AMPK activates the Nrf2-Keap1 pathway to govern dendrite pruning via the insulin pathway in *Drosophila*

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AUTHORS: Liang Yuh Chew, Jianzheng He, Jing Lin Jack Wong, Sheng Li, and Fengwei Yu

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

As you will see, all the referees express great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they comment on the lack of appropriate control for some of the experiments, and they make a number of other useful recommendations. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In Drosophila, dendrite arborization (da) neurons is a well-studied paradigm for neuronal pruning during metamorphosis. Under the control of a pulse of ecdysone, da neurons prune away their larval dendrites by 16h after puparium formation (APF) leaving their axons intact. Potentializing a previous study from the same laboratory (Chew et al., 2021), the authors identify AMP-activated protein kinase (AMPK) as a central player in the ecdysone/Sox14 genetic cascade leading to dendrite pruning via the Nrf2-Keap1 pathway. Interestingly, and in accordance with a previous work on insect fat body involvement in growth rate (Yuan et al. 2020), the authors show that AMPK is acting through the inhibition of the insulin pathway for da dendrite pruning.

This study reveals an important mechanism to promote dendrite pruning. The manuscript is generally clearly written and the experiments used for the dissection of the genetic and biochemical pathways leading from EcR-B1 to the final effectors are well designed, but nevertheless some issues need to be addressed and are outlined below.

1) It is surprising that AMPK, which is under the control of Sox14, is activating hdc although hdc expression was described as independent of Sox14 (Loncle & Williams, 2012). The authors should discuss this.
2) Overexpression of CncC partially rescues the pruning defects of AMPK RNAi. The authors concluded that the rescue is partial because of the lack of Mical expression. It can also be due, in addition, to the lack of Hdc expression. The authors should rescue completely AMPK RNAi with both O/E of CncC and Mical if they want to make this conclusion.
3) In Figure 1F one dose (and not two as in E) of O/E of AMPKKR is rescued by O/E of AMPK. A control showing the mutant phenotype with only one dose of O/E of AMPKKR associated with one dose of O/E of control should be described.
4) The authors are using as O/E of control the UAS-MicalNT. This needs explanation.
5) The control RNAi is not described. This should be done.
6) All the experiments with the UAS-AMPKTD, a constitutively active form of AMPK (AMPKCA) show no effects. The authors should describe, in whatever system, an effect of AMPKCA to make sure that the stock is correct.

Comments for the author

In Drosophila, dendrite arborization (da) neurons is a well-studied paradigm for neuronal pruning during metamorphosis. Under the control of a pulse of ecdysone, da neurons prune away their larval dendrites by 16h after puparium formation (APF) leaving their axons intact. Potentializing a previous study from the same laboratory (Chew et al., 2021), the authors identify AMP-activated protein kinase (AMPK) as a central player in the ecdysone/Sox14 genetic cascade leading to dendrite pruning via the Nrf2-Keap1 pathway. Interestingly, and in accordance with a previous work on insect fat body involvement in growth rate (Yuan et al. 2020), the authors show that AMPK is acting through the inhibition of the insulin pathway for da dendrite pruning.

This study reveals an important mechanism to promote dendrite pruning. The manuscript is generally clearly written and the experiments used for the dissection of the genetic and biochemical pathways leading from EcR-B1 to the final effectors are well designed, but nevertheless some issues need to be addressed and are outlined below.

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Reviewer 2

Advance summary and potential significance to field

Main points Using an RNAi screen, the authors identify AMPK as a new regulator of ddaC dendrite pruning. The Nrf2-Keap1 pathway has previously been shown to promote proteasomal degradation and thus dendritic pruning downstream of ecdysone signaling (Chew et al., 2021). In this paper, the authors show that AMPK activates Hdc, Mical, and the Nrf2-Keap1 to execute dendrite pruning. Finally, the authors show AMPK activates Nrf2-Keap1 by partially inhibiting the insulin signaling pathway.
Overall Thoughts
- Mostly scientifically well conducted. Clear images and quantifications.
- Lacking biological impact. AMPK has been shown to play a role in ddaC dendrite pruning (Marzano et al., 2021). Although the authors cite this paper in the discussion, the fact the AMPK is known to be a regulator for ddaC dendrite pruning, unfortunately, lowers the impact of this paper. Nrf2-Keap1 is a known regulator of dendrite pruning (Chew et al., 2021), and the authors put AMPK upstream of Nrf2-Keap1. The role of insulin as a ddaC dendrite pruning inhibitor is well known (Wong et al., 2013), and the authors simply added this to the AMPK and Nrf2-Keap1 pathway. Showing that AMPK does not play a role in MB pruning could be significant, as it could demonstrate that it is specific to ddaC dendrite pruning. Something with a greater biological impact. Including a known component alongside other known components in the ddaC dendrite pruning pathway is important, but not significant enough for Development.
Thoughts on each section of the paper AMPK is cell-autonomously required for dendrite pruning of ddaC neurons
- Unfortunately, this is not novel (Marzano et al., 2021). AMPK has been shown to cell-autonomously block ddaC dendrite pruning.
- The authors should be careful and use proper controls and equal the number of Gal4 to UAS ratios when performing experiments.
o I believe the control for the AMPK RNAi in Figure 1 (B) should be an independent RNAi with UAS-Dcr in the background.
o For Figure 1 (E) the genotype is w*; ppk-Gal4, UAS-mCD8GFP / ppk-Gal4, UASmCD8GFP; UAS-AMPKKR / UAS-AMPKKR, and for (F) w*; ppk-Gal4, UAS-mCD8GFP / UAS-AMPK; UASAMPKKR / ppk-Gal4, UAS-mCD8GFP. As I have underlined the rescue has 1 less copy of the kinase-dead version. The mutant rescue makes this less of an issue, but it is very important to match the ratio of Gal4 and UAS numbers. Especially in this case where perhaps 1 copy less of the kinase-dead form could be weaker and lead to a more dendrite pruning and be misinterpreted as a rescue.
- For Sup Figure 1, I would like to see UAS-AMPK added. It is used as a rescue in Figure1 the authors should show it has no precocious pruning defects.
AMPK is required for the expression of Mical and Hdc
- Using a Mical-specific transcriptional reporter the authors show that AMPK-dependent loss of Mical is transcriptional. However, AMPK has been shown to reduce the overall rate of translation (Marzano et al., 2021). Hdc could be reduced due to reduced translation rates. The authors should clearly state this in the manuscript.
Rpn7, a regulatory subunit of the 26S proteasome, is a downstream target of Nrf2-Keap1 pathway during dendrite pruning
- Proteasomal protein degradation requires ATP and AMPK pruning defect is enhanced with single loss of proteasome pathway mutants (Marzano et al., 2021). So, this result is not surprising.
- Nrf2-Keap1 has been shown to work through the proteasome pathway (Chew et al., 2021), and the authors identified a good tool to confirm this, but this has a little biological impact. Does Rbn7 loss result in pruning defects?
- For most of the figures starting from Figure 2, it is hard to know what developmental time each figure is representing. The authors do state the timing in the text and figure legends but would be helpful if they could add this to the figures.

AMPK is required for the activation of Nrf2-Keap1 pathway prior to dendrite pruning
- Could be all translational. Are there any pruning genes that are not downregulated in an AMPK knockdown? How could one distinguish between direct activation and global translational defects?
- Nrf2-Keap1 has been reported to play a role in ddaC dendrite pruning through proteasomal degradation (Chew et al., 2021). gstD1-lacZ represents the antioxidant response pathway that has been shown to not play a role in ddaC dendrite pruning. So, although gstD1-lacZ could be a valid reporter for Nrf2-Keap1 signaling, it has no biological significance. It should perhaps be supplementary figures.

AMPK regulates dendrite pruning partly via activating Nrf2-Keap1 pathway
- For Figure 4C- Cnc mutants have an increase of ubiquitinated proteins. Can OE of Cnc just result in the clearance of ubiquitinated proteins? How is this direct? Does AMPK RNAi in a Cnc mutant background have similar ubiquitinated proteins?
- Overexpression of the full-length Mical was sufficient to rescue dendrite pruning defects in sox14 mutants (Kirilly et al., 2009). Can the over-expression of mical and cnc completely rescue the AMPK RNAi phenotype? This will show that Nrf2-Keap1 and mical are the main components downstream of AMPK.

Insulin pathway negatively regulates the Nrf2-Keap1 pathway during dendrite pruning
- One main point of the text is that insulin pathway negatively regulates the Nrf2-Keap1 pathway during dendrite pruning. However, the paper they cite (Wong et al., 2013) shows that Cullin1-based E3 ligase facilitates ddaC dendrite pruning primarily through inactivation of the InR/PI3K/TOR pathway. How does cullin1 complex fit into the model of the authors? What is the relationship between Cullin1 and AMPK?
AMPK promotes dendrite pruning at least partly via inhibiting insulin pathway
- Overexpression of the full-length Mical was stuffiest to rescue dendrite pruning defects in sox14 mutants (Kirilly et al., 2009). Can the overexpression of mical and inactivation of the insulin signaling pathway rescue the AMPK RNAi phenotype?

The AMPK-insulin pathway axis is required for ecdysone signaling to activate Nrf2-Keap1 pathway
- Figure 7C is not surprising- similar experiments were performed before (Wong et al., 2013).

Comments for the author

please see above

Reviewer 3

Advance summary and potential significance to field

The study by Chew et al. presents data demonstrating AMP-activated protein kinase (AMPK) acts in sensory process pruning of ddaC mechanosensory neurons during Drosophila early metamorphosis. These authors previously published the role for AMPK in this process, but here place this enzymatic function in established molecular pathways. The new data build upon the authors' recent work (Chew et al., 2021) showing the molting hormone ecdysone drives the Nrf2-Keap1 pathway to promote proteasomal degradation during pruning. Using careful genetics, the authors demonstrate AMPK works cell autonomously upstream of the Nrf2-Keap1 pathway and functions to partially inhibit the insulin pathway, known to inhibit the Nrf2-Keap1 pathway and to be negatively regulated by ecdysone signaling. Although the AMP involvement is far from clear, the authors show an AMPK role in upregulation of Mical/Headcase levels. Overall, this new study by Chew et al. effectively further elucidates a pathway that governs sensory process pruning, and the quality of the data appears generally good.

Comments for the author

Major Points 1. In Figure 1, the authors define 3 pruning stages (severing, fragmentation and clearance), but make no attempt to characterize where AMPK function acts in this process. The

authors should test the phenotype distribution for each genotype and present these analyses in revised figures.
2. AMPK is an evolutionarily-conserved metabolic energy sensor activated by ATP deprivation. In this context, a fat body role is well established, but it is hard to envision a comparable role in neuronal pruning. It is important that the authors establish this basic mechanistic connection.
3. A recent paper (Marzano et al., 2021) showed ecdysone-driven AMPK function promotes oxidative phosphorylation. Is the same happening here? Does this provide the missing energy link? The authors should employ the published AMPK sensor to help answer this question.
4. Mical/ Headcase functions are unknown. Can the authors comment on this missing link? The effect of AMPK on Mical expression is speculated to be via transcriptional regulation. The authors need to demonstrate this. Likewise, for AMPK regulation of the fly Nrf2 homolog CncC.
5. The paper states "the mical1-LacZ transgene fully resembled the temporal expression of mical in ddaC neurons." However, expression was not shown. The paper states Mical is "upregulated in ddaC neurons at the wandering (wL3) and white prepupal (WP) stages", but mical1-LacZ is shown absent in wL3 (Fig. S3B).
The authors should show comparative expression patterns.

Minor Points:
1. In Figures 1B-H, the authors should clarify that the red arrows point to ddaC somata.
2. In Figure 5, the authors hyperactivated the insulin pathway using both O/E InRCA and O/E Akt separately to assess expression of gstD1-lacZ and Rpn7 levels but they did not do the same for the hyperactivation of the TOR pathway. Is there a reason only O/E S6KCA was used to assess gstD1-lacZ expression while O/E TOR was used to assess Rpn7 and Ub levels?
3. O/E Control and akt RNAi images are missing from Figure S7C.
4. In Figure S7B-C, the authors chose to inhibit the insulin pathway by overexpressing PI3KDN, yet they inhibited the insulin pathway by overexpressing InRDN in Figure 6B. Why was O/E InRDN not used to assess if insulin inhibition alters gstD1-lacZ expression and Rpn7 levels?
5. There are numerous typographical and grammatical errors throughout the manuscript that need to be addressed.

First revision

Author response to reviewers' comments

We greatly appreciate the reviewers' positive assessment and constructive suggestions on our manuscript. All the concerns raised by the reviewers have been addressed with additional experiments. We have summarized several major improvements made and included our point-by-point response to the reviewers' comments.

Major improvements in our revised manuscript are listed as follows:

1. We show that Hdc levels were also reduced in AMPK RNAi neurons overexpressing CncC. Thus, the partial rescue of the AMPK RNAi phenotypes by CncC overexpression is due to the lack of both Mical and Hdc expression. (Reviewer #1)
2. We have now included a proper control with one copy of *UAS-AMPK^{KR}* and *UAS-control* as well as the rescue experiment with one copy of *UAS-AMPK^{KR}* and *UAS-AMPK*. Indeed, the AMPK^{KR} defects were fully rescued by the expression of wild-type AMPK, compared to those control neurons. (Reviewer #1 and #2)
3. We have showed that knockdown of Rpn7, via two distinct RNAi lines (*rpn7* RNAi #1, BL#34787; #2, v101467), led to consistent dendrite pruning defects. (Reviewer #2)
4. We show that CncC protein levels were not reduced but significantly increased in AMPK RNAi or AMPK^{KR}-expressing ddaC neurons at 6 h APF. In addition, Sox14 and EcR expression levels were not downregulated in AMPK RNAi and AMPK^{KR}-expressing ddaC neurons. Thus, these data argue against the possibility that AMPK regulates global gene translation as proposed by the Rumpf paper. (Reviewer #2)
5. We have recombined the transgene *UAS-CncC* with *UAS-control*, *UAS-Mical*, or *UAS-InR^{DN}*, and introduced them in the AMPK RNAi background. Co-overexpression of Mical with CncC or InR^{DN} almost completely rescued the AMPK RNAi phenotypes, compared to

Mical, CncC or InR^{DN} overexpression alone. These new data support the conclusion that AMPK regulates dendrite pruning via both Mical and CncC/Insulin pathway. (Reviewer #2)

6. We have also included the expression pattern of Mical from eL3 to WP. Indeed, Mical upregulation coincided with the *mical1-LacZ* expression at the WP stage. (Reviewer #3)

We believe that we have made substantial improvements on our manuscript. We hope that the editor and reviewers will find our revised paper now suitable for publication in *Development*.

Reviewer 1 Advance Summary and Potential Significance to Field:

In Drosophila, dendrite arborization (da) neurons is a well-studied paradigm for neuronal pruning during metamorphosis. Under the control of a pulse of ecdysone, da neurons prune away their larval dendrites by 16h after puparium formation (APF) leaving their axons intact. Potentializing a previous study from the same laboratory (Chew et al., 2021), the authors identify AMP-activated protein kinase (AMPK) as a central player in the ecdysone/Sox14 genetic cascade leading to dendrite pruning via the Nrf2-Keap1 pathway. Interestingly, and in accordance with a previous work on insect fat body involvement in growth rate (Yuan et al. 2020), the authors show that AMPK is acting through the inhibition of the insulin pathway for da dendrite pruning.

This study reveals an important mechanism to promote dendrite pruning. The manuscript is generally clearly written and the experiments used for the dissection of the genetic and biochemical pathways leading from EcR-B1 to the final effectors are well designed, but nevertheless some issues need to be addressed and are outlined below.

We are very grateful to the reviewer for the positive assessment and helpful suggestions.

1) It is surprising that AMPK, which is under the control of Sox14, is activating hdc although hdc expression was described as independent of Sox14 (Loncle & Williams, 2012). The authors should discuss this.

We have now included the discussion in the revised text (p7-8)--"Mical is an F-actin- disassembly factor which regulates cytoskeletal disassembly in axonal growth and bristle elongation. However, whether Mical regulates F-actin disassembly during dendrite pruning remains unknown. Headcase is upregulated during dendrite pruning, and its exact function during dendrite pruning remains to be determined. While Mical upregulation depends on both EcR and Sox14, Hdc expression is regulated by EcR but not by Sox14. Our data suggest that AMPK might modulate the expression of various pruning factors downstream of both EcR and Sox14.".

2) Overexpression of CncC partially rescues the pruning defects of AMPK RNAi. The authors concluded that the rescue is partial because of the lack of Mical expression. It can also be due, in addition, to the lack of Hdc expression. The authors should rescue completely AMPK RNAi with both O/E of CncC and Mical if they want to make this conclusion.

We agree with the reviewer that the partial rescue could be also due to the lack of Hdc expression. Indeed, Hdc levels were also reduced in AMPK RNAi neurons overexpressing CncC (Fig S6C). We have modified the statement in the revised text (p11).

Moreover, we have also recombined two transgenes UAS-CncC and UAS-Mical to conduct the rescue experiments in the AMPK RNAi background. Co-overexpression of CncC and Mical indeed exhibited almost full rescue of the AMPK RNAi phenotypes, compared to Mical or CncC overexpression alone (Fig S6D). This new data support that AMPK regulates dendrite pruning via both Mical and CncC (p11-12).

3) In Figure 1F one dose (and not two as in E) of O/E of AMPKKR is rescued by O/E of AMPK. A control showing the mutant phenotype with only one dose of O/E of AMPKKR associated with one dose of O/E of control should be described.

We have now corrected the mistake by adding the precise control. Actually, in our initial experiments we had conducted a proper control experiment with one copy of *UAS-AMPK^{KR}* and

UAS-control, in parallel to the rescue experiment with one copy of *UAS-AMPK*^{KR} and *UAS- AMPK*. Indeed, the AMPK^{KR} defects were fully rescued by the expression of wild-type AMPK, compared to those control neurons. We have now included this control image and quantification in the revised Figure 1F, J, K and text (p6).

4) The authors are using as O/E of control the UAS-MicalNT. This needs explanation.

We have now included the description "the non-functional N-terminal Mical (Mical^{NT}) control (Kirilly, et al., 2008)" for the overexpression control (*UAS-Mical*^{NT}) in the revised text (p6) as well as the Materials and Methods section (p20).

5) The control RNAi is not described. This should be done.

The control RNAi line is v25271 (*y-tub37C*) from VDRC. *y-tub37C* is expressed only in germ cells but not in somatic cells (including neurons). We have now included the information in the revised text and Materials & Methods section (p6 and p21).

6) All the experiments with the UAS-AMPKTD, a constitutively active form of AMPK (AMPKCA) show no effects. The authors should describe, in whatever system, an effect of AMPKCA to make sure that the stock is correct.

In this study, we used the same line that worked well in the fat body in our previous study (Yuan, et al., *PNAS* 2020). In the fat body, AMPK^{TD} (also known as AMPK^{CA}) overexpression greatly impeded insulin pathway during the larval feeding stages (Yuan, et al., *PNAS* 2020).

Reviewer 2 Advance Summary and Potential Significance to Field:

Main points

Using an RNAi screen, the authors identify AMPK as a new regulator of ddaC dendrite pruning. The Nrf2-Keap1 pathway has previously been shown to promote proteasomal degradation and thus dendritic pruning downstream of ecdysone signaling (Chew et al., 2021). In this paper, the authors show that AMPK activates Hdc, Mical, and the Nrf2-Keap1 to execute dendrite pruning. Finally, the authors show AMPK activates Nrf2-Keap1 by partially inhibiting the insulin signaling pathway.

Reviewer 2 Comments for the Author:

Overall Thoughts

-Mostly scientifically well conducted. Clear images and quantifications.

We thank the reviewer for the positive comments on the quality of our work.

-Lacking biological impact. AMPK has been shown to play a role in ddaC dendrite pruning (Marzano et al., 2021). Although the authors cite this paper in the discussion, the fact the AMPK is known to be a regulator for ddaC dendrite pruning, unfortunately, lowers the impact of this paper.

We respectfully disagree with the reviewer on the comments on lack of the biological impact of our study. In our study, we focus on the novel mechanism by which AMPK promotes dendrite pruning via activating Nrf2-Keap1 pathway. Moreover, AMPK activates Nrf2-Keap1 pathway via antagonizing insulin pathway during dendrite pruning. **Thus, this manuscript highlights a novel mechanism of AMPK action, which is completely different from that described in the Rumpf paper (Marzano et al., *Cell Rep*. 2021; published on 16th Nov 2021).**
The Rumpf paper mainly describes the mechanism whereby AMPK promotes pyruvate flux to increase use of noncarbohydrate TCA cycle fuels during dendrite pruning. They proposed that AMPK may regulate general translation of the pruning factors, e.g. Mical. However, this possibility has not been verified in their study. Importantly, our findings suggest that AMPK does not affect general translation of all pruning factors, instead, may also regulate *mical* transcription. **First,** although Mical expression was reduced, Sox14 and EcR expression levels were not downregulated

in AMPK RNAi or AMPK^{KR}-expressing neurons (Figure S2). **Second,** CncC protein levels were not reduced but significantly increased in AMPK RNAi or AMPK^{KR}- expressing ddaC neurons at 6 h APF (Figure S5A). Our data indicate that general gene translation is not impaired upon AMPK depletion. **Finally,** we generated a *mical1*-lacZ reporter which clearly shows that LacZ levels were downregulated in AMPK RNAi or AMPK^{KR}- expressing neurons (LacZ is a relatively small protein with 1029 aa) (Figure S3), suggesting that AMPK likely activates Mical expression via transcription. Thus, our above-mentioned data provide additional new insights into AMPK functions.

Finally, the scientific community is moving away from mere competition (on who publishes the work the first) to reproducibility of the published work. We notice that Development journal also offers a similar editorial policy on "scoop protection". The only overlap of our manuscript with the Rumpf paper is the phenotypic analysis of AMPK in dendrite pruning. We are delighted that we found similar AMPK loss-of-function phenotypes as described in the Rumpf paper, which demonstrates that these data are highly reproducible. Importantly, the mechanism shown in our manuscript is distinct from that in the Rumpf paper. In addition, we submitted our initial manuscript within 2 months of publication of the Rumpf paper. Therefore, we are confident on the novelty and biological impact of this study.

Nrf2-Keap1 is a known regulator of dendrite pruning (Chew et al., 2021), and the authors put AMPK upstream of Nrf2-Keap1. The role of insulin as a ddaC dendrite pruning inhibitor is well known (Wong et al., 2013), and the authors simply added this to the AMPK and Nrf2-Keap1 pathway.

Our previous papers show the important roles of insulin and Nrf2-Keap1 pathways in dendrite pruning (Wong et al., *PLOS Biol*. 2013; Chew et al., *Cell Rep*. 2021). However, whether these two pathways are connected during dendrite pruning was unknown. In the current manuscript, **we have identified, for the first time, that the Nrf2-Keap1 pathway functions downstream of insulin signaling pathway in *Drosophila*.** We have also identified the novel link between AMPK and Nrf2-Keap1 pathway during dendrite pruning. **In our opinion, these novel links, which are unknown in other systems and organisms, are impactful and significant**, as they can be potentially applied to other biological contexts, for example, ageing and cancer. AMPK, insulin pathway or Nrf2-Keap1 pathway are individually critical for ageing and tumorigenesis.

Showing that AMPK does not play a role in MB pruning could be significant, as it could demonstrate that it is specific to ddaC dendrite pruning. Something with a greater biological impact.

Whether AMPK has a role in mushroom body (MB) axon pruning is important and interesting. However, we respectfully disagree with the reviewer that a specific role in ddaC dendrite pruning means a greater biological impact. For example, EcR and Sox14 are critical for both ddaC dendrite pruning and MB axon pruning (Lee, et al. *Neuron* 2000; Williams, et al., *Development* 2005; Kirilly, et al., *Nature Neurosci*. 2008), whereas Mical is specifically required for ddaC dendrite pruning (Kirilly, et al., *Nature Neurosci*. 2008).

A manuscript on the role of AMPK in axon pruning of MB γ neurons is currently under preparation by another laboratory (personal communications). Consistently, our unpublished data also indicate an important role of AMPK in axon pruning of MB γ neurons (see below). Thus, AMPK plays pivotal roles in two distinct modes of neuronal pruning, similar to EcR and Sox14. As we hope to focus on AMPK's role in ddaC dendrite pruning but not MB axon pruning in our manuscript, we have decided not to include the MB γ neuron data in this manuscript.

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Including a known component alongside other known components in the ddaC dendrite pruning pathway is important, but not significant enough for Development.

We respectfully disagree with the reviewer on this point. As we have argued above, we believe

providing novel links among various pathways is important and impactful, which will contribute to the understanding of a complete regulatory network. Ultimately, these new knowledges would potentially contribute to identification of potential therapeutic targets for neurological disorders. Overall, the new findings and conclusions in this manuscript could be conserved in distinct biological contexts in *Drosophila* as well as analogous pruning processes in mammals. We hope that the reviewer will agree with us on the importance and general impact of this study.

Thoughts on each section of the paper

AMPK is cell-autonomously required for dendrite pruning of ddaC neurons
-Unfortunately, this is not novel (Marzano et al., 2021). AMPK has been shown to cell-autonomously block ddaC dendrite pruning.

Please refer to our reply to the above-mentioned point on the novelty of this study in the first point.

-The authors should be careful and use proper controls and equal the number of Gal4 to UAS ratios when performing experiments.

oI believe the control for the AMPK RNAi in Figure 1 (B) should be an independent RNAi with UAS-Dcr in the background.

In our initial experiments, we had conducted the AMPK RNAi experiment together with the control RNAi line v25271 (*y-tub37C*) from VDRC. *y-tub37C* is expressed only in germ cells but not in somatic cells (including neurons). We have now included the information in the revised text (p6) and an image for the control RNAi with *UAS-Dcr2* in the revised Figure 1B.

In addition, we have also included *UAS-control* overexpression as a precise control for *UAS-AMPK^{KR}* overexpression, as well as FRT19A MARCM clones as MARCM controls for *ampk^{D2}* MARCM analysis (p6 and Figure 1J, K). We do not include these control images, as there is limited space in Figure 1.

oFor Figure 1 (E) the genotype is w*; ppk-Gal4, UAS-mCD8GFP / ppk-Gal4, UASmCD8GFP; UAS-AMPKKR / UAS-AMPKKR, and for (F) w*; ppk-Gal4, UAS-mCD8GFP / UAS-AMPK; UASAMPKKR / ppk-Gal4, UAS-mCD8GFP. As I have underlined the rescue has 1 less copy of the kinase-dead version. The mutant rescue makes this less of an issue, but it is very important to match the ratio of Gal4 and UAS numbers. Especially in this case where perhaps 1 copy less of the kinase-dead form could be weaker and lead to a more dendrite pruning and be misinterpreted as a rescue.

We have now corrected the mistake by adding the precise control. Actually in our initial experiments we had conducted a proper control experiment with one copy of *UAS-AMPK^{KR}* and *UAS-control*, in parallel to the rescue experiment with one copy of *UAS-AMPK^{KR}* and *UAS- AMPK*. Indeed, the AMPK^{KR} defects were fully rescued by the expression of wild-type AMPK, compared to those control neurons. We have now included this control image and quantification in the revised Figure 1F, J, K and text (p6).

-For Sup Figure 1, I would like to see UAS-AMPK added. It is used as a rescue in Figure1 the authors should show it has no precocious pruning defects.

We have now repeated the experiments for overexpression of AMPK or AMPK^{CA} with an overexpression control. Overexpression of AMPK or AMPK^{CA} neither promoted precocious dendrite pruning at 6 h APF nor inhibited dendrite pruning at 16 h APF (p6) (Figure S1B).

AMPK is required for the expression of Mical and Hdc
-Using a Mical-specific transcriptional reporter the authors show that AMPK-dependent loss of Mical is transcriptional. However, AMPK has been shown to reduce the overall rate of translation (Marzano et al., 2021). Hdc could be reduced due to reduced translation rates. The authors should clearly state this in the manuscript.

We have now included two sentences “Reduced Mical and Hdc levels could be due to impaired

translation rate in AMPK RNAi neurons (Marzano et al., 2021).” and “It complements with a recent study suggesting that AMPK might regulate Mical translation during dendrite pruning (Marzano et al., 2021).” in the revised Results part (p7-8).

While Mical and Hdc upregulation was impaired, upregulation of EcR-B1 and Sox14 protein levels was not altered in AMPK RNAi or AMPK^{KR}-expressing ddaC neurons at WP stage. We have also added the sentence “arguing against a recent study showing that AMPK regulates general gene translation in ddaC neurons (Marzano et al., 2021).” (p7).

Rpn7, a regulatory subunit of the 26S proteasome, is a downstream target of Nrf2-Keap1 pathway during dendrite pruning
-Proteasomal protein degradation requires ATP and AMPK pruning defect is enhanced with single loss of proteasome pathway mutants (Marzano et al., 2021). So, this result is not surprising.

The paper by Marzano et al., 2021 shows the genetic interaction between AMPK and proteasome degradation pathway during dendrite pruning. Their interpretation is that AMPK regulates ATP homeostasis, and proteasomal degradation is an ATP-consuming process. However, their paper did not address the question of how AMPK regulates proteasomal degradation pathway. In our study, we provide the explanations on this question. **First,** AMPK promotes Rpn7 expression and proteasomal degradation via facilitating Nrf2-Keap1 pathway and suppressing insulin pathway; **Second,** insulin pathway negatively regulates Rpn7 expression and proteasomal degradation via inhibiting Nrf2-Keap1 pathway. Thus, our study further advances the understanding of AMPK function in regulating proteasome degradation pathway.

-Nrf2-Keap1 has been shown to work through the proteasome pathway (Chew et al., 2021), and the authors identified a good tool to confirm this, but this has a little biological impact.

Please refer to our reply to the previous point too. By using Rpn7 as a reliable tool, we further demonstrate a clear relationship among AMPK, Insulin pathway, Nrf2-Keap1 pathway, and proteasomal degradation. **First,** AMPK promotes Rpn7 expression and proteasomal degradation via facilitating Nrf2-Keap1 pathway and suppressing insulin pathway; **Second,** insulin pathway negatively regulates Rpn7 expression and proteasomal degradation via inhibiting Nrf2-Keap1 pathway. With the Rpn7 tool, we provide concrete evidence showing how AMPK regulates proteasomal degradation during dendrite pruning. **In our opinion, this is the first time convincingly showing the mechanism whereby AMPK regulates proteasomal degradation pathway via modulation of Nrf2-Keap1 pathway and Insulin pathway.**

Does Rbn7 loss result in pruning defects?

Knockdown of Rpn7, via two distinct RNAi lines (*rpn7* RNAi #1, BL#34787; #2, v101467), led to consistent dendrite pruning defects (p9). We have now included the data in the revised Figure S4B.

-For most of the figures starting from Figure 2, it is hard to know what developmental time each figure is representing. The authors do state the timing in the text and figure legends but would be helpful if they could add this to the figures.

We have now indicated the developmental stages in all the revised figures.

AMPK is required for the activation of Nrf2-Keap1 pathway prior to dendrite pruning
-Could be all translational.

We have now showed that CncC protein levels were not reduced but significantly increased in AMPK RNAi or AMPK^{KR}-expressing ddaC neurons at 6 h APF. Thus, AMPK unlikely promotes CncC translation, instead, regulates CncC activity via suppressing insulin pathway. **Our data argue against the statement “loss of AMPK causes the defects in the translation of pruning factors” by the Rumpf paper (Marzano et al., 2021).** We have now included the CncC expression data in Figure S5A and the text (p10).

Are there any pruning genes that are not downregulated in an AMPK knockdown? How could one

distinguish between direct activation and global translational defects?

Our findings argue against the possibility that AMPK regulates global gene translation as proposed by the Rumpf paper. We show that in addition to CncC expression (Figure S5A, as shown in the previous point), Sox14 and EcR expression levels were not downregulated in AMPK RNAi and $AMPK^{KR}$-expressing ddaC neurons (Figure S2A-B), although Mical expression was downregulated (Figure S2C). These data suggest that pruning gene translation is not globally impaired upon AMPK depletion.

Moreover, we also generated a *mical1*-lacZ reporter which clearly shows that LacZ levels were downregulated in AMPK RNAi and $AMPK^{KR}$-expressing neurons (LacZ is a relatively small protein with 1029 aa) (Figure S3E), suggesting that AMPK is required to activate Mical expression likely via transcription. Thus, AMPK specifically activates the expression of two pruning factors Mical and Hdc, rather than that of EcR, Sox14 and CncC.

-Nrf2-Keap1 has been reported to play a role in ddaC dendrite pruning through proteasomal degradation (Chew et al., 2021). gstD1-lacZ represents the antioxidant response pathway that has been shown to not play a role in ddaC dendrite pruning. So, although gstD1-lacZ could be a valid reporter for Nrf2-Keap1 signaling, it has no biological significance. It should perhaps be supplementary figures.

By using two reporters (*gstD-lacZ and rpn7)*, we could provide more holistic information about the activation of Nrf2-Keap1 pathway by insulin-TOR pathway. Since *gstD-lacZ* is the most widely used reporter of Nrf2-Keap1 pathway, our results with both markers could be more interesting to general audiences who are studying the crosstalk between Nrf2-Keap1 and insulin pathways in other biological contexts. Thus, we hope to keep the *gstD-lacZ* images in the major figures.

-For Figure 4C- Cnc mutants have an increase of ubiquitinated proteins. Can OE of Cnc just result in the clearance of ubiquitinated proteins? How is this direct?

We guess that the reviewer raised the question of whether CncC overexpression directly results in the clearance of ubiquitinated proteins in AMPK RNAi neurons. In this study, we show that overexpression of CncC upregulated the expression of the proteasomal subunit Rpn7, thereby leading to the clearance of ubiquitinated proteins in AMPK RNAi neurons. Our previous real-time PCR study also shows that the transcription factor CncC promotes the transcription of various proteasomal subunits, including *rpn3*, *rpn11*, *prosa4*, *prosa7*, *prosβ1*, *prosβ2*, *prosβ5* (Chew, et al., 2021). Taken together, our studies suggest that CncC overexpression directly upregulates the proteasomal degradation machinery to clear the ubiquitinated proteins in AMPK RNAi neurons.

As a control, we have also overexpressed CncC in $Rab5^{DN}$-overexpressing ddaC neurons. $Rab5^{DN}$ overexpression disrupts endo-lysosomal degradation pathway and leads to accumulation of ubiquitinated protein aggregates in enlarged endosomes (Zhang et al., *Dev Cell* 2014). Interestingly, CncC overexpression failed to clear the ubiquitinated protein aggregates in $Rab5^{DN}$-overexpressing ddaC neurons (Fig S6A). Thus, CncC specifically regulates proteasomal protein degradation but not endo-lysosomal protein degradation (p11).

Does AMPK RNAi in a Cnc mutant background have similar ubiquitinated proteins?

We have now conducted double RNAi knockdown for AMPK and CncC. Double knockdown of AMPK and CncC showed a similar extent of ubiquitinated protein aggregates to CncC single RNAi knockdown, consistent with the conclusion that AMPK and CncC acts in the same pathway to control proteasomal degradation. We have now included the data in the revised Fig S6B (p11).

-Overexpression of the full-length Mical was sufficient to rescue dendrite pruning defects in sox14 mutants (Kirilly et al., 2009). Can the over-expression of mical and cnc completely rescue the AMPK RNAi phenotype? This will show that Nrf2-Keap1 and mical are the main components downstream of AMPK.

We have now recombined two transgenes *UAS-CncC* and *UAS-Mical*, and introduced them in the AMPK RNAi background. Co-overexpression of CncC and Mical exhibited almost full rescue of the

AMPK RNAi phenotypes, compared to Mical or CncC overexpression alone. This new data supports the conclusion that AMPK regulates dendrite pruning via both Mical and Nrf2- Keap1 pathway. We have now included the data in the revised Fig S6D and text (p11-12).

Insulin pathway negatively regulates the Nrf2-Keap1 pathway during dendrite pruning
-One main point of the text is that insulin pathway negatively regulates the Nrf2-Keap1 pathway during dendrite pruning. However, the paper they cite (Wong et al., 2013) shows that Cullin1-based E3 ligase facilitates ddaC dendrite pruning primarily through inactivation of the InR/PI3K/TOR pathway. How does cullin1 complex fit into the model of the authors? What is the relationship between Cullin1 and AMPK?

We previously showed that the Cullin1-based E3 ligase inactivates insulin pathway by targeting Akt for ubiquitination (Wong et al., *PLOS Biol*. 2013). In the present study, we further found that the metabolic regulator AMPK inactivates insulin-TOR pathway likely via its known target TOR during dendrite pruning. Both AMPK and Cullin1 E3 ligase act downstream of ecdysone signaling. Therefore, AMPK and Cullin1 E3 ligase might act independently to ensure inactivation of insulin-TOR pathway during the larval-pupal transition, thereby facilitating dendrite pruning. The relationship between Cullin1 and AMPK is beyond the scope of this current study and awaits further investigation in the future.

AMPK promotes dendrite pruning at least partly via inhibiting insulin pathway
-Overexpression of the full-length Mical was stuffiest to rescue dendrite pruning defects in sox14 mutants (Kirilly et al., 2009). Can the overexpression of mical and inactivation of the insulin signaling pathway rescue the AMPK RNAi phenotype?

We have now recombined two transgenes *UAS-InR*^{DN} and *UAS-Mical* to conduct the rescue experiments in the AMPK RNAi background. Co-overexpression of InR^{DN} and Mical almost completely rescued the AMPK RNAi phenotypes, compared to either Mical or InR^{DN} overexpression alone. This new data support that AMPK regulates dendrite pruning via both Mical and Insulin pathway. We have now included the new data in the revised text (p14) and Figure S10C.

The AMPK-insulin pathway axis is required for ecdysone signaling to activate Nrf2-Keap1 pathway
-Figure 7C is not surprising- similar experiments were performed before (Wong et al., 2013).

No similar experiments shown in Figure 7C were performed in our previous paper (Wong et al., 2013). To our knowledge, this is the first time for us to conduct the InR^{CA}/Akt and Sox14 co-overexpression experiments. With these genetic interaction data, we strengthen the model in Figure 7D showing that AMPK-insulin pathway axis is required for ecdysone signaling to activate Nrf2-Keap1 pathway and thereby induce dendrite pruning.

Reviewer 3 Advance Summary and Potential Significance to Field:

The study by Chew et al. presents data demonstrating AMP-activated protein kinase (AMPK) acts in sensory process pruning of ddaC mechanosensory neurons during Drosophila early metamorphosis. These authors previously published the role for AMPK in this process, but here place this enzymatic function in established molecular pathways. The new data build upon the authors' recent work (Chew et al., 2021) showing the molting hormone ecdysone drives the Nrf2-Keap1 pathway to promote proteasomal degradation during pruning. Using careful genetics, the authors demonstrate AMPK works cell autonomously upstream of the Nrf2-Keap1 pathway and functions to partially inhibit the insulin pathway, known to inhibit the Nrf2-Keap1 pathway and to be negatively regulated by ecdysone signaling. Although the AMP involvement is far from clear, the authors show an AMPK role in upregulation of Mical/Headcase levels. Overall, this new study by Chew et al. effectively further elucidates a pathway that governs sensory process pruning, and the quality of the data appears generally good.

We are grateful to the reviewer for the positive assessment and helpful suggestions.

Reviewer 3 Comments for the Author:

Major Points

1. In Figure 1, the authors define 3 pruning stages (severing, fragmentation and clearance), but make no attempt to characterize where AMPK function acts in this process. The authors should test the phenotype distribution for each genotype, and present these analyses in revised figures.

We have now included the quantification chart showing severing defects and fragmentation defects in Figure S1A, as well as the definition in the Materials and Methods section (p23). In this study we focused on a cell-autonomous role of AMPK in dendrite severing and fragmentation. Since the clearance process is cell-non-autonomously mediated by the surrounding cells, we did not assess the clearance defects in this study.

2. AMPK is an evolutionarily-conserved metabolic energy sensor activated by ATP deprivation. In this context, a fat body role is well established, but it is hard to envision a comparable role in neuronal pruning. It is important that the authors establish this basic mechanistic connection.

We thank the reviewer for recognizing our study important and mechanistic.

3. A recent paper (Marzano et al., 2021) showed ecdysone-driven AMPK function promotes oxidative phosphorylation. Is the same happening here? Does this provide the missing energy link? The authors should employ the published AMPK sensor to help answer this question.

The Rumpf paper (Marzano et al., 2021) mainly describes that AMPK promotes pyruvate flux to increase use of noncarbohydrate TCA cycle fuels during dendrite pruning. In our study, we show that **first,** AMPK promotes dendrite pruning via activating Nrf2-Keap1 pathway; **second,** AMPK activates Nrf2-Keap1 pathway via antagonizing insulin pathway during dendrite pruning. Thus, this manuscript highlights a novel mechanism of AMPK action, which differs from the Rumpf paper (published on 16th Nov 2021) (Marzano et al., 2021). Therefore, we feel it is out of the scope of this manuscript to address the energy link and repeat the role of AMPK on oxidative phosphorylation that has been described in the Rumpf paper.

4. Mical/ Headcase functions are unknown. Can the authors comment on this missing link?

Mical is an F-actin-disassembly factor which regulates cytoskeletal disassembly in axonal growth and bristle elongation (Hung, et al. *Nature* 2010). However, whether Mical regulates F-action disassembly during dendrite pruning remains unknown. Headcase is upregulated by the ecdysone receptor EcR during dendrite pruning, and its exact function during dendrite pruning remains to be determined (Loncle, et al., *J Neurosci.* 2012). We have now included these comments in the revised text (p7).

The effect of AMPK on Mical expression is speculated to be via transcriptional regulation. The authors need to demonstrate this. Likewise, for AMPK regulation of the fly Nrf2 homolog CncC.

Our *mical1-lacZ* data suggest that AMPK regulates Mical expression via transcriptional regulation. Further *in vivo* experiment, such as *in vivo* fluorescence in situ hybridization (FISH) assay, is extremely challenging in our system, as there is only one ddaC neuron each hemi- segment.

As for CncC, CncC protein levels were not reduced but significantly increased in AMPK RNAi or AMPK^{KR}-expressing ddaC neurons at 6 h APF. Thus, AMPK unlikely promotes CncC translation, instead, regulates CncC activity via suppressing insulin pathway. This data also argues against the statement “loss of AMPK causes the defects in the translation of pruning factors” by the Rumpf paper (Marzano et al., 2021). We have now included the CncC expression data in Figure S5A and the text (p10).

5. The paper states “the mical1-LacZ transgene fully resembled the temporal expression of mical in ddaC neurons.” However, expression was not shown. The paper states Mical is “upregulated in ddaC neurons at the wandering (wL3) and white prepupal (WP) stages”, but mical1-LacZ is shown absent in wL3 (Fig. S3B). The authors should show comparative expression patterns.

We apologize for the imprecise description in the original text. We previously show that Mical expression is upregulated only from WP stage, whereas EcR is expressed from wL3 stage onward (Kirilly et al., *Nat Neurosci*. 2009). We have deleted "wandering wL3" in the revised text (p7). We have also included the expression pattern of Mical from eL3 to WP. Indeed, Mical upregulation coincided with the *mical1-LacZ* expression at the WP stage (Figure S3C).

Minor Points:

1. In Figures 1B-H, the authors should clarify that the red arrows point to ddaC somata.

We have now added the sentence "Red arrowheads point to the somata of ddaC neurons" in the legends of Figure 1 as well as other figures if required.

2. In Figure 5, the authors hyperactivated the insulin pathway using both O/E InRCA and O/E Akt separately to assess expression of gstD1-lacZ and Rpn7 levels, but they did not do the same for the hyperactivation of the TOR pathway. Is there a reason only O/E S6KCA was used to assess gstD1-lacZ expression while O/E TOR was used to assess Rpn7 and Ub levels?

We found that overexpression of $S6K^{CA}$ suppressed *gstD1-lacZ* expression (Figure 5A) without affecting Rpn7 expression (unpublished data). TOR overexpression downregulated Rpn7 expression (Figure 5B) and led to elevated Ub levels (Figure 5C) but did not affect *gstD-lacZ* levels (unpublished data). Moreover, our unpublished data show that while TOR^{TED} (dominant negative form) overexpression abolished the expression of phosphorylated RpS6, a readout of TOR activity, TOR overexpression did not enhance phosphorylated RpS6 levels. Thus, TOR overexpression is not sufficient to activate S6K, which can explain why TOR overexpression did not suppress *gstD1-lacZ* expression. Moreover, we have recently found that TOR overexpression is sufficient to alter Rpn7 expression and Ub levels via another downstream pathway which is independent of S6K function. We hope to continue to work on this downstream pathway and present these new findings in a separate study.

3.O/E Control and akt RNAi images are missing from Figure S7C.

We did not include the images in the original Figure S7B and S7C to save some space. We have now included these two control images in the revised Figure S7B and S7C.

4. In Figure S7B-C, the authors chose to inhibit the insulin pathway by overexpressing PI3KDN, yet they inhibited the insulin pathway by overexpressing InRDN in Figure 6B. Why was O/E InRDN not used to assess if insulin inhibition alters gstD1-lacZ expression and Rpn7 levels?

We had conducted both InR^{DN} and $PI3K^{DN}$ overexpression experiments to assess if insulin inhibition alters *gstD1-lacZ* expression and Rpn7 levels. Overexpression of InR^{DN}, like $PI3K^{DN}$, did not augment the expression levels of *gstD1-lacZ* and Rpn7 at 6 h APF, supporting the conclusion that inhibition of insulin pathway is not sufficient for the activation of Nrf2-Keap1 pathway in ddaC neurons. However, InR^{DN} overexpression caused a significant reduction in *gstD1-lacZ* levels without affecting Rpn7 levels. We speculate that InR^{DN} overexpression may have a non-specific effect on *gstD1-lacZ* expression. Like $PI3K^{DN}$, InR^{DN} overexpression did not affect the expression of Rpn7, which is important for dendrite pruning. Thus, these InR^{DN} data do not compromise the conclusion that inhibition of insulin pathway is not sufficient for the activation of Nrf2-Keap1 pathway in ddaC neurons. We have now included the InR^{DN} images and quantification in the revised Figure S7B-C.

5. There are numerous typographical and grammatical errors throughout the manuscript that need to be addressed.

We thank the reviewer for this point. We have carefully proofread the entire manuscript for several times.

Second decision letter

MS ID#: DEVELOP/2022/200536

MS TITLE: AMPK activates the Nrf2-Keap1 pathway to govern dendrite pruning via insulin pathway in Drosophila

AUTHORS: Liang Yuh Chew, Jianzheng He, Jing Lin Jack Wong, Sheng Li, and Fengwei Yu
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

In Drosophila, dendrite arborization (da) neurons is a well-studied paradigm for neuronal pruning during metamorphosis. Under the control of a pulse of ecdysone, da neurons prune away their larval dendrites by 16h after puparium formation (APF) leaving their axons intact. Potentializing a previous study from the same laboratory (Chew et al., 2021), the authors identify AMP-activated protein kinase (AMPK) as a central player in the ecdysone/Sox14 genetic cascade leading to dendrite pruning via the Nrf2-Keap1 pathway. Interestingly, and in accordance with a previous work on insect fat body involvement in growth rate (Yuan et al. 2020), the authors show that AMPK is acting through the inhibition of the insulin pathway for da dendrite pruning.

This study reveals an important mechanism to promote dendrite pruning. The manuscript is generally clearly written and the experiments used for the dissection of the genetic and biochemical pathways leading from EcR-B1 to the final effectors are well designed, but nevertheless some issues need to be addressed and are outlined below.

Comments for the author

The authors have conducted additional experiments to address my concerns satisfactorily. They have also clearly addressed all my previous comments in this revised version of the manuscript. Thus, I fully support its publication now. Jean-Maurice Dura

Reviewer 2

Advance summary and potential significance to field

I think the authors have done a good job addressing our major concerns. In response to our suggestions, the did a number of experiments and did some rewriting that helped clarify things. These include directly addressing the other AMPK paper, and showing that AMPK is not specific for all translation. Even though they did not identify AMPK first what they did for the mechanism is likely sufficient as a new advance. (I fundamentally disagree with the lead author's claim that it doesn't matter who did what first--it does.) Finally, it was reassuring that they could fully rescue the AMPK phenotypes (Figure 6D).

Comments for the author

Please see above.