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Two-Round Ca^{2+} transients in papillae by mechanical stimulation induces metamorphosis in the ascidian *Ciona intestinalis* type A

Maiki K. Wakai, Mitsuru J. Nakamura, Satoshi Sawai, Kohji Hotta and Kotaro Oka

Article citation details
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Review timeline

Original submission:	2 April 2020
1st revised submission:	19 May 2020
2nd revised submission:	4 January 2021
3rd revised submission:	21 January 2021
Final acceptance:	21 January 2021

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSPB-2020-0738.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
Wakai et al. is a very interesting study, using a novel method to immobilize ascidian larva and manipulate its papilla mechanically, coupled to calcium imaging by Gcamp. This allowed the authors to describe for the first time several steps and phases of calcium dynamics in metamorphosis in ascidians. It is especially intriguing that they report calcium dynamics not only in neural tissue but also in other tissues involved in metamorphosis like epidermis or gut. These findings are novel and very interesting for the understanding of larva metamorphosis, which is not well understood. I recommend this to be published with very minor revisions following my comments:
1. The "two-step" is misleading because there seems to be several steps that the authors have divided into two phases, which is confusing. It would be better to alter the title to emphasize the diversity of calcium transients shown here.
2. There is very little discussion of connection between Phase I and Phase II. How do the authors think these phases relate to one another? Is it that there is a threshold of calcium in Phase I needed to activate Phase II? This is what it suggested, but there was no clear discussion of this in the discussion. If the authors believe there is no causal connection, then this should also be discussed.

Review form: Reviewer 2

Recommendation
Major revision is needed (please make suggestions in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Good

General interest: Is the paper of sufficient general interest?
Acceptable

Quality of the paper: Is the overall quality of the paper suitable?
Acceptable

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

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Is it accessible?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
The experiments are well designed and this during one of the most difficult stages of the ascidian life cycle. The authors described well (timing and tissues identification) the CA2+ transient in larval trunk but did not try to integrate this process with the previous identified and published molecular events that occur during the ascidian metamorphosis. The paper lacks functional approach and the bibliography cited in the manuscript is sometimes not updated or not the most accurate. Without additional data, this paper is not appropriate for publication, some major revisions are suggested in the following report. (See Appendix A)

Review form: Reviewer 3

Recommendation
Major revision is needed (please make suggestions in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

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Is it accessible?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
The manuscript by Wakai et al. describes in great detail the Ca2+ transients in Ciona larvae that follow substrate adhesion and links them to tissue remodeling at the beginning of metamorphosis to the juvenile. This paper is in continuation of a previous study by the same lab looking at Ca2+ transients during earlier embryonic stages upon mRNA injection of a the GCaMP6s sensor and using sophisticated equipment to achieve important resolution in time and optical sensitivity. In this paper the authors approach the more difficult swimming larvae that are normally challenging to image due to their tail movements, a problem the authors solve nicely by adhering the larvae laterally to the culture dish. Papillar substrate adhesion is then mimicked artificially for defined lengths of time. By such controlled manipulations the authors obtain continuous time lapse movies to define two phases of Ca2+ transients that are functionally linked and required for metamorphic tissue remodeling (i.e. tail retraction).

The manuscript provides important novel insights about the role of Ca2+ transients to link external cues to inner signals that control metamorphosis through precise timing and targeting of specific tissues. The study opens the way to study the role of Ca2+ transients in other organisms at an equivalent developmental stage. The manuscript is well written, clearly understandable and appropriately structured. I would recommended publication given the following points are adressed to improve the overall quality.

Major points
- The authors nicely show that even short papillar stimulation produces the Phase I Ca2+ transients. Tail retraction, however, requires longer stimulation and is associated with slower Phase II Ca2+ transients. To verify that Phase II Ca2+ transients are causal for tail retraction they could be artificially blocked during Phase II despite a sustained papillar stimulation. For this, Ca2+ retrieval, blockers or chelators could be utilized.
- The importance of these types of Ca2+ transients is discussed very little in a more global context and examples in other species would be nice where such mechanism possibly apply (unless nothing is known – then this may be stated, too). Would it be possible to make any predictions from the presented data ?

Minor points
- Backward movements of epidermal cells are suggested to happen just prior to tail

retraction – this piece of data is not very convincingly presented. Arrows could help pointing on respective cells in Supplement movie 2 and relative fix points/cells not moving backwards may be compared.
- A scheme and/or bright field image would help in Fig.S1 to recognize tissues and structures.
- Fig.1 legend: the sentence ‚The larval tail can avoid papillary adhesion' is unclear.
- Fig.2 legend, second line ‚up until adhesion' is unclear (wrong?) in this context. (H), (I) the ‚blue' triangles seem to be rather ‚black'.
- Fig. 3B would need contours in all the fluorescent images.
- In Fig.5, the larvae in A and B seem to be different stages as seen by the shape of the trunc and the area of the preoral lobe, that is not well comparable/defined.
- (The A/P orientation of Fig.6 is inverted. This may be ok though.)
- Table S1 and Fig.6 may need labeling of the x/y axes.
- Fig. S4 and Table S3: please explain better in the legend what the values, numbers and abbreviations represent and how they were generated.
- Fig.6 legend (6): replace ‚whole epidermal cells' by ‚entire epidermis', for example.
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- Same for various typos (‚tail regressiontail regression' on p.3 at least 3-4 times)
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- The dorsal subregion of the posterior sensory vesicle is not ‚within' the epidermis but rather ‚beneath' (p.7 and twice on p.9).
- Please correct what pATEN should stand for (p.10 bottom).
- Several references are incomplete, lack the year or page numbers etc.

Decision letter (RSPB-2020-0738.R0)

01-May-2020

Dear Dr Hotta:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

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When submitting your revision please upload a file under "Response to Referees" - in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

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If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

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It is a condition of publication that you make available the data and research materials supporting the results in the article. Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

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http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

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All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

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Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Professor Gary Carvalho
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Below you will find our comments from the reviewers. All appreciated the challenging experimental approach, but have made explicit requests in shoring up the claims made in the paper. We would like to offer you the opportunity to thoroughly address the reviewer's concerns.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
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Referee: 2

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Referee: 3

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- Please correct what pATEN should stand for (p.10 bottom).
- Several references are incomplete, lack the year or page numbers etc.

Author's Response to Decision Letter for (RSPB-2020-0738.R0)

See Appendix B.

RSPB-2020-0738.R1 (Revision)

Review form: Reviewer 2

Recommendation
Major revision is needed (please make suggestions in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Good

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors well reply to almost of my concerns and better integratd their results in previous one on metamorphosis.
However, I am still not convinced by the reply of the authors on the U0126 and Fox G experiment. With a good timing for the MEK inhibitor addition it is a feasible experiment , It's really not challenging. It is routine experiment in Ciona community.
Otherwise the Fox G morpholino is still an option.

Review form: Reviewer 3

Recommendation
Accept as is

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors have adressed my concerns sufficiently for publication.

Decision letter (RSPB-2020-0738.R1)

05-Jun-2020

Dear Dr Hotta:

I am writing to inform you that your manuscript # RSPB-2020-0738.R1 entitled "Two-Round Ca²⁺ Transients in Papillae by Mechanical Stimulation Induces Metamorphosis in the Ascidian, Ciona robusta." has been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. It is unusual that we provide another round of opportunity for major changes, but there is a consensus, that your manuscript does contain potential. However, as you will see from the 2nd referee, and I endorse the concern, your response in relation to the lack of a functional demonstration in your work, and empirical support, does compromise the current robustness and likely impact of inferences. I would ask for your careful consideration, in relation to the request for additional data. however, I do recognise that the request may go beyond your original plans, and therefore be beyond the scope for the current manuscript, and you may decide on an alternative publication route. If you do choose to resubmit your manuscript, please upload the following:

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Sincerely,

Professor Gary Carvalho
Editor, Proceedings B
proceedingsb@royalsociety.org

Reviewer(s)' Comments to Author:
Referee: 3

Comments to the Author(s)
The authors have adressed my concerns sufficiently for publication.

Referee: 2

Comments to the Author(s)
The authors well reply to almost of my concerns and better integratd their results in previous one on metamorphosis.
However, I am still not convinced by the reply of the authors on the U0126 and Fox G experiment. With a good timing for the MEK inhibitor addition it is a feasible experiment , It's really not challenging. It is routine experiment in Ciona community.
Otherwise the Fox G morpholino is still an option.

Author's Response to Decision Letter for (RSPB-2020-0738.R1)

See Appendix C.

RSPB-2020-3207.R0

Review form: Reviewer 2

Recommendation
Accept as is

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors have adressed all my concerns and the manuscript could be published in this last revised form.

Decision letter (RSPB-2020-3207.R0)

20-Jan-2021

Dear Dr Hotta

I am pleased to inform you that your Review manuscript RSPB-2020-3207 entitled "Two-Round Ca²⁺ Transients in Papillae by Mechanical Stimulation Induces Metamorphosis in the Ascidian, Ciona intestinalis type A" has been accepted for publication in Proceedings B.

The referee(s) do not recommend any further changes. Therefore, please proof-read your manuscript carefully and upload your final files for publication. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let me know immediately.

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All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the

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Once again, thank you for submitting your manuscript to Proceedings B and I look forward to receiving your final version. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,
Professor Gary Carvalho
mailto:proceedingsb@royalsociety.org

Associate Editor
Board Member
Comments to Author:
We are pleased to inform you that your article has been accepted with minor revisions. Please keep an eye on your email for further information in proceeding towards publication.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
The authors have addressed all my concerns and the manuscript could be published in this last revised form.

Sincerely,
Proceedings B
mailto: proceedingsb@royalsociety.org

Decision letter (RSPB-2020-3207.R1)

21-Jan-2021

Dear Dr Hotta

I am pleased to inform you that your manuscript entitled "Two-Round Ca²⁺ Transients in Papillae by Mechanical Stimulation Induces Metamorphosis in the Ascidian, Ciona intestinalis type A" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

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If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

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All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

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Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,
Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

The authors investigate how larval adhesion through adhesive papillae triggers metamorphosis onset and tail regression in the ascidian *Ciona robusta*. More precisely they aim to investigate how larvae sense mechanical cues during settlement. They first developed an experimental system that allows manage larva adhesion management and they follow CA2+ modulation in the trunk of the larva using a calcium sensor protein. They identify two calcium transients called phase I and phase II, both affect papillae but also endoderm and epidermis respectively. The first phase depends on mechanical stimulus of the papillae and the second precedes the onset of tail regression and is accompanied by backward epidermis trunk movement.

The experiments are well designed and this during one of the most difficult stages of the ascidian life cycle. The authors described well (timing and tissues identification) the CA2+ transient in larval trunk but did not try to integrate this process with the previous identified and published molecular events that occur during the ascidian metamorphosis. The paper lacks functional approach and the bibliography cited in the manuscript is sometimes not updated or not the most accurate. Without additional data, this paper is not appropriate for publication, some major revisions are suggested in the following report.

Major point:

Abstract

The sentence, “Our results indicate that the papillae sense a mechanical cue and two-step Ca^{2+} transient in papillae transmits the Ca^{2+} signaling to different tissues, which subsequently induces metamorphosis”, is confusing. Since the fixation of the larvae through adhesive papillae is the first event of metamorphosis, the second part of the sentence is not correct “which subsequently induces metamorphosis”. Moreover, what the authors observed is Ca2+ transients (not signaling) which subsequently induces tail regression which is one of the events of metamorphosis. In addition, in the last sentence of the background part the authors mentioned “…. we have observed Ca2+ dynamics during metamorphosis…..” which confirms the inaccuracy of this sentence.

Figures

The authors used 3 microscopy methods (Materials and methods (e)). So, in each figure legend in which microscopic observations are presented, the microscopy method should be mentioned. It is mentioned only in figure S2 and video S4 legends.

Results:

(b) the authors reported a trunk epidermal movement towards the posterior at the onset of tail regression illustrated by figure S2 and Video S1 from 0:09:41.360.

The figure S2 is not convincing, first because the labelling fluctuates during the experiment (due to CA2+ oscillation), then it misses the complete picture of the trunk at 11.2 mn. The authors should give a spatial reference to compare the 2 time points and visualize the movement. Concerning the video S2, here again given the definition and the big fluctuation in fluorescence due to CA2+ modulation, it is not possible to visualize correctly this backward movement. The authors should use other labelling

with fixed intensity to illustrate this movement. In addition to generalize this event as "….the first observable change in the initiation of *C. robusta* metamorphosis….." the authors have to better illustrate and then repeat this observation on a statistical representative number of metamorphic larva.

Discussion

Papillary cells activated by mechanical stimulation. In this paragraph the authors mention FoxG expressed in larval papillae as the potential regulator of the mechanosensing process in this tissue during adhesion. During embryogenesis, previous FoxG expression is reported in neural plat cells under the control of the MAPK/ERK signaling pathway (Liu and Satou, Nat Commun. 2019; 10: 4911). In addition, the MAPK ERK is activated in papillae during the swimming period (Chambon et al., Development 2007 134: 1203-1219). Testing the possibility of reactivation of FoxG expression by MAPK/ERK signaling in palps before adhesion is an easy experiment to conduct, and will give rise to a simple functional study using the classical MAPK inhibitor U0126 and evaluate the effect on mechanosensing and CA2+ transient. In addition to fill the missing functional aspect of the manuscript it will also integrate the CA2+ transients in the previously reported molecular events of the ascidians metamorphosis instead of just add a new one.

The last part of the discussion concludes on the tail epidermis contraction hypothesis to explain the tail regression during ascidian metamorphosis, a numerous other hypothesis were proposed and a major mechanism experimentally proved was reported, which is based on apoptosis (Chambon et al., 2002 and 2007 Krasovec et al., 2019) and none of them were discussed here.

Minor points:

Background:

Sentence 1, the authors mixed names of class, phylum and barnacles, it should necessary to homogenize the names to the same classification level.

Sentence 3, "Therefore, it was thought that a specific organ is responsive to external cues and transduces them to internal organs for the subsequent metamorphosis". Some references are needed here to justify this assumption.

Line 10: "After the papillae-mediated adhesion to a substrate, ascidian metamorphosis is characterized by tail regression [7,14]" Chambon et al., Development 2002 129: 3105-3114 is a much more accurate reference.

Line 13 regression tail repeated twice two times.

Results

Figure 2: Gut to define undifferentiated endoderm in larva is not appropriate.

Legend figure 1 (E): “the posterior trunk epidermis moved backward” remove this sentence, it is not observable on this figure, so it should not be mentioned in the figure legend.

Appendix B

Response to editor and reviewers' comments

The response is written in red.

Associate Editor
Board Member: 1
Comments to Author:
Below you will find our comments from the reviewers. All appreciated the challenging experimental approach, but have made explicit requests in shoring up the claims made in the paper. We would like to offer you the opportunity to thoroughly address the reviewer's concerns.

Dear Dr. Carvalho: Associate Editor

We really thank you for taking your time to our paper and offering us the opportunity to improve our manuscript. We carefully considered the reviewers' comments and modified our manuscript accordingly. We addressed all comments below. We have sincerely endeavoured to deal with reviewers' every concern in great detail. We are, however, sorry that considering previous studies and our unpublished results, some requested experiments cannot be achieved by their technical difficulties. Instead of that, we showed detailed explanation for each request to convince reviewers, and we hope our responses will satisfy the reviewers. With the changes to our final manuscript, we hereby resubmit our manuscript for your evaluation again. We trust our revised manuscript is suitable for publication. Thank you once again for your consideration of our paper.

Sincerely,

Kohji HOTTA and Maiki K. WAKAI

Reviewer(s)' Comments to Author:

Referee: 1
Comments to the Author(s)
Wakai et al. is a very interesting study, using a novel method to immobilize ascidian larva and manipulate its papilla mechanically, coupled to calcium imaging by Gcamp. This allowed the authors to describe for the first time several steps and phases of calcium dynamics in metamorphosis in ascidians. It is especially intriguing that they report calcium dynamics not only in neural tissue but also in other tissues involved in metamorphosis like epidermis or gut. These findings are novel and very interesting for the understanding of larva metamorphosis, which is not well understood. I recommend this to be published with very minor revisions following my comments:

Thank you for your encouraging comments on our paper. We feel the comments have helped us explain our findings clearly. We carefully considered your comments and provided the answers to each in red below.

1. The "two-step" is misleading because there seems to be several steps that the authors

have divided into two phases, which is confusing. It would be better to alter the title to emphasize the diversity of calcium transients shown here.
Thank you for your pointing out of the confusion of the title. We changed the title as "Two-Round Ca^{2+} Transients in Papillae by Mechanical Stimulation Induces Metamorphosis in the Ascidian, *Ciona robusta*.".

2. There is very little discussion of connection between Phase I and Phase II. How do the authors think these phases relate to one another? Is it that there is a threshold of calcium in Phase I needed to activate Phase II? This is what it suggested, but there was no clear discussion of this in the discussion. If the authors believe there is no causal connection, then this should also be discussed.
As for connection between Phase I and Phase II, we added discussion below as a second paragraph in Discussion.
"Is there a causal connection between Phase I and Phase II? We consider there is a *temporal threshold* of Ca^{2+} in Phase I that is prerequisite for activation of Phase II. From our results, less than 10s stimulation induce only Phase I whereas average 12 min continuous stimulation induced both Phase I and Phase II Ca^{2+} transients. In our experimental system, Phase II is only observed after Phase I occurs (Table 1). Therefore, we think Phase I and II are tightly coupled also in natural condition".

We wish to thank you again for your valuable comments.

Referee: 2
Comments to the Author(s)
The authors investigate how larval adhesion through adhesive papillae triggers metamorphosis onset and tail regression in the ascidian Ciona robusta. More precisely they aim to investigate how larvae sense mechanical cues during settlement. They first developed an experimental system that allows manage larva adhesion management and they follow CA2+ modulation in the trunk of the larva using a calcium sensor protein. They identify two calcium transients called phase I and phase II, both affect papillae but also endoderm and epidermis respectively. The first phase depends on mechanical stimulus of the papillae and the second precedes the onset of tail regression and is accompanied by backward epidermis trunk movement.
The experiments are well designed and this during one of the most difficult stages of the ascidian life cycle. The authors described well (timing and tissues identification) the CA2+ transient in larval trunk but did not try to integrate this process with the previous identified and published molecular events that occur during the ascidian metamorphosis. The paper lacks functional approach and the bibliography cited in the manuscript is sometimes not updated or not the most accurate. Without additional data, this paper is not appropriate for publication, some major revisions are suggested in the following report.
We wish to express our strong appreciation to you for your valuable advices on our paper. The comments have helped us to integrate our manuscript with the previous knowledge. We carefully considered your comments and offered the explanations to each in red below. According to your advices, we integrated our manuscript with the previously identified and published molecular events by adding appropriate references.

Major point:
Abstract
The sentence, "Our results indicate that the papillae sense a mechanical cue and two-step Ca2+ transient in papillae transmits the Ca2+ signaling to different tissues, which subsequently induces metamorphosis", is confusing. Since the fixation of the larvae through adhesive papillae is the first event of metamorphosis, the second part of the sentence is not correct "which subsequently induces metamorphosis". Moreover, what the authors observed is Ca2+ transients (not signaling) which subsequently induces tail

regression which is one of the events of metamorphosis. In addition, in the last sentence of the background part the authors mentioned "…. we have observed Ca2+ dynamics during metamorphosis….." which confirms the inaccuracy of this sentence.

Thank you for your suggestion. We changed the last 2 sentences in Abstract according to your advice as follows.

"Our results indicate that the papillae sense a mechanical cue and two-round Ca^{2+} transients in papillae transmits the internal metamorphic signals to different tissues, which subsequently induces tail regression which is one of the events of metamorphosis. Our study will help elucidate the internal mechanism of metamorphosis in marine invertebrate larvae in response to environmental cues".

In response to your suggestion, we also revised the penultimate sentence of the background part as follows.

"…we have observed Ca^{2+} dynamics at the beginning of the metamorphosis".

Figures

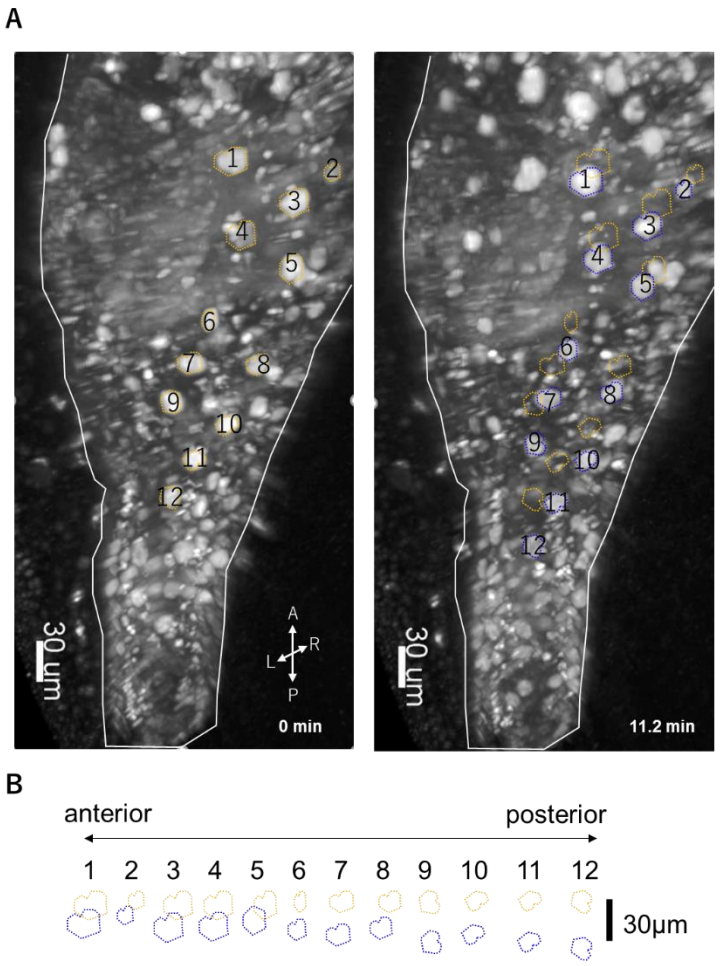
The authors used 3 microscopy methods (Materials and methods (e)). So, in each figure legend in which microscopic observations are presented, the microscopy method should be mentioned. It is mentioned only in figure S2 and video S4 legends.

In each figure legend, we added the microscopy method.

Results:

(b) the authors reported a trunk epidermal movement towards the posterior at the onset of tail regression illustrated by figure S2 and Video S1 from 0:09:41.360. The figure S2 is not convincing, first because the labelling fluctuates during the experiment (due to CA2+ oscillation), then it misses the complete picture of the trunk at 11.2 mn. The authors should give a spatial reference to compare the 2 time points and visualize the movement. Concerning the video S2, here again given the definition and the big fluctuation in fluorescence due to CA2+ modulation, it is not possible to visualize correctly this backward movement. The authors should use other labelling with fixed intensity to illustrate this movement. In addition to generalize this event as "….the first observable change in the initiation of C. robusta metamorphosis….." the authors have to better illustrate and then repeat this observation on a statistical representative number of metamorphic larva.

As we received your suggestion, we prepared data for easily understanding the trunk epidermal movement. The complete pictures of the posterior trunk region were added to Fig. S2 as follows. It helps convince you of the trunk epidermal movement with the spatial references of whole trunk.



The legend Fig. S2 changed as follows.
"(A) Epidermal cells labelled by H2B-GCaMP6s were visualised by LSM. The trunk epidermal cells (yellow dotted regions) moved posteriorly (blue dotted regions) 11.2 min later during the initiation of tail regression. The epidermal cells are numbered in order from the anterior part. (B) The distance of the movement of each cell in (A)."
In addition, we showed additional movie as Video S2. We added the following Video S2 legend.
"In this video, each nucleus was labelled with GCaMP6s-H2B and visualized by Light Sheet Microscopy (LSM). The distance of movement of each cell was measured in Fig. S2."

Discussion
Papillary cells activated by mechanical stimulation. In this paragraph the authors mention FoxG expressed in larval papillae as the potential regulator of the mechanosensing process in this tissue during adhesion. During embryogenesis, previous FoxG expression is reported in neural plat cells under the control of the MAPK/ERK signaling pathway (Liu and Satou, Nat Commun. 2019; 10: 4911). In addition, the MAPK ERK is activated in papillae during the swimming period (Chambon et al., Development 2007 134: 1203-

1219). Testing the possibility of reactivation of FoxG expression by MAPK/ERK signaling in palps before adhesion is an easy experiment to conduct, and will give rise to a simple functional study using the classical MAPK inhibitor U0126 and evaluate the effect on mechanosensing and CA2+ transient. In addition to fill the missing functional aspect of the manuscript it will also integrate the CA2+ transients in the previously reported molecular events of the ascidians metamorphosis instead of just add a new one.
Thank you for your nice idea and for the list of appropriate references. We added the possibility of the reactivation of FoxG which might be regulated one of previously proposed ascidian metamorphosis mechanism, MAPK/ERK pathway in the last part of Discussion as follows.
"During embryogenesis, FoxG expression in neural plate cells are controlled by the MAPK/ERK. In addition, the MAPK/ERK signalling is also activated in papillae during the swimming period [36]. The reactivation of FoxG in papillae by MAPK/ERK signalling before adhesion might integrate the Ca^{2+} transients in the previously reported molecular events of the ascidian metamorphosis. Future studies will elucidate the mechanosensing molecule that is the downstream target of FoxG."
And we also thank you the experimental ideas for supporting our discussion. The suppression experiments using U0126 could give a variety of results because the pathway has many points on which this compound affects (Kourakis et al., Dev Biol. 2007 312(1): 245-257, Sakabe et al., Dev Growth Differ. 2006 48(6): 391-400, and Hudson et al., Development 2003 130: 147-159). Actually, in our laboratory, we have been dealt with such kind of inhibitory compounds and we know that unexpected results can be obtained without working to the intended pathway (Mizotani et al., Biochem Biophys Res Commun. 2015 463(4): 656-660). Therefore, we don't think you will be convinced with any positive or negative results.

The last part of the discussion concludes on the tail epidermis contraction hypothesis to explain the tail regression during ascidian metamorphosis, a numerous other hypothesis were proposed and a major mechanism experimentally proved was reported, which is based on apoptosis (Chambon et al., 2002 and 2007 Krasovec et al., 2019) and none of them were discussed here.
Thank you for letting us know important references. We discussed the other previous possibility of the tail epidermis contraction as a penultimate paragraph in Discussion as follows.
"Other hypothesis of the tail epidermis contraction to explain the tail regression during ascidian metamorphosis is based on apoptosis[17, 36, 39]. Krasovec et al., (2019) observed that the tail regression depends on a postero-anterior wave of a caspase-dependent apoptosis coupled with a contraction event. This apoptosis wave might be triggered by the Phase II wave-like Ca^{2+} propagations in epidermis."

"17. Chambon J-P, Soule J, Pomies P, Fort P, Sahuquet A, Alexandre D, Mangeat P-H, Baghdiguian S. 2002 Tail regression in Ciona intestinalis (Prochordate) involves a Caspase-dependent apoptosis event associated with ERK activation. *Development* **126**, 5809–5818.

36. Chambon JP, Nakayama A, Takamura K, McDougall A, Satoh N. 2007 ERK- and JNK-signalling regulate gene networks that stimulate metamorphosis and apoptosis in tail tissue of ascidian tadpoles. Development 134, 1203–1219. (doi:10.1242/dev.002220)

39. Krasovec G, Robine K, Quéinnec E, Karaiskou A, Chambon JP. 2019 Ci-hox12

tail gradient precedes and participates in the control of the apoptotic-dependent tail regression during Ciona larva metamorphosis. *Dev. Biol.* (doi:10.1016/j.ydbio.2018.12.010)"

Minor points:
Background:
Sentence 1, the authors mixed names of class, phylum and barnacles, it should necessary to homogenize the names to the same classification level.
We clarified Sentence 1 as follows.
"The swimming larvae of marine invertebrates, including crustacea (barnacles), molluscs (bivalve molluscs), ascidians, echinoderms (sea urchins and sea cucumber) and Annelida (polychaetes), eventually settle into the substratum and begin metamorphosis [1–5]."

Sentence 3, "Therefore, it was thought that a specific organ is responsive to external cues and transduces them to internal organs for the subsequent metamorphosis". Some references are needed here to justify this assumption.
We added some references to Sentence 3.
"Therefore, it was thought that a specific organ is responsive to external cues and transduces them to internal organs for the subsequent metamorphosis [12–14]."

"12. Hadfield MG, Meleshkevitch EA, Boudko DY. 2000 The apical sensory organ of a gastropod veliger is a receptor for settlement cues. *Biol. Bull.* **198**, 67–76. (doi:10.2307/1542804)

13. Murabe N, Hatoyama H, Komatsu M, Kaneko H, Nakajima Y. 2007 Adhesive papillae on the brachiolar arms of brachiolaria larvae in two starfishes, Asterina pectinifera and Asterias amurensis, are sensors for metamorphic inducing factor(s). *Dev. Growth Differ.* **49**, 647–656. (doi:10.1111/j.1440-169X.2007.00959.x)

14. Leise EM, Kempf SC, Durham NR, Gifondorwa DJ. 2004 Induction of metamorphosis in the marine gastropod Ilyanassa obsoleta: 5HT, NO and programmed cell death. In *Acta Biologica Hungarica*, pp. 293–300. Acta Biol Hung. (doi:10.1556/ABiol.55.2004.1-4.35)"

Line 10: "After the papillae-mediated adhesion to a substrate, ascidian metamorphosis is characterized by tail regression [7,14]" Chambon et al., Development 2002 129: 3105-3114 is a much more accurate reference.
With your suggestion, we changed to more suitable reference as follows.
"After the papillae-mediated adhesion to a substrate, ascidian metamorphosis is characterised by tail regression [7,17]."

"17. Chambon J-P, Soule J, Pomies P, Fort P, Sahuquet A, Alexandre D, Mangeat P-H, Baghdiguian S. 2002 Tail regression in Ciona intestinalis (Prochordate) involves a Caspase-dependent apoptosis event associated with ERK activation. *Development* **126**, 5809–5818."

Line 13 regression tail repeated twice two times.
We revised the typographic error.

Results
Figure 2: Gut to define undifferentiated endoderm in larva is not appropriate.
Thank you for your comment. The term ‘Gut’ changed to ‘digestive tract’ according to previous study.

Legend figure 1 (E): “the posterior trunk epidermis moved backward” remove this sentence, it is not observable on this figure, so it should not be mentioned in the figure legend.
We agree with your advice. We removed the mentioned sentence.

Thank you again for your comments on our paper. We trust that the revised manuscript is suitable for publication.

Referee: 3
Comments to the Author(s)
The manuscript by Wakai et al. describes in great detail the Ca2+ transients in Ciona larvae that follow substrate adhesion and links them to tissue remodeling at the beginning of metamorphosis to the juvenile. This paper is in continuation of a previous study by the same lab looking at Ca2+ transients during earlier embryonic stages upon mRNA injection of a the GCaMP6s sensor and using sophisticated equipment to achieve important resolution in time and optical sensitivity. In this paper the authors approach the more difficult swimming larvae that are normally challenging to image due to their tail movements, a problem the authors solve nicely by adhering the larvae laterally to the culture dish.

Papillar substrate adhesion is then mimicked artificially for defined lengths of time. By such controlled manipulations the authors obtain continuous time lapse movies to define two phases of Ca2+ transients that are functionally linked and required for metamorphic tissue remodeling (i.e. tail retraction).

The manuscript provides important novel insights about the role of Ca2+ transients to link external cues to inner signals that control metamorphosis through precise timing and targeting of specific tissues. The study opens the way to study the role of Ca2+ transients in other organisms at an equivalent developmental stage. The manuscript is well written, clearly understandable and appropriately structured. I would recommended publication given the following points are adressed to improve the overall quality.

We wish to express our appreciation to you for your insightful advices which have helped us significantly improve the paper. We carefully considered your comments and offered the explanations to each in red below.

Major points
- The authors nicely show that even short papillar stimulation produces the Phase I Ca2+ transients. Tail retraction, however, requires longer stimulation and is associated with slower Phase II Ca2+ transients. To verify that Phase II Ca2+ transients are causal for tail retraction they could be artificially blocked during Phase II despite a sustained papillar stimulation. For this, Ca2+ retrieval, blockers or chelators could be utilized.
We thank you for this suggestion, but we concern no one can technically realize the experiment that you requested. We cannot prepare any effective methods for suppressing Ca^{2+} changes in spatio-temporary for answering your comment; first, we don’t identify cells in which Ca^{2+} increases and Ca^{2+} resources so we do not choose appropriate inhibitors. Second, the tissues keep change in the developmental process and it is difficult

for a specific application of inhibitors to the appropriate cell. Third, we need to block Ca^{2+} increase within a narrow time window. Ca^{2+} increase in Phase II can be blocked within the time between Phase I and Phase II which is between 7 sec and 3 min from the start of adhesion. However, any drug is effective as soon as it is added or activated in previous researches. Especially, it is difficult for ascidian to bring drugs into the internal body due to their hard tunic of epidermis. For instance, the other researchers waited a day to get results after pharmacological treatments (Kamiya et al., 2014). It is possible to block whole animal Ca^{2+} influx with a few hours by calcium and magnesium free artificial seawater, mibefradil or nifedipine (Akahoshi et al., Dev Biol. 2017 431(2): 205-214, Abdul-Wajid et al., Cell Rep. 2015 13(4): 829-839). However, our research needs *Ca^{2+} blocking at the specific time and in specific cells*. The experiments using inhibitors from outside cannot satisfy the spatio-temporal requirements. Therefore, we find the technical problem that present methods cannot solve no matter how long we spend a time. We would like to realize it by an experiment that controls the Ca^{2+} influx spatiotemporally using optogenetics methods in future.

- The importance of these types of Ca2+ transients is discussed very little in a more global context and examples in other species would be nice where such mechanism possibly apply (unless nothing is known – then this may be stated, too). Would it be possible to make any predictions from the presented data ?

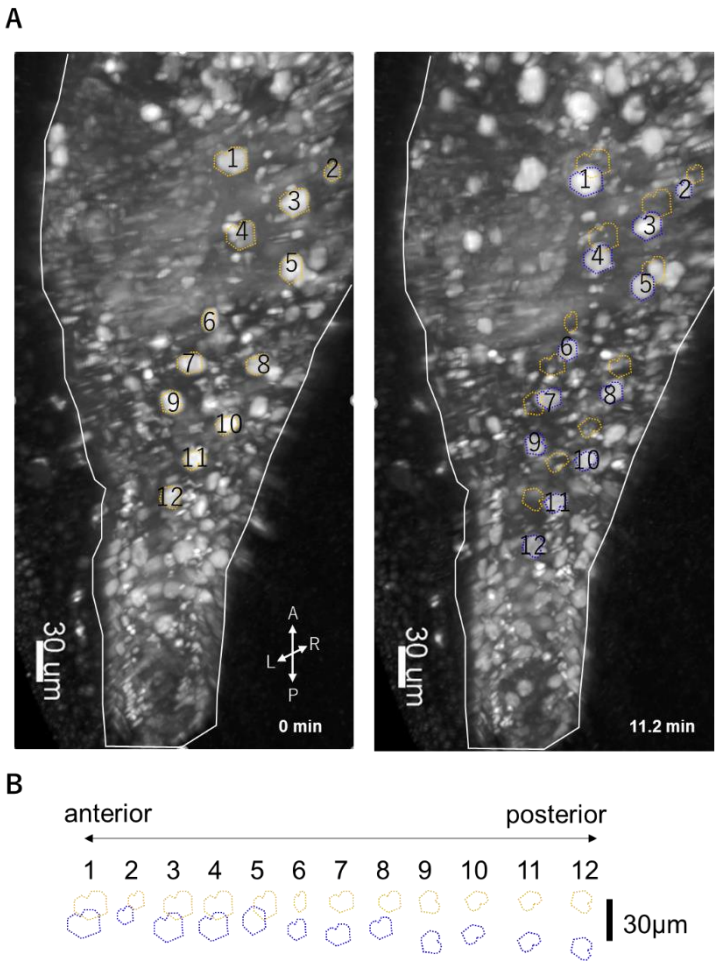
Thank you for your advice. We stated as follows in the last part of Discussion.

“Similar epithelial-conduction model of metamorphic signal propagation has been proposed for the hydrozoan cnidarian *Mitrocomella polydiademata* [36]. Our method developed in this study can be applied to other species to test whether the Ca^{2+} transients that cause metamorphosis are conserved in other marine invertebrates.”

Minor points

- Backward movements of epidermal cells are suggested to happen just prior to tail retraction – this piece of data is not very convincingly presented. Arrows could help pointing on respective cells in Supplement movie 2 and relative fix points/cells not moving backwards may be compared.

In order to respond to your suggestions, we prepared data for easily understanding the trunk epidermal movement. The complete pictures of the posterior trunk region were added to Fig. S2 as follows. It helps convince you of the trunk epidermal movement with the spatial references of whole trunk.

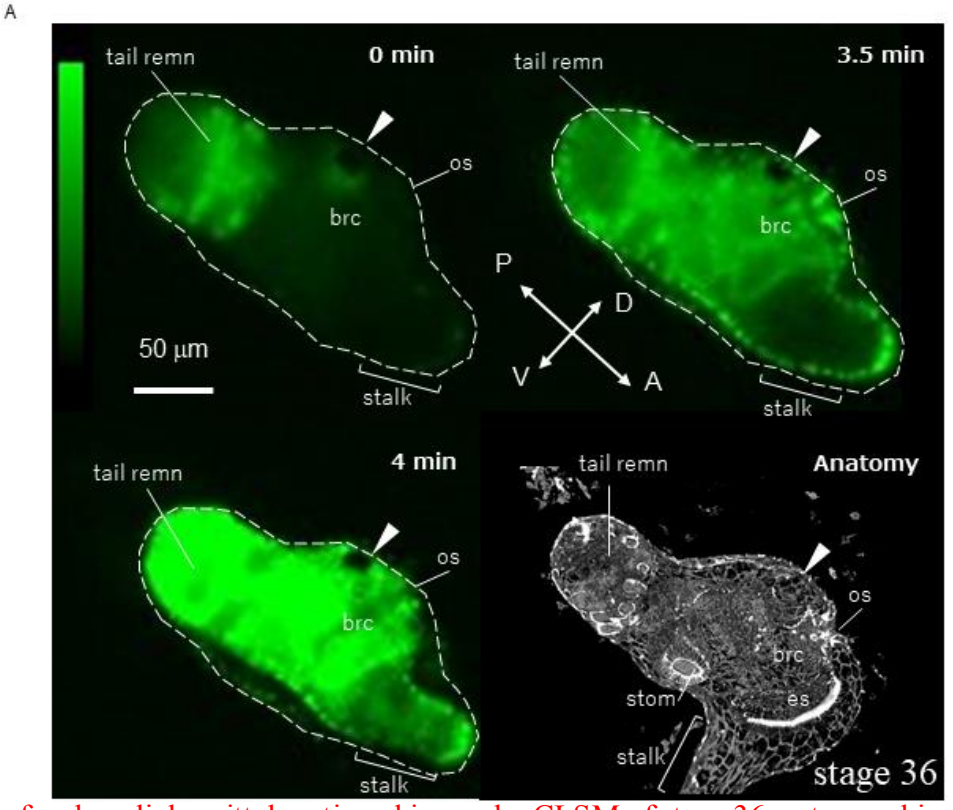


The legend Fig. S2 changed as follows.
"(A) Epidermal cells labelled by H2B-GCaMP6s were visualised by LSM. The trunk epidermal cells (yellow dotted regions) moved posteriorly (blue dotted regions) 11.2 min later during the initiation of tail regression. The epidermal cells are numbered in order from the anterior part. (B) The distance of the movement of each cell in (A)."
In addition, we showed additional movie as Video S2. We added the following Video S2 legend.
"In this video, each nucleus was labelled with GCaMP6s-H2B and visualized by Light Sheet Microscopy (LSM). The distance of movement of each cell was measured in Fig. S2."

- A scheme and/or bright field image would help in Fig.S1 to recognize tissues and structures.

We added a following CLSM image and legend in Fig.S1 for understanding anatomical structures.

Fig. S1 Ca2+ worked after metamorphosis



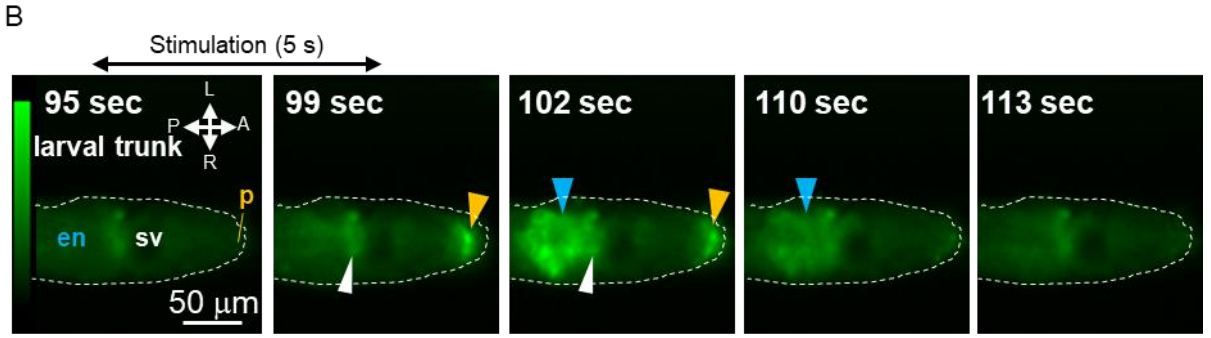
"Confocal medial sagittal sectioned image by CLSM of stage 36 metamorphing larva was shown as anatomical information. Arrowheads show the position of the larval brain remnant. brc: branchial chamber; es: endostyle; os: oral siphon; tail remn: tail remnant."

- Fig.1 legend: the sentence ‚The larval tail can avoid papillary adhesion' is unclear.
We revised the sentence as "The papillae can avoid adhesion."

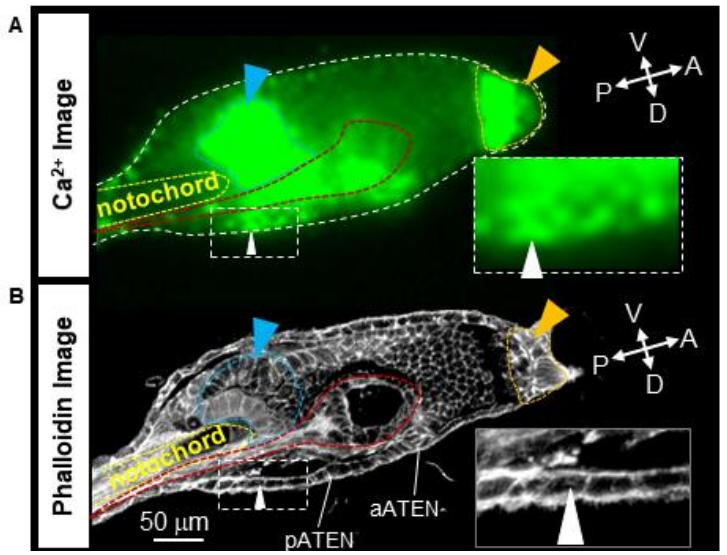
- Fig.2 legend, second line ‚up until adhesion' is unclear (wrong?) in this context. (H), (I) the ‚blue' triangles seem to be rather ‚black'.
We revised the sentence as follows.
"(A)-(F) Ca^{2+} dynamics observed by FM in *C. robusta* from the larval period to adhesion period."
Regarding the expression of the triangle colour, because in our paper all results of the timing of adhesion is represented by a dark blue triangle (Fig. 2, Table S1, Fig. S3), we unified with 'dark blue triangle' as follows.
Fig.2 legend, second line , "In Phase I, short Ca^{2+} transient was observed in both the papillae and endoderm (I) following adhesion (dark blue triangle)."
Fig. S2 legend, second sentence, "The Ca^{2+} transient in palps and trunk in Phase I following adhesion (dark blue triangle)"

- Fig. 3B would need contours in all the fluorescent images.

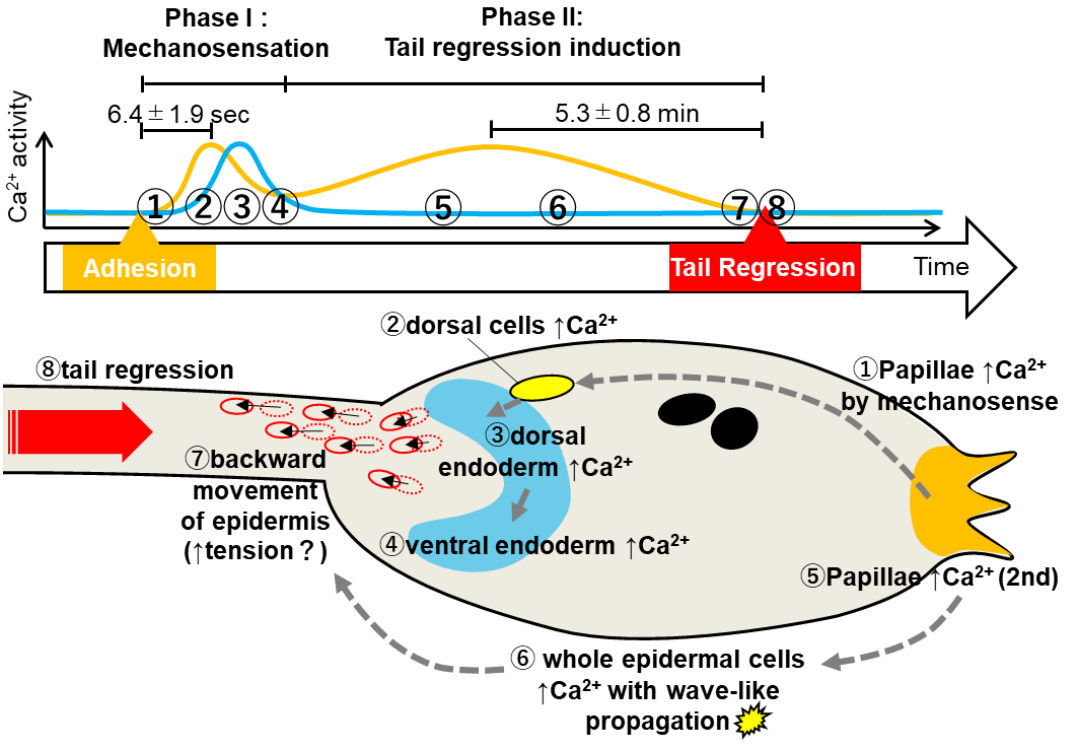
We added contours as you suggested.



- In Fig.5, the larvae in A and B seem to be different stages as seen by the shape of the trunc and the area of the preoral lobe, that is not well comparable/defined.
We replaced to more suitable picture as Fig.5B.



- (The A/P orientation of Fig.6 is inverted. This may be ok though.)
We changed A/P orientation of Fig.6 below.



- Table S1 and Fig.6 may need labeling of the x/y axes.
We added labelling to Table S1 and Fig. 6.

Table S1

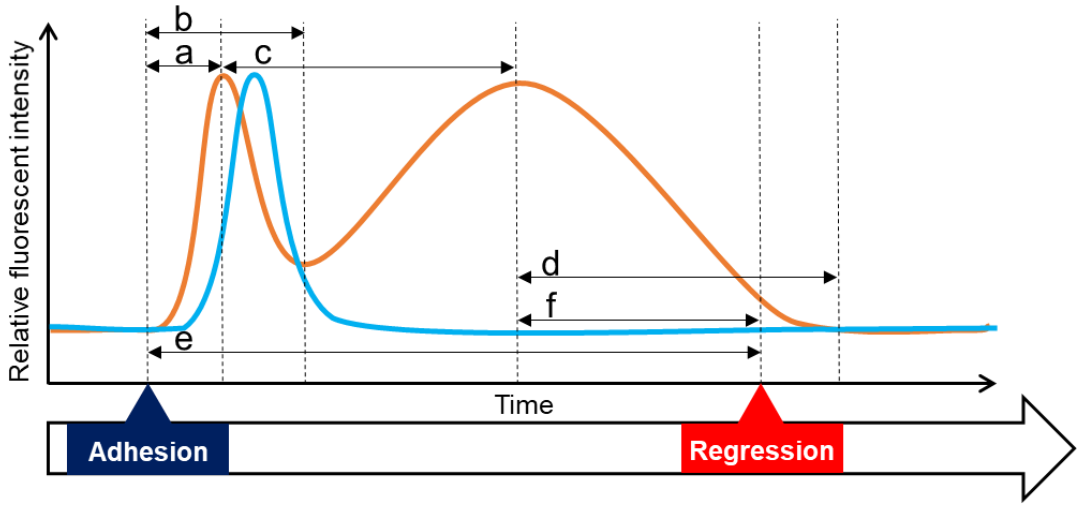


Fig. 6 is shown above.

- Fig. S4 and Table S3: please explain better in the legend what the values, numbers and abbreviations represent and how they were generated.
We added sentences below to the previous legend Fig. S4.
"Each number (#1 - #6) indicates a sample number of larva which completed artificial tail regression. We calculated the correlation coefficient between the relative fluorescent

intensity of papillae in Phase I and that of the trunk region with each time frame delay which is represented on the horizontal axis. ”
For Table S3, we added following sentences to the previous explanation.
“The Ca^{2+} waveform of papillae and the trunk region in Phase I matches most with the delay. Each number indicates a sample number of larva in Fig. S4. Standard error of the mean; SEM.”

- Fig.6 legend (6): replace ‚whole epidermal cells’ by ‚entire epidermis’, for example.
We revised as you advised.
“(6) Ca^{2+} increase in entire epidermis with wave-like propagation.”

- Inconsistencies in the text on descriptions of tissue locations or stages need proof reading and correction. Examples: p.6 and Fig. S1 legend – decide on stage 36 or 37
We unified as stage 36 and revised Fig. S1 legend as follows.
“(A) Ca^{2+} dynamics of *C. robusta* larvae (52 hpf, stage 36 in the body axis rotation period) observed by FM.”

- Same for various typos (‚tail regressiontail regression’ on p.3 at least 3-4 times)
We revised them. Thank for your critical reading.

- Some English corrections may be good (including in the title?), ‚the’ is often lacking.
Thank you for your suggestion. We revised English.

- The dorsal subregion of the posterior sensory vesicle is not ‚within’ the epidermis but rather ‚beneath’ (p.7 and twice on p.9).
We change the name of the cells as “The dorsal subregion corresponded to cells located dorsally above the neck region of the central nervous system and the epidermal region” by careful observation.

- Please correct what pATEN should stand for (p.10 bottom).
We amended to “posterior apical trunk epidermal neuron (pATEN)”.

- Several references are incomplete, lack the year or page numbers etc.
Thank you for your careful review. We revised references.

We wish to thank you again for your comments. We trust that the revised manuscript is suitable for publication.

Appendix C

Response to reviewers' comments
The response is written in red.

Reviewer(s)' Comments to Author:

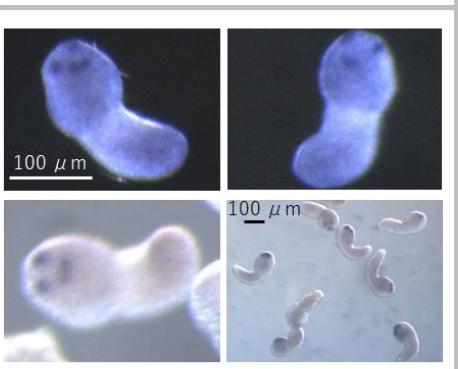
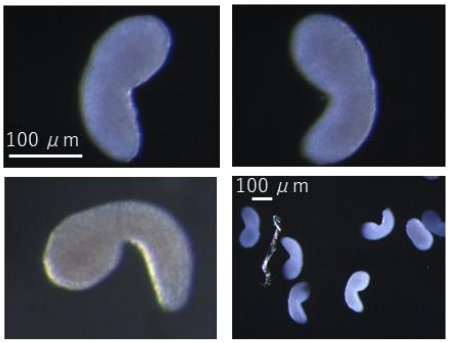
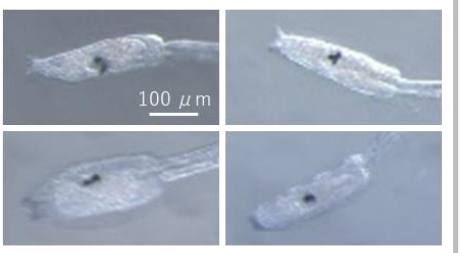
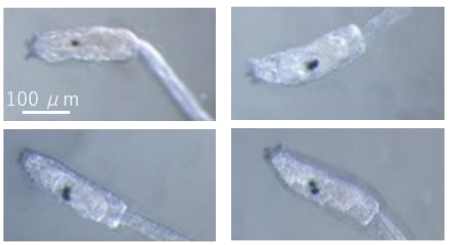
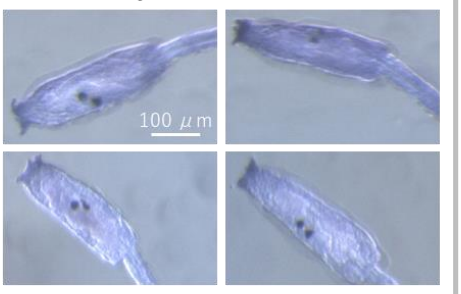
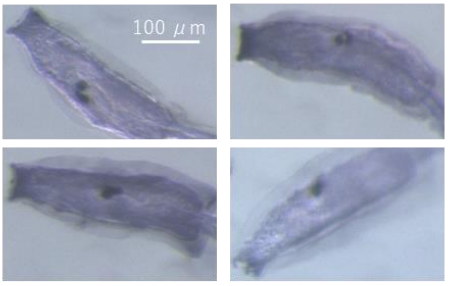
Referee: 3
Comments to the Author(s)
The authors have adressed my concerns sufficiently for publication.

Thank you again for your encouraging comments on our paper. We feel the comments have helped us explain our findings clearly.

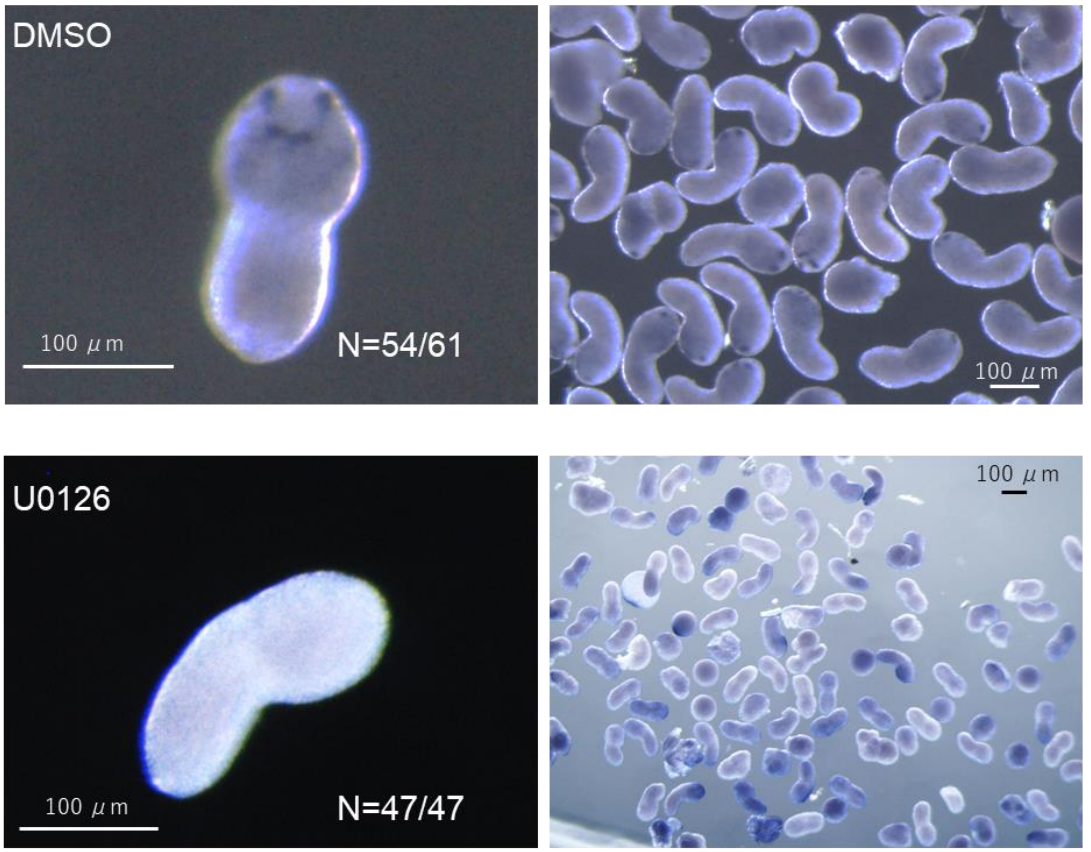
Referee: 2
Comments to the Author(s)
The authors well reply to almost of my concerns and better integratd their results in previous one on metamorphosis.However, I am still not convinced by the reply of the authors on the U0126 and Fox G experiment. With a good timing for the MEK inhibitor addition it is a feasible experiment , It's really not challenging. It is routine experiment in Ciona community. Otherwise the Fox G morpholino is still an option.

We wish to express our appreciation to you for your insightful advices which have helped us significantly improve the paper. We carefully considered your comments and offered the additional data to each in red below.

We tried experiments referee #2 mentioned and collected data from part of modified experiments. First, we show *Foxg in situ* data in the control tailbud and larva in order to check whether *Foxg* is expressed in the larval papillae. We used 8.5 to 9.5 hpf samples for the tailbud and 26 to 30 hpf samples for the larva, respectively. According to the data of the tailbud, it is clear that *Foxg* specifically expressed at the papillae. However, in the larvae, no specific signal could be detected. The signal with the antisense probe was same to that with the sense probe. It is suggested that *Foxg* is not expressed in the larval papillae in larva.

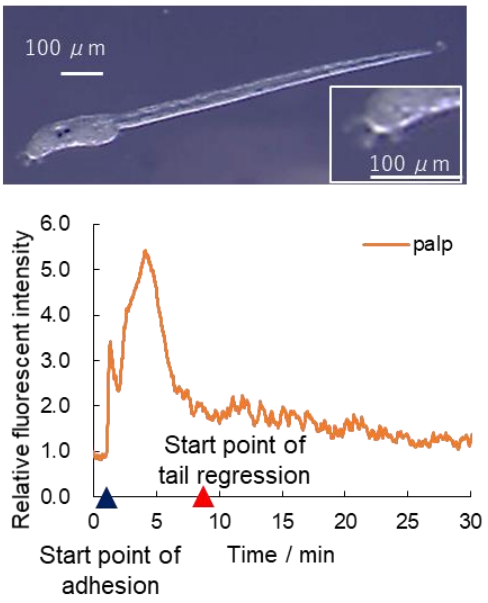
Foxg expression	Ctrl	
	Antisense	Sense
Tailbud		
Larva	30 min detection 	30 min detection 
	Over 1 day detection 	Over 1 day detection 

To inhibit *Foxq* expression in early tailbud stage, 3 different kinds of experiment were performed: U0126 treatment, *Foxq* MO (e2i2: splicing blocker), *Foxq* MO (-11: translation blocker). We confirmed that *Foxq* expression at the papillae disappeared by the treatment of 4 μM U0126 from 6 or 6.5 hpf. On the other hand, *Foxg* expression remained after the treatment of DMSO (as positive control).

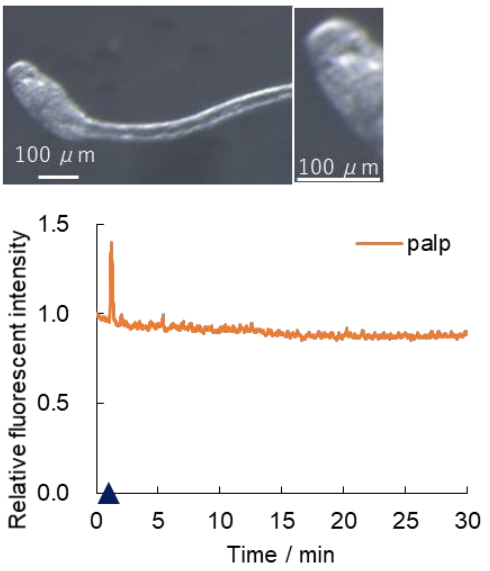


DMSO-treated larva showed the same results as the control larva which showed the second Ca^{2+} transient in Phase II and subsequent tail regression. On the other hand, U0126 treated larva had no palp protrusions (N = 9/9) and neither the second Ca^{2+} transient nor tail regression occurred (N = 5/9). Moreover, we injected *Foxq* MO (splicing blocker) with GCaMP6s and observed Ca^{2+} dynamics. *Foxg* splicing-blocked larva had no palp protrusions (N = 13/13) and neither the second Ca^{2+} transient nor tail regression occurred (N= 10/13). Furthermore, larva which was injected *Foxg* MO (translation blocker) had no palp protrusions and neither the second Ca^{2+} transient nor tail regression occurred (N=4/4). In some samples, Phase I-like short Ca^{2+} transients were observed from epidermis (undifferentiated papillae region) caused by mechanosensation. The table below summarised these results and the number of samples under each condition. The graph of the Ca^{2+} intensity in other samples is shown in **Supplementary Figures for Reviewers** at the end of this document.

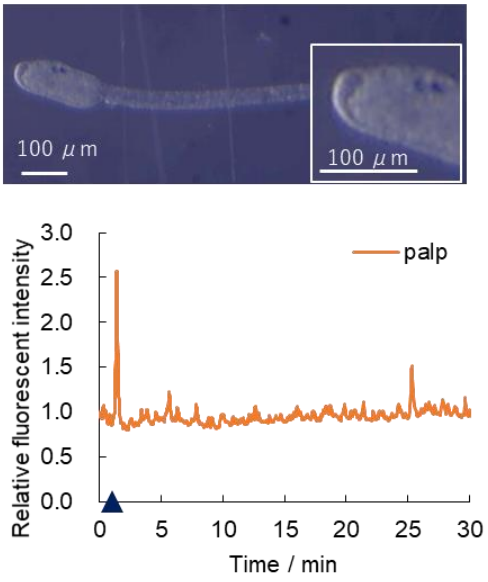
DMSO



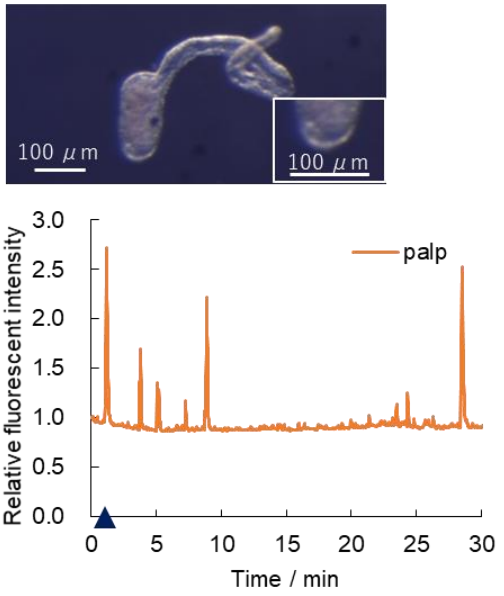
U0126



***Foxg* MO splicing blocker**



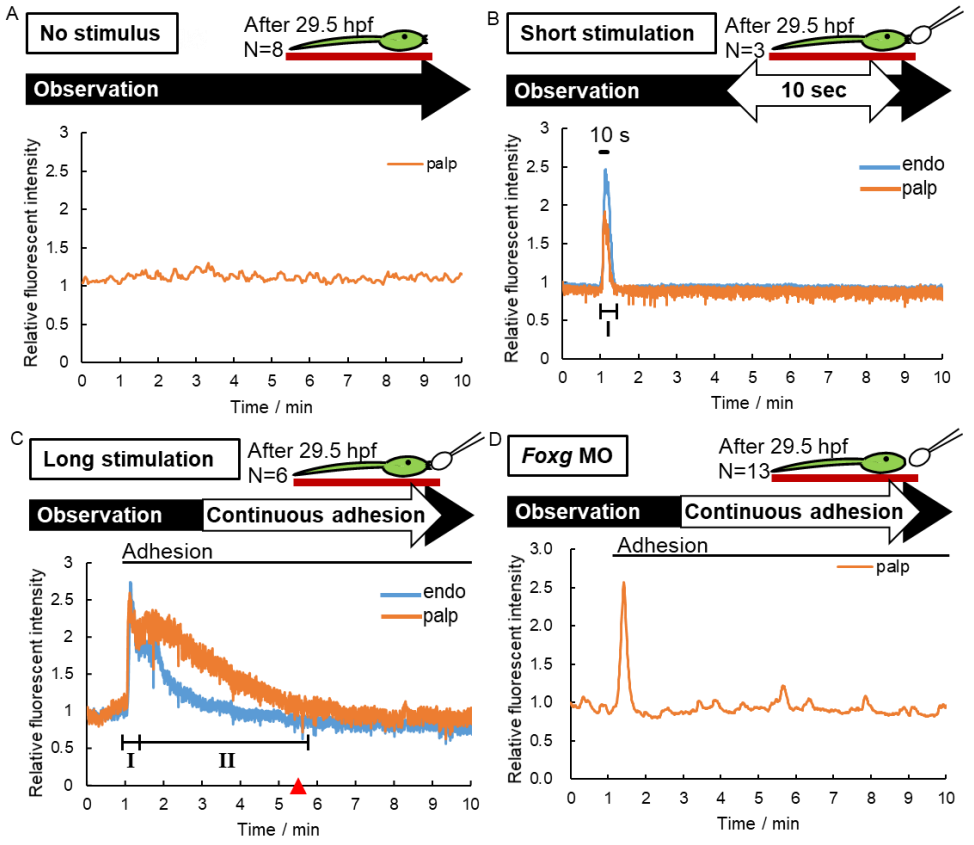
***Foxg* MO translation blocker**



	Continuous stimulation				
	Ctrl	DMSO	U0126	*Foxg* MO (e2i2)	*Foxg* MO (-11)
Phase Ⅱ	6/6	3/3	0/9	0/13	0/4
Tail regression	6/6	3/3	4/9	3/13	0/4

These results revealed that the second Ca^{2+} transient in Phase II has never occurred and tail regression was inhibited in all 3 different *Foxq*-inhibiting experiments. We concluded from this that the second Ca^{2+} transient in Phase II is necessary for tail regression and that *Foxq* is required for maturely functional papillae and induction of the second Ca^{2+} transient. To summarize, ERK dependent *Foxq* expression in papillae is necessary for the functional differentiation of the papillae which enable to induce the second Ca^{2+} transient and tail regression.

The results of U0126 and *Foxq* MO are redundant, so only the result of *Foxq* MO (e2i2) is added in Fig. 4 and Table 1 of the revised manuscript as follows. Other parts of the text that have changed due to this revise are also shown below.



	Stimulus			
	0 sec	10 sec	continuous	
	Ctrl			*Foxg* MO
Phase I	0/8	3/3	6/6	4/13
Phase II	0/8	0/3	6/6	0/13
Tail regression	0/8	0/3	6/6	3/13

We added sentence below in BACKGROUND in line 57.
"*Foxg* is expressed at the papillae under ERK pathway [22]."

We added sentences below in MATERIAL AND METHODS in line 91-93.
"To knockdown *Foxg*, *Foxg* morpholino antisense oligo 5'-AGTGCTGAACTTATAATCTACCTGT-3' was injected with the mRNA of *GCaMP6s*. The specificity of MO has been previously confirmed [22]. *Foxg* MO was gifted from Dr. Yutaka Satou."

We added sentences as follows in RESULTS in line 250-258.
"In addition, to clarify how the papillae differentiation is associated with the induction of Ca^{2+} transient in Phase I and Phase II and subsequent tail regression, we examined the dynamics of Ca^{2+} transient and tail regression in *Foxg* knockdown larva. It has been reported that *Foxg* is expressed in larval papillae where it functions to specify the papillae as sensory neurons [22]. During embryogenesis, *Foxg* expression in neural plate cells are controlled by the MAPK/ERK. In *Foxg* knockdown larva, short Ca^{2+} transients were observed at the anterior trunk epidermis under continuous stimulation. However, neither the second Ca^{2+} transient in Phase II nor tail regression was observed (Fig. 4D; Table 1). This result suggests that *Foxg* is required for generation of the second Ca^{2+} transient and tail regression."

We added sentences as follows in DISCUSSION in line 315-317.
"However, inhibition of the specification of the papilla by *Foxg* MO decoupled them (Fig. 4D). Further studies of papillary sensory neurons will provide a better understanding of the mechanisms that will cause Phase II."

We erased a sentence below in DISCUSSION in line 326-327.
"Since ACCs and CCs are not neurons, we plan to determine if they sense a mechanical stimulus in the future."

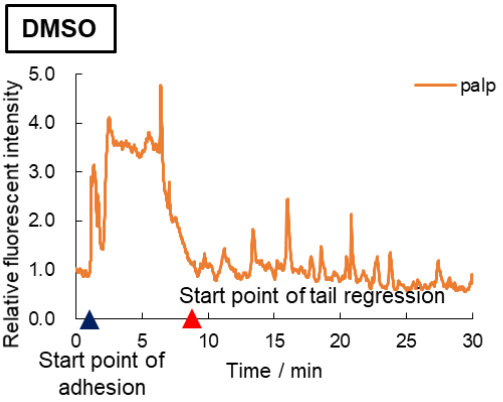
We added a sentence as follows in DISCUSSION in line 328-329.
"Although ACC and CC are not neurons, we need to determine whether they sense mechanical stimuli."

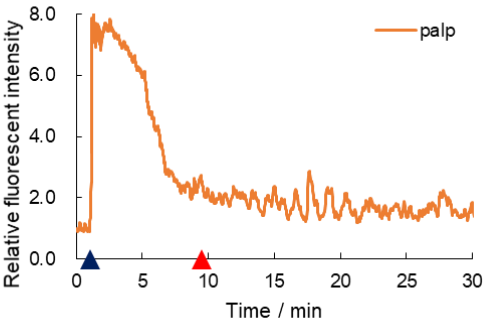
We erased sentences below in DISCUSSION in line 330-336.
"FoxG is expressed in larval papillae where it functions to specify the papillae as sensory neurons [22]. During embryogenesis, FoxG expression in neural plate cells are controlled by the MAPK/ERK. In addition, the MAPK/ERK signalling is also activated in papillae during the swimming period [39]. The reactivation of FoxG in papillae by MAPK/ERK signalling before adhesion might integrate the Ca^{2+} transients in the previously reported molecular events of the ascidian metamorphosis. Future studies will elucidate the mechanosensing molecule that is the downstream target of FoxG."

We added sentences below in Acknowledgements in line 369-371.
"We express our appreciation to Dr. Yutaka Satou for providing MOs against *Foxg*. We thank Dr. Jean-Philippe Chambon for providing experimental protocols."

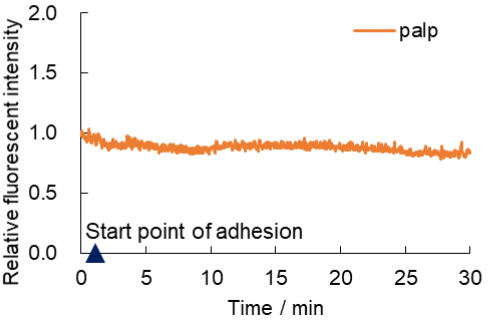
We added following sentences in FIGURE LEDGENDS of Fig.4 in line 426-428.
"(D) Ca^{2+} dynamics in *Foxg* MO injected larva under continuous adhesion. Stimulation began at 1 min (black bar)."

Supplementary Figures for Reviewers





U0126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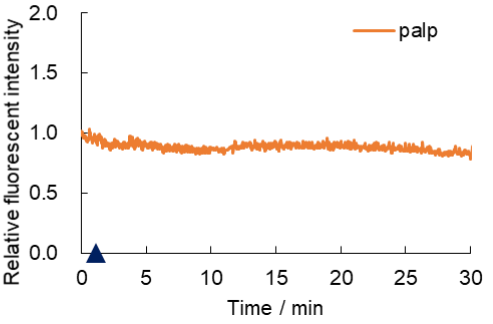
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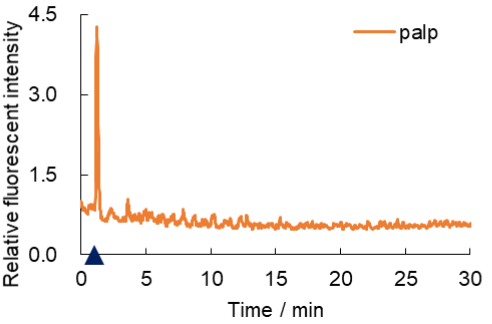
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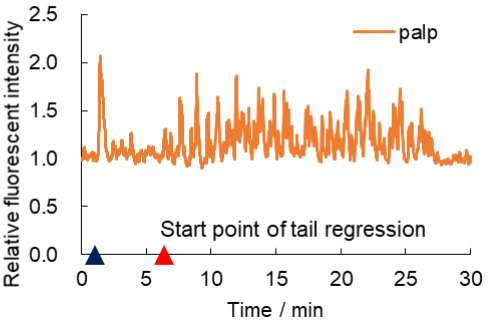
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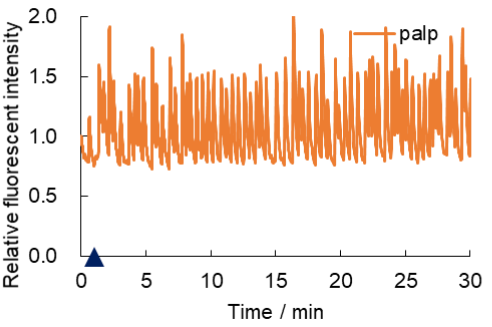
Time / min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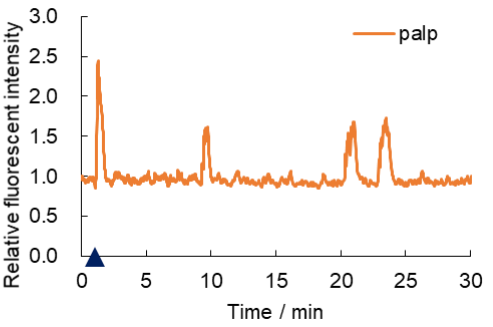
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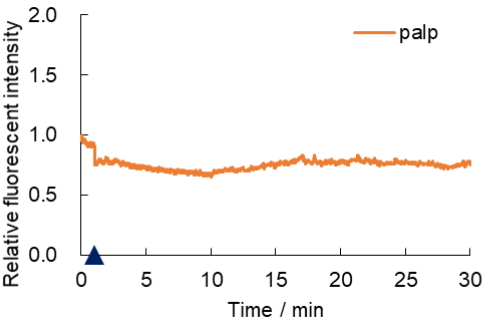
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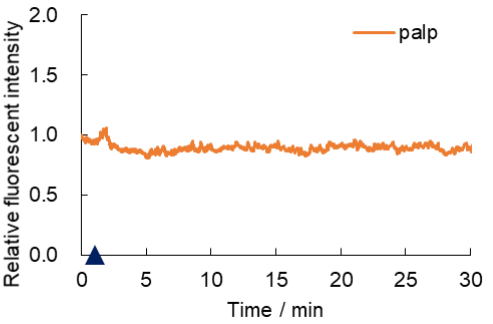
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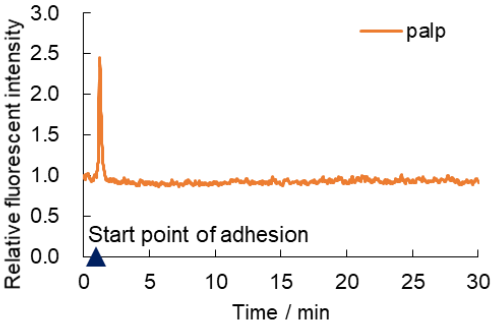
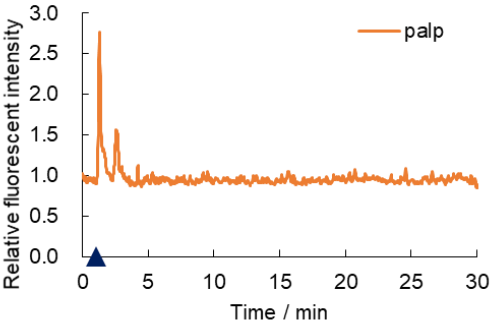
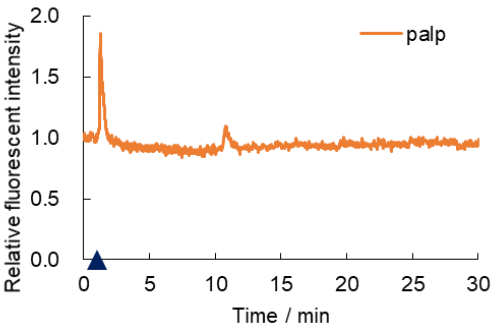
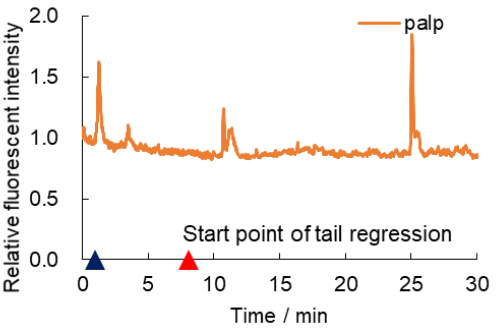
Time / min

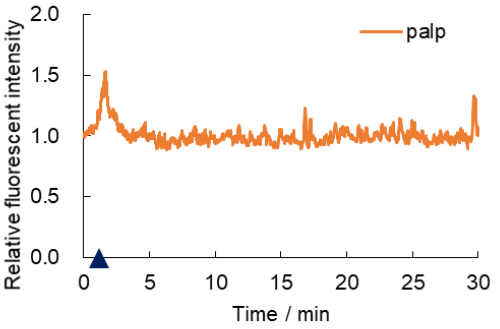
***Foxg* MO splicing blocker**

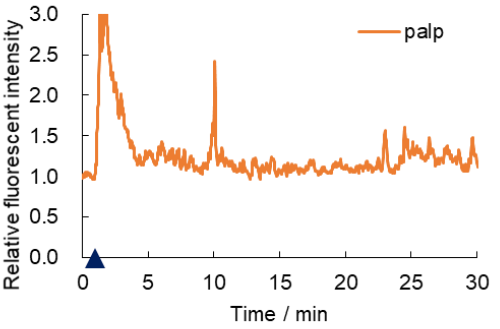


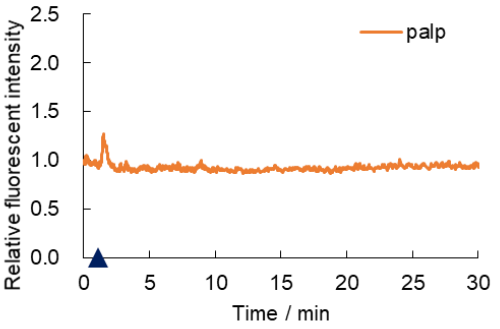


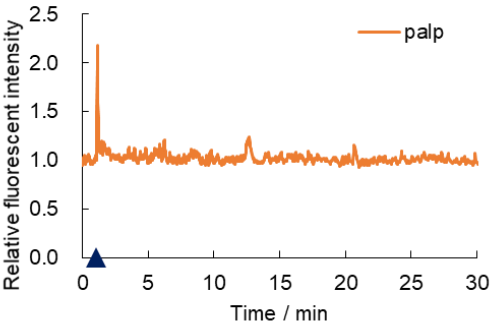


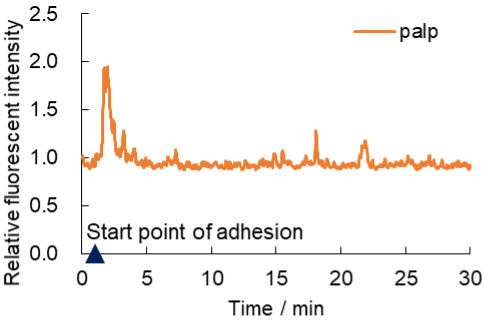








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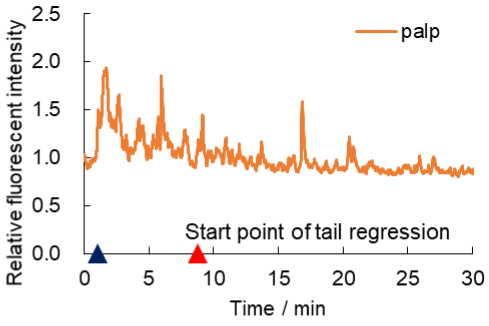
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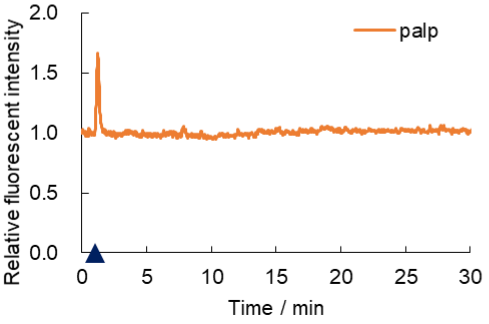
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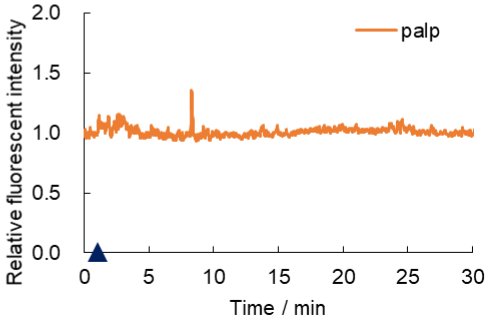
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Time / min

***Foxg* MO translation blocker**