Peer Review File

Manuscript Title: Molecular evidence of anteroposterior patterning in adult echinoderms

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

Echinoderms are a sister group to hemichordates, embedded within the deuterostome clade of bilaterians. Yet in spite of having bilaterally mirror-image symmetric ancestors, the echinoderms display a unique, typically five-fold, radial symmetry. The origin of this novel body plan has been long debated, but remains an unsolved problem. The current study from Chris Lowe and co-workers, makes use of a suite of anterior-posterior axial pattering genes, with conserved patterning roles in other bilaterians, examining how they are expressed in the developing sea star Patiria miniate. They find that the relative spatial expression of these markers in the ectoderm indeed shows similarity with other deuterostomes, with the deuterosome A-P axis recapitulated along the medial-lateral axis of each ray. While the study is conceptually fairly simple, the conclusions are important and will be of wide-spread interest. I have only minor comments for the authors to address prior to publication:

1) Abstract: claiming echinoderms as the "most derived animal group" is both unnecessary and a bit of a stretch.

2) Given that P. miniata is not a common model organism, it would be helpful for readers to provide histological sections along each of the axes used in the RNA tomography experiments.

3) Fig 2: M-L, L-R, and Left-right are used to denote the M-L. Would be clearer to pick just one in both the figure and caption.

4) For the RNA tomography, it would be nice to have a supplemental figure showing more information on clustering and aggregation of sections (show PCA plots etc). Are the aggregated clusters uniform in the number of sections combined together? How does the size of the aggregated clusters in each axis compared to the size of anatomical features (e.g. the podia). The methods section says the number of clusters was chosen based on "biological significance." It's a bit unclear what this means.

5) Fig 2E M-L axis has 7 dimensions but 14 dimensions in Fig S3. I assume the left and right were merged to make a single medial to lateral axis, but the left and right parts of the arms don't seem fully symmetric based on gene expression in fig S3. Are any of the conserved A-P genes the authors looked at asymmetric in the M-L dimension?

6) In some of the HCR in situs, the blue and white can be hard to distinguish when expression level is low (tlx, foxag, rx).

7) Fig 3b is interesting. Would be interesting (but maybe not necessary) to stain for a few other A-P patterning genes during metamorphosis. Is the whole A-P patterning system activated during metamorphosis and not in the Brachiolaria? Is anything known about A-P patterning in the Brachiolaria? This might be relevant to whether the juvenile P. miniata is coopting A-P patterning genes in a novel way, or is maintaining expression during remodeling implying a more direct homology of the A-P patterning system deployment in the pentaradial body plan.

8) Line 232/233: "co-option of of gene regulatory networks into the development of novel structures usually involves a limited number of genes" I am not sure this statement is true (just two examples: https://doi.org/10.1186/s12915-021-01182-2, https://doi.org/10.7554/eLife.43828)

9)This whole section (line 230-237) is a bit shaky – if the developmental program that induces anterior to posterior expression of these genes is linked through regulatory interactions, co-option of the entire network is still a reasonable possibility. For example, nested colinear expression of hox genes in appendages such as limbs in tetrapods is probably an ancient co-option of the hox regulatory program from the main body axis. It seems the authors are arguing here that the ectodermal A-P program in P. miniata is directly homologous to the bilaterian A-P program, which I'm not sure is unambiguously supported by the evidence provided. The lack of a trunk program, different expression pattern of some expected genes (dlx, lhx2/9/, emx, otp, sfrp3/4, engrailed), lack of clear continuity with earlier A-P developmental programs in Brachiolaria, difference in Hox expression compared to other bilaterians, and the unique medio-lateral orientation of the program keep open the possibility that this could still be an instance of co-option.

10) Line 250: "our model" are the authors referring to their model organism or experimental framework? It's a bit unclear what the claim is here.

11) Line 244: It would be helpful if the authors could clarify in the text how their model differs from Adachi et al., 2018. This paper reports that "P. japonica orthologs of the medial markers are expressed in the ambulacral ectoderm of the rudiment; and few P. japonica orthologs of the posterior markers are expressed in ectoderm"

12) Lin 246 in methods section: It doesn't make sense to me to summarize expression profiles to a single expression peak for genes that have multimodal expression profiles, which are likely tied to the presence of multiple anatomical structures in each axis. Can the authors explain this a bit further?

Referee #2 (Remarks to the Author):

Review: Formery et al. on echinoderm body plan

I'm not qualified to judge the methodological details, so my comments are on the general nature and significance of the results and their interpretation. With that caveat, my view is that this is unquestionably a landmark paper in zoology, resolving issues of echinoderm body plan that have puzzled comparative, developmental and evolutionary biologists since the 19th century. The work demonstrates the power of mapping subdomains of the anterior head region across phyla, using hemichordates as a reference point and more genes, to achieve greater resolution any previous work that I know of. The exposition of both the background information, as to the problem to be solved and the various hypotheses, and the results, are clearly explained, and the figures are informative and easily comprehensible. The paper is in my view in the top rank of papers that Nature should ideally be publishing. I have one substantive concern (see item 1, below), but otherwise have no reservations about publishing it in this form.

As to impact, for echinoderms themselves, this rewrites the book when it comes to interpreting the fossil forms, because we can now attach developmental meaning to the morphology. In a broader context, a divergence in body plan of this magnitude is a test of ideas relating to evolutionary contingency, and is of special interest when vertebrates are part of the story, as they are for anything related to ancestral deuterostomes. Evolutionary contingency is relatively new as a field of study, with an emerging theoretical focus, and here we have an example of contingency on a

macro scale more dramatic than perhaps any other in animal evolution. This is the stuff of future textbooks. The key question for me is why echinoderms, after a radical makeover at the developmental level, are otherwise conservative in how that innovation was exploited, whereas chordates, while keeping to the ancestral body plan, have spawned the larger and more successful radiation. One can think of this in terms of scenarios, but contingency is a slippery issue (as much history as science as one author recently put it) and the larger message, if there is one here, is itself a bit of a puzzle.

What few suggestions I have below, though requiring attention, are in no way intended to detract from the authors' achievement, which is impressive by any measure.

1. There is one lapse in scholarship that needs correcting. This concerns the paper by Adachi et al. (2018) on Peronella, which is lumped (line 69) with other papers favouring the stacking hypothesis. My reading of Adachi et al. is (and was in 2018) that they have taken the loss of registry (decoupling) idea to heart and deal with patterning as something that can proceed independently in the ectoderm irrespective of what may be going on internally with Hox genes and the like. This is how I would interpret the statement on pg. 1299 "that echinoderm rays are outgrowths around the adult mouth" and on pg. 1302, that "...echinoderm rays are likely outgrowths of appendages around the adult mouth," although "appendages" in this usage is perhaps an unfortunate choice of term. And, having far fewer markers, Adachi et al. map most of the ray tissue to collar level ectoderm, so a good deal of the logic and conceptual power of this new interpretation by Formery et al. is missing.

This does, however, lead me to view these new results in a somewhat different light than the way the authors have expressed it, that rather than the tomography results being surprising (line 119), they are perhaps better viewed as a validation of an existing line of argument, by Adachi et al., with proof of the model (the achievement of the present work) depending on mapping more of the relevant genes in far more detail. I would point out also that for non-specialists such as myself, it would be useful if the authors would clarify the role of the tomography results as the project unfolded, e.g. as to how much of the in situ work could have gone ahead regardless, or perhaps was being done in parallel. The way the paper is currently written gives the impression that it was the tomography results that set the project in motion, which leaves me wondering if a point is being stretched mainly for narrative convenience.

2. There is also then a slight problem with categorizing models as AP, OA, and ML (lines 204-218). The stacking model belongs in the OA category so long as the body is considered as a solid object. As soon as you restrict the patterning to ectoderm, the OA and ML models converge depending on how you choose to treat the aboral ectoderm, whether as a participant (a full ectodermal OA model would imply this) or as sitting passively on the sidelines (the more restricted ML model), literally with no skin in the game. I think any confusion this might potentially cause can be avoided with some careful rewording in spots. Options like "mapping to a solid body" versus "mapping to individual tissue layers" spring to mind.

3. Minor points and typos

line 73: plural "needs"

line 189: change "than" to "as"?

line 234: re. the reference to 18 or 22 genes carrying anterior identity. I count 20 listed in the first 3 of the 4 groups enumerated in the text (lines 146-188). Removing FezF and dbx for their spotty expression leaves 18, so I would have said 18 of 20, leaving me puzzled as to which genes I've missed out (maybe engrailed and sfrp3/4 are included in the 22?). Ensuring that the counts are internally consistent would remove a potential source of reader confusion.

line 430: it’s a yellow asterisk in my copy

line 440 (Suppl. Section): change “a” to “at”

Referee #3 (Remarks to the Author):

The manuscript of Formery et al is addressing a fundamental zoological question that is puzzling to every student of biology when they hear about anatomical details of urchins and related animals. How are the body axes of an adult sea star related to the clear bilateral axes of other bilateralsymmetric animals? There are not yet clear answers available and several hypotheses have been phrased of which some are more convincing than the others. Using RNA Tomography and in situ hybridization the authors aim for answering this question and discriminate between the existing hypotheses. However, their results are unexpected and very surprising since they are not in line with everything that has been proposed before. The approach is solid and the work is well done. And from the topic and how the work was performed I do in principle support the publication in Nature. However, I have few remarks about the interpretation and would like to see some further investigations or better, analysis of the present dataset.

Puzzling to me is of course that the A-P patterning genes are expressed along a surprising axis: from the mediolateral ambulacral-system (anterior) toward the lateral along the medial-lateral axis of the sea star arms (posterior). The result is convincing from the data support, but so surprising that also the authors struggle with the interpretation. (“Our model offers a powerful tool to establish robust regional homologies between classes and an independent dataset to test existing competing hypotheses.”)

If somebody would give me these data in hand, I would at first glance assume that a very interesting case of the co-option of an existing gene cascade has happened during evolution of this unique, pentaradial body plan. I assume the authors have been gone through this and were also facing similar comments from the audience when publicly presenting the data. Therefore, I assume, the authors provide a disclaimer in the manuscript why this is not a case of a cooption: "However, co-option of gene regulatory networks into the development of novel structures usually involves a limited genes number of there are to our knowledge no examples of co-option of the entire anterior part of the AP patterning program.”
In principle, I dont really buy this argumentation. There can always be new findings and the new “A-P axis” is along a body part that is a novelty and hard to compare with bilaterians. The problem of axis homology reminds me of the discussions in anthozoan cnidarians in which several homeodomain genes are expressed along the directive axis - an axis in which early on also the BMP/chordin axis is aligned. Looking at the ectodermal patterning genes - a subset of the genes that are studied here - are expressed along the oral-aboral axis. This example shows that determining axis homology is not so easy. However, I think to contribute to a solution here, and to improve the paper and the question of axis homology massively is to consider also the dorsoventral patterning system. The authors are well experienced in this (see e.g. Lowe et al 2006 PLOS Biol) and at least in the RNA Tomography datasets the genes of question should be found. A few more in situs can make the story perfect. Is the axis perpendicular to the anteroposterior axis? Where would it be? How can the body plan in the lineage to Echinodermata got modified?
It would be great if the authors walk down this road.

few remarks:

- Please provide a schematic drawing (text-book and lecture quality) about how this axis would be

allocated when the presented results are transferred to the different echinoderm taxa (holothurians, urchins, crinoids).

please remove from the manuscript evolutionary misconceptions, such as:

“the most derived animal group” - this lays in the eyes of the beholder. its totally arbitrary. I would say Sacculina or Gastrotricha is the most derived animal group (if I would join this conversation…).
“evolutionary trend” - what shall this be? a directed evolution?
"similarities across all deuterostomes phyla, including echinoderms.

Referee #4 (Remarks to the Author):

Formery et al. use spatial transcriptomics (RNA tomography) and in situ hybridization in the sea star to identify patterning similarities between echinoderms and other deuterostomes. Importantly, the authors also performed a genome assembly. Specifically, the authors observe that genes involved in anteroposterior patterning of the ectoderm in bilateral deuterostomes have an equivalent pattern in the pentaradial body plan of echinoderms, with the midline of each ray corresponding to an anterior position and the lateral parts to a more posterior identity. Furthermore, they do not find an ectodermal territory expressing the bilaterian trunk patterning program, suggesting that the sea star ectoderm is patterned exclusively by head-like programs.

This is an interesting paper that address a fundamental question in zoology. The manuscript is well written, and the questions and hypotheses are clearly formulated. However, other reviewers will be better qualified than me to evaluate the novelty and importance of the biological results. Overall, the authors present a relatively small study, with little complexity regarding the experimental and computational methodology, and I was surprised that the authors (for instance) did not expand the study other germ layers besides the ectoderm, included additional stages or species, or performed a more systematic analysis of the RNA tomography data. As presented, I would consider the manuscript too preliminary for publication in Nature.

Major points

1. The only transcriptome-wide analysis of the RNA tomography data is done in Fig. 2d. The analysis is nicely done, but it would be useful to match the observed seven clusters to distinct anatomical territories. This could be used as a starting point to e.g. find other AP genes besides the investigated 36 genes, or to find other spatial programs besides anterior genes that contribute to the medial ambulacra.

2. It did not become clear to me why the authors selected only 36 genes for the analysis of the RNA tomography data. It seems to defeat the purpose of a spatial transcriptomics experiment if, in the end, the number of analyzed genes is so small that in situs can be done for all of them. Ultimately, it's probably not a black-and-white question whether the bilaterian AP program is reused in echinoderms or whether the genes have been co-opted into novel developmental roles, so it will be critical to base the assessment on a large number of genes.

3. There seem to be no replicates for the RNA tomography experiment. This is probably fine, but it would at least be good to compare the RNA tomography results to the in situ data for the 36 selected genes. As presented, it is difficult to see to which degree the results are in agreement (taking into account the limited resolution of the RNA tomography data).

4. I'm surprised that the authors decided to limit the scope of the RNA tomography analysis to one species and one time point, and focusing further on the ectoderm. These organisms are fascinating because of the incredible changes in shape and geometry they undergo during metamorphosis,

and the temporal development of such a configuration would be an interesting aspect to study. With the experimental and computational approaches established (including transcriptome and genome assembly), it would seem to be fairly straightforward to broaden the scope of the manuscript. In my opinion, the manuscript would gain immensely if the authors could e.g. quantify the use of AP genes in spatial patterning over time, identify similarities and differences between the germ layers, and demonstrate the generality of the findings by adding another echinoderm species. (While RNA tomography might not be ideal for other species, HCR could demonstrate whether the same principles hold).

Minor points

5. How were the 36 genes of interest selected?

6. Methods, line 247/248: I did not understand the approach for identifying peak positions. What are the replicates that the authors refer to, and what is their criterion for defining whether there is a single peak?

7. The authors seem to use two different stages for the spatial transcriptomics and in situ analysis (young juveniles for the RNA tomography analysis, and post-metamorphic juveniles for HCR). The title refers to adult animals. This should be explained better.

8. Figure S3 is probably interesting, but it was placed in the supplement and is not commented on much.

9. Figure 3 shows some nice images, but it is a bit difficult to know what to conclude from them. Especially because no profiles, density maps or quantitative graphs are shown.

Author Rebuttals to Initial Comments:

We thank the reviewers for their insightful and helpful comments - we have now substantially revised the document and added new data that we think have greatly improved the manuscript and addressed some of the concerns. We commented on the issues raised by each reviewer in order below (in blue).

Referee #1 (Remarks to the Author):

Echinoderms are a sister group to hemichordates, embedded within the deuterostome clade of bilaterians. Yet in spite of having bilaterally mirror-image symmetric ancestors, the echinoderms display a unique, typically five-fold, radial symmetry. The origin of this novel body plan has been long debated, but remains an unsolved problem. The current study from Chris Lowe and co-workers, makes use of a suite of anterior-posterior axial patterning genes, with conserved patterning roles in other bilaterians, examining how they are expressed in the developing sea star Patiria miniata. They find that the relative spatial expression of these markers in the ectoderm indeed shows similarity with other deuterostomes, with the deuterostome A-P axis recapitulated along the medial-lateral axis of each ray. While the study is conceptually fairly simple, the conclusions are important and will be of wide-spread interest. I have only minor comments for the authors to address prior to publication:

1) Abstract: claiming echinoderms as the “most derived animal group” is both unnecessary and a bit of a stretch.

Thanks. We removed this claim.

2) Given that P. miniata is not a common model organism, it would be helpful for readers to provide histological sections along each of the axes used in the RNA tomography experiments.

To help the reader with the unfamiliar anatomy of adult sea stars, we performed micro-CT scans on young *Patiria miniata* juveniles of the same size class as those used for the RNA tomography. These data are now shown as 3D reconstructions (Fig. 2a; Extended Data Fig. 1; Supplementary Fig. 1) and as a movie (Supplementary Fig. 2) to help visualize the sequential cryosections in the 3 dimensions of the arms. Micro-CT allows the anatomy of the sea star to be reconstructed in three dimensions, which makes it easier to understand the organization of the animal compared to histological sections. The micro-CT scans were segmented to highlight the main anatomical structures that are mentioned in the manuscript (nervous system, water vascular system, muscles, digestive tract, endoskeleton). The micro-CT scans and mesh files generated from the 3D reconstruction were deposited in a public repository.

3) Fig 2: M-L, L-R, and Left-right are used to denote the M-L. Would be clearer to pick just one in both the figure and caption.

Thanks, we agree that this was confusing, and in the revised manuscript we have now used M-L (medio-lateral) throughout to refer to this axis.

4) For the RNA tomography, it would be nice to have a supplemental figure showing more information on clustering and aggregation of sections (show PCA plots etc). Are the aggregated clusters uniform in the number of sections combined together? How does the size of the aggregated clusters in each axis compared to the size of anatomical features (e.g. the podia). The methods section says the number of clusters was chosen based on "biological significance." It's a bit unclear what this means.

We have added a summary diagram in Supplementary Fig. 3 recapitulating how sections were aggregated from the cryosection step up to the data analysis step. This diagram also includes the size of the sections for each dimension and the size of the intact animal along the three dimensions. The P-D, O-A and M-L sections used in the data analysis correspond to 970 µm, 500 µm and 300 µm, respectively. This can be used as a starting point to compare the size of the RNA tomography sections to various anatomical features.

The original version of the manuscript only included pictures of the blockface taken as we performed the sections as a way of relating the actual anatomy and the RNA tomography. These pictures were lacking a proper scale and were difficult to interpret. We kept these pictures as part of Supplementary Fig. 3, but have now added micro-CT reconstructions in Figure 2a, Extended Data Fig. 1c,d, Supplementary Fig. 1, and Supplementary Fig. 2, as noted above, which provide a much better presentation of the animal's anatomy. It is important to keep in mind that while the specimens used for micro-CT and those used for the RNA tomography are distinct and are not exactly the same size, the relative proportions of the main anatomical features, and the order of magnitudes are similar. These micro-CT reconstructions are now presented side by side with the anatomical characterization of the RNA tomography in Extended Data Fig. 1, so the reader can visually correlate these two types of data.

To relate the RNA tomography and the actual anatomy, individual podia fall below the resolution of the RNA tomography sections, but as another example we can consider the medio-lateral extent of the radial nerve cords and medial muscles in comparison to the width of the arms. Based on the marker gene expression data presented in Extended Data Fig. 1g, the medial sections of the arm corresponding to the extent of the radial nerve cord is three sections wide, while the extent of the medial muscles are five sections wide. This represents 900 µm and 1.5 mm thickness, respectively, of a total medio-lateral size of 4.8 mm, that is 19% and 31% of the arm width, respectively. Using micro-CT reconstructions we can calculate the same ratio using the width of the arm in its wider portion. This gives 16% for the medio-lateral extent of the radial nerve cords, and 35% for that of the medial muscles, which is close to the measurement obtained from the RNA tomography. The consistency between the anatomical 3D model and the RNA tomography has been indicated in the method section.

Regarding the number of relevant clusters within the RNA tomography dataset, previous datasets have been clustered using k-mean clustering (see Junker et al., 2014).

The predetermined value for k corresponded to the number of anatomical features that were visually identified on the Spearman correlation matrix of the sections. In our case, the correlation matrices show two main anatomical features along the P-D axis (the disk including digestive tract and the tip without digestive tract), two main features along the O-A axis (oral/aboral epidermis and internal organs) and three main features along the M-L axis (epidermis, pyloric caeca and midline). While this naively suggests that 2×2×3=12 could be an appropriate number of clusters for k-mean clustering, this reasoning does not account for the lack of independence of gene expression between different dimensions.

To select a reasonable number of clusters we used the silhouette index, which is a method to analyze the consistency of clusters across a dataset. We evaluated the silhouette index for between 2 and 12 clusters and found that 7 was the appropriate number to use. Similarly, we cut our hierarchical clustering dendrogram to generate between 2 and 12 clusters, and observed that using 7 clusters allowed us to test the 12 hypothetical trends of expression while avoiding redundancy. The rationale for picking 7 clusters is now better explained in the method section.

All this being said, given that the RNA tomography dataset is in general very homogeneous, the number of clusters picked is to some extent arbitrary, and choosing a different number of clusters would not change the outcome of the analyses presented here.

5) Fig 2E M-L axis has 7 dimensions but 14 dimensions in Fig S3. I assume the left and right were merged to make a single medial to lateral axis, but the left and right parts of the arms don't seem fully symmetric based on gene expression in fig S3. Are any of the conserved A-P genes the authors looked at asymmetric in the M-L dimension?

We now refer to the left to right sectioning orientation as medio-lateral throughout the entire manuscript to avoid any confusion. Consequently, we also modify Fig. 2d (previously Fig. 2e) to show the entire range of the M-L dimension and not just the average of the left and right side. In general, while expression profiles are not perfectly symmetrical, the position of the peaks is usually consistent. Notable exceptions include for instance *tlx*, which is clearly offset to the right side. Based on expression data from tissue marker genes (Extended Data Fig. 1g), the genes that appear to have a marked asymmetrical distribution are those associated with the digestive tract. We assume that this asymmetry results from mechanical pressure during the dissection of the arm prior to sectioning. Indeed, pyloric caeca are not embedded into the body wall like the nervous system or the muscles, but are rather loosely suspended in the coelomic cavity by mesenteries. Therefore, it is possible that the pyloric caeca were displaced from the initial position during the dissection. As a result, any AP patterning gene that may have had an additional expression domain in the pyloric caeca might have a biased profile as well. However, we believe that this does not change the outcome of the analysis, since most AP genes are symmetrical in the M-L dimension (as confirmed by HCRs), and since such biases have been accounted for by averaging the left and right side of the arm prior to the calculation of the correlations.

6) In some of the HCR in situs, the blue and white can be hard to distinguish when expression level is low (tlx, foxG, rx).

We tried different other colors and white on blue appeared to give the best contrast. To solve this problem, we increased the contrast for the problematic pictures and hope that Reviewer 1 will agree that these are now easier to visualize.

7) Fig 3b is interesting. Would be interesting (but maybe not necessary) to stain for a few other A-P patterning genes during metamorphosis. Is the whole A-P patterning system activated during metamorphosis and not in the Brachiolaria? Is anything known about A-P patterning in the Brachiolaria? This might be relevant to whether the juvenile P. miniata is coopting A-P patterning genes in a novel way, or is maintaining expression during remodeling implying a more direct homology of the A-P patterning system deployment in the pentaradial body plan.

Thanks for this suggestion, which we agree is an interesting addition. We have done additional work and added a new Supplementary Fig. 9 with expression patterns through metamorphosis for *six3/6*, *nkx2.1* and *dmbx*. In addition to *pax6* and *otx*, these offer a representative view of how the two anterior-most groups of genes are expressed during the transition from bilateral to pentaradial symmetry. Interestingly, *six3/6*, *nkx2.1* and *dmbx* all have larval-specific expression domains (in the axocoel, mouth and esophagus, respectively), but the expression in the future adult tissues is turned on independently of these larval expression domains at the beginning of the metamorphosis. Therefore, it seems that in *Patiria* there is no correlation between larval patterning and adult patterning, which does not help to solve the question of the possible co-option of the AP patterning system in adults. In asteroids, there is strong morphological uncoupling of adult and larval body plans, with most of the larval tissue likely incorporated into the aboral, interradial territory during a fast and dramatic metamorphosis. In other echinoderm groups, like holothuroids, the link between larval and adult patterning through metamorphosis is far more obvious. Selection has likely driven uncoupling of body plans to different degrees: extant groups vary significantly to the extent of how the bilateral larva transitions to the pentaradial adult. Ancestral developmental strategies may have been far more linked during metamorphosis, so the lack of patterning continuity between larva and adult does not invalidate hypotheses of adult axial homology.

Of note, we also tried to survey several other genes during metamorphosis, in particular more posterior ones such as *gbx*, *pax2/5/8* and *hox1*, but none of these were detected during metamorphosis, and these genes are only turned on later in the juvenile.

8) Line 232/233: “co-option of of gene regulatory networks into the development of novel structures usually involves a limited number of genes” I am not sure this statement is true (just two examples:
https://doi.org/10.1186/s12915-021-01182-2, https://doi.org/10.7554/eLife.43828)

9) This whole section (line 230-237) is a bit shaky – if the developmental program that induces anterior to posterior expression of these genes is linked through regulatory interactions, co-option of the entire network is still a reasonable possibility. For example, nested colinear expression of hox genes in appendages such as limbs in tetrapods is probably an ancient co-option of the hox regulatory program from the main body axis. It seems the authors are arguing here that the ectodermal A-P program in *P. miniata* is directly homologous to the bilaterian A-P program, which I'm not sure is unambiguously supported by the evidence provided. The lack of a trunk program, different expression pattern of some expected genes (dlx, lhx2/9/, emx, otp, sfrp3/4, engrailed), lack of clear continuity with earlier A-P developmental programs in Brachiolaria, difference in Hox expression compared to other bilaterians, and the unique medio-lateral orientation of the program keep open the possibility that this could still be an instance of co-option.

For comments 8 and 9 - We have revised the discussion to better acknowledge the possibility of co-option. The argument that co-option usually involves a small set of genes is indeed debatable. Limb and digit patterning involves the redeployment of only posterior members of the Hox cluster, while co-option of head patterning genes into limbs is only a small number of inter-regulated transcription factors (Lemons et al 2011). What constitutes a small or large number of genes is a matter of perspective – one of the papers that Reviewer 1 noted as an example of co-option involving a large number of genes (Neal et al., 2022) was cited in our original submission as showing that co-option usually involves a small number of genes. Since this is therefore not a hard-and-fast criterion, we have removed this argument. Similarly, the comparison between hemichordates and vertebrates also shows a variety of expression differences in the deployment of genes shared with other bilaterian groups, such as the absence of *irx*/*fezF* boundary in hemichordates. In such vastly different body plans and over macroevolutionary time frames we should not expect a perfect conservation of patterning networks, but this does not invalidate comparisons.

There are still some arguments that lead us to favor the homology scenario over a co-option scenario.

- First, the patterning system which defines distinct anatomical regions along the AP axis of chordates or hemichordates is not a single autoregulating network, but rather a suite of interacting modules, each of one has its regulatory logic (See Pani et al., 2012; Yao, Minor et al., 2016). We have a hard time imagining a mechanism by which recruitment could co-opt and deploy these interacting networks in a similar fashion. Not impossible, but not likely in our opinion.
- In addition, we can't think of an example where co-option into a novel role is then followed by loss of the ancestral role. In our case, a series of co-options would need to occur to deploy the AP patterning network into a novel structure - the ambulacral ectoderm, then the ancestral role in AP patterning would then be lost. Again, perhaps not impossible, but not likely.

We suggest in the revised discussion of the manuscript that revisiting the fossil record in light of these findings may allow us to further test between these two scenarios.

10) Line 250: “our model” are the authors referring to their model organism or experimental framework? It’s a bit unclear what the claim is here.

We have clarified that we meant our model relating the echinoderm body plan to the deuterostome/bilaterian AP axis.

11) Line 244: It would be helpful if the authors could clarify in the text how their model differs from Adachi et al., 2018. This paper reports that “P. japonica orthologs of the medial markers are expressed in the ambulacral ectoderm of the rudiment; and few P. japonica orthologs of the posterior markers are expressed in ectoderm”

We thank Reviewer 1 for addressing this important point. Adachi et al. is a major study on echinoderm body plan that came to two conclusions. First, they identified that the ambulacral ectoderm has essentially an anterior identity, and second, they concluded that there is no evidence for an ectoderm carrying a posterior identity in the sand dollar *P. japonica*. We have modified the manuscript to more clearly highlight the findings from Adachi and coauthors and how it contributed to the development of our hypotheses. However, there are important differences between our findings and those of Adachi et al., which likely result from the more exhaustive gene sampling performed in our study and the use of fluorescent *in situ* hybridizations that allows a much finer mapping of gene expression to the anatomical structures than the colorimetric *in situs* used in *P. japonica*. The first difference between our study and that of Adachi is that they identified the ambulacral ectoderm as carrying an axial identity similar to that of the hemichordate collar (that is, to the vertebrate midbrain). By contrast, we propose that the collar identity corresponds to the podia epidermis, while the medial ambulacral ectoderm harboring the central nervous system has an identity more similar to that of the hemichordate proboscis (that is, to the vertebrate forebrain). This leads to the second difference, which is that Adachi et al. did not identify the medial to lateral deployment of the AP registry. This is important because if the ambulacral ectoderm has a homogenous anterior identity, as suggested by the conclusions of Adachi et al., it can be interpreted as the anterior compartment of the stacking hypothesis. By contrast, recognizing that the AP patterning genes are deployed along the medio-lateral dimension means that the polarity of the AP axis in pentaradial echinoderms is perpendicular to that of the mesoderm stack, and so at least from an ectoderm point of view, our model is incompatible with the stacking hypothesis.

12) Line 246 in the methods section: It doesn’t make sense to me to summarize expression profiles to a single expression peak for genes that have multimodal expression profiles, which are likely tied to the presence of multiple anatomical structures in each axis. Can the authors explain this a bit further?

Previously, we used the expression z-score to define a probability for the location of an expression maximum and averaged the results of the sampling to determine a

single, pseudo-peak position that was then used in the correlation analysis. As pointed out by Reviewer 1, this averaging was problematic for genes with bimodal expression profiles. We have replaced this original analysis with two new methods that address this issue, both presented in Fig. 2e and detailed in the methods.

The first method simply uses the location across the RNA tomography dimensions of the maximum z-score value as a readout of the location of a given gene. We then computed the correlation between these positions and the order in which AP patterning related genes are expressed along the *Saccoglossus kowalevskii* AP axis based on published expression patterns. This raw correlation has the advantage of simplicity but could be affected by (1) the choice of a single location for genes with bimodal expression patterns, and (2) the detailed choice of gene ordering (since several genes are more or less coincident in some regions of *S. kowalevskii*).

The second method that we present in parallel attempts to compensate for these potential biases. In this method, for each gene we sampled a location using the expression z-score as a probability. To obtain a null model for the distribution of correlations, we simultaneously shuffled gene order along the AP axis and computed Spearman correlation coefficient for 10^6 samples from this joint distribution. By sampling from the z-score distribution we avoid the pitfalls of using a single averaged value for the peak position of each gene, which is clearly problematic for bimodal distributions. The average correlation value for the 10^6 replications is lower than the raw correlation value. The distribution of correlations for the M-L dimension, however, remains significantly superior (two-sided Wilcoxon rank test, $p < 0.0001$) to the correlations for the P-D and O-A dimensions.

Referee #2 (Remarks to the Author):

I'm not qualified to judge the methodological details, so my comments are on the general nature and significance of the results and their interpretation. With that caveat, my view is that this is unquestionably a landmark paper in zoology, resolving issues of echinoderm body plan that have puzzled comparative, developmental and evolutionary biologists since the 19th century. The work demonstrates the power of mapping subdomains of the anterior head region across phyla, using hemichordates as a reference point and more genes, to achieve greater resolution than any previous work that I know of. The exposition of both the background information, as to the problem to be solved and the various hypotheses, and the results, are clearly explained, and the figures are informative and easily comprehensible. The paper is in my view in the top rank of papers that Nature should ideally be publishing. I have one substantive concern (see item 1, below), but otherwise have no reservations about publishing it in this form.

As to impact, for echinoderms themselves, this rewrites the book when it comes to interpreting the fossil forms, because we can now attach developmental meaning to the morphology. In a broader context, a divergence in body plan of this magnitude is a test of ideas relating to evolutionary contingency, and is of special interest when vertebrates are part of the story, as they are for anything related to ancestral deuterostomes. Evolutionary contingency is relatively new as a field of study, with an emerging theoretical focus, and here we have an example of contingency on a macro scale more dramatic than perhaps any other in animal evolution. This is the stuff of future textbooks. The key question for me is why echinoderms, after a radical makeover at the developmental level, are otherwise conservative in how that innovation was exploited, whereas chordates, while keeping to the ancestral body plan, have spawned the larger and more successful radiation. One can think of this in terms of scenarios, but contingency is a slippery issue (as much history as science as one author recently put it) and the larger message, if there is one here, is itself a bit of a puzzle.

What few suggestions I have below, though requiring attention, are in no way intended to detract from the authors' achievement, which is impressive by any measure.

Thank you for these enthusiastic comments!

1) There is one lapse in scholarship that needs correcting. This concerns the paper by Adachi et al. (2018) on Peronella, which is lumped (line 69) with other papers favouring the stacking hypothesis. My reading of Adachi et al. is (and was in 2018) that they have taken the loss of registry (decoupling) idea to heart and deal with patterning as something that can proceed independently in the ectoderm irrespective of what may be going on internally with Hox genes and the like. This is how I would interpret the statement on pg. 1299 "that echinoderm rays are outgrowths around the adult mouth" and on pg. 1302, that "...echinoderm rays are likely outgrowths of appendages around the adult mouth," although "appendages" in this usage is perhaps an unfortunate choice of term. And,

having far fewer markers, Adachi et al. map most of the ray tissue to collar level ectoderm, so a good deal of the logic and conceptual power of this new interpretation by Formery et al. is missing.

This does, however, lead me to view these new results in a somewhat different light than the way the authors have expressed it, that rather than the tomography results being surprising (line 119), they are perhaps better viewed as a validation of an existing line of argument, by Adachi et al., with proof of the model (the achievement of the present work) depending on mapping more of the relevant genes in far more detail.

Thanks for emphasizing the importance of Adachi et al., and the need to more clearly relate our findings to this landmark study of echinoderm body plan evolution. As pointed out by Reviewer 2, the results of Adachi et al. are essential because they (1) corroborated the decoupling between germ layers proposed by Lacalli, (2) predicted the absence of an equivalent to a trunk, and (3) noted the anterior nature of the oral ectoderm. All these ideas are confirmed in our study, and we have modified our manuscript in several places to give Adachi and colleagues more appropriate credit.

There are, however, several differences between our results and those of Adachi et al., and also differences in our interpretation of these findings.

Adachi and colleagues identified the ambulacral ectoderm as anterior, but they derived it from the collar ectoderm of hemichordates and supposed that most anterior parts of the AP registry are lost during the metamorphosis. In our view, the ambulacral ectoderm that corresponds to the collar is only its lateral part, while its medial part corresponds to the anterior-most AP registry of hemichordates, as shown by the expression of *nkx2.1*, *rx* or *fzd5/8* which are exclusively restricted to the proboscis in hemichordates. As for posterior genes, besides *pax2/5/8* (which in *P. japonica* is expressed in the spine rudiments, that is, in the interambulacral ectoderm), the expression of other posterior genes were reported in the mesoderm by Adachi et al.. Therefore, it seems to us that Adachi and colleagues did not predict the medial to lateral deployment of the anterior part of the AP patterning system. This might have resulted from an insufficient gene sampling, as Reviewer 2 suggests, but maybe also because of the lower resolution of whole mount chromogenic *in situ* hybridization that they performed compared to fluorescent ones, and most likely because the echinoid rudiment is a small structure nested within the larva which makes it anatomically more difficult to investigate compared to the ectoderm of a juvenile sea star.

Consequently, a literal interpretation of Adachi et al. would be that the ambulacral ectoderm has a uniform collar-like identity. This is why we initially classified Adachi as a study supporting the stacking hypothesis, since their findings are in line with the idea of David and Mooi that the oral ectoderm represents the most anterior layer of the AP stacking, with the other layers being mesoderm layers. However, we agree with Reviewer 2 that this interpretation of Adachi et al. is a little forced and we removed this paper from the citations supporting the stacking model. Please note that a strict interpretation of the Adachi et al. model predicts that correlations along the oral-aboral axis should dominate our RNA tomography dataset. Yet instead we find that medio-lateral correlations

dominate. We therefore believe that the medio-lateral deployment of the anterior part of the AP registry revealed through RNA tomography correlations, and confirmed by our gene expression patterns, is novel and unexpected.

I would point out also that for non-specialists such as myself, it would be useful if the authors would clarify the role of the tomography results as the project unfolded, e.g. as to how much of the in situ work could have gone ahead regardless, or perhaps was being done in parallel. The way the paper is currently written gives the impression that it was the tomography results that set the project in motion, which leaves me wondering if a point is being stretched mainly for narrative convenience.

While we had limited expression patterns from conventional colorimetric *in situs* prior to the invention of the HCR method, these were technically challenging in *Patiria* juveniles and this approach was largely unsuccessful for most genes. To comprehensively survey a large number of AP patterning genes, we started over using the systematic RNA tomography approach. Only after the RNA tomography data were obtained did we revisit *in situ* hybridization by taking advantage of the new HCR method to reinvestigate gene expression patterns. So leading with the RNA tomography results is not just a matter of narrative convenience. In addition, comparing gene expression datasets across taxa that have only little or no morphological similarities is not straightforward. For instance, gene expression comparisons between chordates and hemichordates have been the object of controversies (see Holland et al., 2013) even though both share the same basic bilateral organization with a head and a trunk that can be unambiguously identified. To avoid similar issues, we sought an unbiased way to test the polarity of the AP registry in echinoderms, and that's why we decided to use RNA tomography. It is true that the inner-ambulacral model could have been proposed based only on gene expression data, but to unambiguously assess that it is a better explanation than other hypotheses previously proposed required a statistical approach. As such, we believe that RNA tomography provides a quantitative aspect that is much more powerful at testing how distinct hypotheses fit with reality than *in situ* hybridization interpretation alone. In any event we don't think that inverting the order in which the RNA tomography and the gene expression patterns are presented in our manuscript would make our point clearer.

2) There is also then a slight problem with categorizing models as AP, OA, and ML (lines 204-218). The stacking model belongs in the OA category so long as the body is considered as a solid object. As soon as you restrict the patterning to ectoderm, the OA and ML models converge depending on how you choose to treat the aboral ectoderm, whether as a participant (a full ectodermal OA model would imply this) or as sitting passively on the sidelines (the more restricted ML model), literally with no skin in the game. I think any confusion this might potentially cause can be avoided with some careful rewording in spots. Options like "mapping to a solid body" versus "mapping to individual tissue layers" spring to mind.

This is a very good point and we thank Reviewer 2 for giving us the opportunity to address it. The stacking model has been most clearly defined by David and Mooi in their 2014 paper, as a combination of ectoderm and mesoderm components - that is, as a solid object - but to our knowledge has not been defined independent of mesoderm components. It seems reasonable that axial patterning should be examined at the level of each germ layer independently, which is also the approach taken by Adachi et al. Whether the polarity of the patterning is congruent between germ layers (as for example between chordates ectoderm and mesoderm where colinear Hox genes are expressed in parallel) or not (as it appears to be the case across echinoderm germ layers), is a somewhat different question.

If we restrict the stacking model to its ectodermal component, and hypothesize that the aboral/interradial ectoderm has a posterior identity, then as suggested by Reviewer 2 the stacking model and the medio-lateral model that we propose would converge with the most aboral point being also the most posterior. This being said, both our data and the one from Adachi et al. suggest that the aboral/interradial ectoderm is not posterior in nature. Adachi proposed that the posterior identity of the interradial ectoderm might have been lost during evolution, or that the interradial ectoderm might be an echinoid innovation that doesn't fit at all with ancestral AP patterning schemes. We would argue differently, and more in line with the EAT model proposed by David and Mooi, that the interradial ectoderm is an extra-axial component that is inherited from larval tissues, which is very clear when morphologically following how tissues are reorganized during metamorphosis in both asteroids and echinoids. As such, we think that the AP polarity of the interradial ectoderm is determined during the larval stage but is "passive" from a patterning point of view. at the time of the adult body plan elaboration. In that perspective, it is good to keep in mind that sea star larvae have by itself be described as essentially anterior territories ("head larvae") by Yankura et al. (2010) so that even the interradial ectoderm may never carry a posterior identity. Given length constraints, and all of this being very speculative, we don't think that it should be included in the manuscript discussion. It is however something we are starting to look at as a distinct project. The part of the manuscript presenting the distinct hypotheses has been clarified to avoid this potential confusion.

3) Minor points and typos

line 73: plural "needs"
line 189: change "than" to "as"?
line 430: it's a yellow asterisk in my copy
line 440 (Suppl. Section): change "a" to "at"

Thanks for catching these typos, we have corrected them in the resubmitted manuscript.

line 234: re. the reference to 18 or 22 genes carrying anterior identity. I count 20 listed in the first 3 of the 4 groups enumerated in the text (lines 146-188). Removing FezF and

dbx for their spotty expression leaves 18, so I would have said 18 of 20, leaving me puzzled as to which genes I've missed out (maybe engrailed and sfrp3/4 are included in the 22?). Ensuring that the counts are internally consistent would remove a potential source of reader confusion.

As part of modifying our treatment of the possibility of co-option, we removed from the discussion the argument that the high number of anterior genes involved in patterning the ambulacral ectoderm was supporting homology rather than co-option. Therefore, the confusion mentioned here does not apply anymore.

Referee #3 (Remarks to the Author):

The manuscript of Formery et al is addressing a fundamental zoological question that is puzzling to every student of biology when they hear about anatomical details of urchins and related animals. How are the body axes of an adult sea star related to the clear bilateral axes of other bilateral symmetric animals? There are not yet clear answers available and several hypotheses have been phrased of which some are more convincing than the others. Using RNA Tomography and in situ hybridization the authors aim for answering this question and discriminate between the existing hypotheses. However, their results are unexpected and very surprising since they are not in line with everything that has been proposed before. The approach is solid and the work is well done. And from the topic and how the work was performed I do in principle support the publication in Nature. However, I have few remarks about the interpretation and would like to see some further investigations or better, analysis of the present dataset.

We thank Reviewer 3 for this enthusiastic reading of our manuscript.

Puzzling to me is of course that the A-P patterning genes are expressed along a surprising axis: from the mediolateral ambulacral-system (anterior) toward the lateral along the medial-lateral axis of the sea star arms (posterior). The result is convincing from the data support, but so surprising that also the authors struggle with the interpretation. (“Our model offers a powerful tool to establish robust regional homologies between classes and an independent dataset to test existing competing hypotheses.”)

If somebody would give me these data in hand, I would at first glance assume that a very interesting case of the co-option of an existing gene cascade has happened during evolution of this unique, pentaradial body plan. I assume the authors have been gone through this and were also facing similar comments from the audience when publicly presenting the data. Therefore, I assume, the authors provide a disclaimer in the manuscript why this is not a case of a cooption: "However, co-option of gene regulatory networks into the development of novel structures usually involves a limited genes number of there are to our knowledge no examples of co-option of the entire anterior part of the AP patterning program.”

In principle, I don’t really buy this argumentation. There can always be new findings and the new “A-P axis” is along a body part that is a novelty and hard to compare with bilaterians. The problem of axis homology reminds me of the discussions in anthozoan cnidarians in which several homeodomain genes are expressed along the directive axis - an axis in which early on also the BMP/chordin axis is aligned. Looking at the ectodermal patterning genes - a subset of the genes that are studied here - are expressed along the oral-aboral axis. This example shows that determining axis homology is not so easy. However, I think to contribute to a solution here, and to improve the paper and the question of axis homology massively is to consider also the dorsoventral patterning system. The authors are well experienced in this (see e.g. Lowe et al 2006 PLOS Biol)

and at least in the RNA Tomography datasets the genes of question should be found. A few more in situs can make the story perfect. Is the axis perpendicular to the anteroposterior axis? Where would it be? How can the body plan in the lineage to Echinodermata got modified?

It would be great if the authors walk down this road.

We agree with Reviewer 3 that co-option is a possible interpretation, and we expanded the discussion section to more clearly accept this possibility. However, we still think that homology by descent with modification is a more parsimonious explanation to our findings, based on two arguments.

- First, the patterning system which defines distinct anatomical regions along the AP axis of chordates or hemichordates is not a single autoregulating network, but rather a suite of interacting modules, each of one has its regulatory logic (See Pani et al., 2012; Yao, Minor et al., 2016). We have a hard time imagining a mechanism by which recruitment could co-opt and deploy these interacting networks in a similar fashion. Not impossible, but not likely in our opinion.
- In addition, we can't think of an example where co-option into a novel role is then followed by loss of the ancestral role. In our case, a series of co-options would need to occur to deploy the AP patterning network into a novel structure (the ambulacral ectoderm), then the ancestral role in AP patterning would be lost. Again, perhaps not impossible, but not likely.

We suggest in the final section that revisiting the fossil record in light of these findings may allow us to further test between these scenarios.

We also thank Reviewer 3 for the suggestion to also consider the DV axis, which is now discussed in the manuscript in relation to the new Extended Data Figure 2. We first investigated the DV patterning system using the RNA tomography dataset by examining the expression of upstream signaling genes specifying the deuterostome DV axis (*BMP2/4*, *BMP1*, *ADMP1*, *chordin* and *nodal*). Since specification of the DV axis in bilaterians happens transiently and early in embryogenesis, we wanted to look at how these genes are expressed as early as possible during the formation of the pentaradial body plan. We therefore looked at them through the entire process of metamorphosis and not only at the juvenile stage. Although *chordin* and *nodal* are not expressed, it is clear that components of BMP signaling are used in the development of some aspects of the adult body plan, including the tube feet, as was previously reported by Koop et al. (2017). However, they do not appear to define a clear morphological axis perpendicular to the deployment of the AP patterning related genes.

It is not clear how DV polarity is specified in the adult body plan in species with complex life cycles (i.e. when the adult body plan is formed by metamorphosis and not by embryogenesis), and whether a diffusion based patterning system such as the BMP/chordin axis would work well to transform a larval body plan. For example, we see no obvious role of BMP/chordin during metamorphosis in larval species of indirect-developing hemichordates through metamorphosis. We suspect that the expression of

BMP2/4 in *Patiria* tube feet is more analogous to a late patterning role of BMP. We also looked at transcription factors that are typically involved in downstream patterning along the DV axis, *tbx2/3 and msx*. *Pax3/7*, another target of BMP involved in dorsal patterning, was not found, in agreement with previous studies (Howard-Ashby et al., 2006; Barton-Owen et al., 2018). Altogether, *tbx2/3* and *msx* seem to be involved in patterning the tube feet, which is consistent with a role downstream of BMP. We do not, however, see evidence that they are staggered along a morphological axis.

In addition to DV patterning, we also looked at appendage patterning system, which is another conserved suite of transcription factors that has previously been proposed to be related to echinoderm rays (Panganiban et al., 1997; Hotchkiss, 1998). We chose genes that are distributed along the proximo-distal axis of appendages in vertebrates and arthropods, including *meis*, *pbx* and *sp8/9*. *Dlx*, another gene classically involved in proximo-distal patterning, was not expressed, as already revealed by our analysis of AP patterning genes. Of these three genes, *sp8/9* shows an interesting pattern as it is expressed at the tip of the ambulacra, but is not sufficient to build a case for the evolution of the appendage patterning program in echinoderms. Investigating what are the molecular mechanisms involved in the growth zones of echinoderm rays will be a topic of interest in the future.

Few remarks:

1) Please provide a schematic drawing (text-book and lecture quality) about how this axis would be allocated when the presented results are transferred to the different echinoderm taxa (holothurians, urchins, crinoids).

We have provided schematics generalizing our model to echinoids and crinoids, which are the two other classes for which there are consistent published data. Including our asteroid schematic, these three schematics cover pelmatozoans, echinozoans and asterozoans, which are the three main echinoderm extant clades. We chose not to display any representation of ophiuroids and holothuroids because these classes have anatomical specificities that make any prediction uncertain at this point. In fact, we believe that using our model will actually allow us to test competing models of crown group echinoderm evolution, and this is something that we have started working on.

2) please remove from the manuscript evolutionary misconceptions, such as:

“the most derived animal group” - this lays in the eyes of the beholder. its totally arbitrary. I would say Sacculina or Gastrotricha is the most derived animal group (if I would join this conversation…).
“evolutionary trend” - what shall this be? a directed evolution?
"similarities across all deuterostomes phyla, including echinoderms.

Thanks for catching these issues. We have corrected them throughout the manuscript. For the final point, we have put the offending phrase in parentheses: “similarities across all deuterostomes phyla (including echinoderms)”, which we think will be useful for readers who are less familiar with metazoan phylogeny.

Referee #4 (Remarks to the Author):

Formery et al. use spatial transcriptomics (RNA tomography) and in situ hybridization in the sea star to identify patterning similarities between echinoderms and other deuterostomes. Importantly, the authors also performed a genome assembly. Specifically, the authors observe that genes involved in anteroposterior patterning of the ectoderm in bilateral deuterostomes have an equivalent pattern in the pentaradial body plan of echinoderms, with the midline of each ray corresponding to an anterior position and the lateral parts to a more posterior identity. Furthermore, they do not find an ectodermal territory expressing the bilaterian trunk patterning program, suggesting that the sea star ectoderm is patterned exclusively by head-like programs.

This is an interesting paper that addresses a fundamental question in zoology. The manuscript is well written, and the questions and hypotheses are clearly formulated. However, other reviewers will be better qualified than me to evaluate the novelty and importance of the biological results. Overall, the authors present a relatively small study, with little complexity regarding the experimental and computational methodology, and I was surprised that the authors (for instance) did not expand the study other germ layers besides the ectoderm, included additional stages or species, or performed a more systematic analysis of the RNA tomography data. As presented, I would consider the manuscript too preliminary for publication in Nature.

We thank reviewer 4 for these positive comments.

Major points

1) The only transcriptome-wide analysis of the RNA tomography data is done in Fig. 2d. The analysis is nicely done, but it would be useful to match the observed seven clusters to distinct anatomical territories. This could be used as a starting point to e.g. find other AP genes besides the investigated 36 genes, or to find other spatial programs besides anterior genes that contribute to the medial ambulacra.

We agree with Reviewer 4 that matching the seven clusters to sea star anatomy would provide a deeper characterization of the transcriptional landscape in *Patiria*. Unfortunately, RNA tomography does not discriminate among germ layers – which we now know have very different patterning readouts, so every cluster is a mix of genes expressed in distinct territories along the three dimensions. Because of this, RNA tomography is mostly useful in quantitatively testing hypotheses rather than for identifying genes with conserved roles in ectodermal patterning. To help register RNA tomography data with the fine-scale anatomy of the sea star, we now provide new micro-computed tomography (micro-CT) analyses as part of Fig. 2, Extended Data Fig. 1 and Supplementary Figs. 1 and 2.

Regarding the investigation of other spatial regulatory programs, as suggested by Reviewer 4, we used both the RNA tomography and additional gene expression patterns

to examine the deployment of bilaterian dorsoventral (DV) axial and the limb proximodistal patterning programs. These results are now discussed in the manuscript in reference to Extended Data Fig. 2.

2) It did not become clear to me why the authors selected only 36 genes for the analysis of the RNA tomography data. It seems to defeat the purpose of a spatial transcriptomics experiment if, in the end, the number of analyzed genes is so small that in situs can be done for all of them. Ultimately, it's probably not a black-and-white question whether the bilaterian AP program is reused in echinoderms or whether the genes have been co-opted into novel developmental roles, so it will be critical to base the assessment on a large number of genes.

The 36 AP patterning related genes that we chose to analyze correspond to the extent of the AP genes for which expression patterns are available in *Saccoglossus kowalevskii*, which is the bilaterally symmetric species the most closely related to echinoderms that also has the largest gene expression database. In this context, the reason for a RNA tomography analysis was less to offer a transcriptional 3D atlas of a sea star than to quantify the transcription profiles of these AP patterning genes and allow us to statistically test evolutionary hypotheses related to their spatial deployment. This is relevant because comparisons of *in situ* hybridization patterns across different species are by definition non-quantitative and may be subject to interpretation biases. Here instead RNA tomography provides quantitative data that we can use to test evolutionary hypotheses on axial patterning statistically, beyond the interpretation of *in situ* hybridization of expression patterns.

3) There seem to be no replicates for the RNA tomography experiment. This is probably fine, but it would at least be good to compare the RNA tomography results to the in situ data for the 36 selected genes. As presented, it is difficult to see to which degree the results are in agreement (taking into account the limited resolution of the RNA tomography data).

Thanks for this suggestion. We modified the Figure 2e and replaced it by the actual expression profile of the 36 AP patterning genes, which had originally been placed in Supplementary Fig. 3. This important information is now easier to see and can be compared directly with the expression data.

4) I'm surprised that the authors decided to limit the scope of the RNA tomography analysis to one species and one time point, and focus further on the ectoderm. These organisms are fascinating because of the incredible changes in shape and geometry they undergo during metamorphosis, and the temporal development of such a configuration would be an interesting aspect to study. With the experimental and computational approaches established (including transcriptome and genome assembly), it would seem to be fairly straightforward to broaden the scope of the manuscript. In my opinion, the manuscript would gain immensely if the authors could e.g. quantify the use of AP genes

in spatial patterning over time, identify similarities and differences between the germ layers, and demonstrate the generality of the findings by adding another echinoderm species. (While RNA tomography might not be ideal for other species, HCR could demonstrate whether the same principles hold).

Regarding the focus on one species and time point, although the methods may be extendable to new species, funding and time are not! We agree with Reviewer 4, however, that anatomical features of other echinoderm species may not be appropriate for RNA tomography. In echinoids the ambulacra are curved, making interpretation of sections problematic, while in crinoid and brittle stars it would be challenging to perform a medio-lateral sectioning of a single arm. It is only in sea stars that the three dimensions (P-D, O-A and M-L) are easily accessed. In addition, the value of a comparative project across different classes would be much more interesting in the context of understanding the radiation of echinoderms which has been a controversial topic in the field of paleontology. However, that is a different question. We do refer to gene expression datasets that have been published in echinoids and crinoids and that are consistent with our model.

Regarding the focus on ectoderm, we note that the patterning of the three germ layers carry different levels of information. Historically, comparisons of axial properties across bilaterian animals (essentially arthropods, chordates, annelids and hemichordates) has been done on the basis of ectoderm patterning, which in chordates and arthropods correlate with the regionalization of the central nervous system. The patterning of the endoderm and the mesoderm are of course other interesting topics, but they are relevant to questions that fall outside the scope of our study, and ideally should be addressed in the future.

Minor points

5) How were the 36 genes of interest selected?

The rationale for selecting these 36 genes is to compare the deployment of the AP axis in *Patiria* to the most closely related bilaterally symmetrical species for which there is an extensive characterization of AP patterning related genes: the hemichordate worm *Saccoglossus kowalevskii*. By reviewing the literature on this species, we came up with a list of AP-patterning related genes that have been described with *in situ* hybridization expression patterns. Of those, 36 were found to be expressed in *Patiria*, either in the RNA tomography data or by *in situ* hybridization. Furthermore, as noted in the text, all these genes are known to be largely consistent between chordates and hemichordates in the way they are deployed along the AP axis, providing a robust inference of the ancestral deuterostome deployment of these genes.

6) Methods, line 247/248: I did not understand the approach for identifying peak positions. What are the replicates that the authors refer to, and what is their criterion for defining whether there is a single peak?

Previously, we used the expression z-score to define a probability for the location of an expression maximum and averaged the results of the sampling to determine a single, pseudo-peak position that was then used in the correlation analysis. However, this averaging was problematic. We have replaced this original analysis with two new methods that address this issue and provide a better way to test the correlation between gene ranking and position. These new methods are now both presented in Fig. 2e and detailed in the methods.

First, we simply used the location across the RNA tomography dimensions of the maximum z-score value as a readout of the location for a given gene. We then computed the correlation between these positions and the order in which AP patterning related genes are expressed along the *Saccoglossus kowalevskii* AP axis based on published expression patterns. This raw correlation has the advantage of simplicity but could be affected by (1) the choice of a single location for genes with bimodal expression patterns, and (2) the detailed choice of gene ordering (since several genes are more or less coincident in some regions of *S. kowalevskii*).

Second, to compensate for these potential biases, we sampled each gene location along the RNA dimensions using the expression z-score as a probability. To obtain a null model for the distribution of correlations, we simultaneously shuffled gene order along the AP axis and computed Spearman correlation coefficient for 10^6 samples from this joint distribution. By sampling from the z-score distribution we avoid the pitfalls of using a single averaged value for the peak position of each gene, which is clearly problematic for bimodal distributions. The average correlation value for the 10^6 replications is lower than the raw correlation value, but the distribution of correlations for the M-L dimension remains significantly superior (two-sided Wilcoxon rank test, $p < 0.0001$) to the correlations for the P-D and O-A dimensions.

7) The authors seem to use two different stages for the spatial transcriptomics and in situ analysis (young juveniles for the RNA tomography analysis, and post-metamorphic juveniles for HCR). The title refers to adult animals. This should be explained better.

We agree with Reviewer 3 that this could be confusing. We typically consider both post-metamorphic and young juvenile “stars” to exhibit the pentaradial “adult” body plan in contrast to the bilateral larval body plan. The larger juveniles are not amenable to whole mount in situ hybridization, and smaller, post metamorphic juveniles impractical for RNA tomography. However, their patterning profiles are consistent. To avoid any confusion we have changed the title of the manuscript to “Molecular evidence of anteroposterior patterning in the pentaradial body plan of echinoderms.”

8) Figure S3 is probably interesting, but it was placed in the supplement and is not commented on much.

We have now moved most of what was Supplementary Figure S3 into Extended Data Figure 1g to go along with the new micro-CT dataset.

9) Figure 3 shows some nice images, but it is a bit difficult to know what to conclude from them. Especially because no profiles, density maps or quantitative graphs are shown.

Because hybridization *in situ* hybridization is not a quantitative method, we did not see a rationale for including quantitative graphs in Figure 3. On the other hand, the quantitative aspect is provided by the RNA tomography data, although at a different (but still pentaradial) stage.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have done a nice job of addressing my concerns. I am happy to support publication of this revised submission. It is an important study and will be of considerable interest.

Referee #2 (Remarks to the Author):

I liked the original submission, but the revisions have made it better. The case the authors make for their interpretation is more carefully argued with suitable caveats regarding the alternatives, the logical sequence of experimental work is easier to follow, and the additional data is helpful, not least in addressing the question as to where the dorsoventral axis has gone.

The interpretation advanced by the authors is in my view as convincing as one could hope, and certainly convincing enough to prompt a reconsideration of other aspects of basal deuterostome and early chordate evolution in light of what echinoderms appear to be doing. This is a fascinating and stimulating paper.

Referee #3 (Remarks to the Author):

The authors have addressed all my concerns and provided a sound explanation for the D-V axis topic. I congratulate the authors for this manuscript and it will be a pleasure to integrate it to my lectures in basic zoology.

Referee #4 (Remarks to the Author):

The revised manuscript has been improved substantially, and my comments have been addressed in a satisfactory manner. I do not have any further concerns regarding methodology and conclusions, and I support publication of the manuscript without further changes.