

Molecular evidence for early deuterostome origins of ovarian cell types and neuroendocrine control of reproduction

Corresponding Author: Dr S. Zachary Swartz

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript by Paganos et al. describes the cellular composition of the ovary in the sea star *Patiria miniata* by using a combination of immunofluorescence, HCR and single-cell sequencing analysis. The imaging of HCR and immunostainings in this paper is absolutely stunning and, combined with single-cell data analysis, provide a comprehensive and clear understanding of the diversity and location of the ovarian cell types in sea star. The manuscript includes the description of follicle cells surrounding the oocytes, clusters of germ cell cysts and a network of neurons running through the gonadal epithelium. The single-cell sequencing dataset allows the authors to compare the cell types mainly across deuterostomes and to explore the complement of developmental signalling and neuropeptide ligand and receptor genes present in the different ovarian cell types.

Despite the extraordinary quality of the imaging data and the comprehensive single cell atlas of the ovary, the paper has two major weaknesses:

(i) The WGA and F-actin staining are not convincingly showing that germ cell cysts are connected by cytoplasmic bridges. This claim needs further validation, f.ex. by electron microscopy or experimentally by showing that injected dextran beads get transferred between cyst cells.

(ii) One of the key findings that makes the paper interesting and relevant for a broader audience is the potential existence of an intrinsic neuroendocrine system that controls oogenesis. While the single-cell expression data is intriguing, it is not as conclusive as the authors claim and not supported by any functional data. Based on the single-cell sequencing dataset, nearly all neuropeptides and receptors, with the exception of oxy/vp ligand and its receptor, are expressed in both neurons and germ cysts or additionally in muscles or follicle cells. Therefore, the authors' summary in Fig. 7j oversimplifies the complexity of the expression data and the implied signalling direction is only poorly supported by the data. Experimental validation is necessary to support the existence of a putative HPG axis in the sea star. This could be done f.ex. by injecting some of the conserved neuropeptides of the HPG axis, such as GnRH or Oxytocin/Vasopressin. It would also add a mechanistic aspect to the work that would lift it beyond a 'single-cell sequencing atlas'.

Other points:

- in line 269: Reference to 1b is probably wrong.
- In SFig.1: There is data from 3 different species in this figure and although this is clearly described in the legend, it would be even clearer if the different species were also indicated in the figure panel. Also, it is unclear if the 'surface->lumen' applies to a-d or the whole figure.
- l. 340-343 & SFig. 3b-c: it would be helpful if the authors could clarify which one of the three sample in a-c refers to the three samples described in the text.
- l350-355 include long lists of genes whose relevance remain unclear until some of them are later described in the text. I think the authors could describe their relevance to identify respective cell types already here. Also, it remains unclear if some of these markers were previously published as cell type markers in sea stars or if all of them have only been described in sea urchins or chordates. In any case, the sources of this information should be cited. A table showing their expression in sea stars, sea urchin and a selection of other chordates could also be helpful for getting an overview of the markers and their conservation across species.
- In the same section, the 'immunocytes' need more explanation. On what basis do the authors think that these are immune cells? Have these been previously described in sea star? What is their function?

- In Fig.2a and throughout the paper: it is confusing to name clusters 'germ' and others 'oocytes' as oocytes are of course also germ cells. Please consider renaming 'germ' f.ex. to 'germ cysts'.
- In Fig.2b (and any other dot plot): it would help to indicate which genes have been validated by HCR, and in which figure these can be found. Also, the authors should consider grouping & labelling the genes on the y-axis so that they can easier be found. F.ex. it took me some time to find the *syt1* gene mentioned in l. 382-3.
- Also in Fig.2b (and any other dot plots): Gene annotations do not contain any gene identifier that would allow finding this gene in a gene bank. This is especially important for genes with identical annotations, such as in Fig. S9b, where >25 genes carry the same annotation.
- l. 403-409: It is unclear if the genes mentioned in brackets are referring to the mammalian or sea star genes.
- Fig.3 (and any other Sankey plot figure): the representation of these plots is confusing. First, as sea star is always the reference dataset, it would make sense to always plot it on the left side. Also, it would help to keep the order of clusters identical between plots, and to colour-code the different clusters in groups (follicles cells, oocytes, epithelium...).
- On a conceptual level, the authors seem to have only used ovary single-cell data from other species for comparison with their sea star dataset. Using that strategy, the authors will miss close relationship between cell types in the sea star ovary and cell types that are located in non-ovary tissue in vertebrates. This needs to be addressed.
- Fig.3d: the terms next to the animals are too vague. F.ex., which muscles in flies or fish or mouse? And these are homologous to which muscles in sea star?
- Fig.4a and SFig. 5a: It remains what 'the average score of genes involved in G1/S, G2/M phases of the cell cycle' means. The comparison between 4a and S5a could also become clearer if the clusters and genes were always on the same axes, and if the genes in S5a were grouped in G1/S, G2/M and Meiosis. And as the category G2/M includes 'Meiosis', it remains unclear what the differences are.
- l.474-6; Fig. S7: The authors focus on the difference between the GO enrichment analysis without acknowledging that the top-3 GO categories are identical between cysts and oocytes.
- In Fig.5: Although the authors claim the 'activation of oocyte GRN' (Fig.5I), only cysts-enriched genes are plotted or validated using HCR. The authors should also plot and HCR-validate some genes that become activated only in maturing oocytes.
- l. 499: The selection of signalling pathway is poorly explained. Why those and not others (e.g. FGF, IGF, Hippo/Yap?)
- l. 565: The authors claim that F-Salmf is specifically expressed in neuron and germ cell groups, but the dot plot shows highest average expression in an epithelial cluster. Please comment.
- Fig.7c could be clearer in grouping/highlighting neuropeptide/receptor pairs.
- l.673: there is any comparative information missing for the claim that sea star follicle cell types are conserved to distinct follicle cell populations in protostomes.
- l. 692-96: This hypothesis on the roles of RA, Wnt TGF β and Hh pathway in self-renewal-meitotic entry decision is overly speculative.
- l. 699-709: I suggest moving this information on HPG signalling to the introduction as it is key to understand the relevance of the different neuropeptides in the results part.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

This interesting manuscript presents the use of single-cell RNA sequencing (scRNA-seq) to identify cell populations in the ovary of the sea star *Patiria miniata*, a non-chordate deuterostome. The authors combine this approach with high-resolution 3D imaging to investigate the spatial organization of the identified cell types. They argue that their data provide molecular evidence for the pre-chordate origins of ovarian cell types and for neuroendocrine control of reproduction.

Specifically, the authors identify putative oogonial stem cells (OSCs), granulosa-like oocyte support cells, and theca-like cells. They also provide evidence supporting the existence of an intrinsic neuroendocrine system that may regulate oogenesis and predate the vertebrate hypothalamic-pituitary-gonadal axis. The evolutionary origins of ovarian cell types and neuroendocrine regulation of reproduction is of broad interest. While several claims are well supported by the data, others rely on relatively limited evidence.

Major Comments/Suggestions:

1. Oogonial Stem Cells (OSCs)

While it is plausible that OSCs exist in sea stars given the annual production of large numbers of oocytes, the current dataset does not convincingly identify this cell type. If sea stars spawn once per year, OSCs might only be active for a brief period and thus constitute a rare population. The data shown depict probable oocyte progenitors in interconnected cysts—consistent with a stem cell-driven oocyte production model but not definitive proof of OSCs.

In vertebrates, *nanos2* is a specific marker of OSCs. Do sea stars have a *nanos2* ortholog, and is it expressed in the ovary? Please also clarify whether sea star *nanos1* and *nanos3* are orthologous to the vertebrate *nanos1* and *nanos3*. Without more definitive markers or lineage tracing, the authors should refrain from asserting that OSCs have been identified.

2. Follicle Cell Subtypes

The identification of granulosa-like (follicle) cells is compelling, and the presence of subtypes is particularly interesting. In vertebrates, distinct populations of pre-follicle and granulosa cells support germ cells at different stages. However, it is unclear whether any of the sea star follicle subtypes correspond to these vertebrate populations.

Genes such as *irx3*, *irx5*, *lhx9*, *emx2*, and *wt1* are specifically expressed in pre-follicle cells in mammals and zebrafish. Do sea stars have orthologs of these genes, and are they expressed in a specific follicle subtype? If these markers are absent or not differentially expressed, can the authors speculate which population is most analogous to vertebrate pre-follicle cells?

3. Theca-like Cells and Steroidogenesis

Vertebrate theca cells synthesize steroid hormones and provide precursors for estrogen biosynthesis by granulosa cells. The authors report that follicle population III expresses *star* and *cyp17a1*, genes also expressed in vertebrate theca cells. However, key steroidogenic genes such as *cyp11a1* (cholesterol side-chain cleavage enzyme) appear absent.

Are sea stars capable of producing steroid hormones? If so, which ones? Can the authors speculate on the steroid profile produced by theca-like cells?

4. Stromal/Interstitial Cells

In addition to follicle and theca cells, stromal cells provide ovarian structural support. Muscle cells, for instance, may represent a specialized stromal subtype. Could follicle III cells be more closely related to vertebrate stromal cells or represent a common ancestral population? In vertebrates, markers like decorin (*dcn*) and various collagen genes mark stromal-like cells. Are similar genes expressed in sea star follicle III?

Minor Comments/Suggestions

1. Data Sharing

In addition to depositing the data resources on GitHub, I encourage the authors to also consider depositing it on a portal, like the Broad Institute's Single Cell Portal, that allows easy browsing of the data.

2. Line 318

Define "GVBD" at first mention for clarity.

3. Figure S3

In the legend or main text, specify which UMAPs in panel S3d correspond to ovaries from different reproductive stages (recently spawned, early cyst-enriched, mature oocyte-rich).

4. Line 355

Orthologs of *Drosophila piwi1* are typically annotated as *piwil1*. Please revise accordingly.

5. Line 402 / Figure 3

Figure 3 is difficult to interpret without a more detailed legend. Please clarify which datasets and cell types are being compared. Consider adding labeled UMAPs corresponding to each dataset, with cluster numbers that match those in Figure 3.

6. Line 449 / EdU Incorporation

If EdU+ cells are within a germline cyst, all connected cells should exhibit synchronized DNA synthesis and incorporate EdU. However, only a subset appears labeled. Can the authors explain this observation?

Reviewer: Bruce Draper

(Remarks on code availability)

I confirmed that the data and code are uploaded on github, but I did not download or run the code.

Reviewer #3

(Remarks to the Author)

This paper by Paganos et al. examines the cellular composition and gene expression landscape of the adult ovary in the sea star *Patiria miniata* using single-cell RNA sequencing. The authors identified 27 transcriptionally distinct clusters, including germ cells, several follicle cell types, epithelial and muscle cells, and a group of intrinsic neurons. Germ cell populations were categorized into multiple developmental stages, and their inferred progression was supported by spatial transcript detection and EdU labeling.

The authors stated the expression of neuropeptides typically associated with vertebrate reproductive regulation—such as *gnrh*, *kisspeptin*, and *oxy/vp*—in neuronal clusters within the ovary, alongside receptor expression in germline clusters. This suggests the presence of a local signaling network potentially analogous to the vertebrate HPG axis, though its functional equivalence remains to be determined.

The authors also reported expression of steroidogenesis-related genes (*star*, *cyp17a1*) in epithelial and follicle clusters, which may reflect the presence of steroidogenic activity, albeit in the absence of canonical vertebrate pathways. Comparative transcriptomic analyses with model species including zebrafish, mouse, and *Drosophila* indicate partial conservation of transcriptional programs across evolutionarily distant lineages, particularly among follicle and germ cell types.

Collectively, these results offer a cellular and molecular framework for understanding reproductive tissue organization in echinoderms, with implications for the evolution of local neuroendocrine systems.

The methods are appropriate, and the results are convincing and clear. While the cellular and transcriptomic characterization of the sea star ovary provides a valuable dataset, the reviewer has significant concerns regarding the interpretation of these findings in the Discussion and Conclusion sections. The evolutionary and neuroendocrinological implications proposed by the authors relied heavily on selectively interpreted evidence and speculative parallels to vertebrate systems. In their current form, these claims risk misrepresenting the biological significance of the data and may lead to serious misconceptions across multiple disciplines, including evolutionary biology and neuroendocrinology.

1. The authors refer to echinoderms as “pre-chordates,” which is a phylogenetically inaccurate and misleading designation. Echinoderms and chordates are both deuterostomes that diverged from a common ancestor; chordates did not evolve directly from echinoderms. This fundamental error undermines the evolutionary framing of the study and should be corrected. Moreover, the complete absence of discussion regarding hemichordates—an intermediate and highly relevant group—further suggests a lack of evolutionary rigor in the manuscript's phylogenetic interpretation.

2. In keeping with the above, while the inclusion of *Drosophila* may highlight a few conserved transcriptional programs, comparisons with protostomes offer limited relevance to understanding the evolutionary origins of reproductive systems in chordates or deuterostomes more broadly. The manuscript currently overstates the evolutionary significance of these comparisons without adequately framing their phylogenetic distance and limitations.

3. The authors propose that echinoderm ovarian neurons are analogous to elements of the mammalian HPG axis, and speculate that intrinsic neuroendocrine systems may represent an ancestral mode of reproductive control later taken over by centralized neuroendocrine organs. However, this hypothesis, as stated on page 33 (lines 726–731), overextends the data and risks misleading readers. Crucially, the vertebrate HPG axis is centered on pituitary-derived gonadotropins (FSH and LH), which have no homologs in invertebrates. Similarly, sex steroid hormones such as estrogens and androgens—along with their feedback roles—are essential in vertebrate reproduction but remain unidentified in echinoderms, both structurally and functionally. While the presence of genes like *cyp17a1* is interesting, its biological significance cannot be inferred without evidence of active steroidogenesis or corresponding hormonal pathways. The evolutionary argument presented therefore lacks molecular and physiological grounding.

4. In sea stars, oocyte maturation and ovulation are well known to be regulated by the relaxin-like gonad-stimulating peptide (RGP) via induction of 1-methyladenine (1-MeAde) synthesis, as clearly documented in the literature (e.g., Mita, *Biomolecules*, 2023). Moreover, RGP receptors have been identified in *Patiria pectinifera* (Mita et al., *PLoS One*, 2020; Feng et al., *BMC Biol.*, 2025). Despite the central role of the RGP–1-MeAde axis in echinoderm reproduction, this study makes no mention of these molecules in either the results or discussion. This omission significantly weakens the physiological context and interpretive relevance of the findings.

Conversely, the authors focus on vertebrate-type peptides such as kisspeptin and GnRH (or their corazonin-related homologs), yet there is currently no experimental evidence that these neuropeptides are involved in oocyte maturation or ovulation in invertebrates. For example, in the ascidian *Ciona*, a basal chordate, recent studies have shown that tachykinin is involved in early follicular growth, vasopressin in oocyte maturation, and vasopressin and cholecystokinin in ovulation (Satake and Sasakura, *Mol Cell Endocrinol*, 2024).

A meaningful comparative analysis should take into account such lineage-specific reproductive control mechanisms across invertebrate deuterostomes. Furthermore, in the vertebrate HPG axis, kisspeptin and GnRH do not act directly on follicular cells; rather, they regulate gonadotropin release from the pituitary. Thus, the detection of kisspeptin or GnRH-related ligands and receptors in the sea star ovary does not, in itself, justify analogies to HPG function. If anything, the findings reported in this study underscore mechanistic differences between echinoderm and vertebrate reproductive regulation, rather than similarities.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised manuscript by Paganon et al. has substantially improved the presentation of the data, especially the SAMPlots, and is more careful in the interpretation of the expression data. Unfortunately, however, it could not resolve whether germ cell cysts are connected, or provide validation for a functional role of neuropeptides in oogenesis.

In addition, the improved representation of the SAMPlots to compare cell cluster expression profiles between species revealed new concerns about the cell type homologies proposed by the authors. Most importantly, it became clear that comparing clusters between sea stars and sea urchins, which should be clear due to their close relationship, does not result in clear, 1:1 cell type homology of the three follicle cell types. This puts into question the validity of any other comparisons of

sea star cell clusters with vertebrates and flies. In addition, the presentation of cluster comparisons seems biased, focussing on similarities and not accounting for or testing the possibility of evolutionary convergence. Overall, the cell type comparison part is shallow and oversimplified, likely as the dataset does not have the predictive power to reconstruct cell type evolution between sea stars, sea urchins and other bilaterians in a rigorous way.

The authors have added an experiment using a Tgf- β inhibitor, but this experiment lacks essential validation experiments and does not provide any meaningful mechanistic or evolutionary aspects to the manuscript. It is not even mentioned in the discussion.

In summary, the revised manuscript is essentially a high-quality cell atlas of the sea star ovary cells, but does not provide any clear answers to reconstruct the evolution of these cell types beyond the ones that were obvious before (e.g., oocytes are conserved) or any novel mechanistic information on the role of growth or neuroendocrine signalling pathways in sea star oogenesis.

I therefore cannot recognize the value of the revised manuscript for a broader readership and unfortunately cannot recommend publication in Nature Communications.

Detailed explanations on major issues:

Cell type comparisons

I thank the authors for using a consistent colour scheme and plotting order for the SAMPlot analysis. I had hoped that it would facilitate the interpretation of these plots, but in contrast, it increased my doubts about the interpretation of this data. I have two major concerns in their interpretation:

1) The results are described in a very shallow manner and seem biased to highlight similarities that suggest evolutionary conservation, while ignoring similarities between clusters that do not fit a scenario of evolutionary conservation.

For example, the authors write this simple and vague summary of description of the follicle cell similarities in the results part: 'We also identified similarities between the three sea star follicle cell groups and their counterparts in fruit fly and sea urchin'

In reality, the situation is much more complex:

I would expect that the sea star-sea urchin comparison works as 'positive control' for the whole analysis, but the authors give it similar (or even less) weight than flies or mammals (or even less). Looking more in detail to this comparison, I was worried by the many sea star clusters that do not have 'fitting' corresponding clusters in the sea urchin dataset. F.ex. Follicle II clusters 9-12 are linked to 'oocytes_sp0 cluster of sea urchins (and to oogonia in zebrafish). And the follicle III clusters 23-25 seem to be linked to a large variety of different sea urchin clusters including epithelium and oocytes clusters. In fish, these clusters have strong links to immune cells, refuting a conservation of these follicle cell types between sea star and fish. Comparisons to flies seem dominated by links of nearly all clusters to 'border stretch hemocytes', which is not explained anywhere in the manuscript.

The follicle cell I clusters 5-8 seems to be the only ones consistently linked to follicle cells in sea urchin, fish and flies.

2) Even for follicle cell I, it is unclear which genes drive their similarities, is it transcription factors/signalling genes or effector genes? And could the similarities be resulting from evolutionary convergence? As the function of follicle cells is mainly to provide oocytes with nutrients and protection, they could have recruited similar genes in an independent manner to execute this function.

Insufficient presentation and experimental design of inhibitor experiment:

I. 656: 'with the specific Tgf β inhibitor LDN-193189'

- Reference missing

- The authors ignore the complexity of 'Tgf β signalling', which can occur via Nodal-, Tgf β , Activin- or BMP- receptors, and intracellular via Smad1/5/8 or Smad2/3 signalling. The inhibitor used by authors has been described as specific for ALK2/3 (Activin receptors) in vertebrates, which signals specifically via Smad2/3. All of these details are ignored by the authors.

- The authors show that alk2, a putative target of the LDN-193189 inhibitor, is expressed in oocytes and germ cell nests, and it remains obscure how that fits with the phenotype of disorganised transzonal projections.

- All of these issues disqualify the conclusion that 'the deep evolutionary conservation and functional role of Tgf β signaling pathway components within the ovarian follicle.' (I. 568-60).

No evidence for oogonial stem cells

I.: 568-70: 'These data identified replicating cells within the sea star ovary (Fig. 4f) and support our hypothesis of a self-renewing oogonial stem cell pool in adult sea star ovaries.' This statement is not supported by the data as the authors show no evidence for self-renewal, and all of the EdU+ could just as well represent determined, mitotic oogonia cells. As OSC would need to be uncommitted by definition, the concern is further supported by the authors' own statement that 'Cluster 13 was chosen as the root because its molecular signature and cellular morphology was most similar to uncommitted germ cells' (I. 582ff).

Minor issues:

I. 79: '...explored their evolutionary relationships with other animals' -> '...relationship with ovary cell types of other animals'

I. 83: 'we report gene expression conservation of signaling pathways involved in oogenesis': what does that mean?

Fig.1a: What is the relevance of the 'average lifespan'? I do not see its relevance and is obviously not correlated to presence/absence of adult oogenesis. I would also recommend to add species names, so that it is clearer that the info for *Drosophila* is not representative of the entire protostome taxon.

I.370-71: Synaptotagmin-1 staining is not visible in Fig. 1d

I. 380: 'average size of 8-16 μm ': I do not understand what that means, as a range is not an average. Also, 'size' is imprecise, I assume the authors mean diameter? These numbers are also confusing as on the graph in S2a, there are values beyond these range, especially $>16\mu\text{m}$. I suggest reporting a $\text{mean} \pm \text{s.e.m.}$ and indicating the mean on the graph.

I. 381: (Fig. 1g, i)-> (Fig. 1g, h).

I. 385: Phalloidin does not label all actin, but only F-actin, so please change 'actin' -> 'F-actin' throughout the manuscript.

I. 403: 'While sea stars conserve three nanos genes homologous to those in mammals and other organisms': syntax, gene nomenclature (->italic)

I. 410: 'We found that the smallest nanos1+ oocytes are located next to germ cell nests and are within the range of 17-42 μm in diameter': how can 'the smallest nanos1+ oocytes' have such a broad range?

I. 421-3: how are pre-GVBD defined? And in Fig. 1m, the authors propose three stages of oocytes, but a clear description of how these stages are distinguished and/or defined is missing.

Fig. 2a/S3: why is there a different colour code for the same clusters between these figures?

Fig. 2i needs annotations

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The authors have adequately addressed my previous comments/suggestions.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

The authors have substantially improved the manuscript in response to my previous comments regarding the RGP-1-MeAde axis and the broader neuroendocrine context of echinoderm reproduction.

The reviewer appreciate that the revised Discussion now clearly acknowledges the established role of the relaxin-like gonad-stimulating peptide (RGP/rgpp) and 1-methyladenine (1-MeAde) in sea star oocyte maturation, and places the new data within this well-characterized physiological framework. The additional analysis of rgpp and rgppR expression in the single-cell dataset, together with the spatiotemporal mapping presented in Figure S9b, is particularly valuable. The finding that rgpp is predominantly expressed in epithelium, oocytes, and muscles, whereas rgppR is restricted to specific epithelial, neuronal, and follicle III (theca-like) clusters, provides an important cellular context for the canonical RGP-1-MeAde pathway.

In particular, the observation that only one follicle cell cluster, corresponding to theca-like follicle III cells, expresses rgppR is a very interesting result. This strongly suggests that this follicle cell subtype is a prime candidate for the 1-MeAde-producing population in *Patiria miniata*, and it nicely integrates classical physiological work with the new single-cell atlas. I would, however, encourage the authors to phrase this conclusion in slightly more explicitly tentative terms (e.g., "strongly suggests" or "is a likely candidate for the follicle cell type that produces 1-methyladenine"), to make clear that the current evidence is based on receptor expression patterns rather than direct measurement of 1-MeAde synthesis in defined cell types.

More broadly, the expanded Discussion on neuroendocrine control in invertebrates (including ascidians and other lineages) now provides an appropriate comparative framework for interpreting the presence of GnRH-, kisspeptin-, vasopressin/oxytocin-, and PrRP-like signaling systems in the sea star ovary. The language describing a "hypothalamic-like and gonadotropic-like intrinsic ovarian neuroendocrine signaling system" is now much more clearly framed as a hypothesis and as a mechanistic novelty that is distinct from the vertebrate HPG axis. For complete clarity, I would only suggest slightly softening the sentence stating that "the absence of hypothalamus and pituitary gland is substituted by ovarian neurons," for example by rephrasing as "may be partially substituted" or "could be functionally compensated, at least in part, by ovarian neurons," to emphasize the speculative nature of this proposal.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I sincerely thank the authors for their detailed response, clarifications and for performing additional experiments to validate the use of the chemical inhibitor LDN-193189. These efforts address several of the previously raised concerns. However, some of my key points remain insufficiently addressed and require revision.

Cell type comparisons

I thank the authors for their clarifications regarding my concerns about the SAMap analysis. I agree that a strict 1:1 correspondence between sea star and sea urchin datasets is neither expected nor necessary, given both evolutionary distance and technical variability.

However, my concern was not based on an expectation of strict correspondence, but rather on how the results are described and interpreted. This issue remains central, as it underpins a key claim of the manuscript—namely, the proposed early deuterostome origin of ovarian cell types. The current presentation appears selective, emphasizing similarities that support evolutionary conservation while not adequately addressing patterns that are inconsistent with this interpretation. I must therefore insist that this point is addressed more rigorously.

While I agree with the authors that the sea star–sea urchin comparison represents the most informative available reference, it is currently absent from the revised Results and Discussion of the manuscript. This comparison should be incorporated as a central component of both the Results and Discussion. In particular, the manuscript should address the following points:

- The claim that follicle_II cluster cells are 'echinoderm-specific', suggesting conservation across echinoderms (l. 831). As previously noted, there is a strong link between the sea star follicle_II cluster to a sea urchin oocyte cluster. This observation suggests a lack of conservation across echinoderms and should be explicitly discussed.

- The interpretation of sea star follicle_III clusters as expressing genes 'typically found in ... theca cells in mammals' (l. 832), leading to the conclusion that 'our results also suggest possible evolutionary origins for theca and granulosa cells that predate the divergence of Ambulacraria and chordates' (l. 835–837). This conclusion relies primarily on comparisons to mammals and is not supported by comparisons within echinoderms or with other non-mammalian taxa.

In the sea urchin-sea star comparison, follicle_III clusters show the strongest links to epithelial and oocyte clusters. This substantially weakens the claim of an pre-chordate origin of theca cells, yet this discrepancy is not discussed. Similarly, in zebrafish, theca cells map to sea star epithelial cells (l. 565–566), while, as noted in my previous review, follicle_III cells show strong links with immune cell clusters. Taken together, these comparisons with both sea urchins and zebrafish contradict the conclusion that theca cell types predate the Ambulacraria–chordate divergence.

In addition, I previously noted that the sea urchin–fly comparison appears dominated by links to 'border stretch hemocytes.' Clarification of the biological meaning of this mapping would still be helpful.

The evolutionary conservation of germ cells and oocytes is clear and expected.

In summary, the Results and Discussion of the SAMap analysis should be revised to provide a balanced and comprehensive account of all relevant mappings, including those that do not support evolutionary conservation. While the evidence supporting conservation of follicle_I/granulosa-like cells (e.g., foxL2, gata4, and broadly consistent SAMPlot support) appears convincing, the current data—particularly the absence of key transcription factor signatures and the inconsistency of SAMap mappings—do not, in my view, support equivalent claims for theca cell type conservation.

Tgfb inhibition by LDN-193189

I thank the authors for the additional experiments and clarifications provided in their response. The authors convincingly demonstrate that treatment with the inhibitor LDN-193189 in sea star larvae phenocopies previously observed effects on the BMP receptor-mediated axis formation and suppresses pSMAD1/5/8 phosphorylation.

However, the description of these results still does not adequately reflect the complexity of TGFb signalling, and it remains unclear if the reported combination of TGFb ligands, receptors and Smad proteins may function together in this system. To improve clarity and precision, the authors should explicitly state:

- that tgfb receptor 3 ortholog likely functions as a TGF-beta co-receptor, whose vertebrate orthologs lack intrinsic signalling activity
- that alk2 receptor is a type I Activin receptor that binds BMP ligands and typically signals via Smad1/5/8
- which TGFb receptor types are expected to be inhibited by LDN-193189

In addition, the authors should consistently use 'pSMAD1/5/8' throughout the manuscript to clearly distinguish it from pSMAD2/3 and avoid potential confusion.

These clarifications would substantially strengthen this section.

Figure 1a

I previously questioned the relevance of 'average lifespans' in Fig.1a, and suggested adding species names to clarify that the indicated lifespans are not representative of the entire taxa (f.ex. 'protostomes'). While I appreciate the author's attempt to clarify this point, I respectfully disagree with their proposed revision, which in my view increases confusion.

The added statement 'Average lifespans of the depicted taxa are shown to highlight the rare case of long-lived animals (echinoderms) capable of adult oogenesis.' is problematic. It introduces ambiguity (what is 'long-lived?') and reinforces a misleading narrative. Even under a conservative definition of long-lived (>100 years; which would exclude sea stars), numerous examples of long-lived animals capable of adult oogenesis exist across different taxa, incl. vertebrates (f.ex. reptiles, sharks), annelids (some tube worms), molluscs (famously *Arctica islandica*), as well as cnidarians (corals, some jellyfish) and sponges. In addition, some sea stars and many sea cucumbers, which are also echinoderms, are rather short-

lived (<10 years). This shows that the authors' generalisation of echinoderms as long-lived does not hold, and sea urchins do not represent a unique or rare case.

Furthermore, the authors clarify in their response that each depicted animal represents multiple species or genera. Therefore, absent taxonomic annotation leaves their interpretation ambiguous and potentially misleading.

Additional issues include:

- annotating the root of this tree as 'metazoans' is problematic as the figure includes exclusively bilaterian taxa
- The use of lifespan ranges to represent 'average' lifespans, which is conceptually inconsistent.

With appropriate revisions, the figure could be informative to illustrate the phylogenetic relationships among the species used for the comparative single-cell analysis, and species names should be explicitly included. However, the connection between lifespan and adult oogenesis is presented in a biased and unsupported manner and is not directly relevant to the manuscript. I therefore strongly recommend that this component should be removed.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

The authors have fully addressed comments of the reviewer.

(Remarks on code availability)

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We thank the reviewers for their very helpful and constructive comments. We provide our point-by-point responses below. Changes to the manuscript in this revision have been highlighted in yellow.

Reviewer #1 (Remarks to the Author):

Despite the extraordinary quality of the imaging data and the comprehensive single cell atlas of the ovary, the paper has two major weaknesses: (i) The WGA and F-actin staining are not convincingly showing that germ cell cysts are connected by cytoplasmic bridges. This claim needs further validation, f.ex. by electron microscopy or experimentally by showing that injected dextran beads get transferred between cyst cells.

We appreciate the positive comments about our imaging, and these suggestions for better resolving the question of whether the germ cells are connected by cytoplasmic bridges. We considered the dextran transfer experiment, but the cysts are difficult to identify with DIC imaging and are buried under the outer muscular layer of the ovary, making it extremely difficult to microinject into these ~15 micron cells. We also consulted with multiple experts in electron microscopy, including pFIB-SEM volume imaging. Unfortunately, the consensus was that this approach would be too challenging to achieve in a short timeframe consistent with this manuscript revision, and would instead need to be its own standalone project.

As an alternative, we learned what is a new technique for our lab: pan-expansion microscopy (ExM), which involves the isotropic expansion of the sample with polymers and staining with reactive fluorescent esters to stain total protein. This yields an EM-like image. We further combined this approach with a brand new ExM compatible actin probe (HAK-Actin). These data have been added to Figure 1H,I and Figure S1P. With this approach, we were unable to see membrane connections between oogonia in the nests. We also did not see the circular structures seen with phalloidin staining using the HAK-actin probe. Therefore, while we cannot exclude the possibility of bridges, we have decided to call the structures nests rather than cysts, and to remove the statements about the oogonia being connected by cytoplasmic bridges.

(ii) One of the key findings that makes the paper interesting and relevant for a broader audience is the potential existence of an intrinsic neuroendocrine system that controls oogenesis. While the single-cell expression data is intriguing, it is not as conclusive as the authors claim and not supported by any functional data. Based on the single-cell sequencing dataset, nearly all neuropeptides and receptors, with the exception of oxy/vp ligand and its receptor, are expressed in both neurons and germ cysts or additionally in muscles or follicle cells. Therefore, the authors' summary in Fig. 7j oversimplifies the complexity of the expression data and the implied signalling direction is only poorly supported by the data. Experimental validation is necessary to support the existence of a putative HPG axis in the sea star. This could be done f.ex. by injecting some of the conserved neuropeptides of the HPG axis, such as GNRH or Oxytocin/Vasopressin. It

would also add a mechanistic aspect to the work that would lift it beyond a 'single-cell sequencing atlas'.

*We completely agree that more mechanistic/functional data would improve our study. In response to this comment, we identified the peptide sequences of GnRH (pQIHVKVPGWPG-NH₂, where pQ=pyroglutamate) by comparing *Patiria miniata* sequence to GnRH from a related sea star, where it had been previously characterized (<https://pubmed.ncbi.nlm.nih.gov/29033898/>). In addition, we identified the sequence for PmOxytocin (CLVQDCPEG-NH₂). We had both of these peptides custom synthesized and tested their effects on ovary explants cultured in vitro for 1 week. Unfortunately, we did not observe consistent effects on the morphology of either the somatic or germline cells in the ovary, nor induction of spawning. We hypothesize that these neuropeptides are acting upon ovarian processes over a timescale of months. Therefore, to meaningfully perform these experiments would require long-term genetic knockout studies, but doing so would not be feasible for this revision. We do hope to achieve this in a future study.*

As an alternative, we opted to focus on the TGFbeta pathway and its roles in folliculogenesis, which we feel similarly elevates our study. In brief, we treated in vitro cultured ovaries with a specific TGFbeta receptor inhibitor (LDN-193189) for several days. This resulted in increased numbers of transzonal projections, but they were highly disorganized (see explanation in a comment below), and now include these data in Fig. 6.

We also appreciate the reviewer's point about the possible oversimplification of neuropeptide signaling and the cell types involved. Fig. 7j is intended to summarize the predicted crosstalk between the germ cell nests and the ovarian neurons. However, we understand that within the manuscript the expression domains of the neuropeptides and their respective receptors was presented only in regards to these two cell type groups. To this end we further described the predicted expression domains of the neuropeptides and receptors in the rest of the ovarian cell types and added a statement regarding their pleiotropic role in ovarian physiology that is not limited only to the germ cell nests. In detail we added the following: "Neuropeptides and their receptors are also expressed in additional domains, including follicle cells, muscles, epithelium, and immune tissues. The insulin-like peptide 1 (ilp1) is found in follicle cells and muscles; lqn and gnRhR in muscles; prpp1 in the epithelium, follicle cells II and muscles; F-salmf and L-salmf in the epithelium; prrpR and lqR2 in immune cells; kissR1 and kissR8 in the epithelium; kissR3 in the epithelium, muscles and in follicle III; oxy/vpR in the epithelium and follicle II; salmfR1 and salmfR2 broadly in the epithelium with salmfR1 also expressed in follicle I, muscles and immune cells while salmfR2 in follicle II; and insR in the epithelium, follicle I, and immune cells. These data suggest a pleiotropic role of neuropeptidergic signalling in ovarian physiology (Fig. 7c)." (lines: 712-721)

Other points:

- in line 269: Reference to 1b is probably wrong.

This reference has been removed.

- In SFig.1: There is data from 3 different species in this figure and although this is clearly described in the legend, it would be even clearer if the different species were also indicated in the figure panel. Also, it is unclear if the 'surface<->lumen' applies to a-d or the whole figure.

This has been corrected in the figure.

- I. 340-343 & SFig. 3b-c: it would be helpful if the authors could clarify which one of the three sample in a-c refers to the three samples described in the text.

This has been clarified in the text (lines:445-447)

- I350-355 include long lists of genes whose relevance remain unclear until some of them are later described in the text. I think the authors could describe their relevance to identify respective cell types already here. Also, it remains unclear if some of these markers were previously published as cell type markers in sea stars or if all of them have only been described in sea urchins or chordates. In any case, the sources of this information should be cited. A table showing their expression in sea stars, sea urchin and a selection of other chordates could also be helpful for getting an overview of the markers and their conservation across species.

We have added a supplementary table (Supplementary Table 1) to detail the marker gene selections and their references. This is referenced in the legend for Figure 2, and in the text (lines: 453-455).

- In the same section, the 'immunocytes' need more explanation. On what basis do the authors think that these are immune cells? Have these been previously described in sea star? What is their function?

We added further explanation to this interpretation based on gene expression and prior morphological evidence (lines:460-464).

- In Fig.2a and throughout the paper: it is confusing to name clusters 'germ' and others 'oocytes' as oocytes are of course also germ cells. Please consider renaming 'germ' f.ex. to 'germ cysts'.

We have changed 'germ' to 'germ cell nests.'

- In Fig.2b (and any other dot plot): it would help to indicate which genes have been validated by HCR, and in which figure these can be found. Also, the authors should consider grouping & labelling the genes on the y-axis so that they can easier be found. F.ex. it took me some time to find the syt1 gene mentioned in I. 382-3.

We have added asterisks to the dotplots to indicate genes that have been HCR validated, and explain where the HCRs can be found in the figure legends.

- Also in Fig.2b (and any other dot plots): Gene annotations do not contain any gene identifier that would allow finding this gene in a gene bank. This is especially important for genes with identical annotations, such as in Fig. S9b, where >25 genes carry the same annotation.

We have added LOC identifiers, which allow the reader to search the gene on NCBI.

- I. 403-409: It is unclear if the genes mentioned in brackets are referring to the mammalian or sea star genes.

Genes mentioned in brackets refer to their sea star annotations, which generally correspond to the names in humans.

- Fig.3 (and any other Sankey plot figure): the representation of these plots is confusing. First, as sea star is always the reference dataset, it would make sense to always plot it on the left side. Also, it would help to keep the order of clusters identical between plots, and to colour-code the different clusters in groups (follicles cells, oocytes, epithelium...).

We have made these suggested changes to the Sankey plots.

- On a conceptual level, the authors seem to have only used ovary single-cell data from other species for comparison with their sea star dataset. Using that strategy, the authors will miss close relationship between cell types in the sea star ovary and cell types that are located in non-ovary tissue in vertebrates. This needs to be addressed.

Unfortunately, we are constrained by available single cell datasets and our bandwidth to perform the SAMap analysis, which is not trivial. We opted to compare our dataset to ovaries in several other species where robust data are available, as well as to human pituitary, and the sea urchin larva. The human pituitary is a non ovary tissue that we specifically selected to test the similarity of ovarian neurons to this vertebrate neuropeptide signaling center. The sea urchin larva was chosen as a null set to understand what background cell type similarity looks like and allow us to calibrate our interpretations. We feel that adding additional analysis would be very time consuming and not significantly add to our conclusions.

- Fig.3d: the terms next to the animals are too vague. F.ex., which muscles in flies or fish or mouse? And these are homologous to which muscles in sea star?

We have clarified in the text that these are all cells in ovarian datasets (lines: 520 and 522).

- Fig.4a and SFig. 5a: It remains what 'the average score of genes involved in G1/S, G2/M phases of the cell cycle' means. The comparison between 4a and S5a could also become clearer if the clusters and genes were always on the same axes, and if the genes in S5a were grouped in G1/S, G2/M and Meiosis. And as the category G2/M includes 'Meiosis', it remains unclear what the differences are.

We apologize for the confusion. We now define what average score is in the relevant methods section. We clarified in the text that G2/M is mitosis rather than meiosis and we have modified Fig.S5 so that the genes are grouped in the same way as in Fig4a.

- I.474-6; Fig. S7: The authors focus on the difference between the GO enrichment analysis without acknowledging that the top-3 GO categories are identical between cysts and oocytes.

We apologize for this. It is now acknowledged.

- In Fig.5: Although the authors claim the 'activation of oocyte GRN' (Fig.5I), only cysts-enriched genes are plotted or validated using HCR. The authors should also plot and HCR-validate some genes that become activated only in maturing oocytes.

*We have plotted and performed HCRs for *ddx4*, a gene that, based on this analysis, is enriched exclusively in the small oocytes. We have re-highlighted this in the paragraph associated with Fig. 5. Moreover, Supplementary data 4 contains all the genes that are up- and downregulated in the nests. Similarly, based on the nature of this comparison that compares germ cell nests versus the small oocytes as a reference group all the genes that are downregulated in the germ cell nests are the ones that are upregulated in the small oocytes and vice versa.*

*We agree that additional validation of these differentially expressed genes by HCR would be very valuable. To this end we performed HCRs for *cycA*, which according to our analysis is absent in the germ cell nests and upregulated in the oocytes. This was confirmed by the HCRs. We have added these experiments as additional panels in Fig. 5 accompanied by the relative featureplot of *cycA*. In total, in this figure we have two validations of genes enriched in the oocytes; *ddx4* and *cycA*. Addressing this more comprehensively would likely require its own manuscript and numerous HCRs, but we hope that this addition helps to add confidence in our work here.*

- I. 499: The selection of signalling pathway is poorly explained. Why those and not others (e.g. FGF, IGF, Hippo/Yap?)

*A sentence clarifying why these signaling pathways were chosen was added: "We first mined our atlas for gene groups corresponding to the Wnt, Tgf β , Delta/Notch, Hh, and retinoic acid (RA) signaling pathways, which are important in oocyte development in other organisms (51–54) and in oocyte polarity in *P. miniata* (55)." (lines: 622-625)*

- I. 565: The authors claim that F-Salmf is specifically expressed in neuron and germ cell groups, but the dot plot shows highest average expression in an epithelial cluster. Please comment.

We apologize for this. Indeed it was a typo. We meant L-Salmf and not F-Salmf. Moreover, in the results section we now mention in detail the predicted expression profiles of the neuropeptides/receptors analyzed. (lines: 712-721)

- Fig.7c could be clearer in grouping/highlighting neuropeptide/receptor pairs.

We understand that the initial grouping could make it hard for the reader to follow. Apart from the color-code to indicate ligand/receptor pairs we have also indicated those pairs using brackets.

- I.673: there is any comparative information missing for the claim that sea star follicle cell types are conserved to distinct follicle cell populations in protostomes.

We have added more information on the role of foxl2 in planaria as a representative protostome. Moreover we have added a sentence on our findings regarding the alignment of sea star and Drosophila follicle cell groups.

- I. 692-96: This hypothesis on the roles of RA, Wnt TGFb and Hh pathway in self-renewal-meitotic entry decision is overly speculative.

This is meant just as a hypothesis based on what is known in other organisms. To help make clear that this is speculation, we changed lines 849-852 to read: “Additionally, we speculate that the self-renewal-meiotic entry decision could be regulated by RA, Wnt, Tgfβ, and Hh pathways, whose respective receptors and intracellular components were found expressed in the germ cell nests, but testing this possibility will require future work.”

- I. 699-709: I suggest moving this information on HPG signalling to the introduction as it is key to understand the relevance of the different neuropeptides in the results part.

We thank the reviewer for this suggestion with which we agree. That section is now expanded and moved to the introduction.

Reviewer #2 (Remarks to the Author):

Major Comments/Suggestions:

1. Oogonial Stem Cells (OSCs)

While it is plausible that OSCs exist in sea stars given the annual production of large numbers of oocytes, the current dataset does not convincingly identify this cell type. If sea stars spawn once per year, OSCs might only be active for a brief period and thus constitute a rare population. The data shown depict probable oocyte progenitors in interconnected cysts—consistent with a stem cell-driven oocyte production model but not definitive proof of OSCs.

In vertebrates, nanos2 is a specific marker of OSCs. Do sea stars have a nanos2 ortholog, and is it expressed in the ovary? Please also clarify whether sea star nanos1 and nanos3 are orthologous to the vertebrate nanos1 and nanos3. Without more definitive markers or lineage tracing, the authors should refrain from asserting that OSCs have been identified.

*We appreciate this great point about the seasonality of the animals. Some sea star species, such as our local *Asterias forbesi*, are highly seasonal and only spawn once per year. *Patiria miniata*, however, is preferred in the laboratory because it undergoes oogenesis throughout the year and its ovary appears to be asynchronous. To identify the potential stem cells, we felt that EdU incorporation and proliferation were the best proxy for this. To address this potential ambiguity, we added the following to lines 570-571 of the manuscript: “ However, future work will be required to fully elucidate the seasonality, regulation, and lineage of the germ cells in the ovary”*

We also agree that another marker gene for OSCs would be great, and so we looked more closely at Nanos paralog expression. Sea stars conserve 3 Nanos paralogs (Nos1-

3), including ovarian specific-isoform (Nanos1), identified in a prior ovary de novo transcriptome (<https://doi.org/10.1371/journal.pone.0119627>), and two embryonically expressed paralogs (2 and 3) (<https://doi.org/10.1242/dev.155663>). Unfortunately, the orthology of Nanos orthologs is quite ambiguous. Nanos genes are short, rapidly evolving, and often only single exons in echinoderms. A recent analysis using 6 informatic tools was unable to identify 1:1 orthologs between sea star and human Nanos genes (<https://doi.org/10.1093/database/baab030>). We therefore focused on the ovary expressed isoforms (Nanos1, which is more highly expressed, and Nanos3) in our study. These data can be found in Fig. 1J and Fig. S3E. We did also attempt an HCR for Nanos2, but were unable to detect a signal, consistent with its lack of expression in echinoderm ovarian RNAseq datasets (<https://doi.org/10.1371/journal.pone.0119627>). The ambiguity regarding orthology is now mentioned in the text: “While sea stars conserve three nanos genes homologous to those in mammals and other organisms, their orthology and correspondence to their human counterparts is unclear (44).” (lines: 403-405).

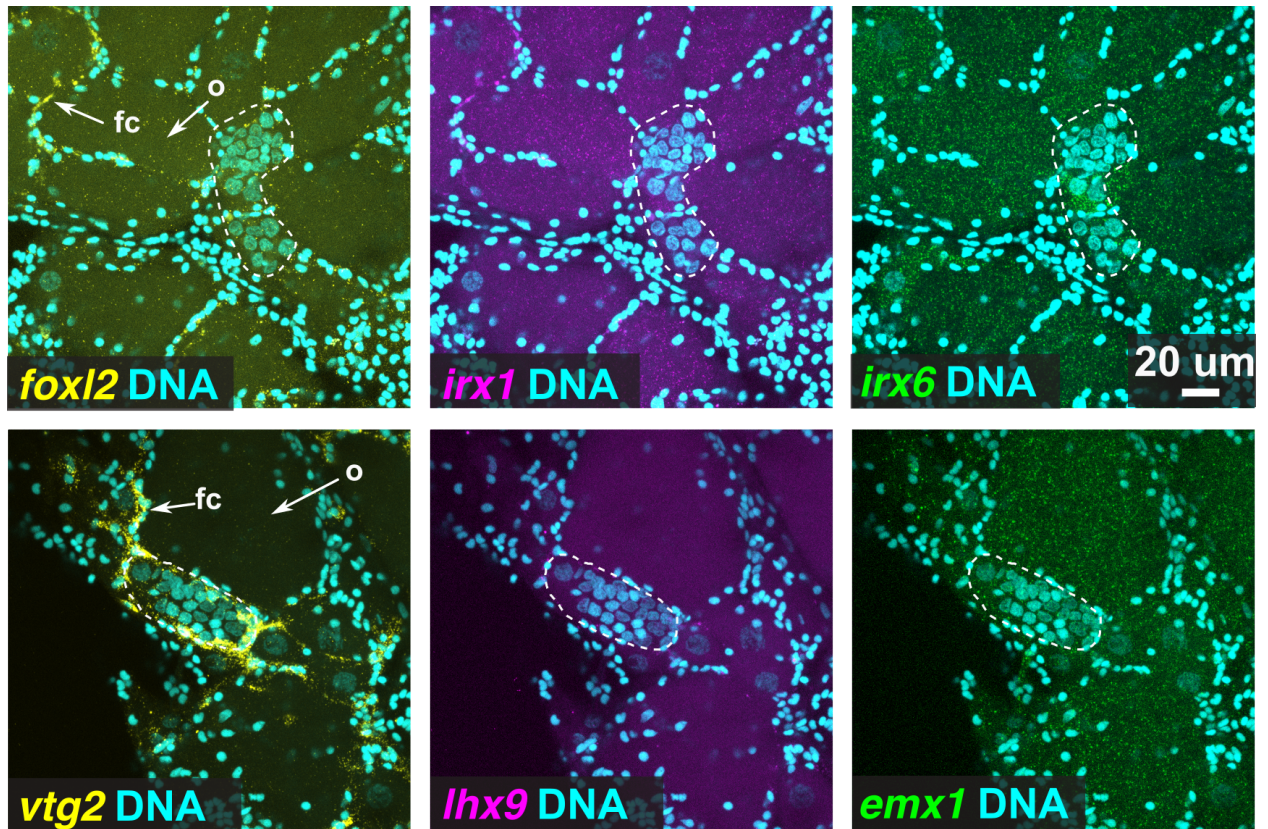
2. Follicle Cell Subtypes

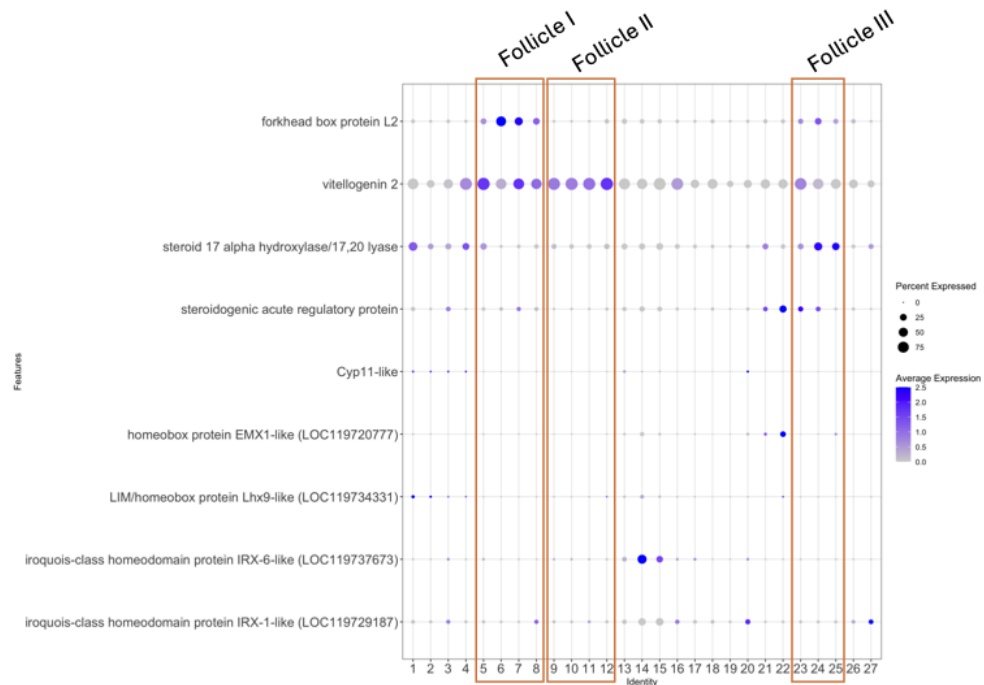
The identification of granulosa-like (follicle) cells is compelling, and the presence of subtypes is particularly interesting. In vertebrates, distinct populations of pre-follicle and granulosa cells support germ cells at different stages. However, it is unclear whether any of the sea star follicle subtypes correspond to these vertebrate populations.

Genes such as *irx3*, *irx5*, *lhx9*, *emx2*, and *wt1* are specifically expressed in pre-follicle cells in mammals and zebrafish. Do sea stars have orthologs of these genes, and are they expressed in a specific follicle subtype? If these markers are absent or not differentially expressed, can the authors speculate which population is most analogous to vertebrate pre-follicle cells?

We agree with the reviewer that identifying the pre-follicle cell population would be very valuable. Our cross-species comparison between the sea star and fly ovarian single cell datasets suggest that the Follicle I (and *foxl2* positive) clusters 6 and 7 align with the fly cluster *dm3* which contains pre-follicle cells. We also appreciate the suggestion of Iroquois homeobox factors, *lhx9*, *emx2*, and *wt1*. We found two *irx* family members: *Irxd1*, expressed in Follicle I, nests, oocytes, Neurons, and Immune cells in our dataset; and *Irxd6*, expressed in germ cell nests. These genes are all expressed at very low levels (see dotplot below), but we made an attempt at HCR for each of them. We find that *Irxd1* is primarily expressed in germ cell nests and early oocytes, and expressed at slightly lower levels in full grown oocytes, consistent with the scRNAseq data. *Irxd6* is expressed in nests and small oocytes, more weakly in large oocytes, and possibly at a very low level in some follicle cells, though the signal is challenging to interpret. *Lhx9* and *Emx1* are possibly expressed in a subset of *Vtg2* positive follicle cells toward the periphery of the inner ovary. Moreover, within the germ lineage *emx1* appears to be also expressed in large oocytes. We include the images here in the response document for the reviewer to see. However, the signals are weak and we are cautious about drawing conclusions and thus have opted not to include them in the revised manuscript. The unclear expression of these genes prevent us from drawing safe conclusions on the pre-follicle cells at this

moment. We might speculate that Cluster 6 may contain cells with a pregranulosa-like state, due to its high expression of FoxL2 but low expression of other markers, but we don't feel confident saying so in the manuscript. We have added this question to the discussion section: "An open question from our work is whether a pool of pre-follicle cells exists that corresponds to the pregranulosa in the mammalian ovary. Future investigation with potential marker genes and lineage analysis will help resolve this question. (lines: 792-795).



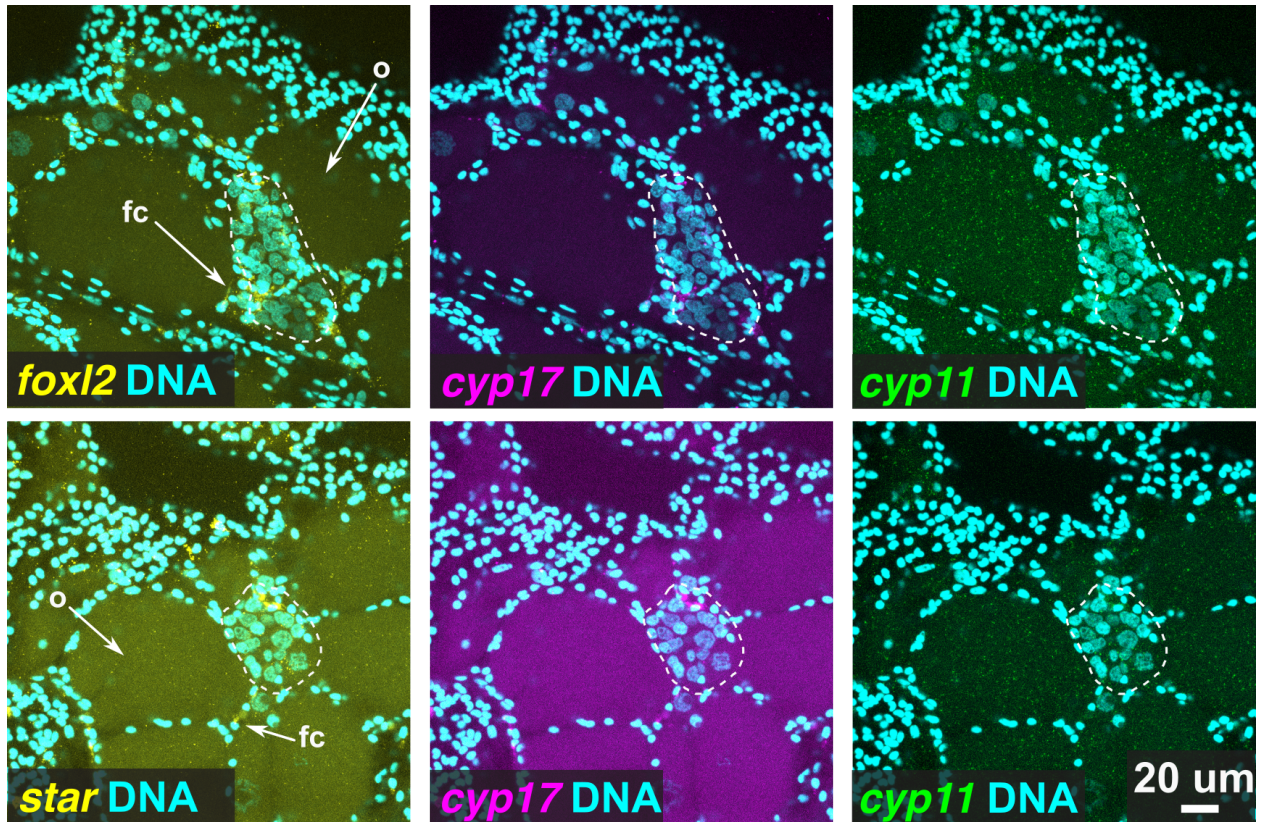


3. Theca-like Cells and Steroidogenesis

Vertebrate theca cells synthesize steroid hormones and provide precursors for estrogen biosynthesis by granulosa cells. The authors report that follicle population III expresses star and cyp17a1, genes also expressed in vertebrate theca cells. However, key steroidogenic genes such as cyp11a1 (cholesterol side-chain cleavage enzyme) appear absent. Are sea stars capable of producing steroid hormones? If so, which ones? Can the authors speculate on the steroid profile produced by theca-like cells?

To address this point, we added new paragraphs in the discussion summarizing what is known regarding steroid sex hormones in sea stars and echinoderms in general and discuss our data in that context (lines: 817-831). In summary previous studies have confirmed that echinoderms produce sex steroids, but their roles are not fully understood. Sea stars, sea urchins, and sea cucumbers can convert cholesterol into sex steroids, with estradiol detected in sea urchins and sea cucumbers. In sea cucumber progesterone and testosterone were also confirmed, along with steroidogenesis genes. Moreover, Estradiol and progesterone influence vitellogenin (vtg) and ovarian development in sea cucumbers, therefore have conserved roles in reproduction. Similarly in sea stars, estrogen treatments enhanced ovarian growth and protein content. Overall, data so far suggest that estrogens and progesterone regulate reproduction and nutrient cycles in sea stars and sea cucumbers.

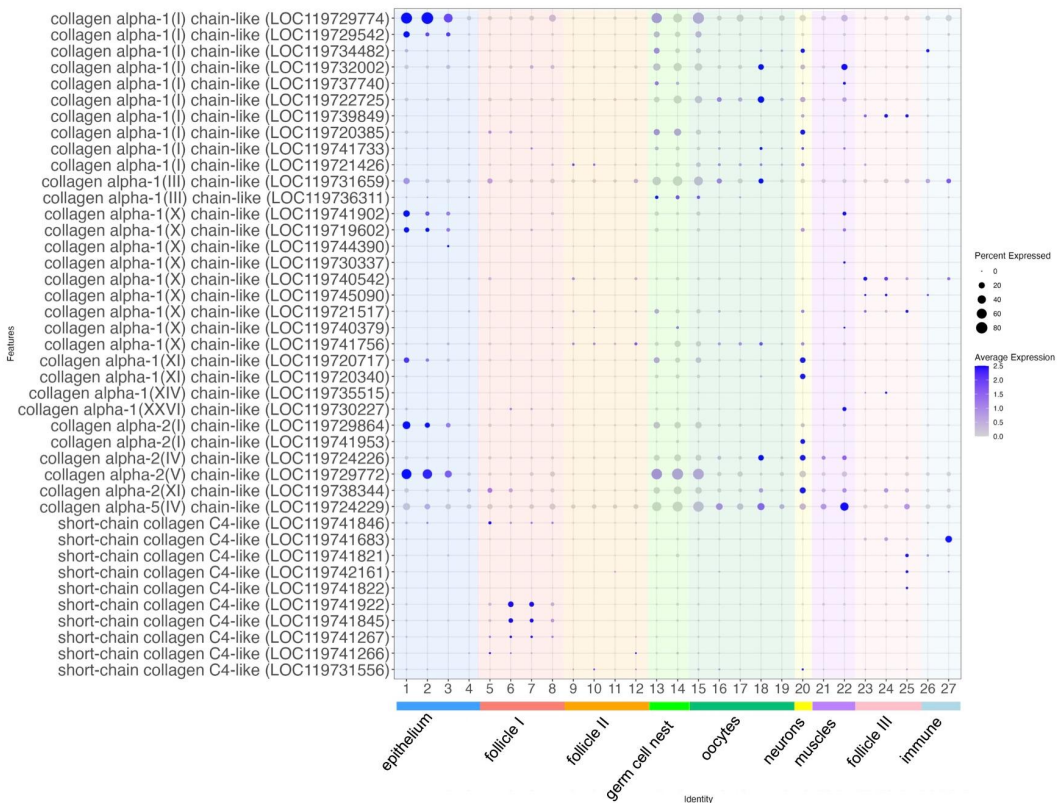
Sea stars do conserve the cyp11a1 gene (cyp11-like), in addition to cyp17a1, but it is not highly expressed in the ovary and its expression is restricted to epithelial clusters (see dotplot in response to the previous point). We also attempted HCR for cyp11a1, cyp17a1, and star, but were not able to detect strong signals (see below). We think this is because of their low expression levels. We do see weak signals for star and cyp17a1 in some follicle cells, but are hesitant to over-interpret these weak signals.



4. Stromal/Interstitial Cells

In addition to follicle and theca cells, stromal cells provide ovarian structural support. Muscle cells, for instance, may represent a specialized stromal subtype. Could follicle III cells be more closely related to vertebrate stromal cells or represent a common ancestral population? In vertebrates, markers like decorin (*dcn*) and various collagen genes mark stromal-like cells. Are similar genes expressed in sea star follicle III?

*Thank you for suggesting this interesting hypothesis. We looked into these potential stromal marker genes but could not find a decorin (*dcn*) ortholog in echinoderms. We also checked a collection of collagen-related genes (the dotplot is included below), and found that overall they tend to be most highly expressed in the epithelial and muscle clusters. A subset of cells in the Follicle I and III clusters also express some of these collagen-related genes. While we are not confident enough to add this to our paper, it does seem possible from these data that some cells in the Follicle clusters may be stromal-like.*



Minor Comments/Suggestions

1. Data Sharing

in addition to depositing the data resources on GitHub, I encourage the authors to also consider depositing it on a portal, like the Broad Institute's Single Cell Portal, that allows easy browsing of the data.

We thank the reviewer for directing us toward the Broad Institute's Single Cell Portal. We have added our study here and hope this increases the accessibility of the data. Genes are searchable by Loc ID, and a table of Loc IDs and their NCBI gene name annotations is provided through a link on the portal in the Data Availability statement.

2. Line 318

Define "GVBD" at first mention for clarity.

We apologize for this. It is now defined at the first mention.

3. Figure S3

In the legend or main text, specify which UMAPs in panel S3d correspond to ovaries from different reproductive stages (recently spawned, early cyst-enriched, mature oocyte-rich).

Thank you for spotting this. We specified within the text which library corresponds to each reproductive stage.

4. Line 355

Orthologs of *Drosophila piwi1* are typically annotated as *piwil1*. Please revise accordingly.

*We revised the *piwil1* naming in the text and the figures.*

5. Line 402 / Figure 3

Figure 3 is difficult to interpret without a more detailed legend. Please clarify which datasets and cell types are being compared. Consider adding labeled UMAPs corresponding to each dataset, with cluster numbers that match those in Figure 3.

We have added the citations in the figure legend for the published scRNAseq datasets used as comparisons. We prefer not to include the UMAP plots from each study here, because we feel that would complicate the figure and would require permission from each journal to reproduce the plots from these studies. The figure legend has been modified to make the interpretation easier.

6. Line 449 / EdU Incorporation

If EdU+ cells are within a germline cyst, all connected cells should exhibit synchronized DNA synthesis and incorporate EdU. However, only a subset appears labeled. Can the authors explain this observation?

This is a very interesting question. One possibility is that only some cells within the nests are transiently interconnected, and that they stochastically fragment, as recently observed in mice (doi: 10.1016/j.cub.2024.10.062). Another possibility is that Cdks and other cell cycle regulators do not transit between these connections. A third option we must consider, especially in light of our new expansion microscopy data in response to Reviewer #1, is that these cells are not interconnected at all. To this point, we have changed our language throughout the manuscript to call these structures 'nests.' Our goal will be to resolve this more fully in future work.

Reviewer: Bruce Draper

Reviewer #3 (Remarks to the Author):

The methods are appropriate, and the results are convincing and clear. While the cellular and transcriptomic characterization of the sea star ovary provides a valuable dataset, the reviewer has significant concerns regarding the interpretation of these findings in the Discussion and Conclusion sections. The evolutionary and neuroendocrinological implications proposed by the authors relied heavily on selectively interpreted evidence and speculative parallels to vertebrate systems. In their current form, these claims risk misrepresenting the biological significance of the data and may lead to serious misconceptions across multiple disciplines, including evolutionary biology and neuroendocrinology.

We thank the reviewer for highlighting this. We have adjusted our language throughout the manuscript to ensure that the biological significance is not misinterpreted. Moreover we have added a paragraph in the discussion clarifying the evolutionary scenarios we propose as well as highlighting that the presence of sea star ovarian neurons is a mechanistic novelty. In detail we added the following:

“This system may be received by germline cells, representing a mechanistic novelty in echinoderms that is distinct from the vertebrate reproductive axis. However, core components of the vertebrate HPG axis, other than FSH and LH which are absent in invertebrates (102), may have been retained in echinoderms, but operate locally within the gonad. In such a scenario, ovarian neurons could be functionally analogous to distinct elements of the mammalian HPG axis. While in mammals the HPG axis flows from the central nervous system, we propose that in sea stars, in addition to regulation by the radial nerves, local neuropeptidergic signaling from ovarian neurons may act directly on germ cells and developing oocytes. In this case, the absence of hypothalamus and pituitary gland is substituted by ovarian neurons. However, further studies are necessary to explore the function of the sea star ovarian neurons, and the directionality of the signaling across cell types.

In summary, we speculate that during animal evolution, the regulation of oogenesis may have initially relied on intrinsic neuroendocrine systems within the gonad itself. As organisms became more complex, this regulatory role may have been gradually co-opted by specialized structures, including centralized nervous systems and endocrine glands.” (lines: 877-892)

1. The authors refer to echinoderms as “pre-chordates,” which is a phylogenetically inaccurate and misleading designation. Echinoderms and chordates are both deuterostomes that diverged from a common ancestor; chordates did not evolve directly from echinoderms. This fundamental error undermines the evolutionary framing of the study and should be corrected. Moreover, the complete absence of discussion regarding hemichordates—an intermediate and highly relevant group—further suggests a lack of evolutionary rigor in the manuscript's phylogenetic interpretation.

Thank you for this important point - we have adjusted our language throughout the manuscript to be more precise with respect to the evolutionary relationships between echinoderms, hemichordates, and deuterostomes. To this end the term pre-chordates was replaced with terms such as non-chordate deuterostome or early deuterostome. Unfortunately, we are unaware of any detailed cellular, molecular, or scRNAseq studies that provide comparative data on the hemichordate ovary. However, we agree that such data will be essential in the future to better resolve the evolutionary histories of ovarian cell types. We have added several lines of text to reflect this point. In detail, we introduced hemichordates as the sister group to echinoderms in the introduction and we adapted our hypothesis of the origin of ovarian cell types in the context of chordate and ambulacraria divergence. Lastly we stressed that future studies involving hemichordates are needed to further investigate the cellular composition and the evolutionary origins of the cell types constituting the gonad.

2. In keeping with the above, while the inclusion of *Drosophila* may highlight a few conserved transcriptional programs, comparisons with protostomes offer limited relevance to understanding the evolutionary origins of reproductive systems in chordates or deuterostomes more broadly. The manuscript currently overstates the evolutionary significance of these comparisons without adequately framing their phylogenetic distance and limitations.

*Thank you for this point. We have broadened our discussion of available protostome data beyond *Drosophila*, which we feel gives better context for our interpretation of deuterostome evolution. For instance in the discussion, we have added several sentences highlighting the expression of a FoxL2 homolog in somatic, possibly follicular cells in the planarian ovary. This further suggests potential conservation between ovarian somatic cells between protostomes and deuterostomes. From a morphological perspective, others have noted striking similarities between follicle cells across distantly related species (see <https://onlinelibrary.wiley.com/doi/10.1002/mrd.23645>). We agree that more protostome data will be needed to further resolve the evolutionary history of these cell types, and now state this in the manuscript. With respect to the germ line, several authors have recently highlighted numerous similarities between ovarian germ cell cysts in protostomes (including *Drosophila*, annelids, and flatworms) and deuterostomes (for instance, see <https://doi.org/10.7554/eLife.83230> and <https://doi.org/10.1007/s00441-009-0788-8>).*

3. The authors propose that echinoderm ovarian neurons are analogous to elements of the mammalian HPG axis, and speculate that intrinsic neuroendocrine systems may represent an ancestral mode of reproductive control later taken over by centralized neuroendocrine organs. However, this hypothesis, as stated on page 33 (lines 726–731), overextends the data and risks misleading readers. Crucially, the vertebrate HPG axis is centered on pituitary-derived gonadotropins (FSH and LH), which have no homologs in invertebrates. Similarly, sex steroid hormones such as estrogens and androgens—along with their feedback roles—are essential in vertebrate reproduction but remain unidentified in echinoderms, both structurally and functionally. While the presence of genes like *cyp17a1* is interesting, its biological significance cannot be inferred without evidence of active steroidogenesis or corresponding hormonal pathways. The evolutionary argument presented therefore lacks molecular and physiological grounding.

Thank you for this point. We have now expanded our discussion of steroid hormones in echinoderms, also in response to Reviewer #2 (lines: 817-831), stated that LH and FSH are not conserved in echinoderms (lines: 879-881), and softened our conclusions overall. In summary, previous studies have confirmed that echinoderms produce sex steroids, but their roles are not fully understood. Sea stars, sea urchins, and sea cucumbers can convert cholesterol into sex steroids, with estradiol detected in sea urchins and sea cucumbers. In sea cucumber progesterone and testosterone were also confirmed, along with steroidogenesis genes. Moreover, Estradiol and progesterone influence vitellogenin (vtg) and ovarian development in sea cucumbers, therefore have conserved roles in reproduction. Similarly in sea stars, estrogen treatments enhanced

ovarian growth and protein content. Overall, data so far suggest that estrogens and progesterone regulate reproduction and nutrient cycles in sea stars and sea cucumbers.

4. In sea stars, oocyte maturation and ovulation are well known to be regulated by the relaxin-like gonad-stimulating peptide (RGP) via induction of 1-methyladenine (1-MeAde) synthesis, as clearly documented in the literature (e.g., Mita, Biomolecules, 2023). Moreover, RGP receptors have been identified in *Patiria pectinifera* (Mita et al., PLoS One, 2020; Feng et al., BMC Biol., 2025). Despite the central role of the RGP–1-MeAde axis in echinoderm reproduction, this study makes no mention of these molecules in either the results or discussion. This omission significantly weakens the physiological context and interpretive relevance of the findings.

Conversely, the authors focus on vertebrate-type peptides such as kisspeptin and GnRH (or their corazonin-related homologs), yet there is currently no experimental evidence that these neuropeptides are involved in oocyte maturation or ovulation in invertebrates. For example, in the ascidian *Ciona*, a basal chordate, recent studies have shown that tachykinin is involved in early follicular growth, vasopressin in oocyte maturation, and vasopressin and cholecystokinin in ovulation (Satake and Sasakura, Mol Cell Endocrinol, 2024).

A meaningful comparative analysis should take into account such lineage-specific reproductive control mechanisms across invertebrate deuterostomes. Furthermore, in the vertebrate HPG axis, kisspeptin and GnRH do not act directly on follicular cells; rather, they regulate gonadotropin release from the pituitary. Thus, the detection of kisspeptin or GnRH-related ligands and receptors in the sea star ovary does not, in itself, justify analogies to HPG function. If anything, the findings reported in this study underscore mechanistic differences between echinoderm and vertebrate reproductive regulation, rather than similarities.

We thank the reviewer for this. We do agree that these were important findings and we regret not discussing prior work on relaxin-like peptide and 1-methyladenine. In the revised version we added the missing information regarding the role of rgpp in oocyte maturation through the stimulation of follicle cells to produce 1-methyladenine in the discussion. We also explored the expression of the ligand and the receptor in our dataset. This allowed us to describe the spatiotemporal profiles of rgpp and its receptor at a single cell resolution in similar cell types as previously reported in another sea star species. These results have been added to Figure S9b. Performing this analysis we found that out of all the follicle cell clusters present in our dataset, only one that corresponds to theca-like cells is expressing the receptor, suggesting that this is the follicle cell type that produces 1-methyladenine. We have adapted our discussion accordingly to include these findings. Moreover we have modified the introduction and discussion to include information on the neuroendocrine control of reproduction in other invertebrates. Regarding the point raised concerning the analogies with the HPG, and in line with the request of reviewer 2, we have significantly modified the discussion,

adapted our language to avoid misinterpretation and highlighted that the presence of such a system represents a mechanistic novelty between echinoderms and vertebrates.

Response to Reviewers round 2

Reviewer #1

Detailed explanations on major issues:

Cell type comparisons

I thank the authors for using a consistent colour scheme and plotting order for the SAMPlot analysis. I had hoped that it would facilitate the interpretation of these plots, but in contrast, it increased my doubts about the interpretation of this data. I have two major concerns in their interpretation:

1) The results are described in a very shallow manner and seem biased to highlight similarities that suggest evolutionary conservation, while ignoring similarities between clusters that do not fit a scenario of evolutionary conservation.

For example, the authors write this simple and vague summary of description of the follicle cell similarities in the results part: 'We also identified similarities between the three sea star follicle cell groups and their counterparts in fruit fly and sea urchin'

In reality, the situation is much more complex:

I would expect that the sea star-sea urchin comparison works as 'positive control' for the whole analysis, but the authors give it similar (or even less) weight than flies or mammals (or even less). Looking more in detail to this comparison, I was worried by the many sea star clusters that do not have 'fitting' corresponding clusters in the sea urchin dataset. F.ex. Follicle II clusters 9-12 are linked to 'oocytes_sp0 cluster of sea urchins (and to oogonia in zebrafish). And the follicle III clusters 23-25 seem to be linked to a large variety of different sea urchin clusters including epithelium and oocytes clusters. In fish, these clusters have strong links to immune cells, refuting a conservation of these follicle cell types between sea star and fish. Comparisons to flies seem dominated by links of nearly all clusters to 'border stretch hemocytes', which is not explained anywhere in the manuscript. The follicle cell I clusters 5-8 seems to be the only ones consistently linked to follicle cells in sea urchin, fish and flies.

We disagree with the premise that ovarian cell types in sea stars and sea urchins should exhibit clear 1:1 correspondence. These lineages diverged ~500 million years ago, making strict cell-type homology, at the level of clusters, gene expression, or cis-regulatory architecture neither expected nor supported by existing evidence. Indeed, even among much more closely related taxa, one-to-one correspondence of cell types is rarely observed. The sea star-sea urchin comparison is by no means a positive control; it is the exploration of cell type repertoires in animals both belonging to a diverse phylum (echinoderms) that share reproductive strategies, but still have completely different physiological features. However, despite their long evolutionary distance, the sea star-sea urchin comparison yields the highest number of ovarian clusters and similarities among all cross-species analyses performed, underscoring that it remains the most informative available reference, building support to the rest of the comparisons. In interpreting these results, one must also consider technical differences between datasets, including distinct

laboratories, protocols, sequencing depth, and cell numbers, as well as the comparatively lower coverage of the available sea urchin atlas, which may obscure or entirely miss relevant cell states. In summary, the absence of strict 1:1 correspondence does not undermine the validity of the comparison, nor does it call into question broader cross-species inferences; rather, it reflects expected evolutionary and technical constraints inherent to such analyses.

2) Even for follicle cell I, it is unclear which genes drive their similarities, is it transcription factors/signalling genes or effector genes? And could the similarities be resulting from evolutionary convergence? As the function of follicle cells is mainly to provide oocytes with nutrients and protection, they could have recruited similar genes in an independent manner to execute this function.

We agree that an important open question is whether the observed similarities are driven by shared transcriptional regulators, signaling pathways, or downstream effector genes, and whether they reflect evolutionary convergence. SAMap cannot discern between these possibilities, as it just tests gene expression similarity across an unbiased set of genes. Indeed, this is an interesting question that we would like to investigate in follow-up manuscripts. In this study, however, our evolutionary focus is on comparing ovarian cell-type repertoires across animals using SAMap, rather than on resolving the underlying regulatory or mechanistic basis of these similarities. As such, we interpret the observed transcriptional similarities at the level of cell-type correspondence, without inferring whether they arise from shared regulatory programs or independent recruitment of functionally similar genes. We added the following to lines 566-568: “Alternatively, these cell state similarities could arise through convergent evolutionary processes and broader species sampling is therefore required to resolve these possibilities.”

Insufficient presentation and experimental design of inhibitor experiment:

I. 656: ‘with the specific Tgfβ inhibitor LDN-193189’

- Reference missing

- The authors ignore the complexity of ‘Tgfβ signalling’, which can occur via Nodal-, Tgfβ, Activin- or BMP- receptors, and intracellular via Smad1/5/8 or Smad2/3 signalling. The inhibitor used by authors has been described as specific for ALK2/3 (Activin receptors) in vertebrates, which signals specifically via Smad2/3. All of these details are ignored by the authors.

- The authors show that alk2, a putative target of the LDN-193189 inhibitor, is expressed in oocytes and germ cell nests, and it remains obscure how that fits with the phenotype of disorganised transzonal projections.

- All of these issues disqualify the conclusion that ‘the deep evolutionary conservation and functional role of Tgfβ signaling pathway components within the ovarian follicle.’ (I. 568-60).

The missing reference regarding the specificity of LDN-193189 was added. We now include additional validation for the tgfb inhibitor LDN-193189. This inhibitor was originally selected because it targets a range of Alk receptors, including Alk 2, which we find expressed in the oocyte lineage. We then tested whether this inhibitor resulted in a characteristic Bmp/Nodal inhibition phenotype in embryos (“radialization”), which has been extensively described in response to disrupting dorsal-ventral and left-right axis specification (Lapraz F, Besnardeau L, Lepage T. Patterning of the dorsal-ventral axis in echinoderms: insights into the evolution of the BMP-chordin signaling network. PLoS Biol. 2009, Luo YJ, Su YH. Opposing nodal and BMP signals regulate left-right asymmetry in the sea urchin larva. PLoS Biol. 2012;10(10):e1001402. doi: 10.1371/journal.pbio.1001402). We found that LDN reproducibly engenders this phenotype. In addition, we tested whether downstream tgfb signaling is disrupted with LDN treatment by staining for phosphorylated smad1/5/8. We found that LDN treatment strongly reduced detectable pSmad signal, which we quantified by nuclear segmentation and pixel intensity measurements. We now include these data in Figure S8. We appreciate the point that tgfb signaling is complex and could involve a range of ligands and receptors. However, systematically testing these genes by knockout would require extensive time and effort that we feel is well beyond the scope of this paper.

No evidence for oogonial stem cells

I.: 568-70: ‘These data identified replicating cells within the sea star ovary (Fig. 4f) and support our hypothesis of a self-renewing oogonial stem cell pool in adult sea star ovaries.’: This statement is not supported by the data as the authors show no evidence for self-renewal, and all of the EdU+ could just as well represent determined, mitotic oogonia cells. As OSC would need to be uncommitted by definition, the concern is further supported by the authors’ own statement that ‘Cluster 13 was chosen as the root because its molecular signature and cellular morphology was most similar to uncommitted germ cells’ (l. 582ff).

We agree with this point and have toned down our language and modified the legend of Figure 4. We also added the following in discussion to mention what the edu positive oogonia could mean. “The unexpected presence of mitotically active oogonia in an adult female is indicative of persistence or reactivation of pre-meiotic germ cells and thus, may imply either altered germ cell differentiation or activity of oogonial stem cells. Future work will be needed to definitively map the germ lineage from stem cell to oocyte.”

Minor issues:

I. 79: ‘..explored their evolutionary relationships with other animals’ -> ‘..relationship with ovary cell types of other animals’

It is now fixed.

I. 83: ‘we report gene expression conservation of signaling pathways involved in oogenesis’: what does that mean?

The sentence was modified accordingly for clarity

Fig.1a: What is the relevance of the ‘average lifespan’? I do not see its relevance and is obviously not correlated to presence/absence of adult oogenesis. I would also recommend to add species names, so that it is clearer that the info for Drosophila is not representative of the entire protostome taxon.

Average lifespan of key experimental systems in reproductive biology is reported to showcase the unique case of echinoderms that are both long-lived and capable of adult oogenesis. The following sentence was added to Figure 1 legend to explain the relevance of the average lifespan. “Average lifespans of the depicted taxa are shown to highlight the rare case of long-lived animals (echinoderms) capable of adult oogenesis.” It is not our intention to claim that each depicted animal is representative of an entire taxon; however, because the depictions correspond to multiple species or even genera, we prefer not to add species names, as this could be misleading or add unnecessary complexity to an introductory figure panel.

I.370-71: Synaptotagmin-1 staining is not visible in Fig. 1d

It is now fixed.

I. 380: ‘average size of 8-16 μm ’: I do not understand what that means, as a range is not an average. Also, ‘size’ is imprecise, I assume the authors mean diameter? These numbers are also confusing as on the graph in S2a, there are values beyond these range, especially $>16\mu\text{m}$. I suggest reporting a mean $\pm$ s.e.m. and indicating the mean on the graph.

We now use both diameter range and diameter mean $\pm$ SD to refer to the various size groups. We chose this instead of SEM as we think communicating information about the distribution of cell sizes (range and standard deviation in addition to mean) is more relevant to understanding these cell size data than the confidence in the measurement of the mean, which is what SEM shows. The relevant text, panels Fig.1m and Fig.S2a-f, figure legends and supplementary data file 2 have been modified accordingly.

I. 381: (Fig. 1g, i)-> (Fig. 1g, h).

The figure reference is correct and intended, therefore no change was made.

I. 385: Phalloidin does not label all actin, but only F-actin, so please change ‘actin’->‘F-actin’ throughout the manuscript.

We prefer not to make this change. While phalloidin selectively binds filamentous actin, the manuscript uses the term “actin” deliberately to describe the actin cytoskeleton as a structural and functional system, rather than exclusively its polymerized state. Moreover, we have clearly stated that using phalloidin we stained for F-actin within the manuscript. We believe that the current terminology is consistent with common usage in the field.

I. 403: 'While sea stars conserve three nanos genes homologous to those in mammals and other organisms': synthax, gene nomenclature (->italic)

It is now fixed.

I. 410: 'We found that the smallest nanos1⁺ oocytes are located next to germ cell nests and are within the range of 17-42 µm in diameter': how can 'the smallest nanos1⁺ oocytes' have such a broad range?

This was addressed in the previous comment as we now report size as diameter mean ± SD. Regarding the broad size range of the smallest nanos1⁺ oocytes, we believe this reflects natural biological variability during oogenesis. Importantly, despite this variation, this oocyte group remains consistently smaller than other nanos1⁺ populations, supporting its classification as the smallest nanos1⁺ group. Nevertheless, the sentence has been modified for clarity. It now reads:

*We found that the young oocytes adjacent to the nests range from 16–42 µm in diameter (**Fig. S2c–d**), with a mean diameter of 29.0 ± 7.1. This population represents the smallest group of young oocytes, constituting primary follicles that are positive for nanos1⁺.*

I. 421-3: how are pre-GVBD defined? And in Fig. 1m, the authors propose three stages of oocytes, but a clear description of how these stages are distinguished and/or defined is missing.

We apologize for this. The sentence was modified accordingly for clarity.

Fig. 2a/S3: why is there a different colour code for the same clusters between these figures?

The difference in the color-code is described in the figure legends and the manuscript. In Figure 2a the color-code corresponds to cell type groups, while in Figure S3 color is indicative of different clusters. Therefore, the usage of the color-code is explained and intended.

Fig. 2i needs annotations

We believe the description within the main manuscript and the figure legend are sufficient for the reader to understand panel 2i. Therefore, we chose not to add additional annotations that would make that panel and the figure in general too busy and the data harder to interpret.

Reviewer #2

The authors have adequately addressed my previous comments/suggestions.

Reviewer #3 (Remarks to the Author):

The authors have substantially improved the manuscript in response to my previous comments regarding the RGP–1-MeAde axis and the broader neuroendocrine context of echinoderm reproduction.

The reviewer appreciate that the revised Discussion now clearly acknowledges the established role of the relaxin-like gonad-stimulating peptide (RGP/rgpp) and 1-methyladenine (1-MeAde) in sea star oocyte maturation, and places the new data within this well-characterized physiological framework. The additional analysis of rgpp and rgppR expression in the single-cell dataset, together with the spatiotemporal mapping presented in Figure S9b, is particularly valuable. The finding that rgpp is predominantly expressed in epithelium, oocytes, and muscles, whereas rgppR is restricted to specific epithelial, neuronal, and follicle III (theca-like) clusters, provides an important cellular context for the canonical RGP–1-MeAde pathway.

In particular, the observation that only one follicle cell cluster, corresponding to theca-like follicle III cells, expresses rgppR is a very interesting result. This strongly suggests that this follicle cell subtype is a prime candidate for the 1-MeAde–producing population in *Patiria miniata*, and it nicely integrates classical physiological work with the new single-cell atlas. I would, however, encourage the authors to phrase this conclusion in slightly more explicitly tentative terms (e.g., “strongly suggests” or “is a likely candidate for the follicle cell type that produces 1-methyladenine”), to make clear that the current evidence is based on receptor expression patterns rather than direct measurement of 1-MeAde synthesis in defined cell types.

We appreciate the reviewer for their positive feedback and we thank them for their suggestion. That part now reads: “Notably, only cluster 25, belonging to the follicle III cell type group (theca-like cells), expressed the Relaxin-like Gonad-Stimulating Peptide receptor (rgppR) (Fig. S9b). This strongly suggests that this population of theca-like cells is a likely candidate for the follicle cell type that produces 1-methyladenine and highlights the subfunctionalization of diverse follicle cell types.”

More broadly, the expanded Discussion on neuroendocrine control in invertebrates (including ascidians and other lineages) now provides an appropriate comparative framework for interpreting the presence of GnRH-, kisspeptin-, vasopressin/oxytocin-, and PrRP-like signaling systems in the sea star ovary. The language describing a “hypothalamic-like and gonadotropic-like intrinsic ovarian neuroendocrine signaling system” is now much more clearly framed as a hypothesis and as a mechanistic novelty that is distinct from the vertebrate HPG axis. For complete clarity, I would only suggest slightly softening the sentence stating that “the absence of hypothalamus and pituitary gland is substituted by ovarian neurons,” for example by rephrasing as “may be partially substituted” or “could be functionally compensated, at least in part, by ovarian neurons,” to emphasize the speculative nature of this proposal.

Again, we thank the reviewer for this suggestion. The sentence now reads: "In this case, the absence of hypothalamus and pituitary gland could be functionally compensated, at least in part, by ovarian neurons."

Response to Reviewers round 3

Reviewer #1 (Remarks to the Author):

I sincerely thank the authors for their detailed response, clarifications and for performing additional experiments to validate the use of the chemical inhibitor LDN-193189. These efforts address several of the previously raised concerns. However, some of my key points remain insufficiently addressed and require revision.

Cell type comparisons

I thank the authors for their clarifications regarding my concerns about the SAMap analysis. I agree that a strict 1:1 correspondence between sea star and sea urchin datasets is neither expected nor necessary, given both evolutionary distance and technical variability.

However, my concern was not based on an expectation of strict correspondence, but rather on how the results are described and interpreted. This issue remains central, as it underpins a key claim of the manuscript —namely, the proposed early deuterostome origin of ovarian cell types. The current presentation appears selective, emphasizing similarities that support evolutionary conservation while not adequately addressing patterns that are inconsistent with this interpretation. I must therefore insist that this point is addressed more rigorously.

While I agree with the authors that the sea star–sea urchin comparison represents the most informative available reference, it is currently absent from the revised Results and Discussion of the manuscript. This comparison should be incorporated as a central component of both the Results and Discussion.

We appreciate the reviewer's point. Therefore, we have revised this section of the Results to clarify this aspect. The revised text now reads as follows: "Overall, our analysis not only reveals a high degree of transcriptional similarity among ovarian cell types across evolutionarily distant echinoderms, yielding the highest number of shared ovarian clusters among all cross-species analyses, but also identifies conserved ovarian cell type programs across phylogenetically distant organisms. These findings suggest that the origins of several of these cell types, including follicle cells, may predate the divergence of chordates and Ambulacraria, which includes echinoderms and hemichordates." We would prefer not to further emphasize these results, as doing so would dilute the focus of our manuscript on ovarian cell type evolution in bilaterians by shifting it toward cell type evolution within echinoderms.

In particular, the manuscript should address the following points:

- **The claim that follicle_II cluster cells are 'echinoderm-specific', suggesting conservation across echinoderms (l. 831). As previously noted, there is a strong link between the sea star follicle_II cluster to a sea urchin oocyte cluster. This observation suggests a lack of conservation across echinoderms and should be explicitly discussed.**

We appreciate the reviewer's comment. We have added a paragraph describing in great detail the alignment of the sea star follicle cell II clusters with both follicle and non-follicle clusters across the ovarian atlases analyzed. Based on the ubiquitous alignment of this cell type group and its clear follicle cell identity, supported by the high expression of the two sea star vtg gene orthologs, we had previously defined it as echinoderm-specific. However, we have now rephrased this to reflect that it is a cell type group with no clear equivalent outside sea stars and that, based on the high expression of vitellogenin gene orthologs, it likely represents cell populations dedicated to vitellogenesis.

- **The interpretation of sea star follicle_III clusters as expressing genes 'typically found in ... theca cells in mammals' (l. 832), leading to the conclusion that 'our results also suggest possible evolutionary origins for theca and granulosa cells that predate the divergence of Ambulacraria and chordates' (l. 835–837). This conclusion relies primarily on comparisons to mammals and is not supported by comparisons within echinoderms or with other non-mammalian taxa.**

In the sea urchin-sea star comparison, follicle_III clusters show the strongest links to epithelial and oocyte clusters. This substantially weakens the claim of an pre-chordate origin of theca cells, yet this discrepancy is not discussed. Similarly, in zebrafish, theca cells map to sea star epithelial cells (l. 565–566), while, as noted in my previous review, follicle_III cells show strong links with immune cell clusters. Taken together, these comparisons with both sea urchins and zebrafish contradict the conclusion that theca cell types predate the Ambulacraria–chordate divergence.

In summary, the Results and Discussion of the SAMap analysis should be revised to provide a balanced and comprehensive account of all relevant mappings, including those that do not support evolutionary conservation. While the evidence supporting conservation of follicle_I/granulosa-like cells (e.g., foxL2, gata4, and broadly consistent SAMPlot support) appears convincing, the current data—particularly the absence of key transcription factor signatures and the inconsistency of SAMap

mappings—do not, in my view, support equivalent claims for theca cell type conservation.

*We appreciate this feedback. We have toned down our language regarding the evolutionary origins of theca cells and clarified the evidence supporting our hypothesis. Similar to our approach for the follicle cluster II group, we have provided detailed information on the mapping of the follicle cells III group against multiple clusters in our cross-species comparisons. We now also clearly state that, despite the lack of SAMap alignment between this group and mammalian theca cells, these clusters remain enriched for genes associated with mammalian theca cells. Therefore, we suggest that this cell type group may represent a **diverged**, sea star **theca-like** cell type.*

The following two sections were added in Results and Discussion respectively to address these issues.

(Results)

According to our cross-species comparisons, sea star follicle cell II and III clusters exhibit puzzling and heterogeneous alignments across species. Follicle cell II clusters align with follicle and oocyte populations in sea urchin and with oocyte populations in zebrafish; with granulosa populations in humans; with follicle-associated and hemocyte populations in Drosophila; and with stromal, epithelial, and granulosa populations in mice. In contrast, follicle cell III clusters align with follicle and epithelial populations, as well as an uncharacterized population (cluster 12) and oocytes, in sea urchin; with immune populations in zebrafish; with granulosa and immune populations in humans; with follicle, follicle-related, and hemocyte populations in Drosophila; and with stromal and immune populations in mice. Taken together, these alignment patterns preclude a confident definition of their identities.

(Discussion)

Here we provide evidence for three distinct sea star follicle cell groups. One group (follicle I) is transcriptionally similar to follicle cells in zebrafish and to granulosa cells in mice and humans, while expressing genes characteristic of granulosa cells. The second group (follicle II) has a poorly defined identity, computationally aligning with a range of follicle cell types as

well as non-follicle types across species, with no clear equivalent outside sea stars. Based on the high expression of vtg genes, we hypothesize that it reflects cell populations dedicated to vitellogenesis. Lastly, the third group (follicle III) also shows ambiguous cross-species similarity, yet expresses genes typically associated with mammalian theca cells; therefore, we speculate that it reflects a possibly diverged sea star theca-like cell type. An open question from our work is whether a pool of pre-follicle cells exists that corresponds to the pregranulosa in the mammalian ovary. Future investigation with potential marker genes and lineage analysis will help resolve this question. Surprisingly, our results also suggest possible evolutionary origins for granulosa and possibly theca cells that predate the divergence of Ambulacraria and chordates.

In addition, I previously noted that the sea urchin–fly comparison appears dominated by links to 'border stretch hemocytes.' Clarification of the biological meaning of this mapping would still be helpful.

Regarding the sea star–fly comparison, the predominance of links to the border, stretch, and hemocyte clusters is indeed an interesting observation. One possible explanation is the uneven resolution between the Drosophila melanogaster ovarian atlas and our sea star ovarian atlas, the latter containing a greater number of cell type groups, which may introduce technical bias. Alternatively, this pattern may reflect shared features between the highly diverse border, stretch, and hemocyte populations and multiple sea star cell types. For instance, several sea star ovarian cell types may express genes associated with functions such as support, epithelial remodeling, migration, or engulfment, which are characteristic of these fly cell types. It is also possible that multiple sea star ovarian cell types express genes related to extracellular matrix organization, adhesion, motility, and phagocytosis. However, in the absence of additional data and given the extensive analysis that addressing this would require, we refrain from further speculation, as we believe this falls beyond the main scope of the manuscript.

The evolutionary conservation of germ cells and oocytes is clear and expected.

Tgfb inhibition by LDN-193189

I thank the authors for the additional experiments and clarifications provided in their response. The authors convincingly demonstrate that treatment with the inhibitor

LDN-193189 in sea star larvae phenocopies previously observed effects on the BMP receptor-mediated axis formation and suppresses pSMAD1/5/8 phosphorylation. However, the description of these results still does not adequately reflect the complexity of TGF β signalling, and it remains unclear if the reported combination of TGF β ligands, receptors and Smad proteins may function together in this system. To improve clarity and precision, the authors should explicitly state:

- that *tgfb* receptor 3 ortholog likely functions as a TGF-beta co-receptor, whose vertebrate orthologs lack intrinsic signalling activity
- that *alk2* receptor is a type I Activin receptor that binds BMP ligands and typically signals via Smad1/5/8
- which TGF β receptor types are expected to be inhibited by LDN-193189

In addition, the authors should consistently use 'pSMAD1/5/8' throughout the manuscript to clearly distinguish it from pSMAD2/3 and avoid potential confusion. These clarifications would substantially strengthen this section.

We understand the reviewer's concerns. We now consistently use "pSMAD1/5" throughout the manuscript to clearly distinguish it from pSMAD2/3, and have rephrased the relevant sections as follows:

We found that the type I Activin TGF β receptor *alk2*, which binds BMP ligands and signals through the intracellular messengers *smad1/5/8*; the *smad5* ortholog itself; and the *tgfb3* ortholog, a likely TGF β co-receptor whose vertebrate counterpart lacks intrinsic activity; are all expressed in both germ cell nests and developing oocytes, validating the scRNA-seq predictions (**Fig. 6d-f**).

To test the role for Tgf β in folliculogenesis, we used the specific Tgf β inhibitor LDN-193189 (56) that selectively inhibits BMP signaling by blocking SMAD1/5/8 activation through targeting type I BMP receptors ALK1, ALK2, ALK3 and ALK6 (56).

Figure 1a

I previously questioned the relevance of 'average lifespans' in Fig.1a, and suggested adding species names to clarify that the indicated lifespans are not representative of the entire taxa (f.ex. 'protostomes'). While I appreciate the author's attempt to clarify this point, I respectfully disagree with their proposed revision, which in my view increases confusion.

The added statement 'Average lifespans of the depicted taxa are shown to highlight the rare case of long-lived animals (echinoderms) capable of adult oogenesis.' is

problematic. It introduces ambiguity (what is 'long-lived'?) and reinforces a misleading narrative. Even under a conservative definition of long-lived (>100 years; which would exclude sea stars), numerous examples of long-lived animals capable of adult oogenesis exist across different taxa, incl. vertebrates (f.ex. reptiles, sharks), annelids (some tube worms), molluscs (famously *Arctica islandica*), as well as cnidarians (corals, some jellyfish) and sponges. In addition, some sea stars and many sea cucumbers, which are also echinoderms, are rather short-lived (<10 years). This shows that the authors' generalisation of echinoderms as long-lived does not hold, and sea urchins do not represent a unique or rare case.

Furthermore, the authors clarify in their response that each depicted animal represents multiple species or genera. Therefore, absent taxonomic annotation leaves their interpretation ambiguous and potentially misleading.

Additional issues include:

- annotating the root of this tree as 'metazoans' is problematic as the figure includes exclusively bilaterian taxa
- The use of lifespan ranges to represent 'average' lifespans, which is conceptually inconsistent.

With appropriate revisions, the figure could be informative to illustrate the phylogenetic relationships among the species used for the comparative single-cell analysis, and species names should be explicitly included. However, the connection between lifespan and adult oogenesis is presented in a biased and unsupported manner and is not directly relevant to the manuscript. I therefore strongly recommend that this component should be removed.

We understand the reviewer's point of view. We have therefore removed the average lifespan element and instead introduced the species names of the animals depicted. Moreover, the tree is now rooted in Bilateria. Additionally, the sentence in regard to echinoderm longevity now reads as: Echinoderms, including sea stars, continuously produce millions of oocytes, and some species have documented lifespans that can exceed a century (15). Moreover, the figure legend corresponding to this panel has been revised and now reads as follows: (a) Phylogenetic tree showing the evolutionary relationships of P. miniata with other bilaterian animals for which ovarian cell type repertoires have been defined using single-cell transcriptomics.

Reviewer #3 (Remarks to the Author):

The authors have fully addressed comments of the reviewer.