# The novel membrane protein Hoka regulates septate junction organization and stem cell homeostasis in the *Drosophila* gut

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**Original submission**

First decision letter

MS ID#: JOCES/2020/257022

MS TITLE: A novel membrane protein Hoka regulates septate junction organization and stem cell homeostasis in the *Drosophila* gut

AUTHORS: Yasushi Izumi, Kyoko Furuse, and Mikio Furuse
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers are enthusiastic but raised some critical points that will require amendments to your manuscript. Many of the comments can be taken care of by textual changes. However, some additional control experiments were suggested. As stated by referee #1, "The authors show that concomitant downregulation of aPKC or yki in EC cells partially suppresses this overproliferation phenotype (Fig. 8). However, they do not present control experiments showing what happens when these proteins are downregulated alone."

I hope that you will be able to make the requested changes, because I would like to be able to accept your paper.

*We are aware that you may be experiencing disruption to the normal running of your lab that makes 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 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. Please attend to all of the reviewers' comments. 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 their manuscript, Izumi and co-authors present a thorough description of the phenotypes observed in mutants for a novel component of the smooth septate junctions (sSJ), the product of the gene hoka. They also show that the protein Hoka localizes to sSJ in Drosophila and makes a complex with other known components of these structures. Their data are original, solid, well presented and their conclusions are sound. I believe that this work contains a nice piece of data and deserves publication. However, the current version of the manuscript presents a few problems that may require some rewriting (and performing a couple of controls).

*Comments for the author*

1) I feel this article provides a good occasion to discuss the evolutionary origins of the Hoka protein. The authors show that the coding sequence for this protein is conserved in the drosophilids, and they have also found an homolog in a single lepidopteran species. What about other butterflies and other insects? Does this protein have a more ancient origin? There are many arthropod genome sequences and transcriptomes available for phylogenetic studies.

2) Unless I have missed something, the two sentences starting on line 257 are very puzzling:

"In the hoka-mutant first-instar larval midgut epithelial cells, Ssk was mislocalized to the apical and lateral membrane (Fig. 3N), and Mesh and Tsp2A were mislocalized along the lateral membrane (Fig. 3O, P). Further, in these larvae, Ssk, Mesh, and Tsp2A were found to be accumulated in the apicolateral region (Fig. 3Q-S)".

Panels N-P show a strong phenotype whereas panels Q-S present what seems a series of wild type distributions. Both panels are labeled as hoxax211. Could the authors explain these differences and what do they mean?

3) The authors show that knocking down hoka in the EC cells of the adult midgut results in ISC overproliferation. This phenotype can have multiple causes, as it has been shown that, for instance, tissue damage induced by bleomycin provokes a similar effect (see Xu et al., 2019). The authors show that concomitant downregulation of aPKC or yki in EC cells partially suppresses this overproliferation phenotype (Fig. 8). However, they do not present control experiments showing what happens when these proteins are downregulated alone. Xu et al. showed that the aPKC RNAi line used by the authors (HMS01411) has a strong effect on ISC proliferation, although in their case they used a different GAL4 driver (Fig S5C in Xu et al., 2019). If aPKC is necessary for proliferation, then it would not be surprising if its downregulation could counter the effects observed upon Hoka knock down. We are left wondering what would happen if any other factor required for cell proliferation would have been tested in the same conditions. I feel that concluding that aPKC and Yki mediate the effects of Hoka is tempting, but a bit premature, because we do not know whether this mediation is specific. I feel that the particularities of aPKC take a disproportionate part in the discussion, considering the weight of the new data presented in this manuscript.

Minor points:

There is a typo in the labeling of panel Fig. 1D, it should be frame shift and not flame shift.

In the figure caption of Figure S1, what are DROSOAN and DROSOER?

Reviewer 2

*Advance summary and potential significance to field*

This paper identifies and investigates a previously uncharacterized protein, Hoka that is part of the Drosophila smooth septate junction (sSJ) complex. The authors identify Hoka through a genetic screen for genes required for sSJ function and show that Hoka encodes a short transmembrane protein containing a Tyr-Thr-Pro-Ala motif. Hoka is conserved within insect species, but unexpectedly not to all arthropods which also have sSJs. Immunoprecipitation and colocalization experiments show that Hoka forms a complex with other sSJ compenents including Ssk, Mesh and Tsp2A, and is required for their localization to sSJs. Knockdown of hoka in the adult midgut leads to intestinal barrier dysfunction, stem cell over proliferation, and epithelial tumors. In hoka-knockdown midguts, aPKC is up-regulated in the cytoplasm and the apical membrane of epithelial cells. Depleting aPKC and yki in hoka-knockdown midguts results in reduced stem cell over proliferation. Together, these results show that Hoka cooperates with the sSJ-proteins Ssk, Mesh, and Tsp2A to organize sSJs, and is required for maintaining intestinal stem cell homeostasis through the regulation of aPKC and Yki activities in the Drosophila midgut.

The experiments in the paper are thorough and well done, and the paper is also well-written. Given that the Drosophila smooth septate junctions are the best model of insect and arthropod smooth septate junctions, which are potential targets for novel classes of insecticides, and that these junctions feed into control of stem cells through differential regulation of aPKC and yki, this paper will be on interest to readers of JCS. Notably, the paper is ready for publication once minor concerns are addressed (Note I selected "No" for the question ". If a revision is invited, would you want to review the revised version before potential publication?" because I don't think responses to my suggested revisions need re-review. If other reviewers have more substantial requests that need re-review, I am happy to re-review the paper.

*Comments for the author*

No major concerns.

Minor concerns:

Discussion top of page 21, lines 482-497: Although the authors qualify their point at the end of the paragraph, it is just not meaningful to compare defects between RNAi knockdown of different genes without basing the discussion on a quantitative measure of the amount of knockdown achieved. Whether a gene is a good target for existing RNAi constructs says nothing about how much of the protein is needed. This paragraph should be rephased using statement along the lines of “It is notable that an 80% reduction in the level of hoka protein by RNAi produced a much weaker defect than an 90% reduction in protein levels of gene X or gene Y by RNAi. These results suggest that sSJ function may be partly maintained in the hoka-deficient midgut”. If the quantitative data is not available for this comparison, the paraph should be deleted. The discussion is plenty long already.

Fig. 4 – It would be helpful if there were some inset panels or additional panels zooming in of the septa (such as those marked by brackets). It is possible to blow the image up to 400% in the PDF viewer to see details, but this is generally inconvenient and is not possible if the reader is looking at the print version.

Fig. 6D There are no error bars on this graph and no “n” value (although the n can be dug out of the figure legend). I would be much better to show the individual data points and the mean such as the authors did in Fig 8C.

**First revision**

Author response to reviewers' comments

We wish to thank all the reviewers for their constructing comments. We hope that we have addressed these comments to their satisfaction and have revised the manuscript accordingly. Below, we discuss all the comments by each referee one by one. The sentences added or modified in the revised manuscript are highlighted by yellow.

Reviewer 1 Comments for the author
Comment
1) I feel this article provides a good occasion to discuss the evolutionary origins of the Hoka protein. The authors show that the coding sequence for this protein is conserved in the drosophilids, and they have also found an homolog in a single lepidopteran species. What about other butterflies and other insects? Does this protein have a more ancient origin? There are many arthropod genome sequences and transcriptomes available for phylogenetic studies.

Response
We agree that the evolutionary origin of Hoka is of interest. In accordance with the reviewer's suggestion, we have performed the sequence alignments of Hoka homolog proteins as shown in revised Fig. S1: the alignment of the sequences for Hoka homologs between Drosophilids is shown in Fig. S1A, and that between Drosophila melanogaster and other insects is shown in Fig. S1B. To answer the reviewer's questions, we have aligned the sequences for Hoka homologs of two lepidopteran species in Fig. S1B. In these sequence alignments, we found several interesting aspects concerning insect Hoka homologs. First, as we mentioned in the original manuscript, three types of Hoka homologs categorized by the number of YPTA motif (one, two, or three) are present in Drosophila (Fig. S1A). Second, holometabolous insects have the same or similar motif: the mosquito and beetle homologs have a single YTPA motif; the butterfly, ant, bee, sawfly, and moth homologs have a single YQPA motif; the flea homolog has a single YTAA motif (Fig. S1B). Third, the YTPA/YQPA/YTAA motif is not present in the homologs of hemimetabolous insects, such as the psyllid, aphid, planthopper, miridae, termite, and thrip. These results suggest that the YTPA/YQPA/YTAA motif was evolutionarily acquired in holometabolous insect linages. According to the findings obtained from the new alignment data, we have added new sentences in the Results (Word: page 8, line 169, pdf: page 8, line 173), Discussion (Word: page 18, line 430, pdf: page 19, line 441), and Fig. S1 legend.

Comment
2) Unless I have missed something, the two sentences starting on line 257 are very puzzling:
"In the hoka-mutant first-instar larval midgut epithelial cells, Ssk was mislocalized to the apical and lateral membrane (Fig. 3N), and Mesh and Tsp2A were mislocalized along the lateral membrane (Fig. 3O, P). Further, in these larvae, Ssk, Mesh, and Tsp2A were found to be accumulated in the apicolateral region (Fig. 3Q-S)".
Panels N-P show a strong phenotype whereas panels Q-S present what seems a series of wild type distributions. Both panels are labeled as hoxax211. Could the authors explain these differences and what do they mean?

Response
As the reviewer pointed out, our explanation of Fig. 3Q-S was insufficient. In the hoka-mutant first-instar larval midgut epithelial cells, Ssk was mislocalized to the apical and lateral membrane (Fig. 3N), and Mesh and Tsp2A were mislocalized along the lateral membrane (Fig. 3O, P). In addition, we occasionally observed the apicolateral accumulation of Ssk, Mesh, and Tsp2A in the mutant midgut epithelial cells as shown in Fig. 3Q–S. Therefore, we suggested that Hoka is required for efficient localization of Ssk, Mesh and Tsp2A to the apicolateral regions in the epithelial cells. To clarify this point, we have modified the original sentence, "Further, in these larvae, Ssk, Mesh, and Tsp2A were found to be accumulated in the apicolateral region (Fig. 3Q–S)" (page 11, line 260) to "We occasionally observed the apicolateral accumulation of Ssk, Mesh, and Tsp2A in the mutant midgut epithelial cells (Fig. 3Q–S)" (Word: revised page11, line 255, pdf: page 11, line 262).

Comment
3) The authors show that knocking down hoka in the EC cells of the adult midgut results in ISC overproliferation. This phenotype can have multiple causes, as it has been shown that, for instance, tissue damage induced by bleomycin provokes a similar effect (see Xu et al., 2019). The authors show that concomitant downregulation of aPKC or yki in EC cells partially suppresses this overproliferation phenotype (Fig. 8). However, they do not present control experiments showing what happens when these proteins are downregulated alone.

Response
As the reviewer pointed out, we did not present control experiments showing what happens aPKC or Yki proteins are downregulated alone. In this revision, we performed control RNAi experiments in ECs: aPKC-RNAi or yki-RNAi in addition to Luc-RNAi, hoka-RNAi together with Luc-RNAi, hoka-RNAi together with aPKC-RNAi, and hoka-RNAi together with yki-RNAi. As shown in the revised Fig. 8L, we recapitulated the observation that expression of hoka-RNAi together with aPKC-RNAi or yki-RNAi in ECs significantly reduced ISC overproliferation, compared to the hoka-RNAi and the control Luc-RNAi midgut. In addition, we observed that expression of aPKC-RNAi alone in ECs do not lead to a significant difference in ISC proliferation, and expression of yki-RNAi moderately increased the proliferation, compared to the Luc-RNAi midgut. According to these observations, we have added the sentence “Expression of aPKC-RNAi driven by Myo1Ats-GAL4 in ECs did not lead to a significant difference in ISC proliferation, whereas expression of yki-RNAi moderately increased the proliferation, compared to the control Luc-RNAi midgut (Fig. 8L).” in the Results (Word: page 17, line 397, pdf: page 18, line 408).

Comment
Xu et al. showed that the aPKC RNAi line used by the authors (HMS01411) has a strong effect on ISC proliferation, although in their case they used a different GAL4 driver (Fig S5C in Xu et al., 2019). If aPKC is necessary for proliferation, then it would not be surprising if its downregulation could counter the effects observed upon Hoka knock down. We are left wondering what would happen if any other factor required for cell proliferation would have been tested in the same conditions. I feel that concluding that aPKC and Yki mediate the effects of Hoka is tempting, but a bit premature, because we do not know whether this mediation is specific. I feel that the particularities of aPKC take a disproportionate part in the discussion, considering the weight of the new data presented in this manuscript.

Response
We thank the reviewer for giving us important suggestions. Xu et al. reported that concomitant depletion of aPKC together with Tsp2A from the progenitor cells (ISCs and enteroblasts) by using esg-GAL4 strongly suppressed ISC over proliferation caused by Tsp2A-depletion (Fig. 6I in Xu et al., 2019). They also showed that concomitant depletion of aPKC together with Tsp2A from the ECs by using Myo1A-GAL4 significantly reduced ISC over proliferation caused by Tsp2A-depletion (Fig. S5I in Xu et al., 2019). Further, they found that aPKC in progenitor cells is involved in increased ISC proliferation caused by bleomycin-induced midgut damage (Fig. S5C in Xu et al., 2019). As the reviewer suggested, these observations imply that aPKC is generally required for ISC proliferation mediated by progenitor cells and ECs. However, since sSJ-proteins in ECs might be affected by bleomycin-induced midgut damage, which may cause aPKC upregulation, we cannot rule out the possibility that aPKC is specifically involved in the ISC proliferation caused by Hoka (and other sSJ-proteins)-deficient ECs. To further understand the issue, as the reviewer mentioned, it will be helpful to examine what would happen if any other factor required for cell proliferation would have been tested in the same conditions. At present, as the reviewer pointed out, the mediation of aPKC in ISC overproliferation caused by hoka-depletion from ECs may not be specific although involvement of aPKC in ISC proliferation itself is intriguing. Accordingly, we agree that “concluding that aPKC and Yki mediate the effects of Hoka is a bit premature”, and that “the particularities of aPKC take a disproportionate part in the discussion”. We have deleted page 23, lines 528-533, 537-539 from Discussion of the original manuscript.

Minor points:
Comment
-There is a typo in the labeling of panel Fig. 1D, it should be frame shift and not flame shift.

Response
We thank the reviewer for pointing out a spelling mistake. We have corrected “flame shift” to “frame shift” in the labeling of panel Fig1D.

Comment
-In the figure caption of Figure S1, what are DROSOAN and DROSOER?

Response
We thank the reviewer for pointing out errors in the caption of Figure S1. DROSOAN and DROSOER mean Drosophila ananassae and Drosophila erecta, respectively. We have mentioned them in the Figure S1 caption.

Reviewer 2 Comments for the author
Minor concerns:
Comment
-Discussion top of page 21, lines 482-497: Although the authors qualify their point at the end of the paragraph, it is just not meaningful to compare defects between RNAi knockdown of different genes without basing the discussion on a quantitative measure of the amount of knockdown achieved. Whether a gene is a good target for existing RNAi constructs says nothing about how much of the protein is needed. This paragraph should be rephased using statement along the lines of “It is notable that an 80% reduction in the level of hoka protein by RNAi produced a much weaker defect than an 90% reduction in protein levels of gene X or gene Y by RNAi. These results suggest that sSJ function may be partly maintained in the hoka-deficient midgut”. If the quantitative data is not available for this comparison, the paraph should be deleted. The discussion is plenty long already.

Response
As the reviewer pointed out, it was not meaningful to compare defects between RNAi knockdown of different genes without quantitative measurements. We do not have the quantitative date that enable us to compare the reduction level of Hoka protein and other sSJ-proteins in the RNAi knockdown experiments. We also agree the reviewer’s opinion that “The discussion is plenty long already”. Hence, we deleted the paragraph of page 21, lines 482-497 from the original manuscript.

Comment
-Fig. 4 – It would be helpful if there were some inset panels or additional panels zooming in of the septa (such as those marked by brackets). It is possible to blow the image up to 400% in the PDF viewer to see details, but this is generally inconvenient and is not possible if the reader is looking at the print version.

Response
As the reviewer pointed out, it was difficult to recognize the septa observed in hoka-mutant epithelial cells of Fig. 4C, D and E. We have zoomed in the regions including the septa marked by brackets in Fig. 4C, D and E and exhibited them as inset panels, Fig. 4C’, D’ and E’.

Comment
-Fig. 6D There are no error bars on this graph and no “n” value (although the n can be dug out of the figure legend). I would be much better to show the individual data points and the mean such as the authors did in Fig 8C.

Response
In original Fig. 6D, the percentage of midgut barrier-defective flies in each genotype was calculated as follows: % of barrier-defective flies = barrier-defective flies / barrier-defective flies + non-barrier-defective flies x 100. Then, the statistical significances between the genotypes were evaluated by the Fisher’s exact tests. In accordance with the reviewer’s comment, we re-analyzed our statistical data. We performed three independent barrier integrity (Smurf) assay in each genotype. We calculated the percentage of midgut barrier-defective flies in each assay, and then calculated the mean percentage and the standard deviation in each genotype (n=3 for each genotype). In the revised Fig. 6D, we have added error bars and “n” value on the graph. In

addition, we have showed the individual data points as the plots and the mean±s.e.m. on the graph. According to the modification of Fig. 6D data, we have modified the legend of Fig. 6D.

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Second decision letter

MS ID#: JOCES/2020/257022

MS TITLE: A novel membrane protein Hoka regulates septate junction organization and stem cell homeostasis in the *Drosophila* gut

AUTHORS: Yasushi Izumi, Kyoko Furuse, and Mikio Furuse
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.