, Morgane Thomas-Chollier, Thierry Lepage, and Denis Thieffry

Many apologies for the delay in obtaining reviews on your revised manuscript. Despite many reminders, we were unable to obtain a review from one of the referees and so I have made a decision based upon the two reviews we have received. 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.

Both referees are largely happy with your revisions and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of 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.

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

Reviewer 1

Advance summary and potential significance to field

The authors have addressed most of the reviewers' comments in their revised manuscript, and the current models can serve as a basis for more detailed simulations in the future. I have a few minor comments listed in detail below regarding points that were not fully addressed.

Comments for the author

1) I recommend rewriting the manuscript to make clear that not all questions set up in the introduction are addressed in this work, e.g. why Nodal and BMP signaling are located at opposite poles despite co-expression on the ventral side.

2) The extended description of the model building procedure is useful, but it does not directly address the consistent comments from all three reviewers. I therefore recommend to clearly mention the challenges that were encountered and to expose sensitive parameters that are crucial

for the model to work. This would be particularly helpful in light of the additional changes that were made to the model during the revision process.

3) I recommend to at least discuss the caveat that Noggin and Follistatin, whose role is well characterized in many model systems, will probably have to be considered in future models, and that the dominance idea might have to be reconsidered.

4) I appreciate the new documentation on GINsim, but I agree with Reviewer #2 that an annotation of Fig.3 with references would be very helpful for the readers.

5) Regarding my previous point about Fig. 1F: If possible, please show the ventral view as well for completeness (currently only the lateral view is shown).

6) Univin could be included as a ventral marker in Fig. 2 to help readers connect the markers to the genes shown in Fig. 3.

7) Since the new Materials and Methods section mentions that recombinant mouse BMP-4 protein was used, it would be useful to replace “BMP2/4” with “BMP4” (e.g. in line 503).

Reviewer 2

Advance summary and potential significance to field

The revised manuscript is more accessible and the authors have addressed many of the previous issues. I only have a few more comments that should be addressed before publication.

Comments for the author

1. In the first section of the results describing the model, it should be stated that the linkages can represent protein-protein as well as protein-DNA interactions since the model does not seem to distinguish between the two, which is confusing and should be explained.

2. It is still not clear whether the values of a node represent gene expression or protein activity. This distinction is important when thinking about validating the predictions of this model. For example, the value of nodal is 2 for ventral ectoderm and 1 for ciliary and dorsal ectoderm. However, gene expression is clearly not detected outside of ventral ectoderm, so this value probably refers to protein levels upon diffusion.
But the manuscript in line 247 states that the values reflect gene expression levels of nodal. This is confusing and it should be clarified whether node values represent gene expression levels, protein levels, or signaling activity.

3. It should be mentioned in the results section on model building that default states are assumed where activators are unknown, for example for foxa, which is now only mentioned in the caption of table1.

4. The model assumes nodal values of 2-1-1 in ventral to dorsal ectoderm. Is there any evidence for this in wildtype embryos? The description of the model should include these and other assumptions that are not obvious based on experimental data.

5. Lines 458-464: the oscillation behavior could be overcome by multilevel modeling, but the reason for not including smad6 is not because it cannot be modeled but because its function is not sufficiently understood or not considered to be relevant for the early distinction of the three ectodermal fates.

6. The sentence line 476 is not clear: “Therefore, an asymmetry of panda mRNA or of Panda protein constitutes the main driving signal to allocate cell fates, rather than a change in overall Panda concentration”

7. For the purpose of clarity, a summary paragraph should be included either in the results or discussion section, which describes the key mechanisms that the model predicts to be responsible for the distinct specification of the three ectodermal cell fates in sea urchin embryos.

Second revision

Author response to reviewers' comments

We thank the reviewers for fruitful comments on the manuscript. We present here a revised manuscript submission with the main text modification emphasized in blue.
Please find below our answer to the reviowers' comments.

Responses to Reviewer 1's Comments:
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1) I recommend rewriting the manuscript to make clear that not all questions set up in the introduction are addressed in this work, e.g. why Nodal and BMP signaling are located at opposite poles despite co-expression on the ventral side.

We have rewritten the end of the introduction section to clarify this point (cf. line 141-150).

2) The extended description of the model building procedure is useful, but it does not directly address the consistent comments from all three reviewers. I therefore recommend to clearly mention the challenges that were encountered and to expose sensitive parameters that are crucial for the model to work. This would be particularly helpful in light of the additional changes that were made to the model during the revision process.

We have added a paragraph discussing the critical parameters encountered during modelling steps (lines 420-428).

3) I recommend to at least discuss the caveat that Noggin and Follistatin, whose role is well characterized in many model systems, will probably have to be considered in future models, and that the dominance idea might have to be reconsidered.

We have added a discussion regarding the consideration of Noggin and Follistatin to the model (lines 485-491).

4) I appreciate the new documentation on GINsim, but I agree with Reviewer #2 that an annotation of Fig.3 with references would be very helpful for the readers.

We are now including a Supplemental Table S1 containing the list of original articles used as references for the construction of our unicellular model, especially the different molecular components.

The key references used for the model are : Duboc 2004; Range et al., 2007; Flowers et al., 2004; Yaguchi et al., 2007; Nam et al., 2007; Duboc et al., 2008; Lapraz et al., 2009; Saudemont et al., 2010; Haillot et al., 2015; Lapraz et al., 2015.

5) Regarding my previous point about Fig. 1F: If possible, please show the ventral view as well for completeness (currently only the lateral view is shown).

We apologise for not having updated this figure in our first revised version, the new Fig.1F is now properly integrated in the manuscript.

6) Univin could be included as a ventral marker in Fig. 2 to help readers connect the markers to the genes shown in Fig. 3.

Although univin is ventrally expressed at mesenchyme blastula stage, we chose not to label it as a ventral marker due to its highly dynamic pattern of expression : Indeed, univin expression is dynamic and is only a ventral marker at specific stages of development. univin is uniformly and strongly expressed maternally and during cleavage. At blastula stages, univin is broadly expressed in the whole ectoderm. Around the mesenchyme blastula stage, it is restricted to the ventral ectoderm. At later stages, it is expressed exclusively in the Ciliary band territory (Lapraz et al., 2006).

7) Since the new Materials and Methods section mentions that recombinant mouse BMP-4 protein was used, it would be useful to replace “BMP2/4” with “BMP4” (e.g. in line 503).

We have corrected the text mentioned by the reviewer, as well as Fig. 4.

Answers to Reviewer 2's comments:
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1) In the first section of the results describing the model, it should be stated that the linkages can represent protein-protein as well as protein-DNA interactions since the model does not seem to distinguish between the two, which is confusing and should be explained.

We are describing in more detail the interaction types in the manuscript, as advised by the reviewer (lines 193-195).

2) It is still not clear whether the values of a node represent gene expression or protein activity. This distinction is important when thinking about validating the predictions of this model. For example, the value of nodal is 2 for ventral ectoderm and 1 for ciliary and dorsal ectoderm. However, gene expression is clearly not detected outside of ventral ectoderm, so this value probably refers to protein levels upon diffusion. But the manuscript in line 247 states that the values reflect gene expression levels of nodal. This is confusing and it should be clarified whether node values represent gene expression levels, protein levels, or signaling activity.

We thank the reviewer for pointing out the confusing sentence and we have corrected it (line 254). Indeed Nodal input level represents signalling activity and not gene expression. We have further clarified that the values of input nodes represent protein activity levels (line 195).

3) It should be mentioned in the results section on model building that default states are assumed where activators are unknown, for example for foxa, which is now only mentioned in the caption of table1.

We have clarified the default state selection for nodes with no activator in lines 591-593.

4) The model assumes nodal values of 2-1-1 in ventral to dorsal ectoderm. Is there any evidence for this in wildtype embryos? The description of the model should include these and other assumptions that are not obvious based on experimental data.

We indeed observed a gradient of Nodal mRNA expression (Duboc et al., 2004), which might correlate with protein levels but, as far as we know, there is no direct report of visualization of Nodal protein in the sea urchin embryo. However, We did observe the expression of target genes that presumably depend on nodal levels (Saudemont et al., 2010).

We have added a section on critical parameters of the model to better reflect our key assumptions (lines 420-428).

5) Lines 458-464: the oscillation behavior could be overcome by multilevel modeling, but the reason for not including smad6 is not because it cannot be modeled but because its function is not sufficiently understood or not considered to be relevant for the early distinction of the three ectodermal fates.

We have corrected the referred section (lines 479-483).

6) The sentence line 476 is not clear: “Therefore, an asymmetry of panda mRNA or of Panda protein constitutes the main driving signal to allocate cell fates, rather than a change in overall Panda concentration”

We have rephrased the sentence (lines 503-505).

7) For the purpose of clarity, a summary paragraph should be included either in the results or discussion section, which describes the key mechanisms that the model predicts to be responsible for the distinct specification of the three ectodermal cell fates in sea urchin embryos.

We have added a more detailed paragraph summarizing our results in the Discussion (cf. lines 514-522).

Third decision letter

MS ID#: DEVELOP/2020/189944

MS TITLE: Deciphering and modelling the TGF-&[beta] signalling interplays specifying the dorsal-ventral axis of the sea urchin embryo.

AUTHORS: Swann Floc'hlay, Maria Dolores Molina Jimenez, Celine Hernandez, Emmanuel Haillot, Morgane Thomas-Chollier, Thierry Lepage, and Denis Thieffry
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.