The complete cell lineage and MAPK- and Otx-dependent specification of the dopaminergic cells in the *Ciona* brain

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

As you will see, all the referees are enthusiastic about your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. They make a number of useful points and in particular, they recommend that you include an experimental test of the role of Ephrin in the differentiation between a10.74 and a10.73. On the other hand, the request of referee 3 that you examine the expression of Meis does not seem essential to support the conclusions of the study, and you won't need to perform this for your revision. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

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

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

in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript Oonuma and Kusakabe describe the cell lineage and part of the gene regulatory network leading to dopaminergic (DA) cell specification in ascidian (Ciona) embryos. The DA neurons of ascidians are an interesting cell type forming at least some of the “coronet” cells on the left wall of the larval sensory vesicle that extend bulbous protrusions into the sensory vesicle and may function as a pressure receptor. These cells are thought to be homologous to the coronet cells identified in the hypothalamus of fish. Furthermore, DA cells are transcriptionally unique, forming a discreate group of cells with unique expression of genes including those encoding for the Fer2 transcription factor, DA biosynthetic pathway and secreted neuropeptides (Horie et al 2018). One of the major challenges in ascidian CNS development is linking the cell lineage to the complex gene expression patterns. Identifying the lineage of brain cells has been troublesome due to high cell numbers and because the brain rotates during larval stages. Furthermore, left-right patterning of the brain is disrupted when the chorion is removed from eggs, a standard practice carried out to facilitate embryological manipulation. Oonuma and Kusakabe have developed a technique to express Kaede from neural promoters in chorionated embryos in order to follow cell lineages in embryos with intact left-right patterning. Using this technique, they have previously resolved the developmental origin and lineage of the larval photoreceptors. Here, they provide indisputable evidence that the DA cells arise from both bilateral a9.37 cells. Furthermore, they describe the complete larval DA cell lineage, showing that the a9.37 cells divide along the anterior posterior axis and the posterior daughter cell (a10.73) will finally generate 16 DA cells. Specification of DA cell-fate in a10.73 requires MAPK activity and in the absence of MAPK activation the posterior a10.73 cell appears to adopt the fate of its anterior sister cell, at least at late neurula stages (7.5 hours). Next, the authors show that Otx, expressed in brain precursors for an extended period of development, is required for DA cell specification and for what seems to be an unequal division of a9.37. Taken together with previous studies on neural induction, and patterning of the neural plate, this study unravels a critical part of the puzzle and allows one to understand each phase of DA cell specification, from the start of “neural induction” at blastula stages, to their final “fate restricting” cell division giving rise to a DA cell specific lineage and expression of the DA cohort of genes. This would be impossible to understand without the correct cell lineage analysis reported here. Furthermore, this study also provides a cell count and positional information for every brain precursor of the neural plate as well as a more detailed description of the brain rotation that takes place at larval stages. The manuscript is mostly well written, well presented and includes helpful schematics. I recommend this manuscript for publication in Development following a few modifications.

Comments for the author

Suggested modifications

1) The cell-by-cell lineage labelling that the authors perform should generate quite an interest for ascidian embryologists. With a bit more information, this could provide a basic “atlas” of cell lineage for the brain. Unfortunately, in Figures 1-3 for the panels -X”-, it is difficult to orient exactly where the cells are. For example, looking through the descriptions of the cell descendants and the images, it appears that the a-line derivatives rotate counterclockwise but not so much the A-line cells. Is this correct? It is not clear, therefore, what forms the right side of the anterior-lateral part of the brain vesicle. It also seems that there are great many more cells on the left compared to the right in these images, but this was not reported in Ryan et al, 2016. I wonder if the authors could provide the confocal stacks for these images as supplementary so that readers can see for themselves exactly where the labelled cells are? A schematic drawing like in Figure 7C for each of the different cell lineages would be really helpful to understand the contribution of the different lineages.

2) In lines 149-152, the authors state that within the DA cell population of the sensory vesicle the right a9.37 descendants are anterior and left a9.37 descendants are posterior. In Figure 3D" (right a9.37 Kaede labelled) I think I can see some anti-GFP+ cells that are posterior to the Kaede-Red labelled cells, which would fit with this. However, in Figure 3B" (left a9.37 Kaede labelled) I would expect to see some anterior anti-GFP labelled cells that are not labelled with Kaede-Red, corresponding to the right a9.37 DA cells. Could the authors provide an explanation for why it is not possible to see the Fer>GFP cells that should correspond to the right a9.37 cell. Is this because the Fer2>GFP is inherited in a mosaic way? An explanation should be provided in the text or Figure legend. If the authors have another non-mosaic larva image showing the Kaede-Red unlabeled but anti-GFP+ cells anterior to the Kaede-Red+/anti-GFP+ cells, it would make it easier to appreciate the final positions of the left and right a9.37 descendants.

3) Figure S1 shows beautifully the rotation of the sensory vesicle in a counterclockwise direction from a dorsal view, anterior up. However, since the images are very close up, it is difficult to really appreciate the rotation: the landmarks the authors used to orient the embryo are not visible to the reader. Maybe a schematic drawing of this experiment with landmarks highlighted clearly would help the reader.

4) For all panels in Figure S1, the authors should provide the "n=".

5) Could the authors mention if the isolated cell from the R a9.33 visible in Figure S1D' (arrowhead) is consistent for all labels of R a9.33? (for example, it is not possible to identify this cell in Figure 2H").

6) Lines 174-202. The authors do not always mention the number of descendants for each labelled cell, and it would be better to mention the average number of cells for each cell pair labelled in the main text, so the reader does not have to keep checking the supplementary table.

7) For Figure 5 it would be better to show whether DA cells are lost (or at least loss of Fer2 expression is maintained) following U0126 treatment, using a later marker/stage. It would strengthen the idea that there may be a fate change between a10.74 and a10.73 that is governed by MAPK.

8) Lines 270-273, I do not see the need for "However" when describing FGF9/16/20 expression above and below the a9.37 cells, since it is the same as the published images in Hudson and Yasuo 2005 when the row II cells have divided.

9) FGF-Ephrin part of results and discussion.
The authors write 'To determine whether ephrin-Eph signaling regulates the MAPK pathway….we investigated the expression patterns of ephrin ligands and Eph receptors'. Since knowing the expression pattern by itself would not determine whether ephrin-Eph signaling regulates the MAPK pathway, the authors should revise this sentence. Similarly, I do not understand how the authors can state (lines 287-289) "These results suggest that (1) ephrin-Eph signaling derived from Efna.b and Eph.a is active in both a10.73 and a10.74 cells, and (2) this signaling inhibit the MAPK pathway in a10.74 cells."
It would suffice to say that FGF9 is expressed above and below the a9.37 daughters and that there are also several ephrin ligands and receptors which MAY be involved in generating the differential MAPK between a10.74 and a10.73, but that this would require further investigation. Incidentally, Efna.c may also be a good candidate as it appears to be expressed anterior to and maybe in a10.74 (see Figure S1 in Haupaix et al, 2014).

On lines 391-397, the authors suggest that the potential difference in size (see comment below) between a10.74 ("small") and a10.73 ("large") may account for differential areas of contact with FGF expressing cells, resulting in differential MAPK. However, the authors show that in Otx-MO embryos the unequal cleavage may be lost, but yet the differential MAPK activity persists. Therefore, it seems that this potential unequal cleavage is not required for differential MAPK activity.

Lines 402-404, I see no evidence as to what factors activate or do not activate MAPK in a10.73 cells. Please revise.

Overall, the part of the discussion lines 381 to 406 is rather long, distracting and speculative. I think the authors could consider to remove this part and simply state that more work would be required to understand if and how FGF- and ephrin- signaling might be involved in the differential activity of MAPK between a10.74 and a10.73.

10) Lines 305-309. The authors describe a difference in “cell size” between a10.74 and a10.73, with a10.74 being a smaller sister. However, what the authors are presumably looking at is apical cell surface and nuclear size (which may be an indicator of cell size). The authors should acknowledge in the text what they are looking at (apical cell size and nuclear size) when referring to this potential unequal cleavage.

Line 394, please remove the reference to the volume of these cells, as this was not measured.
11) The term "asymmetric cell division" could imply many different things: cell fate, cell size, organelle or protein segregation etc. When the authors use this term, they should first define what they are referring to. In the discussion the authors discuss ‘asymmetric cell division’ (line 423, line 439) when presumably referring to the possible different cell sizes of a10.73 and a10.74. The term ‘(potential) unequal cleavage’ is better in this case. In addition, in lines 426 and 436, it is not clear if ‘asymmetric cell division’ is referring to an unequal cleavage or segregation of a localized factor from a polarized mother cell (i.e. not dependent upon daughter cell size).

12) First part of the discussion. In Cole and Meinertzhagen 2004 and Taniguchi and Nishida 2004, these authors may not have been strictly looking at the DA cell population, since they were looking at coronet cells. It is not entirely clear, at least to me, if DA cells make only coronet cells, although it seems to be clear that some coronet cells are DA+ (Moret et al, 2005a). Ryan et al, 2016 confirms a number of 19 coronet cells in C.intestinalis, compared to the 16 DA cells reported here in C.robusta. Is this a species difference or is it possible, that that some coronet cells are not DA+ cells and that some DA+ cells are not coronet cells? This may be part of the reason for the differences in lineages reported in this study and the previous studies. The authors may want to discuss in more detail the data linking DA cells to coronet cells.

13) Lines 464 to 480. The rotation of the brain is difficult to follow. For dorsal cells to finish on the left, viewed from the posterior, it should be a counterclockwise rotation rather than clockwise (line 464). In Figure 7C, indeed, with anterior up, both rotations are counterclockwise, so I guess this is a typo. The second rotation (line 471) would be counterclockwise when viewed from the dorsal side with anterior up (it is better to orientate with respect to both axis in the text). Are these two events temporally separable or do the cells rather move at an angle with these two rotations acting more or less concomitantly? Please clarify.
Lines 476 to 480. It is not clear how the movement of the left a9.33 cell (towards the posterior) is consistent with reports that left a8.17 derivatives in Halocynthia are located at the posterior and anterior brain vesicle (Taniguchi and Nishida 2004), since both left a9.33 and a9.34 (L and R, Figure 2) are shown to be posterior here.

14) Line 494. I think the authors should acknowledge that the homologies between regions of the ascidian and vertebrate CNS is not fully agreed.

15) Line 486. Ryan et al, 2016 provide a spectacular description of brain asymmetry in ascidians so the authors might consider to cite again this article here.

16) Please could the authors provide the C.robusta unique gene identifiers for all the genes they study in this manuscript. This would make it much easier to unambiguously identify the genes being discussed, as well as for annotation purposes (for example in ANISEED).

17) It is difficult to precisely stage the embryos shown in the Figures based on the timing. On Figure 7A, along the top the authors could put developmental time plus the precise developmental stage (Hotta et al, 2007; doi:10.1002/dvdy.21188), which would be very helpful.
typo:
Line 258-359 revise to "…is expressed in cells anterior and posterior to a9.37…".

Reviewer 2

Advance summary and potential significance to field

Oonuma and Kusakabe present their analysis of the complete cell lineage of the dopaminergic (DA) cells in the Ciona brain and the deciphering of regulatory mechanisms that specifically generate the DA cells.

To understand cellular and molecular events producing dopaminergic (DA) cells during development, the authors combined the advantages of the ciona embryo (stereotyped development and fixed cell lineage, few neurons in the CNS of the larva, single cluster of DA cells) with a method they previously established to analyze cell lineages in intact chorionated embryos. This method involves the specific labeling of cells of interest in the neural plate using the photoconvertible fluorescent protein Kaede expressed under control of suitable drivers. They report the complete DA cell lineage in a simple chordate brain, showing that DA cells are derived from the a10.73 cell pair of the neural plate. They also decoded details of regulatory mechanisms involved in the DA cell fate specification along this lineage.

By uncovering the complete cell lineage of DA cells in the chordate Ciona embryo, the authors provide a rigid framework to further decrypt the mechanisms controlling the specification of DA cells. The experiments are overall conducted with great care. My overall assessment is that, pending the authors address the concerns discussed below, the paper will be of broad interest for both the developmental biology and the neurobiology communities. I therefore support the publication of this study in Development pending the following concerns are addressed.

Comments for the author

Major concerns:
a. Figure 2 A-G?? shows that when the authors label the a9.37 cell pair using the photoconvertible protein Kaede, all their descendants expressed Fer2>EGFP and therefore gave rise to DA cells. Further down the manuscript, however, they show that only the posterior daughters of the a9.37 cell pair (the a10.73 cell pair) give rise to DA cells. The authors should explain why by labelling the a9.37 pair, they detect no Kaede-Red-labelled nuclei outside of the DA cells expressing Fer2>EGFP.

b. The authors discuss a potential role for ephrin-Eph signalling in the inhibition of the MAPK pathway in a10.74 cells. This should be confirmed by blocking this pathway with available chemical inhibitors.

c. In the discussion section (lines 391-397), the authors propose that the larger surface of contact to FGF-expressing cells of a10.73 explains the asymmetrical activation of ERK in a9.37 daughter cells. This is, however, unlikely as ERK is still only active in a10.73 following Otx knock-down, despite the two a9.37 daughters having the same size. A more probable explanation of what is observed involves differences in the ratio between the surfaces of contact exposed to FGF and Ephrin signalling (Ohta and Satou, PloS Comp. Bio. 2015; Guignard et al., Science 2020). This point should be discussed in the manuscript.

d. The discussion about a possible role of Otx in the asymmetric cell division should mention that this effect may be very indirect as the authors knock down Otx function by microinjecting MO in the egg. The loss of the division inequality of a9.37 could result from much earlier cell fate changes, or from the control of secreted ligand by Otx in neighbours of a9.37.

e. Evolutionary conservation of the DA lineage in ascidians. The authors discuss line 333-335 the conservation of the DA lineage between Halocynthia and Ciona. Are the authors absolutely sure that no single DA cell comes from the a8.17 lineage in Ciona? If so, how do the authors interpret this species difference? Are the expression patterns of Fer2 the same in Ciona and Halocynthia?
More minor issues:
Figure 1 shows unnecessary data concerning the A-lineage: the authors present a detailed labelling of each pair of the A-lineage cells in the neural plate. The spatial distribution of the descendants of each A-line pair is beyond the scope of the paper. Instead, the authors should present a labeling of the whole A-lineage row III/IV cells as they do for rows II/IV. This will suffice to show that DA cells do not develop from the A-lineage. Figures 1 and 2 could be combined and reorganized to first show

a complete labeling of the A-lineage rows I/II, then the complete labeling of the a-lineage cells of rows III/IV, and finally the detailed labeling of individual row III/IV cells.

Line 76, the most quantitative demonstration of the invariance of the ascidian cell lineage is Guignard et al. (10.1126/science.aar5663). It would also be nice to cite Taniguchi and Nishida (2004) in the introduction as the DA lineage was described in Halocynthia before the authors embarked on their study.

For the last row of pictures in figures 1-3, the authors should include either in the legend or in the figure, or both, that the row is a combination between the fluorescence images and a brightfield view of the larvae.

Some results are presented as novel although they are already known from the literature. For example, in Figure 5, dpERK staining is presented as novel although it was already known that ERK is activated in row IIIp (reviewed in Hudson 2016). All confirmatory figures should be moved to the supplementary information.

The section ?Spatial distribution of the descendants of the neural plate cells? could be strongly shortened by suppressing the description of non DA-fated cells, and focusing on the results showing the rotation in the a9.37-lineage cells. Figure 3 does not really show that the descendants of the right a9.37 cell tend to be located at posterior to the left a9.37 descendants. This figure should be replaced with Figure S1 which shows much more convincing evidence by labelling the left side of the embryo with FITC at the 2-cell stage.

Figure 1, Figure 2, and Figure 3: In some cases (e. g. for some descendants of the a9.33 pair, figure 2 B-H??) we see labelled nuclei with Kaede-Red in/very-close-to the EGFP-labelled DA domain. It would be better if the cytoplasmic labelling of DA cells obtained using a Fer2>EGFP was replaced with a nuclear labelling of DA cells. This approach would also allow to precisely count the number of DA cells at the larval stage and to compare this number with the number of descendants of the a9.37 pair labelled with photoconverted Kaede.

Table 1, Table 2 and Table S1: The authors do not describe what S.E. means.

Figure 5: In the control condition (DMSO), we see that the Acsal1 marker gene is also expressed anteriorly to the a10.74 cells. In the UO126 condition, which is supposed to convert the posterior a10.73 to an a10.74 anterior fate, we see that the more anterior expression pattern of Acsal1 is also lost. How could the authors explain this observation?

The authors should indicate precisely how they reconstruct the outline of each cell to compute its volume and determine the inequality of the a9.37 divisions.

Figure AH: the progeny of a10.74 should be highlighted.

The number of experiments performed is generally, but not always indicated (e. g. Figs 4 and 5).

As it stands this article will be very difficult to digitalize and enter into databases. A major issue is that the genes mentioned are not clearly indicated (gene names change over time?). A reference to their KH gene model ID is necessary. In addition, clone IDs are incomplete. For instance, the clone mentioned for fer2 should be R1CiGC44e22 and not GC44e22 in order to be found in the main ascidian database, Aniseed.

Reviewer 3

Advance summary and potential significance to field

In this manuscript Oonuma and Kusakabe determine the complete cell lineage of the Ciona larval dopaminergic (DA) cells. In addition, they provide evidence for the contribution of the MAPK pathway and the transcription factor Otx in the specification of the DA cells.

This study is of significance to the Ciona community as it provides conclusive evidence for the cell lineage of the DA cells. It is also of broader interest to the developmental neurobiology community because Ciona is one of the very few model systems which can be used in order to study the developmental mechanisms that give rise to dopaminergic cells at single cell resolution. The findings of Oonuma and Kusakabe enhance our understanding of the evolution of DA cells, since they highlight some similarities and differences in the molecular mechanisms underlying the development of DA cells between ascidians and vertebrates.

Comments for the author

Major points to address:
Regarding the cell lineage tracing experiments:
1.It would be great if the authors could generate one or more supplemental figures showing maximal projections from more photoconverted animals that they have imaged but haven't shown in Figures 1-3. This would strengthen the authors' statement in line 145 that: "there was little position variation of the labelled cells amongst individuals".
2.The authors should include additional panels in the main Figures 1-3 showing the non-photoconverted Kaede (green) signal. This would allow for the more comprehensive reporting of their experimental results, while it would also help to exclude the possibility that there are Kaede-green cells that colocalize with Fer2(+) DA cells. However, both in the current manuscript and the Oonuma et al 2016 paper, they don't show at the larva stage Kaede –green cells. My first reaction was that this could be due to their fixation protocol (methanol based from what I gather), which would quench most fluorescent signals. But on the other hand as far as I can tell the Kaede-red signal is unaffected. So if the authors have the green signal channel they should include that, if not they should consider devising an experimental strategy that would allow them to show Kaede-green, Kaede-red and Fer2(+) cells. For example a live experiments with Kaede and Fer2>BFP (to avoide spectral overlap issues). To clarify if the authors were to design a new experiment this would only be required for the a9.37/a9.37 lineage tracing, which is the most important experiment for their conclusions in my view.

3. Figure 1C-C'' The Fer2>GFP signal seems to be somewhat different when compared to that of the other animals shown in the figure (e.g. expression above the ocellus and otolith, process(es) extending posteriorly. The authors can comment on this or even better show more Maximal projections from different animals (see point 1).

4.In Figure 3 I am expecting to see/count approximately 8 Kaede-red cells and16 Fer2(+) cells in each of the panels B', B'', D' and D''. I can roughly count 8 Kaede-red cells in each panel as the authors report but I am not sure I can find 16 Fer2(+) cells. Note that the Fer2 positive cluster looks rather different in B'-B'' and D'-D''. Even without counting one would expect to roughly see double the number of yellow cells compared to the magenta cells in each panel. This is not really the case. Since these are maximal projections it is possible that a number of Fer2(+) cells are 'sandwitched' together and thus hardly distinguishable. Can the authors provide alternative views (e.g. orthogonal views) of this data.
Regarding the cell movements during development:
The authors go to some effort to report and discuss cell movements that occur in the brain vesicle during development. Their conclusions are based on the start and end positions of photoconverted cells and on an experiment combining FITC labelling and Fer2 mRNA detection during 3 different time-points. In principle, I do not disagree with the authors conclusions (they seem logical), but I do feel that it would have been much more convincing to show these movements using live time-lapse imaging in combination with nuclei tracking (Kaede-NLS signal) using confocal microscopy and relevant analysis software e.g. IMARIS or FIJI. Now an alternative would be to enrich their Figure S1 with more time points and different views (A-C'' only dorsal views are provided and they are high magnification so there are no clear landmarks/frame of reference since the FITC+ cells are changing in shape and/or are moving). Note that for Figure S1 the number of animals used is not provided.
Another aspect that the authors tackle in their manuscript is the contribution of MAPK signalling and Otx in specifying DA cell fate. Here are some points that I would like the authors to address:

5. Lines 232-233: The authors suggest that Meis is expressed in both a10.73 and a10.74 cells. Figure S2 A' supports their conclusion with respect to a10.73 cells but the signal observed in a10.74 is very

weak (not different to the staining levels outside the white boxes) at least in the picture shown in Figure S2A'. Maybe the can provide a more clear example. Alternatively they can take a quantitative approach to illustrate that the signal in the a10.74 box is stronger than the background with the caveat that WISH experiments are not necessarily the most robust when it comes to quantitation.

6. The authors examine the role of the MAPK pathway in DA cell fate. They show that treatment with U0126 abolishes Fer2 mRNA expression (figure 5). The authors should test whether Meis mRNA is also downregulated following U0126 treatment and whether as a consequence of Fer2 downregulation, TH expression is abolished. These are some relatively simple in situs to do but they could be potentially informative on whether MAPK pathway is required only for Fer2 expression or whether it also affects Meis expression. The TH in situ would be a 'positive' control confirmatory of the authors conclusions.

7. The authors come up with two possible explanations for the observation of MAPK activation only in a10.73 cells (lines 365-368). They seem to favour the idea that other factors besides FGF ligands activate the MAPK pathway in a10.73 cells. Their opinion is supported by in situs of potential molecular players (Fgf9/16/20, Fgfr, Efna.b, Eph.a, Eph.c.) However, they do not address either of these possibilities with functional experiments. For example, before downplaying FGF's involvement they should use the inhibitor SU5402 (there may be other alternatives) and determine whether in their hands FGF signalling inhibition alters dERK signal, or Fer2/Acsal1 expression in a10.73 a10.74 cells. Regarding the prediction that Eph.a may be involved in the inhibition of the MAPK pathway in a10.74 (discussed lines 373-380) this could also be tested by means of an Eph.a dominant negative or a knock-down. In short, approximately 2.5 pages of the discussion are dedicated on what might activate the MAPK pathway in a10.73 cells but the corresponding experimental evidence provided in the manuscript is rather limited. At least one of the two proposed scenarios should be tested.
8. The authors should check whether knockdown of Otx affects Meis expression. In addition, the authors provide strong evidence that MAPK signalling is not affected by Otx knockdown. However, the influence of MAPK on Otx has not been tested. So they authors should investigate this by determining whether U0126 treatment affect Otx expression.

Minor points:
9. I gather that Fer2>EGFP is in fact referring to Fer2>G-GECO1.1. Maybe the authors can clarify this as it may cause some confusion to readers and people who may request this construct in the future.

10. In Figure 2 panels L-L'' there are 2 white arrowheads pointing to a single large nucleus. I might have missed it but I don't see anywhere an explanation as to what the authors are trying to point out.

11. The authors should include Unique Gene IDs and/or Gene Model IDs for all the genes used in their study.

First revision

Author response to reviewers' comments

Response to Reviewers:

Following is a detailed description of our responses to the reviewer's comments and other changes in the revised manuscripts.

Reviewer 1:

1. We prepared serial optical section movies of confocal images that show the detailed distribution of the labeled cells in the brain vesicle (Movies 1-9). These videos and our

manuscript (lines 152-210) revealed that derivatives of the labeled cells except for a9.33/a9.33- and a9.37/a9.37-lineage cells were located at the left and right sides of the brain vesicle, while descendants of a9.33/a9.33 and a9.37/a9.37 cells distributed to only the left side of the brain vesicle. Although we agree that a schematic drawing like in Figure 7C for each of the different cell lineages would be helpful to understand the contribution of the different lineages, we focus on a9.33/a9.33- and a9.37/a9.37-lineage cells because the present manuscript mainly describes and discusses development of these lineage cells with high-precision analyses.

2. We prepared optimal images for Figure 3 (Fig. 3A-D"). By adding the images of the non-photoconverted fluorescence of Kaede in Figure 3, we revealed that the right a9.37 descendants locate anteriorly and left a9.37 descendants locate posteriorly within the DA cell population. We added this explanation in the Results section (lines 141-149). Moreover, although the mosaic distribution of *Fer2*>EGFP (renamed *Fer2*>EGFPv in the revised manuscript) construct may occur, we used only embryos exhibiting fluorescence of Kaede-green in the neural plate of most a-line or A-line cells and collected the larvae expressing GFP widely in the brain vesicle.

3. For the images shown in original Figure S1A-C", which are now included in new Figure 3 (F-F", H-H", J-J"), we added images at lower magnification merged with a bright field image, which allowed us to show the outline of the trunk region of the tailbud embryos (Fig. 3E-J"). We also draw the mid line of the embryos in these images (Fig. 3E-J").

4. In all images of original Figure S1 (Fig. 3E-J" and Fig. S2A,B in the revised manuscript), we added the number of examples (n=).

5. In the original version of Figure 2H, the cell adjacent to the ocellus pigment derived from the right a9.33 was not visible because the z-stack slices did not include it. So, we changed the image so that the merged image now includes all descendants of the right a9.33 cell.

6. We added and mentioned the number of descendants for each labelled cell (lines 185, 194-196).

7. According to the reviewer's suggestion, we added the new data showing that *Fer2* expression at the mid neurula and the mid tailbud stages was lost in the embryos treated with MEK inhibitor U0126 from the late gastrula stage (new Fig. S5; lines 265-267). Because Horie et al. (2018) suggested that *Fer2* is required for expression of the DA marker genes such as *Th*, it is expected that the DA cells are not differentiated in the embryos treated with U0126.

8. We omitted "However" in the lines 270-273 of the previous manuscript (line 283-285 in the revised manuscript).

9. We agree with the reviewer's opinion. Accordingly, we greatly reduced the volume of this section and made it much more concise (lines 388-398). We also conducted a new experiment in which a dominant negative form of the *Eph.a* receptor was overexpressed in embryos and detected dpERK in these embryos. We added the results of this experiment and discussed the regulation of MAPK pathway in a10.73 cells (Fig. S4B, C; lines 296-302, 392-394). Although the difference in cell size
seemed to be lost in *Otx*-MO embryos, it remains unclear if the contact ratio between cells expressing FGF ligand and receptor was influenced by *Otx* knockdown. Therefore, we remained the description about the possibility that the contact ratio is related to the activation of MAPK pathway in a10.73 cells in a concise manner (lines 396-398).

10. The reviewer suspects that the indicators of cell size we used were apical cell surface and nuclear size. In fact, we estimated and compared cell sizes as follows. We outlined the cell-cell boundary based on the cytoplasmic immunofluorescent signals of dpERK in the confocal image viewed from the dorsal (apical) side. We described this in the text (lines 324-326).
The reviewer suggested to remove the reference to the volume of these cells, as this was not

measured. In the revised manuscript, we measured the cell volume by visualizing cell membranes and making 3D-reconstructions of cells. The method is described in Materials and methods and the data is shown in Fig. S8.

11. We have changed “asymmetric cell division” to “unequal cleavage” or “unequal cell division” (lines 421,428,433) and also replaced the citation and the explanation (Hawkins and Garriga, 1998; Knoblich, 2008) with a report that explains unequal cleavage in ascidian embryos (Negishi and Nishida, 2018) (lines 423-426).

12. Although previous studies reported the number of the coronet cells and our present study revealed the number of DA cells, it remains unclear whether all coronet cells are composed of only all DA cells in *C. robusta*. However, because it was reported that the number of the coronet cells is 16 and that DA cell has a coronet protrusion (Ryan et al., 2016; Ryan and Meinertzhagen, 2019; Moret et al., 2005), all coronet cells may be all DA cells. We newly discussed the relationship between the coronet and the DA cells (lines 351-354). As for the number of coronet cells in *C. intestinalis* reported by Ryan et al. (2016), it is actually 16 but not 19 (see Table 3 of Ryan et al. 2016).

13. The reviewer pointed out that the rotation of the brain is difficult to follow in the previous manuscript. Accordingly, we revised Figure 7C by adding an illustration
that further explain movement of brain cells. Although it is not clear whether the three rotations observed in the present study are separable, these three events seem to occur simultaneously (lines 476-479).
Regarding the consistency of cell movements between *Ciona* and *Halocynthia*, we omitted the corresponding sentence because we do not have enough evidence (line 465).

14. We agree with this comment and revised the sentence (lines 489-493).

15. We cited Ryan et al. (2016) in this sentence (lines 480-482). We also added Taniguchi and Nishida (2004) in the same sentence.

16. In the revised manuscript, we provide KH gene model IDs and Gene collection IDs for every genes to precisely identify them in Materials and methods (lines 570-575).

17. We added the FABA stages (Hotta et al., 2007) in Figure 7A.

18. We fixed the typos the reviewer pointed out (lines 384-386).

Reviewer 2:
Major concerns

a. Because we stacked a part of the optical section images but not all images, Figure 2H- H" did not show no Kaede-Red-labelled nuclei outside of the DA cells expressing Fer2>EGFP when a9.37 cells were labeled. Therefore, we prepared the confocal stack movies that show the all optical section images in each labeled cells of the neural plate (Movies 1-11).

b. We used the dominant negative form of *Eph.a* receptor instead of chemical inhibitors. We added the results that show the detection of dpERK in embryos overexpressing the dominant negative form of *Eph.a* receptor (Fig. S4B,C) (lines 298-302).

c. Although the different cell size seemes to be lost in Otx-MO embryos, it remains unclear if the contact ratio between cells expressing FGF ligand and receptor was influenced by *Otx* knockdown. Therefore, we still consider the possibility that the contact ratio involves the MAPK pathway activation in a10.73 cells.

d. According to the reviewer’s suggestion, we revised the manuscript stating that the role of *Otx* on the asymmetric cell division (unequal cleavage) may be indirect (lines 428,429).

e. By chasing the labeled cells, we showed that a9.33 and a9.34 cells, daughter cells of a8.17

cell, do not give rise to the DA cells in *Ciona* larva. Therefore we are sure that no single DA cell comes from the a8.17 lineage in *Ciona*.
In *Halocynthia*, only an uncharacterized antigen (Hpr-1) has been used as the coronet cell marker (Taniguchi and Nishida, 2004) and other molecular markers, such as *Th* and *Fer2* have not been identified. So, we think further investigations are required to precisely understand the species differences of the cell lineage of the DA/coronet cells. We added sentences describing these points (lines 358-362).

More minor issues:

1. The reviewer argued that we show unnecessary data concerning the A-lineage and that the spatial distribution of the descendants of each A-line pair is beyond the scope of the paper. However, we wanted to exclude the possibility that the DA cells are not derived from the A-lineage cells completely. It also remained unclear how the A- lineage cells that give rise to the brain vesicle at the tailbud stage are distributed by the larval stage although the distribution of the brain vesicle cells until the tailbud stages was reported. Besides, another reviewer (comment #1 of reviewer 1) supports the inclusion of data of each of the different cell lineages, which would be helpful to understand the contribution of the different lineages. Therefore, we remained the data about the A-lineage in Figure 1.

2. We cited these two references (lines 75-80).

3. We added the description that the rows of the figure1-3 is a combination between the fluorescence images and a bright-field view of the larvae in each figure legend (lines 866,877,887).

4. We appreciate this comment and moved the Figure 5A-A" to supplemental figure (Fig. S4).

5. We shortened the explanation of the non-DA cells in the section "Spatial distribution of the descendants of the neural plate cells" (lines 194-202). We also changed the images in Figure 3 and added the images in Fig. S1A-C" into Figure 3 to show that the descendants of the right a9.37 cell tend to be located at posterior to the left a9.37 descendants.

6. We appreciate the suggestion. But we would like to keep using the Fer2>EGFP construct because the fluorescence of EGFP detected in the cytoplasm of DA cells and we could distinguish the fluorescence of Kaede-red from EGFP fluorescence.

7. We described what S.E. means in the table legends (Table 1, 2, S1) (lines 944,947).

8. The detected signal anteriorly to the a10.74 cells in the control embryos is maternal expression of *Acsal1* in the endoderm. *Acsal1* mRNA was maternally expressed until the gastrula stages and there are also embryos that *Acsal1* mRNA was detected after the gastrulation in both the control and U0126 conditions. We reduced the z-stack slice number of images in the control (Fig. 5C,C').

9. We newly measured the cell volume of a10.73 and a10.74 cells (Fig. S8) and suggest the unequal cleavage between these two cells.

10. We highlighted the progeny of a10.74 cells in Fig. 4H.

11. We described the number of embryos in Fig. 5 and the legend for Fig. 4 (lines 584,585).

12. According to the comment, we described the KH gene model IDs and correct Gene Collection IDs (lines 570-575).

Reviewer 3: Major points

1. To show little position variation of the labelled cells amongst individuals, we added the results of the labelled cells of the different individuals in the supplemental figure (new Fig.

S1).

2. We newly chased the left and right a9.37 cells by the photoconverted fluorescence of Kaede and added the images detecting the Kaede-green signal in Figure 3 (Fig. 3B', D'). Although each left and right a9.37 cell but not both a9.37 cells was labeled in these specimens, these results revealed that the number of cells labeled by Kaede- green and -red was consistent with our present findings that DA cells are equally derived from the left and right a9.37 cells (Table 1).

3. We replaced and showed the images from different animals in Figure 1C-C".

4. We newly made serial optical section movies of confocal images of the labeled cells to count the Kaede-red positive cells (Movies 10,11). We also provided the number of the labeled cells in Table 1.

Regarding the cell movements during development:
We thank the reviewer for the suggestion and we agree that live time-lapse imaging provide us for better information to show the cell movement, too. We attempted to do time-lapse imaging using the confocal microscopy, but we could not chase the labeled cells because the rotation of embryos from the neurula to tailbud stages. Therefore, we used the fixed embryos. We also added the number of examples for each data shown in the original supplemental Figure 1, which are now included in Fig. 3 (original Fig. S1A-C") and Fig. S2 (original Fig. S1D,D').

5. We replaced the images showing the expression of *Meis* at the neurula stage (Fig. S3).

6. We thank the reviewer for the suggestion. However, because we focused on the regulatory mechanisms of *Fer2* expression that is more specific to the DA-lineage cells than *Meis*, we did not newly examine the *Meis* expression pattern in the embryos treated with U0126. We did not also investigate the *Th* expression in U0126-treated embryos because Horie et al. (2018) reported that *Th* reporter was not expressed in the *Fer2* morphants. Loss of *Th* expression in the embryos treated with U0126 is predicted.

7. We could not examine the activation of MAPK pathway in the embryos treated with inhibitor of FGF receptor, SU5402, because the cell division in the row III of the neural plate was inhibited by the chemical. But we newly described the results in the manuscript (data not shown) (lines 262-265). We also added the results that the detection of dpERK in the embryos overexpressing the dominant negative form of *Eph.a* receptor in the neural plate (Fig. S4B,C) (lines 298-302).

8. We agree with this comment. However, we did not examine whether knockdown of *Otx* affects *Meis* expression because our present study focused on the regulatory mechanisms of *Fer2* expression that show the specific expression in the DA-lineage cells. We investigated whether MAPK pathway regulate the expression of *Otx* in the DA-lineage cells by the treatment with U0126 (Fig. 6E,F) (lines 319-321).

Minor points:

9. We added explanation that G-GECO1.1 is one of the EGFP variants in initiation of the results section and referred "G-GECO1.1" to "EGFPv" for distinguishing GECO1.1 from EGFP (line 122).

10. We removed the arrowheads in Figure 2L-L".

11. We added and described unique gene collection IDs and KH gene model IDs for all the genes in the Materials and Methods section (lines 570-575).

Other changes:

1. We added movie titles and captions in the supplemental file. We moved the legends of supplementary figures to the supplementary information file.

2. We revised references section (lines 677-679,687-690,708-711,751,752,813-815).

3. The descriptions of PH-GFP and pSP-*Etr*>dn*Eph.a* were added (lines 521- 548,620,621).

Second decision letter

MS ID#: DEVELOP/2020/198754

MS TITLE: The complete cell lineage and MAPK- and Otx-dependent specification of the dopaminergic cells in the *Ciona* brain

AUTHORS: Kouhei Oonuma and Takehiro G. Kusakabe

I have now received of two of the referees who reviewed the earlier version of your manuscript and I have reached a decision. The reports are appended below and you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining suggestions and comments of the two referees. Please attend to all these comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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

Reviewer 2

Advance summary and potential significance to field

Oonuma and Kusakabe present their revised version of their analysis of the complete cell lineage of the dopaminergic (DA) cells in the Ciona brain and the deciphering of regulatory mechanisms that specifically generate the DA cells. Thanks to the comments of the different reviewers and the revisions of authors which have overall been conducted with care, the quality of the manuscript has really been improved and I therefore support the publication of this study in Development.

Comments for the author

Three minor revisions that do not require much effort would improve the manuscript:
1) The use of hpf is confusing for non-specialists (and varies with the temperature..). I suggest indicating whenever possible the Hotta stage in addition to the more precise but less intuitive hpf.
2) Fig S4: The results presented here to show that the ephrin-Eph signaling does not inhibit the MAPK pathway in the a10.74 cells could be more convincing if for S4-B and for S4-C the dp-ERK channels were also presented alone (not merged), allowing to see clearly that there is no signal in the a10.74 cells.
3) Fig S4: The overall dp-ERK stainings are quite different between the control panels A" and B. For example, the 4 cells anterior to the a10.74 cells in A" show a very intense signal which contrast to the weaker signal observed in the same cells in B, and also in C. How do the authors explain these differences? could they reflect slightly different developmental stages? If the immunostaining of dp-

ERK is variable, how can we be sure that there is no effect of the dnEph.a in the a10.74 cells? It would be useful if the authors could discuss this point.

Reviewer 3

Advance summary and potential significance to field

In this manuscript Oonuma and Kusakabe have leveraged some of the advantages of Ciona (i.e. the ability to obtain very accurate cell lineages and understand cell fate at the single-cell level) to obtain mechanistic insight on the specification of dopaminergic cells. Their work is of substantial importance to the field as they have determined the complete cell lineage of the Ciona larva dopaminergic cells. In addition, they show that the MAPK signalling pathway and the transcription factor Otx are essential for the expression of Fer2, an important transcription factor that regulates dopamine synthesis genes.

Comments for the author

I am mostly satisfied with the revisions performed by the authors and their responses to my comments. I understand the challenges of performing the suggested live imaging experiments(response to reviewer 3 point 4)and appreciate the fact that the authors attempted this experiment, even if not successful. Therefore, I am happy to recommend this manuscript for publication in Development with a couple of minor changes.
Minor points:
1.There is no statistical test for Figure S8. Even though visually it seems like there is a very clear difference between a10.73 and a10.74 it would be wise to have some stats confirming this.
2. In their response to reviewers the authors mention that in Figure 3 E,G,I they highlight the embryo mid line (i am assuming they are referring to the yellow dashed line in these panels) but there is no explanation of what this line indicates in the main text or the figure legend. It would be great if the authors could add this in the figure legend.

Second revision

Author response to reviewers' comments

Following is a detailed description of our responses to the reviewer’s comments and other changes in the revised manuscripts.

Reviewer 2:

1. According to the reviewer’s suggestion, we added information of FABA stages corresponding with embryonic stages in "hpf" in Figures (Fig. 3-6,S3-S6). We also explained the use of these two types of the developmental stages in the materials and methods section (lines 513-514).

2. We prepared and added images of both nucleus and the dpERK signal to Figure S4 (new Fig. S4B,B’,C,C’). These images clearly show that MAPK pathway was not active in a10.74 cells of the embryos in which dn*Eph.a* was overexpressed.

3. We noticed that there was a moderate variation of dpERK signal intensity among control embryos particularly in those four cells in the row anteriorly adjacent to the a10.74 cells. This variation seems to be due to the difference in phases of the cell cycle of these cells. Cells just after the division tend to exhibit a weaker dpERK signal, which is the case for the specimen shown in Fig.

S4B. We think this is the main reason why dpERK patterns looked different between Fig. S4A" and B. However, dpERK signals were constantly observed in other cells, particularly in the a10.65 and a10.97 cells located left and right sides of the a10.73 cells. In the embryos overexpressing dn*Eph.a*, dpERK signals in these cells were stronger than in control embryos, whereas dpERK was not detected in the a10.74 cells. This result was highly reproducible (n=25).

Reviewer 3:

Minor points:

1. We performed an unpaired Student's *t*-test for comparison of the cell volume between a10.73 and a10.74 cells and added this explanation to "materials and methods" (lines 629-631) and the Figure S8 legend.

2. We added the explanation of yellow dashed lines indicating the mid line of the embryos in Figure 3 E,G,I to the figure legend (lines 896-897).

Other changes:

1. We added the explanation of "iTB" and "lN" in the legend for Figure S3.

Third decision letter

MS ID#: DEVELOP/2020/198754

MS TITLE: The complete cell lineage and MAPK- and Otx-dependent specification of the dopaminergic cells in the *Ciona* brain

AUTHORS: Kouhei Oonuma and Takehiro G. Kusakabe
ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.