

# PP2A-dependent TFEB activation is blocked by PIKfyve-induced mTORC1 activity

Junya Hasegawa, Emi Tokuda, Yao Yao, Takehiko Sasaki, Ken Inoki, and Lois Weisman

*Corresponding author(s): Junya Hasegawa, Tokyo Medical and Dental University*

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*Editor-in-Chief: Matthew Welch*

## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E21-06-0309

TITLE: PIKfyve maintains TFEB in its inactive state in the cytoplasm via inhibition of PP2A-dependent dephosphorylation of TFEB

Dear Dr. Hasegawa:

The review of your manuscript is now complete and the reviewers' comments follow below. As you will see, the reviewers felt that your manuscript was potentially of interest to the readership of MBoC but they raised a number of concerns that need to be addressed.

In view of these comments we cannot accept the manuscript in its current form but hope that you can resolve these issues in a revised manuscript. Please note that the manuscript will be sent back to the original reviewers so it will be important to address all their comments in full.

Thank you for submitting your work to MBoC.

Sincerely,

Robert Parton  
Monitoring Editor  
Molecular Biology of the Cell

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Dear Dr. Hasegawa,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office ([mboc@ascb.org](mailto:mboc@ascb.org)).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at [mboc@ascb.org](mailto:mboc@ascb.org).

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors ([www.molbiolcell.org/info-for-authors](http://www.molbiolcell.org/info-for-authors)). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: [Link Not Available](#)

Please contact us with any questions at [mboc@ascb.org](mailto:mboc@ascb.org).

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office  
[mbc@ascb.org](mailto:mbc@ascb.org)

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Reviewer #1 (Remarks to the Author):

This brief manuscript reports on the interesting observation that pharmacological inhibition of PIKfyve, a lipid kinase that produces PI3,5P2 (and possibly PI5P) in the late endosomal/ lysosomal system, causes the nuclear translocation of the lysosomal transcription factor TFEB. They further show that TFEB nuclear translocation under conditions of impaired PIKfyve activity is due to PP2A-mediated dephosphorylation of TFEB. PIKfyve activity loss also affects dephosphorylation of eIF4E but not other PP2A substrates. Based on these data the authors propose that PIKfyve serves to repress TFEB activity by inhibiting PP2A-mediated TFEB dephosphorylation at S211.

The manuscript contains a nice set of high-quality data that are of potential interest to the readers of MBoC. Some aspects need to be corroborated by additional experiments as detailed below.

1. Essentially all conclusions of this study are based on acute inhibitor experiments. Hence, the pharmacological data using Apilimod and YM should be corroborated by PIKfyve or VAC14 KD or KO, i.e. using cells from available cKO models.
2. I feel uneasy about the results derived from the application of VPS4IN1. The inhibitor is known to be unstable/ loses activity upon frozen storage. Besides 1uM seems borderline for complete inhibition of Vps34. As these results are somewhat peripheral to the message of the paper, I suggest to eliminate these data from the present Ms.
3. The effects of PP2A KD seem weaker than those of OA treatment. Does this indicate off-target activity of OA or poor efficiency? Please clarify. Moreover, rescue experiments for PP2AC are missing. Likewise, the data in Fig. 6 miss a control OE using inactive PP2AC.
4. What is the effect of Apilimod-mediated inhibition on the expression of autophagy/ lysosomal genes? One would expect upregulation of TFEB targets but this would need to be shown. What is the effect of PIKfyve loss or inhibition on other MITF family members such as TFE3? Is their activity similarly affected by PIKfyve inhibition?
5. The present dataset does not provide any insights into the physiological regulation of PP2A/ TFEB by PIKfyve, e.g. the role of nutrients, oxygen, or other kinds of stress (e.g. osmotic stress). For example, is PIKfyve activity repressed under conditions known to activate TFEB? Conversely, does hyperactivation of PIKfyve affect TFEB localization and/ or activity? Some data along this line would greatly increase the impact of the present story, although it may not be mandatory for publication.
6. The mechanism involved in the PIKfyve mediated regulation of TFEB activity remains uncertain. While the data clearly argue that PP2A is involved, the relationship between PP2A and PI(3,5)P2 is unclear. In the absence of additional data at the very least some speculation should be included in the discussion to guide further experiments.
7. Statistics: I suggest to plot individual data points rather than mere bar diagrams of the mean for all quantifications.

Reviewer #2 (Remarks to the Author):

In this manuscript, Hasegawa and colleagues investigated the role of the lipid kinase PIKfyve as a negative regulator of transcription factor EB (TFEB). The authors show that pharmacological inhibition of PIKfyve results in TFEB de-phosphorylation and nuclear translocation without affecting the phosphorylation of the mTORC1 substrate S6K. The authors further show that inhibition of PP2A, but not of calcineurin, rescues TFEB de-phosphorylation in cells treated with PIKfyve inhibitors. Based on these data, they conclude that PIKfyve regulates TFEB via modulation of PP2A activity.

Although the evidence that PIKfyve inhibition promotes TFEB activation without affecting mTORC1 activity is not novel (PMID: 29661845), the mechanism underlying this finding has remained elusive. Thus, elucidating how PIKfyve controls TFEB is potentially interesting. The manuscript is well-designed and experiments are well conducted. However, I found that the main conclusion of the authors (PIKfyve modulates TFEB by regulating PP2A activity) is not supported by solid data. The authors did not convincingly show that PIKfyve inhibition induces PP2A activation. In addition, they did not consider possible alternative mechanisms responsible for TFEB modulation by PIKfyve.

In order to clarify these points, the authors should address the following issues:

1. PIKfyve and PP2A:

A major weakness of this manuscript is the lack of formal evidence showing that PIKfyve inhibition affects PP2A activity. Although the authors clearly show that PP2A inhibition rescues TFEB phosphorylation upon treatment of cells with PIKfyve inhibitors, this finding by itself does not support the conclusion that PIKfyve inhibition promotes PP2A activation. Previous evidence has shown that TFEB continuously shuttles between the cytosol and the nucleus (PMID: 30120233 and 29992949), thus suggesting the presence of a constitutively active phosphatase that promotes TFEB nuclear translocation even in well-fed

cells. Thus, blocking the activity of a constitutive phosphatase would impair TFEB de-phosphorylation even if such activity is not induced by PIKfyve inhibition. Supporting this, the authors show that PIKfyve inhibition only weakly affected the phosphorylation of the PP2a substrate eIF4F and had no effect on the phosphorylation of other well-characterized PP2A substrates (ERK and GSK3). Together these data suggest that PIKfyve inhibition does not affect PP2A activity per-se and that pharmacological inhibition of PP2A impairs constitutive, rather than PIKfyve-induced, TFEB de-phosphorylation, thus preventing its nuclear translocation. Other in-depth mechanistic assays to measure apilimod-induced substrate-specific PP2A activity (e.g. in vitro phosphatase assays, PP2A-substrate binding, etc) are needed to formally prove that PP2A is activated by PIKfyve inhibition and to support the authors' claim that PIKfyve controls PP2A activity.

As an alternative, the authors should revise their conclusions and consider alternative mechanisms (see point 2).

## 2. PIKfyve and mTORC1:

As the authors mentioned in their introduction, a recent manuscript showed that TFEB, unlike the well-characterized mTORC1 substrates S6K and 4E-BP1, is phosphorylated by mTORC1 via a substrate-specific mechanism that is independent from Rheb and is dependent on the activity of RagC/D GTPases (PMID: 32612235). Interestingly, this pathway has been shown to be selectively modulated by perturbations of the lysosomal membrane (PMID: 32989250). Thus, the finding that PIKfyve inhibition did not affect the phosphorylation of S6K is not sufficient to claim that the effect of PIKfyve on TFEB is mTORC1-independent. It is possible, instead, that PIKfyve inhibition specifically prevents mTORC1-mediated phosphorylation of TFEB, via inhibition of RagC/D GTPases, without affecting the phosphorylation of other mTORC1 substrates. This hypothesis should be tested experimentally using specific approaches. For instance, does PIKfyve inhibition induce TFEB de-phosphorylation and nuclear translocation in cells expressing constitutively active RagC? Does PIKfyve inhibition impair either TFEB-RagC binding or TFEB lysosomal localization (which occurs upon RagC-binding) in either fed or Torin-treated cells? If the experiments above show positive results, it would be also interesting to determine whether overexpression of the FLCN-FNIP1 complex, which acts as a RagC/D activator, restores TFEB phosphorylation and cytosolic localization in cells treated with PIKfyve inhibitors.

3. Page 10, lane 186: Commenting the experiment shown in Fig 2A, the authors claim that PIKfyve inhibition impairs TFEB phosphorylation "despite the fact that other mTORC1 substrates, p-p70S6K, p-4E-BP1, and p-ULK1 are observed". However, in Figure 2 I could not find any blot showing the phosphorylation status of S6K, 4E-BP1 or ULK1. The authors should add these blots to figure 2A and show that, in the same experiment, PIKfyve inhibition impairs TFEB phosphorylation without affecting other mTORC1 substrates. In addition, the effect of PIKfyve inhibition on mTOR lysosomal localization, which is mainly dependent on RagA/B activation status, should be determined.

4. The role of PIKfyve in the modulation of TFEB has been only explored using PIKfyve inhibitors. The authors should corroborate at least some of their findings using genetic approaches (e.g. knock-down or knock-out).

## 5. References:

The role for PIKfyve in the modulation of TFEB has been previously described (Choy et al., 2018). However, the authors cite this reference for the first time on lane 119 referring to a role of PIKfyve in the maintenance of lysosomal homeostasis, without ever mentioning its role in the modulation of TFEB. The authors should properly acknowledge these findings in their introduction.

In addition, many references throughout the text are out of context and need to be fixed. Here a few examples:

Lane 77: one or more references describing how mTORC1 is regulated by Rag and Rheb GTPases should be added.

Lane 80: The evidence that TFEB is an mTORC1 substrate was first reported in manuscripts PMID: 22576015, 22343943 and 22692423, which therefore should be added in that section instead of Vega-Rubin-de-Celis et al. 2017.

Lane 87: the reference by Shen and Mizushima, 2014 (a review on autophagy), appears out of context when referring to TFEB and TFE3 (the same also applies to lane 174). Please consider substituting it with a more appropriate reference. In addition, the manuscript PMID: 24448649 was the first to report a role for TFE3 in lysosomal biogenesis and autophagy and should be added here.

Lane 89: as for lane 80, references PMID: 22576015, 22343943 and 22692423 first showed that TFEB is phosphorylated by mTORC1 and should be all added here.

6. As stated above, experiments in Figure 1 are not novel. Similarly, experiments in Figure 6 are not novel (PMID: 29945972) and are not informative to prove that PIKfyve modulates TFEB via PP2A. Finally, experiments in Figure 3 are negative data. I would suggest moving these data in supplementary figures.



November 19, 2021

Dr. Robert Parton  
Monitoring Editor  
Molecular Biology of the Cell

Manuscript# E21-06-0309

Dear Dr. Robert Parton,

We thank you and the referees for your efforts in the review of our manuscript E21-06-0309. Based on the well-informed questions from the reviewers, we made significant progress since the original submission. In light of our new findings, we changed the title of the manuscript to “PIKfyve maintains TFEB in its inactive state by enabling mTORC1 phosphorylation of TFEB at a site that is also a target of an opposing phosphatase, PP2A”. Briefly, the revised manuscript reveals that TFEB becomes dephosphorylated by release of mTOR from TFEB, while simultaneously maintaining the association of phosphatase PP2A to TFEB. Together, this results in the dephosphorylation of TFEB in PIKfyve suppressed cells. These findings provides important novel mechanistic insights into how PI(3,5)P<sub>2</sub> and/or PI5P regulates the intracellular signaling.

As part of the revision, we added a new author, Emi Tokuda, who contributed to obtaining the new data. Detailed responses to each comment are attached below. We hope that this paper is now suitable for publication in *Molecular Biology of the Cell*.

Sincerely,

Junya Hasegawa  
Lois S. Weisman

## **Reply to reviewers:**

**We would like to thank both reviewers for his/her valuable comments on our manuscript. All the comments and suggestions were very helpful and insightful. We hope that this revised manuscript will be judged suitable for publication.**

## **Specific response:**

(Reviewer's comments are in italics.)

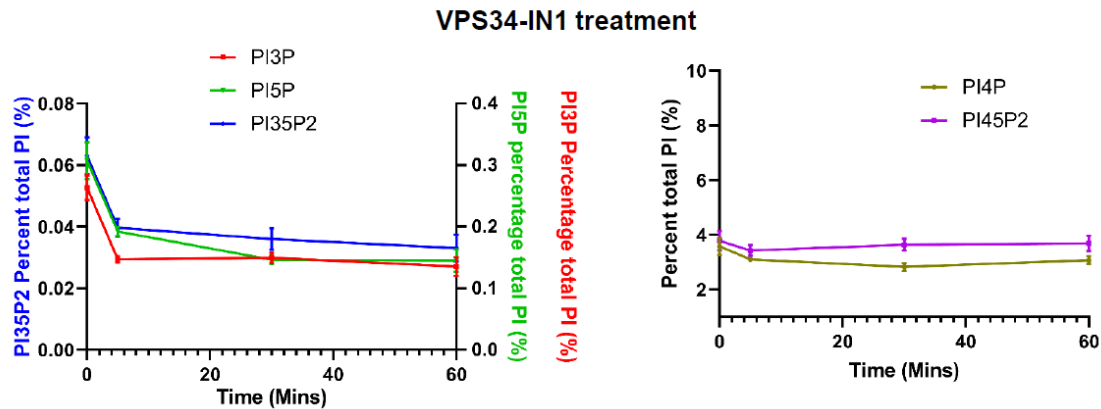
### **Reviewer #1**

*1. Essentially all conclusions of this study are based on acute inhibitor experiments. Hence, the pharmacological data using Apilimod and YM should be corroborated by PIKFYVE or VAC14 KD or KO, i.e. using cells from available cKO models.*

**We added new data showing that PIKfyve knockdown also induces the dephosphorylation of TFEB as well as the nuclear localization of TFEB (Figure 2, C-E).**

*2. I feel uneasy about the results derived from the application of VPS4IN1. The inhibitor is known to be unstable/ loses activity upon frozen storage. Besides 1uM seems borderline for complete inhibition of Vps34. As these results are somewhat peripheral to the message of the paper, I suggest to eliminate these data from the present Ms.*

**We have extensive experience with VPS34IN1, and store VPS34IN1 in small aliquots to avoid freeze-thaw cycles. In a recent manuscript on BioRxiv we show the effect of VPS34-IN1 on PI3P and PI(3,5)P2 levels (<https://www.biorxiv.org/content/10.1101/2021.05.25.445615v1>). In Figure S6 of that paper (also shown below), we found that treatment of cells with VPS34-IN1 over 120 minutes decreases the levels of PI3P and PI(3,5)P2 to half of their normal levels. These are the same conditions that we used in this current manuscript. This shows that the inhibitor is potent and lowers PI3P levels, as well as lowering PI(3,5)P2 levels.**



3. The effects of PP2A KD seem weaker than those of OA treatment. Does this indicate off-target activity of OA or poor efficiency? Please clarify. Moreover, rescue experiments for PP2AC are missing. Likewise, the data in Fig. 6 miss a control OE using inactive PP2AC.

Okadaic acid is known to have off-target effects which is why we also included PP2A KD experiments. However, PP2A knockdown as well as okadaic acid treatment for a long time affects cell survival. In this manuscript, we transfected siRNAs against PPP2CA and PPP2CB into HeLa cells for only 48 hours to avoid secondary effects (Figure 4). However, we found that transfection with the siRNAs for over 72 hours was deleterious to the cells (data not shown). Therefore, we analyzed the effects of partial KD of PP2A. Importantly, as suggested, we added data from rescue experiments (Supplemental figure 8) and the data showing that overexpression of wild-type PPP2CA, but not the inactive D85N mutant, results in the dephosphorylation of TFEB (Figure 5).

4. What is the effect of Apilimod-mediated inhibition on the expression of autophagy/lysosomal genes? One would expect upregulation of TFEB targets but this would need to be shown. What is the effect of PIKfyve loss or inhibition on other MITF family members such as TFE3? Is their activity similarly affected by PIKfyve inhibition?

We agree that this is an important question. We performed real-time PCR to test



**the effect of PIKfyve inhibitors on the expression of TFEB target genes. We found that each of the lysosomal and autophagic genes tested were upregulated in apilimod- or YM201636-treated cells (Supplemental figure 4). Furthermore, we found that PIKfyve inhibitors induce the nuclear translocation of TFE3 (Supplemental figure 3), suggesting that the mechanism by which PIKfyve controls the localization of TFE3 is similar to that of TFEB.**

*5. The present dataset does not provide any insights into the physiological regulation of PP2A/ TFEB by PIKFYVE, e.g. the role of nutrients, oxygen, or other kinds of stress (e.g.osmotic stress). For example, is PIKFYVE activity repressed under conditions known to activate TFEB? Conversely, does hyperactivation of PIKFYVE affect TFEB localiaztion and/ or activity? Some data along this line would greatly increase the impact of the present story, although it may not be mandatory for publication.*

**We now add new data which reveal that the expression of the PIKfyve-KYA hyperactive mutant, which elevates the levels of PI(3,5)P2 and/or PI5P, suppresses the nuclear localization of TFEB during starvation (Supplemental figure 5). This result, combined with data showing that PIKfyve inhibition causes the nuclear translocation of TFEB, indicates that the intracellular levels of PI(3,5)P2 or PI5P contribute to controlling the nuclear localization of TFEB.**

*6. The mechanism involved in the PIKFYVE mediated regulation of TFEB activity remains uncertain. While the data clearly argue that PP2A is involved, the relationship between PP2A and PI(3,5)P2 is unclear. In the absence of additional data at the very least some speculation should be included in the discussion to guide further experiments.*

**We agree that in the original manuscript, the role of PP2A during PIKfyve suppression was not clear. We now find that while mTORC1 is not globally inhibited during inhibition of PIKfyve, TORC1 activity on TFEB is impaired. We added these new findings (Figures 6 and 7) in this revised manuscript. We found that the interaction between TFEB and mTOR is suppressed in PIKfyve-inhibited cells (Figure 7). Moreover, the localization of mTOR is shifted from lysosomes to the cytoplasm during PIKfyve suppression (Figure 7). In contrast, the binding of**

**TFEB with PP2A remains intact during apilimod treatment. These data suggest that the intracellular levels of PI(3,5)P2 and/or PI5P likely contribute to the localization of mTORC1. When PIKfyve is inhibited, mTORC1 is dissociated from TFEB, but the binding of TFEB with PP2A is maintained. Thus, the dephosphorylation of TFEB by PP2A becomes dominant over the potential to be phosphorylated by mTORC1.**

*7. Statistics: I suggest to plot individual data points rather than mere bar diagrams of the mean for all quantifications.*

**We now present individual data points in all the graphs.**

## **Reviewer #2**

### *1. PIKfyve and PP2A:*

*A major weakness of this manuscript is the lack of formal evidence showing that PIKfyve inhibition affects PP2A activity. Although the authors clearly show that PP2A inhibition rescues TFEB phosphorylation upon treatment of cells with PIKfyve inhibitors, this finding by itself does not support the conclusion that PIKfyve inhibition promotes PP2A activation. Previous evidence has shown that TFEB continuously shuttles between the cytosol and the nucleus (PMID: 30120233 and 29992949), thus suggesting the presence of a constitutively active phosphatase that promotes TFEB nuclear translocation even in well-fed cells. Thus, blocking the activity of a constitutive phosphatase would impair TFEB de-phosphorylation even if such activity is not induced by PIKfyve inhibition. Supporting this, the authors show that PIKfyve inhibition only weakly affected the phosphorylation of the PP2a substrate eIF4F and had no effect on the phosphorylation of other well-characterized PP2A substrates (ERK and GSK3). Together these data suggest that PIKfyve inhibition does not affect PP2A activity per-se and that pharmacological inhibition of PP2A impairs constitutive, rather than PIKfyve-induced, TFEB de-phosphorylation, thus preventing its nuclear translocation. Other in-depth mechanistic assays to measure apilimod-induced substrate-specific PP2A activity (e.g. in vitro phosphatase assays, PP2A-substrate binding, etc) are needed to formally prove that PP2A is activated by PIKfyve inhibition and to support*

*the authors' claim that PIKfyve controls PP2A activity.*

*As an alternative, the authors should revise their conclusions and consider alternative mechanisms (see point 2).*

**We agree with these comments. During the revision of this manuscript, we uncovered evidence that suggests that the change in TFEB phosphorylation may be due to a defect in the interaction of mTORC1 with TFEB. Our new data in Figure 7 shows that PIKfyve inhibition disrupts the interaction between TFEB and mTORC1, but does not impact TFEB interaction with PP2A. Moreover, we find a significant decrease in mTOR association with the lysosome upon PIKfyve inhibition (Figure 7, E and F). These new findings suggest a model that PI(3,5)P<sub>2</sub> and/or PI5P are required for mTORC1 association with TFEB, and also contribute to the localization of mTORC1.**

## *2. PIKfyve and mTORC1:*

*As the authors mentioned in their introduction, a recent manuscript showed that TFEB, unlike the well-characterized mTORC1 substrates S6K and 4E-BP1, is phosphorylated by mTORC1 via a substrate-specific mechanism that is independent from Rheb and is dependent on the activity of RagC/D GTPases (PMID: 32612235). Interestingly, this pathway has been shown to be selectively modulated by perturbations of the lysosomal membrane (PMID: 32989250). Thus, the finding that PIKfyve inhibition did not affect the phosphorylation of S6K is not sufficient to claim that the effect of PIKfyve on TFEB is mTORC1-independent. It is possible, instead, that PIKfyve inhibition specifically prevents mTORC1-mediated phosphorylation of TFEB, via inhibition of RagC/D GTPases, without affecting the phosphorylation of other mTORC1 substrates. This hypothesis should be tested experimentally using specific approaches. For instance, does PIKfyve inhibition induce TFEB de-phosphorylation and nuclear translocation in cells expressing constitutively active RagC? Does PIKfyve inhibition impair either TFEB-RagC binding or TFEB lysosomal localization (which occurs upon RagC-binding) in either fed or Torin-treated cells? If the experiments above show positive results, it would be also interesting to determine whether overexpression of the FLCN-FNIP1 complex, which acts as a RagC/D activator, restores TFEB phosphorylation and cytosolic localization in cells treated with PIKfyve inhibitors.*

**These are important questions. As suggested, we tested the impact of PIKfyve**

**inhibition on TFEB localization in cells expressing RagA (QL) and RagC (SN) mutants (constitutively active). Importantly, we found that dephosphorylation of TFEB by apilimod was suppressed by expression of the constitutively active forms of Rag proteins (Figure 6). This suggests that PIKfyve may act upstream of Rag small GTPases to support lysosomal mTORC1 localization and TFEB phosphorylation. These new findings raise the possibility that PIKfyve inhibition may reduce the activity of Rag GTPases and the subsequent release of mTORC1 from the Rag GTPase complex. This possibility as well as the potential involvement of other regulators including the FLCN-FNIP1 complex are presented in the discussion section.**

*3. Page 10, lane 186: Commenting the experiment shown in Fig 2A, the authors claim that PIKfyve inhibition impairs TFEB phosphorylation "despite the fact that other mTORC1 substrates, p-p70S6K, p-4E-BP1, and p-ULK1 are observed". However, in Figure 2 I could not find any blot showing the phosphorylation status of S6K, 4E-BP1 or ULK1. The authors should add these blots to figure 2A and show that, in the same experiment, PIKfyve inhibition impairs TFEB phosphorylation without affecting other mTORC1 substrates. In addition, the effect of PIKfyve inhibition on mTOR lysosomal localization, which is mainly dependent on RagA/B activation status, should be determined.*

**Immunoblots of (phospho-TFEB and TFEB) shown in old Figure 2A were performed in the same experiments with the other mTORC1 substrates, and are now included in Figure 1A .**

**In addition, we added new key results that RagA/C activation suppresses the dephosphorylation of TFEB by apilimod treatment (Figure 6). In addition, we showed that apilimod treatment causes the cytoplasmic localization of mTOR (Figure 7, E and F). These data suggest that PIKfyve may act upstream of Rag small GTPases and support lysosomal mTORC1 localization and mTOR interaction with TFEB.**

*4. The role of PIKfyve in the modulation of TFEB has been only explored using PIKfyve inhibitors. The authors should corroborate at least some of their findings using genetic approaches (e.g. knock-down or knock-out).*

**We added data showing that PIKfyve knockdown also induces the phosphorylation of TFEB as well as the nuclear localization of TFEB (Figure 2, C-E).**

*5. References:*

*The role for PIKfyve in the modulation of TFEB has been previously described (Choy et al., 2018). However, the authors cite this reference for the first time on lane 119 referring to a role of PIKfyve in the maintenance of lysosomal homeostasis, without ever mentioning its role in the modulation of TFEB. The authors should properly acknowledge these findings in their introduction.*

**We have addressed this inadvertent omission. We have added this citation and previous findings to the introduction. “Moreover, inhibition of PIKfyve induces the nuclear translocation of TFEB (Choy et al., 2018), however, the molecular mechanisms underlying PIKfyve-dependent regulation of TFEB regulation were unclear.”**

*In addition, many references throughout the text are out of context and need to be fixed. Here a few examples:*

*Lane 77: one or more references describing how mTORC1 is regulated by Rag and Rheb GTPases should be added.*

*Lane 80: The evidence that TFEB is an mTORC1 substrate was first reported in manuscripts PMID: 22576015, 22343943 and 22692423, which therefore should be added in that section instead of Vega-Rubin-de-Celis et al. 2017.*

*Lane 87: the reference by Shen and Mizushima, 2014 (a review on autophagy), appears out of context when referring to TFEB and TFE3 (the same also applies to lane 174). Please consider substituting it with a more appropriate reference. In addition, the manuscript PMID: 24448649 was the first to report a role for TFE3 in lysosomal biogenesis and autophagy and should be added here.*

*Lane 89: as for lane 80, references PMID: 22576015, 22343943 and 22692423 first showed that TFEB is phosphorylated by mTORC1 and should be all added here.*

**Thank you for your comments. We have added and corrected the corresponding references in the text.**

6. As stated above, experiments in Figure 1 are not novel. Similarly, experiments in Figure 6 are not novel (PMID: 29945972) and are not informative to prove that PIKfyve modulates TFEB via PP2A. Finally, experiments in Figure 3 are negative data. I would suggest moving these data in supplementary figures.

**We moved “Figure 3” to “Supplemental Figure 6”. While Figures 1 and 6 contain some data that has been reported previously, they also contain new information that is key to the current manuscript. While it was previously shown that inhibition of PIKfyve did not impact the phosphorylation status of p70S6K, to our knowledge, additional mTORC1 substrates, 4E-BP1 and ULK1 were not previously tested. That these also were not impacted provides a broader view of the impact of PIKfyve inhibition on mTORC1 activity. In addition, the data showing that factors in serum do not affect the stability of apilimod or contribute to the lack of effect of apilimod on mTORC1 activity (Figure 1C) are important.**

**While it was previously shown that activation of PPP2CA plays a role in the activation TFEB during arsenite-induced oxidative stress, it was not clear whether this role was particular to those conditions. Moreover, to our knowledge, that paper (PMID: 29945972) is currently the only publication to suggest that PP2A acts on mTORC1 sites on TFEB. Current reviews still cite calcineurin as the only phosphatase that acts on TFEB. In addition, while Martina et al. show that wild-type PPP2CA plays a role in the dephosphorylation of TFEB, in Figure 6, we show the impact of the inactive mutant, D85N.**

RE: Manuscript #E21-06-0309R

TITLE: "PIKfyve maintains TFEB in its inactive state by enabling mTORC1 phosphorylation of TFEB at a site that is also a target of an opposing phosphatase, PP2A"

Dear Dr. Hasegawa,

I am very pleased to say that the reviewers are now positive about your revised manuscript. Reviewer 2 did suggest some text improvements, including clarifying throughout the manuscript the role of PIKfyve on mTORC1 vs PP2A activity, and with these minor revisions I would be happy to accept the manuscript for publication. I look forward to receiving a suitably revised manuscript.

Best regards,

Rob Parton

Monitoring Editor  
Molecular Biology of the Cell

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Dear Dr. Hasegawa,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office ([mboc@ascb.org](mailto:mboc@ascb.org)).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at [mboc@ascb.org](mailto:mboc@ascb.org).

In preparing your revised manuscript, please follow the instruction in the Information for Authors ([www.molbiolcell.org/info-for-authors](http://www.molbiolcell.org/info-for-authors)). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):  
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Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at [www.molbiolcell.org/science-sketches](http://www.molbiolcell.org/science-sketches). Please contact [mboc@ascb.org](mailto:mboc@ascb.org) if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office  
[mbc@ascb.org](mailto:mbc@ascb.org)

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Reviewer #1 (Remarks to the Author):

The authors have done a beautiful job in revising their Ms. I now enthusiastically endorse publication in MBoC.

Reviewer #2 (Remarks to the Author):

The authors addressed all my previous concerns. I found the manuscript greatly improved. In my opinion there is only one major concern that raises confusion about the role of PIKfyve on mTORC1 vs PP2A activity. While the authors clearly state in their rebuttal that PIKfyve inhibition prevents mTORC1-mediated phosphorylation of TFEB without affecting PP2A activity (which in my opinion is in agreement with the data), in the manuscript they instead mention several times that PIKfyve inhibition also induces PP2A activation (see lanes 268, 284, 299, 362 for a few examples). Based on the new results provided by the authors in this revision, the latter conclusion is wrong and should be corrected. The authors should clearly state in the manuscript that PIKfyve inhibition affects TFEB activity by preventing its mTORC1-mediated phosphorylation. On the contrary, PP2a activity, although required to induce TFEB de-phosphorylation, is constitutive and is not affected by PIKfyve inhibition. This is an important point that is required to provide a clear message to the paper. In this respect, I also believe that the title of the manuscript should be changed.

Finally, I have some minor concerns/suggestions:

Lane 84: The authors state: "In response to amino acid stimulation, the guanosine triphosphate (GTP)-bound small GTPase protein Rag heterodimer, which consists of RagA or RagB and RagC or RagD, is activated and recruits cytoplasmic mTORC1 to the surface of the lysosomal membrane".

This sentence is misleading as the Rag GTPases are known to form an active dimer when RagA/B are GTP-bound and RagC/D are GDP-bound. The sentence should be adjusted accordingly.

Lane 100: "Under nutrient-rich conditions, TFEB is phosphorylated at Ser-142 and Ser-211 by mTORC1, which promotes TFEB binding to 14-3-3 proteins (Roczniak-Ferguson et al., 2012) and/or its localization to lysosomes through binding to activated Rag GTPase proteins (Settembre et al., 2012a; Martina and Puertollano, 2013; Martina et al., 2014)".

This sentence is misleading to me for two reasons: first, TFEB phosphorylation at S142 does not promote 14-3-3 binding but promotes its nuclear export (PMID: 30120233, 29992949). Second, phosphorylation does not promote localization to lysosomes and Rag GTPase binding. On the contrary, it is binding to Rag GTPases that serves as a substrate-recruitment mechanism that allows TFEB phosphorylation (PMID: 32612235, 23401004). The authors should rephrase this sentence accordingly.

Lane 106: "In the absence of growth factors and amino acids, TFEB is dephosphorylated and translocates to the nucleus...". TFEB is known to be insensitive to growth factor availability. The authors may want to say instead: "In the absence of nutrients, TFEB..."

Lane 360: "Our studies suggest that PIKfyve may enhance mTORC1-dependent TFEB phosphorylation by acting upstream or in parallel to Rag GTPases".

The evidence that the expression of active Rag GTPases prevents apilimod-induced TFEB nuclear translocation indicates that PIKfyve acts upstream, rather than in parallel, to Rag GTPases.





December 22, 2021

Dr. Robert Parton  
Monitoring Editor  
Molecular Biology of the Cell

Manuscript# E21-06-0309R

Dear Dr. Robert Parton,

We thank you and the referees for your efforts in the review of our manuscript E21-06-0309R. Based on suggestions from the reviewer, we made several suggested changes to the text of the manuscript. In addition, along the lines suggested by the reviewer, we changed the title of the manuscript to “PP2A-dependent TFEB activation is blocked by PIKfyve-induced mTORC1 activity”.

We thank the reviewer for their multiple informative comments. Detailed responses to each comment are attached below. We believe that the new alterations have improved the manuscript, and hope that this paper is now suitable for publication in *Molecular Biology of the Cell*.

Sincerely,

Junya Hasegawa  
Lois S. Weisman

## **Reply to reviewers:**

We thank both reviewers for their efforts on our revised manuscript. We hope that the further revisions outlined below have improved the manuscript and that the manuscript is now suitable for publication.

### **Reviewer #2**

*In my opinion there is only one major concern that raises confusion about the role of PIKfyve on mTORC1 vs PP2A activity. While the authors clearly state in their rebuttal that PIKfyve inhibition prevents mTORC1-mediated phosphorylation of TFEB without affecting PP2A activity (which in my opinion is in agreement with the data), in the manuscript they instead mention several times that PIKfyve inhibition also induces PP2A activation (see lanes 268, 284, 299, 362 for a few examples). Based on the new results provided by the authors in this revision, the latter conclusion is wrong and should be corrected. The authors should clearly state in the manuscript that PIKfyve inhibition affects TFEB activity by preventing its mTORC1-mediated phosphorylation. On the contrary, PP2a activity, although required to induce TFEB de-phosphorylation, is constitutive and is not affected by PIKfyve inhibition. This is an important point that is required to provide a clear message to the paper. In this respect, I also believe that the title of the manuscript should be changed.*

We agree with these points. We now clarify in multiple places throughout the manuscript that PIKfyve inhibition is not promoting PP2A activity but rather is acting via loss of mTORC1 activity on TFEB.

*Lane 84: The authors state: "In response to amino acid stimulation, the guanosine triphosphate (GTP)-bound small GTPase protein Rag heterodimer, which consists of RagA or RagB and RagC or RagD, is activated and recruits cytoplasmic mTORC1 to the surface of the lysosomal membrane".*

*This sentence is misleading as the Rag GTPases are known to form an active dimer when RagA/B are GTP-bound and RagC/D are GDP-bound. The sentence should be adjusted accordingly.*

We agree. We made the suggested changes. This section now reads "In response to

amino acid stimulation, the Rag small GTPases which exist as heterodimers, become active when the complex contains GTP-bound RagA or RagB with GDP-bound RagC or RagD.”

*Lane 100: "Under nutrient-rich conditions, TFEB is phosphorylated at Ser-142 and Ser-211 by mTORC1, which promotes TFEB binding to 14-3-3 proteins (Roczniak-Ferguson et al., 2012) and/or its localization to lysosomes through binding to activated Rag GTPase proteins (Settembre et al., 2012a; Martina and Puertollano, 2013; Martina et al., 2014)".*

*This sentence is misleading to me for two reasons: first, TFEB phosphorylation at S142 does not promote 14-3-3 binding but promotes its nuclear export (PMID: 30120233, 29992949). Second, phosphorylation does not promote localization to lysosomes and Rag GTPase binding. On the contrary, it is binding to Rag GTPases that serves as a substrate-recruitment mechanism that allows TFEB phosphorylation (PMID: 32612235, 23401004). The authors should rephrase this sentence accordingly.*

Yes, these are critical points. We have now changed this section to read “Under nutrient-rich conditions, TFEB binding to Rag GTPases results in a specific substrate-dependent recruitment mechanism and results in TORC1-dependent phosphorylation of TFEB (Napolitano *et al.*, 2020). mTORC1 phosphorylates TFEB at multiple sites including Ser-142 and Ser-211 (Roczniak-Ferguson *et al.*, 2012; Settembre *et al.*, 2012). TFEB phosphorylation at Ser-211 promotes TFEB binding to 14-3-3 proteins (Settembre *et al.*, 2012; Martina and Puertollano, 2013; Martina *et al.*, 2014) and this phosphorylation likely masks a nuclear localization signal, and results in the retention of TFEB in the cytoplasm (Roczniak-Ferguson *et al.*, 2012). In contrast, phosphorylation at Ser-142 induces the nuclear export of TFEB (Li *et al.*, 2018; Napolitano *et al.*, 2018).” In addition, we added the following sentence to the discussion “In support of this hypothesis, mTORC1 phosphorylation of TFEB requires the amino-acid-mediated activation of Rag GTPases, but is insensitive to Rheb (Napolitano *et al.*, 2020).”.

Note also that we added (PMID 32612235 and 23401004).

*Lane 106: "In the absence of growth factors and amino acids, TFEB is dephosphorylated and translocates to the nucleus..."*

*TFEB is known to be insensitive to growth factor availability. The authors may want to say instead: "In the absence of nutrients, TFEB..."*

Thank you for pointing this out. We changed "In the absence of growth factors and amino acids," to "In the absence of nutrients,"

*Lane 360: "Our studies suggest that PIKfyve may enhance mTORC1-dependent TFEB phosphorylation by acting upstream or in parallel to Rag GTPases".*

*The evidence that the expression of active Rag GTPases prevents apilimod-induced TFEB nuclear translocation indicates that PIKfyve acts upstream, rather than in parallel, to Rag GTPases.*

Yes, we agree, and changed the manuscript accordingly.

RE: Manuscript #E21-06-0309RR

TITLE: "PP2A-dependent TFEB activation is blocked by PIKfyve-induced mTORC1 activity"

Dear Dr. Hasegawa:

I am pleased to accept your manuscript for publication in *Molecular Biology of the Cell*.

Sincerely,  
Robert Parton  
Monitoring Editor  
*Molecular Biology of the Cell*

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Dear Dr. Hasegawa:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at [www.molbiolcell.org/toc/mboc/0/0](http://www.molbiolcell.org/toc/mboc/0/0) is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office  
[mbc@ascb.org](mailto:mbc@ascb.org)

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