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PAQR3 controls autophagy by integrating AMPK signaling to enhance ATG14L-associated PI3K activity

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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.)

1st Editorial Decision   23 September 2015

Thank you for submitting your manuscript entitled 'PAQR3 controls autophagy by enhancing ATG14L-associated PI3K complex formation via AMPK-mediated phosphorylation'. I have now received reports from all referees, which are enclosed below.

As you will see, the referees acknowledge that your findings will be of interest to the field. However, they raise a number of concerns, which need to be addressed to better support your conclusions and to strengthen your manuscript. Given the interest in the topic, I would like to invite you to submit a revised version of the manuscript to us, addressing all issues raised by the referees.

Importantly,
- Referee #1 notes that the relationship of PAQR3 to NRBF2 needs to be analyzed.
- More information on the interactions and more direct data for this are needed (see reports from referee #1 and #2).
- Referee #2 further notes that the localization data need to be extended.
- A potential neuronal phenotype of the knock-out mice should be analyzed (see report from referee #2) and discussed.
- The requested controls from referee #3 need to be fully addressed.
- Referee #2 also would appreciate more data regarding the mechanism underlying increased Vps34 activity in the context of PAQR3. I don't know whether you already have data at hand addressing this concern, but anything that you can add would clearly strengthen your manuscript.

Please note that a revised version will be sent back to all referees, and that I would need strong

support from them in order to move forward with the paper here. Please contact me in case of other questions regarding the revision of your manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

This is a very interesting study that reports PAQR3 as a component of Atg14L associated beclin 1-VPS34 complex that regulate autophagic activity via AMPK-mediated phosphorylation. As a Golgi resident protein, PAQR3 seems to provide a scaffold for VPS34-beclin 1 complex and integrate AMPK signaling for autophagy regulation in response to cellular energy levels. The study provides insight into autophagy regulation through AMP-VPS34 in addition to known AMPK-ULK1 signaling. Coincidently, the structural and functional role for PAQR3 in regulating VPS34 component assembly and kinase activity resembles largely another autophagy related protein NRBF2, which is a yeast Atg38 homologue reported previously by at least four groups (Interestingly, the current paper has not mentioned this protein at all). The reviewer is wondering if the two proteins PAQR3 and NRBF2 are related at all. For example, are PAQR3 and NRBF2 functionally redundant? Whether the two proteins are both required for holding the VPS34 Atg14L complex together?

Other concerns:

1. In Figure S1D, rapamysin-induced autophagy is effectively inhibited in PAQR3 deleted cells, suggesting that PAQR3 is required for autophagy induced by mTOR inhibition. Paradoxically the authors claimed that the regulation of PAQR3 on autophagy is mTOR independent.

2. The authors claimed that PAQR3, Atg14L and beclin 1 are in a "ternary" protein complex. What is the exact evidence for the "ternary" complex without structural analysis?

3. The mapping of the binding domains in PAQR3, Atg14L or beclin 1 proteins were performed in cell culture. Therefore, it is unclear whether they directly interact with each other. Other proteins could mediate PAQR3 interaction with Atg14L or beclin 1. It is also hardly convincing that Atg14L and beclin 1 occupy a narrow 40 aa N-terminal sequence of PAQR3 without structural evidence.

4. There is no introduction of PAQR3 KO mice (e.g. literature) at all. If it is the first time to characterize the KO mice, necessary information is required for the confirmation of a true KO and any phenotypes (if there is, such as developmental or adult) should also be mentioned.

5. There are numerous places that have awkward expression, such as "capacitance", "PI3P machine", etc. The authors are recommended to consult native English speakers for appropriate expression.

Referee #2:

Xu et al. showed that a Golgi-localized transmembrane protein PAQR3 functions as a scaffold protein that facilitates formation of ATG14 but not UVRAG-linked VPS34 complex, leading to enhanced PI3P production and autophagy activation. In addition, AMPK phosphorylates PAQR3 at T32 and switches on PI3P production after glucose starvation. Deletion of PAQR3 in mice leads to reduction of exercise-induced autophagy. The study is potentially interesting given the novelty and specific regulation of autophagy specific PI3K activity. The data quality is good in general. However, several concerns need to to be addressed before publication.

1. The localization of PAQR3 is not clear. The authors showed that GFP-PAQR3 almost perfectly colocalizes with LC3 in Fig. 1A, but very limited colocalization of GFP-PAQR3 with LC3 in Fig. S1F. How to explain these discrepancies? Does PAQR3 normally localize to Golgi? These staining

should be shown in Fig. 1A as controls. If the translocation of PAQR3 from Golgi to autophagosomes upon glucose starvation is clear, that will become a strong supporting evidence for their hypothesis.

2. The authors concluded that PAQR3 is a constitutive component of the ATG14/Beclin1/VPS34 complex, mutually exclusive with UVRAG, mainly based on co-IP experiments. However, PAQR3 is not presented as a stoichiometric subunit of PI3KC3 in published literatures (Sun PNAS 2008, Matsunaga NCB 2009, Zhong NCB 2009). The conclusion could be strengthened if the authors are able to detect ATG14/Beclin1/VPS34 as stoichiometric subunits of PAQR3 cellular complex. Also, in vitro pull-down assay using recombinant proteins will help to elucidate if these interactions are direct.

3. If PAQR3 is a constitutive component of the ATG14/Beclin1/VPS34 complex even in the absence of autophagic stress, can the authors detect the Golgi localization of ATG14/Beclin1/VPS34 complex? How is PAQR3 attracted to autophagosomes/ phagophores upon glucose starvation?

4. PAQR3 T32 phosphorylation by AMPK is potentially important. However, how this modification affect PI3K lipid kinase activity is not elaborated in this study. The detailed mechanism will be helpful to clarify the significance of this phosphorylation event.

5. The authors showed that PAQR3 is required for exercise-induced autophagy. How about the neurological phenotype of PAQR3 knockout mice? The neurodegenerative phenotypes are well documented in autophagy deficient mice.

Minor points:

6. In Figure 1F, it is striking to observe essentially no LC3 puncta in PAQR3 KO cells upon GS, since residual LC3 lipidation could still be observed (Fig. 1B). Is this image representative?

7. In Fig. 1D, quantitative analysis of autophagic vacuoles in EM images should be performed.

8. In Fig. 2H, left bottom panel, one mutant (del41-60) is missing. Is this mutant protein expressed?

9. In Fig. 3F, this experiment lacks a negative control, seems all presented proteins are reduced upon depletion. This could be a non-specific artifact caused by antibody depletion.

10. In Fig. 7F, this data is not convincing. Fraction 1 and 2 could be protein aggregates rather then real protein complexes. Can the authors repeat the fractionation assay in ATG14 or Beclin1 knockdown cells? It is not well documented if the complex could be disrupted by missing certain subunits.

Referee #3:

The manuscript reports that the protein PAQR3 has a pro-macroautophagic role upstream of PI3P synthesis. PI3P is a signaling lipid essential for autophagosome formation. The authors went on to show that the transmembrane protein PAQR3 is an interaction partner of the class III PI3K complex 1. The class III PI3K complex 1 contains the VPS34, VPS15, Beclin1 and ATG14 subunits. The authors show that PAQR3 interacts via two adjacent regions in its N-terminus with Beclin1 and ATG14, respectively. The interactions appear to promote the assembly of the complex and to stimulate PI3K activity. In contrast, assembly of the class III PI3K complex 2 which contain the UVRAG subunit instead of ATG14 is not promoted by PAQR3. PAQR3 is phosphorylated by AMPK on threonine 32. This phosphorylation is not required for the PI3K complex assembly activity but for the lipid kinase stimulatory activity of PAQR3. Phosphorylation of T32 is also shown to be required for the autophagy promoting effect of PAQR3 as measured by LC3-II and p62 accumulation. Finally, the authors show that PAQR3 is required for exercise-induced autophagy in skeletal muscle and liver tissue (again as measured by LC3-II and p62 accumulation) and that in the absence of PAQR3 the subunits of the class III PI3K complex 1 are shifted to lower molecular weight fraction.

In summary the manuscript by Xu et al. is a very solid and well-conducted study. The data are of very high quality. The paper will be interesting for people working on autophagy and the PI3K in particular. I have only a few comments:

1. The authors write "In contrast, the autophagic activity of a PAQR3-stably transfected cell line was profoundly augmented compared to the wild type cells (Figure S1E)." In a recent study it was shown that PAQR3 overexpression causes disruption of the Golgi (Hewavitharana, Cell Signal, 2015 PMID: 26327583). Could the elevated level of autophagy seen in PAQR3 overexpressing cells be the results of Golgi fragmentation and ER stress caused by overexpression of a transmembrane protein? Is the Golgi apparatus intact the cells the authors used?

2. Most of the experiments are done with glucose starvation whereas amino acid starvation is the autophagic inducer most commonly used in the autophagy field. In fact the authors show that PAQR3 is required for full amino-acid and rapamycin-induced autophagy (Figure S1). Is T32A phosphorylation also important for amino-acid starvation-induced autophagy and increased PI3K activity?

3. The authors measure autophagic activity by accumulation of LC3-II (the lipidated form of LC3). However, in their western blots only one LC3 band is visible. How do the authors know that this is LC3-II and not LC3-I (the non-lipidated form).

4. The authors used a N-terminal fragment of PAQR3, combined it with ATG14L and added AMPK to show that AMPK directly phosphorylates PAQR3 (Figure 5E). It is not clear to me how Flag-ATG14L was purified for this experiment (please add to the methods) but the authors should include an additional control where they add Flag-ATG14L and a catalytic dead AMPK (or no AMPK) in order to exclude that the phosphorylation they detect is not mediated by another kinase co-purifying with Flag-ATG14L.

5. The experiment shown in Figure 6G in which the authors show the impact of the PAQR3 T32A mutation should be repeated with the inclusion of a lysosomal inhibitor such as chlorquine or bafilomycin.

6. In Figure 7F the authors show gel filtration profiles of the PI3K complex subunits using lysates from wild type and PAQR3 deficient livers. The result indicates that PAQR3 is required for the presence of these subunits in fractions 1 and 2. If PAQR3 functions as scaffold, as suggested by the authors the protein should also be present in these fractions. Can the authors provide evidence for this?

7. The authors write on page 22 "PAQR3-deleted mice had an apparent reduction in exercise-induced autophagy as assayed by immunohistochemical staining for LC3 in both skeletal muscle and liver (Figure 7A~7B)". An increase in the LC3 level does not directly show enhanced autophagy. Thus the authors should tone down this statement.

8. The authors should show the expression levels of the PAQR3 T32A and T32E mutants compared to the wild type protein.

9. On page 16, first line S4B must be S2B.

1st Revision - authors' response   13 December 2015

Response to Reviewers

Reviewer #1:

This is a very interesting study that reports PAQR3 as a component of Atg14L associated Beclin1-VPS34 complex that regulate autophagic activity via AMPK-mediated phosphorylation. As a Golgi resident protein, PAQR3 seems to provide a scaffold for VPS34-beclin 1 complex and integrate AMPK signaling for autophagy regulation in response to cellular energy levels. The study provides insight into autophagy regulation through AMP-VPS34 in addition to known AMPK-ULK1 signaling. Coincidently, the structural and functional role for PAQR3 in regulating VPS34 component assembly and kinase activity resembles largely another autophagy related protein NRBF2, which is a yeast Atg38 homologue reported previously by at least four groups (Interestingly, the current paper has not mentioned this protein at all). The reviewer is wondering if the two proteins PAQR3 and NRBF2 are related at all. For example, are PAQR3 and NRBF2 functionally redundant? Whether the two proteins are both required for holding the VPS34 Atg14L complex together?

Response: Thanks for the great question. Based on our experiments, PAQR3 and NRBF2 are not completely functionally redundant. PAQR3 and NRBF2 have both overlapping and different functions in holding the ATG14L-linked VPS34 complex together. Our conclusions are supported by following experimental findings. We have added the discussion about the relationship between PAQR3 and NRBF2 in the revised manuscript (page 30, lines 7-14).

(1) PAQR3 and NRBF2 are not completely functionally redundant in regulating class III PI3K activity.

Firstly, in accordance with a previous report (*Nat Commun. 2014. PMID:24849286*), we found that NRBF2 knockdown could dramatically suppress the activity of ATG14L-associated class III PI3K (Appendix Fig S7A, also shown here). Remarkably, not only shRNA-resistant NRBF2, but also PAQR3 reconstitution can completely rescue the impaired ATG14L-linked VPS34 activity in NRBF2 knockdown cells (Appendix Fig S7A), suggesting PAQR3 and NRBF2 have overlapping functions in regulating class III PI3K. Secondly, stably overexpressed NRBF2 can only partially rescue the decreased ATG14L-linked VPS34 activity in PAQR3-knockdown cells (Appendix Fig S7B), indicating that PAQR3 and NRBF2 are not completely functionally redundant in regulating class III PI3K activity. Thirdly, the ATG14L-linked VPS34 activity in PAQR3- and NRBF2-double knockdown cells was suppressed to a greater extent than that in the cells with either PAQR3- or NRBF2-knockdown alone (Appendix Fig S7C). This result further supported that PAQR3 and NRBF2 are both required for modulating ATG14L-linked class III PI3K activity.
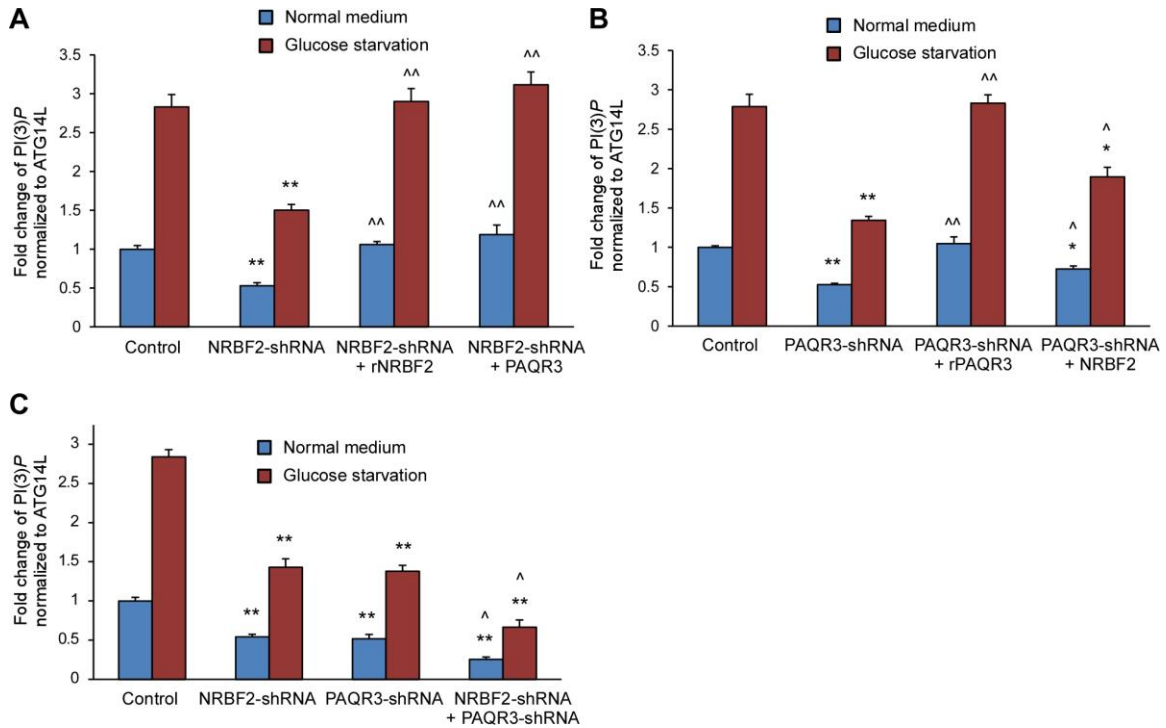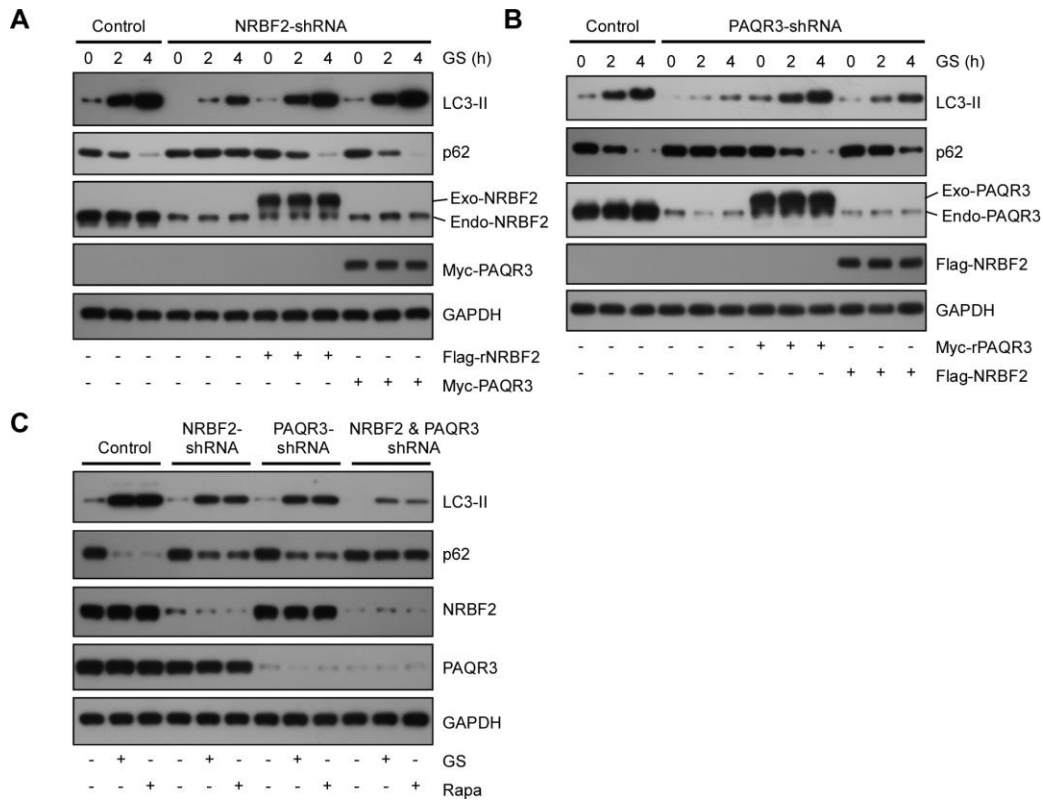
Appendix Fig S7. PAQR3 and NRBF2 are both required for modulating ATG14L-linked class III PI3K activity.

(A~C) NRBF2-knockdown HeLa cells were infected with lentivirus expressing PAQR3 and shRNA-resistant NRBF2 respectively as indicated (A). PAQR3-knockdown HeLa cells were infected with lentivirus expressing NRBF2 and shRNA-resistant PAQR3 respectively as indicated (B). HeLa cells were infected with NRBF2- or PAQR3-knockdown lentivirus as indicated (C). After glucose starvation (GS) for 4 h, ATG14L-linked VPS34 complexes were immunoprecipitated from the cell lysates by ATG14L antibody. The PI(3)P level was determined by a quantitative ELISA assay. The PI(3)P level was normalized to the amount of ATG14L in this assay (n = 5; * for $p < 0.05$ and ** for $p < 0.01$ as compared with the first group with the same treatment; ^ for $p < 0.05$ and ^^ for $p < 0.01$ as compared with the second group with the same treatment).

(2) PAQR3 and NRBF2 are not completely functionally redundant in modulating autophagic activity.

As shown in Appendix Figure S8A, glucose starvation-induced LC3-II accumulation and p62 degradation were dramatically ameliorated by NRBF2 knockdown in HeLa cells, indicating that NRBF2 is required for autophagy activation. Remarkably, the suppressed autophagic markers in NRBF2 knockdown cells can be totally reversed by shRNA-resistant NRBF2 or PAQR3 overexpression (Appendix Fig S8A). However, stably overexpressed NRBF2 can only partially rescue the decreased autophagic activity in PAQR3 knockdown cells (Appendix Fig S8B). These data, therefore, demonstrated that PAQR3 can be recognized as a compensatory substitute for NRBF2 upon autophagy regulation to a certain extent, but PAQR3 and NRBF2 are not completely functionally redundant upon autophagy regulation. Furthermore, simultaneous knockdown PAQR3

and NRBF2 resulted in the lowest autophagic activity compared with that in the cells with either PAQR3 or NRBF2 knockdown alone (Appendix Fig S8C). Therefore, PAQR3 and NRBF2 are both necessary to coordinately regulate autophagic activity.

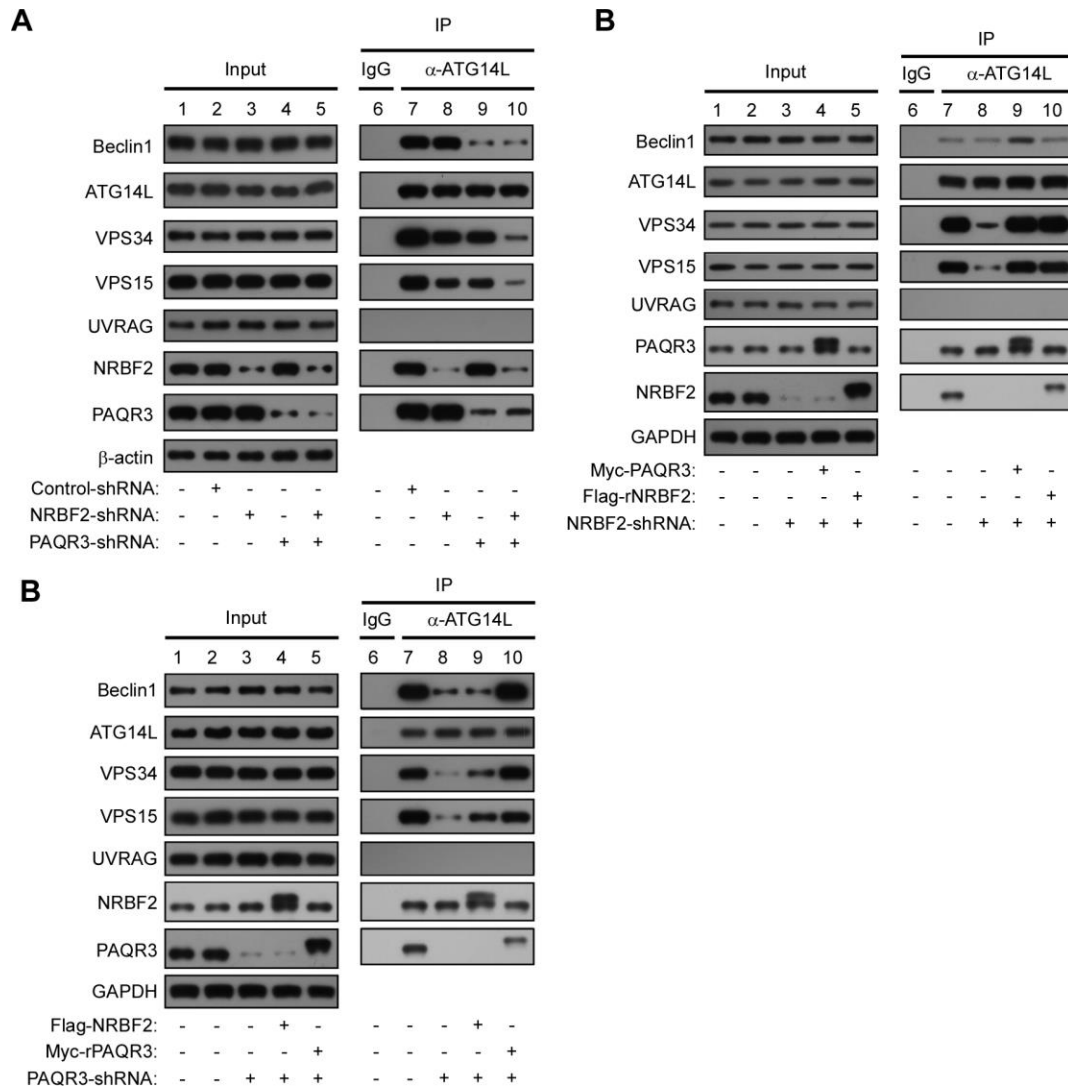**Appendix Fig S8. PAQR3 and NRBF2 are both necessary to coordinately regulate autophagic activity**

(A) NRBF2-knockdown HeLa cells were infected with lentivirus expressing PAQR3 and shRNA-resistant NRBF2 respectively. After glucose starvation (GS) for 2 h or 4 h, cell lysates were subjected to immunoblotting (IB) analysis with the indicated antibodies. (B) PAQR3-knockdown HeLa cells were infected with lentivirus expressing NRBF2 and shRNA-resistant PAQR3 respectively. After glucose starvation (GS) for 2 h or 4 h, cell lysates were subjected to IB analysis with the indicated antibodies. (C) HeLa cells were infected with NRBF2- or PAQR3-knockdown lentivirus as indicated. After glucose starvation or rapamycin (50 nM) treatment for 4 h, whole-cell lysates were harvested for IB analysis with indicated antibodies.

(3) PAQR3 and NRBF2 are both required for ATG14L-linked VPS34 complex assembly.

To further investigate the underlying mechanism by which PAQR3 and NRBF2 regulate the activity of class III PI3K activity respectively, we examined whether PAQR3 and NRBF2 are both required for holding ATG14L-linked VPS34 complex together. As

shown in Appendix Fig S9A, the protein levels of ATG14L, Beclin-1, VPS34, VPS15, and UVRAG were not altered in PAQR3- or NRBF2-knockdown cells. However, endogenous ATG14L pulled down much less VPS34 and VPS15 after PAQR3 or NRBF2 was silenced (Appendix Fig S9A). Additionally, the interactions between ATG14L and VPS15/VPS34 were ameliorated to a greater extent in PAQR3- and NRBF2-double knockdown cells. These data, therefore, indicated that these two proteins are both needed for ATG14L-associated VPS34 assembly, in spite of their functional overlaps. However, it is worth mentioning that, NRBF2 was not required for the interaction between Beclin-1 and ATG14L (Appendix Fig S9A, lanes 7 and 8), consistent with a previous report (*J Biol Chem. 2014. PMID:25086043*). In contrast, the interaction between Beclin-1 and ATG14L was decreased sharply in PAQR3-knockdown cells (Appendix Fig S9A, lanes 9 and 10). These data suggested that PAQR3 and NRBF2 participate in ATG14L-associated VPS34 assembly in different manners. Furthermore, we investigated whether PAQR3 and NRBF2 are functionally redundant in modulating ATG14L-Beclin1-VPS15-VPS34 association. In NRBF2-knockdown cells, PAQR3 reconstitution could almost totally rescue the interaction between ATG14L and VPS15/VPS34 (Appendix Fig S9B, lanes 8 and 9). Nevertheless, in PAQR3-knockdown cells, NRBF2 can only moderately reverse the reduced interaction between ATG14L and VPS15/VPS34, while the impaired ATG14L-Beclin1 association was minimally restored by NRBF2 overexpression (Appendix Fig S9C, lanes 8 and 9). In conclusion, although PAQR3 and NRBF2 share functional overlaps in class III PI3K assembly, they are both required for holding the ATG14L-associated VPS34 complex together

Appendix Fig S9. PAQR3 and NRBF2 are both required for ATG14L-linked VPS34 complex assembly.

(A) HeLa cells were infected with PAQR3 and NRBF2 knockdown lentivirus as indicated. The cell lysates were used for immunoblotting (IB) and immunoprecipitation (IP) analysis with the indicated antibodies.

(B) NRBF2-knockdown HeLa cells were infected with lentivirus expressing PAQR3 and shRNA-resistant NRBF2 respectively.

(C) PAQR3-knockdown HeLa cells were infected with lentivirus expressing NRBF2 and shRNA-resistant PAQR3 respectively. The cell lysates were used immunoblotting (IB) and immunoprecipitation (IP) with the indicated antibodies.

Other concerns:

1. In Figure S1D, rapamycin-induced autophagy is effectively inhibited in PAQR3 deleted cells, suggesting that PAQR3 is required for autophagy induced by mTOR inhibition. Paradoxically the authors claimed that the regulation of PAQR3 on autophagy is mTOR independent.

Response: Thanks for raising this issue. Based on our results in Fig EV1D and EV1E of the revised manuscript (i.e., Figure S1C and S1D of the previous manuscript), we agree with the reviewer's summary that PAQR3 is required for autophagy induced by mTOR inhibition. However, our description that "the regulation of PAQR3 on autophagy is mTOR independent" is based on the unchanged phosphorylation level of 4-EBP1, a direct downstream effector of mTORC1, in PAQR3-deleted cells as compared with the wild type cells under various autophagy-promoting conditions (Figs 1B and EV1B-E). From this perspective, we believe that the regulation of PAQR3 on autophagy is not due to altering mTOR activity itself. Coincidentally, another research group made a similar conclusion by conducting a similar assay (*Cell. 2013. PMID:24034250*). Nevertheless, we agree with reviewer that our previous description that "the regulation of PAQR3 on autophagy is independent of mTOR pathway" might mislead the readers. In order to avoid ambiguity, we have changed this description to "the regulation of PAQR3 on autophagy is not due to an alteration of mTOR activity itself" in the revised manuscript (page 8, lines 14-15).

2. The authors claimed that PAQR3, Atg14L and beclin 1 are in a "ternary" protein complex. What is the exact evidence for the "ternary" complex without structural analysis?

Response: Thanks for the great question. As shown in Figure 4A, we demonstrated that "PAQR3, ATG14L and Beclin1 formed a ternary complex" as revealed by a two-step coimmunoprecipitation assay. This experimental method has been well accepted in the biochemistry field to examine whether three different proteins can exist in the same complex simultaneously (for example: *Cancer Cell. 2008. PMID:18772112; Nat Cell Biol. 2009. PMID:19448627*). However, the term "ternary" might be an inappropriate description confusing the readers, so we have changed it into "PAQR3, ATG14L and Beclin1 could co-exist in the same complex simultaneously" in the revised manuscript (page 15, lines 9-11). We have also deleted the use of "ternary" in the manuscript. In addition, we agree with the reviewer that this conclusion could be strengthened by structural analysis of this complex. In fact, our group has been trying very hard to solve this problem. Unfortunately, until now, we have not yet obtained the crystal structure of PAQR3, as membrane proteins have been proven to be extremely difficult to study owing to their hydrophobic surfaces, flexibility and lack of stability in structural analysis (*Nat Methods. 2010. PMID: 20508636; Curr Opin Struct Biol. 2008. PMID: 18674618*). Alternatively, we detected the cellular co-localization of PAQR3, Beclin1 and ATG14L to better understand this complex. Consistent with previous reports (*Nat Cell Biol. 2009. PMID: 19270696; Cell Res. 2012. PMID:22310240*), we found that both ATG14L and Beclin1 are mainly diffusely distributed in the cytosol when transfected alone (Fig EV2A, first two lines). However, upon co-expression of PAQR3 with ATG14L and Beclin1,

these three proteins co-localized well both under normal medium and glucose starvation (Fig EV2E), even though PAQR3 exhibited as punctiform distribution after glucose starvation. These data further supported that PAQR3, Beclin1 and ATG14L could exist in the same complex simultaneously. We have added these data into Figs EV2A and EV2E in the revised manuscript. Thanks again for understanding the difficulty in crystal structural analysis of PAQR3 protein.

Fig EV2A, first two panels
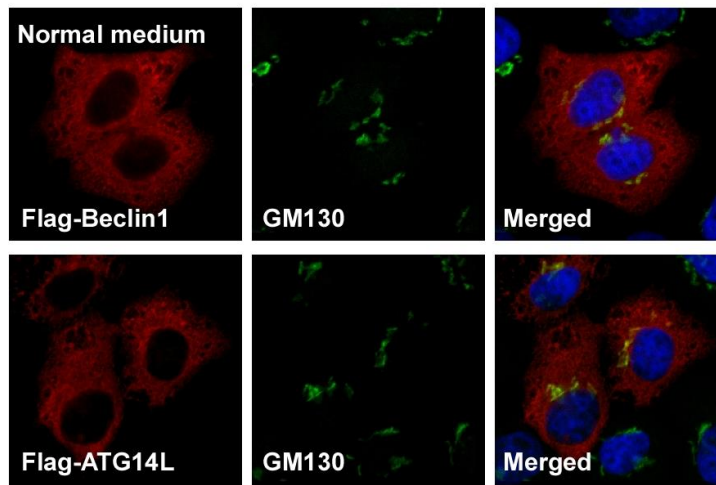
Figure EV2E
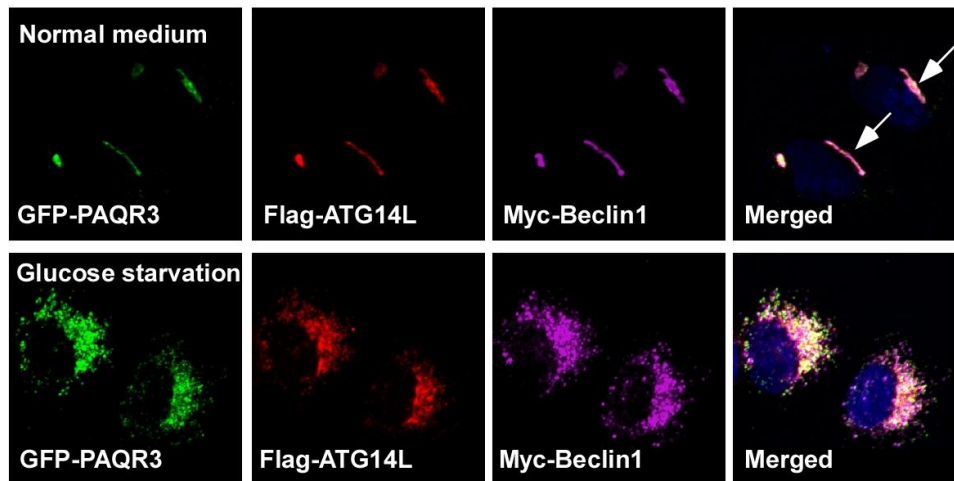
Figure EV2A. (first two panels) HeLa cells were transfected with the plasmids as indicated, followed by immunofluorescence staining with the indicated antibodies.

Figure EV2E. HeLa cells were co-transfected with GFP-fused PAQR3, Flag-tagged ATG14L and Myc-tagged Beclin1 simultaneously. At 24 h after the transfection, the cells were fixed for immunofluorescence staining before or after glucose starvation as indicated. The arrow indicates apparent colocalization of ATG14L and Beclin1 with PAQR3.

3. The mapping of the binding domains in PAQR3, Atg14L or Beclin1 proteins were performed in cell culture. Therefore, it is unclear whether they directly interact with each other. Other proteins could mediate PAQR3 interaction with Atg14L or Beclin1. It is also hardly convincing that Atg14L and Beclin1 occupy a narrow 40 aa N-terminal sequence of PAQR3 without structural evidence.

Response: Thank you for raising this issue. We did following experiments to address this issue.

(1) For your first concern, we think that *in vitro* GST pull down assay is one of the best ways to clarify the direct interaction of PAQR3 with ATG14L or Beclin-1. However, we could hardly purify full-length Beclin1 and ATG14L in bacterial expression system, likely due to the instability or poor solution behavior of these two proteins (as mentioned in reported papers, such as *Cell. 2013. PMID:23332761; Cell Res. 2012. PMID:22310240; Proc Natl Acad Sci U S A. 2011. PMID:21518905*). Also, we have not obtained the purified full-length PAQR3 in bacterial expression system, perhaps owing to the hydrophobic surfaces, flexibility and instability of membrane proteins (*Nat Methods. 2010. PMID: 20508636; Curr Opin Struct Biol. 2008. PMID: 18674618*). Alternatively, based on our findings shown in Figure 4C and 4D for mapping the interacting domains, we purified following proteins including GST-tagged 1-71 aa of PAQR3, His-tagged 181~492 aa of ATG14L and His-tagged 1~88 aa of Beclin1 respectively. Our new data revealed that the GST-fused PAQR3 NH$_2$-terminal 71 aa domain, but not GST alone, could pull down His-tagged 181~492 aa of ATG14L (Fig 4E), indicating a direct interaction between PAQR3 and ATG14L. In contrast, PAQR3 could not associate with Beclin1 directly, suggesting that other proteins/molecules might be needed to mediate PAQR3 interaction with Beclin1 as nicely pointed out by the reviewer. These data have been included in Figure 4E of the revised manuscript.
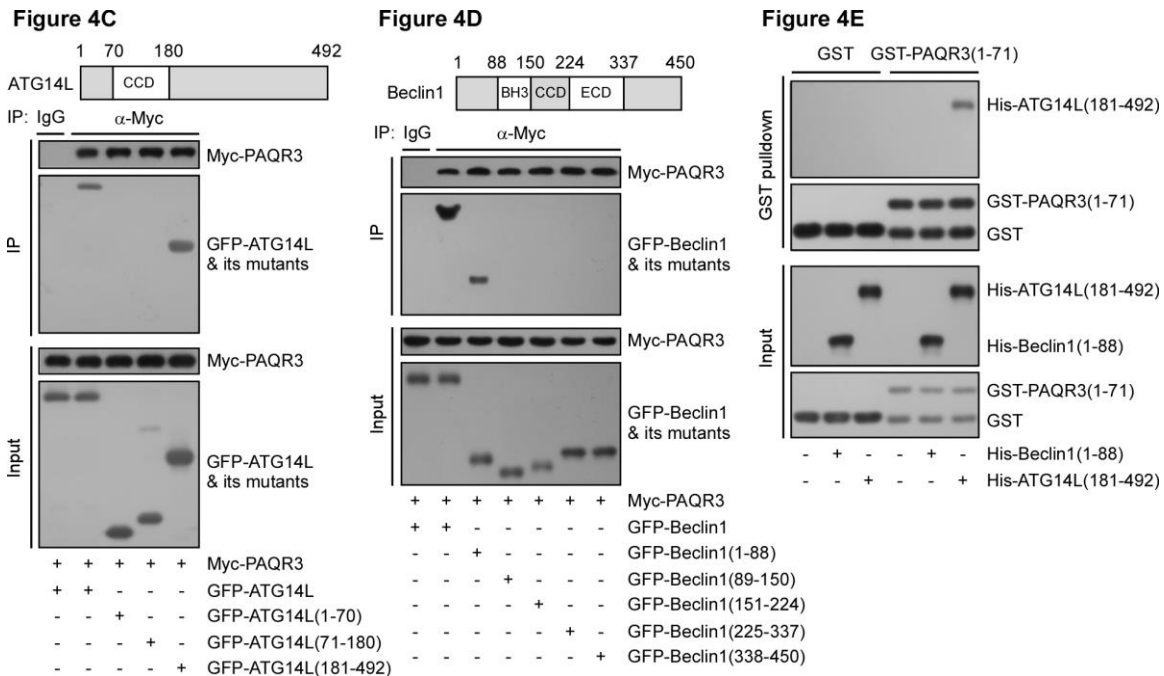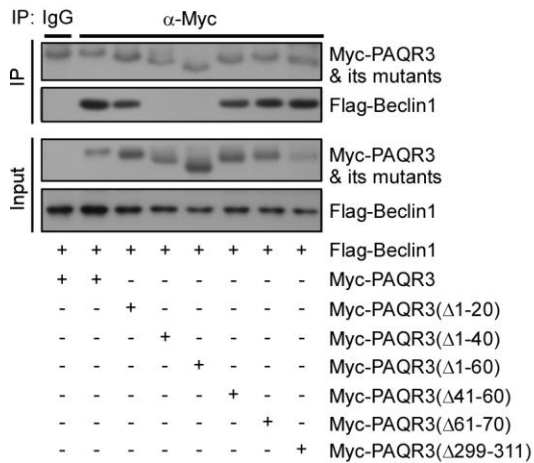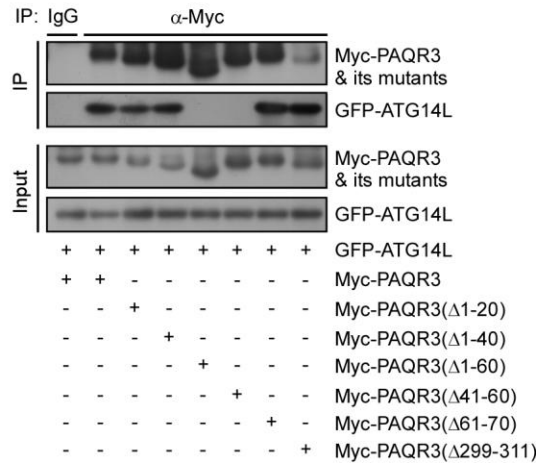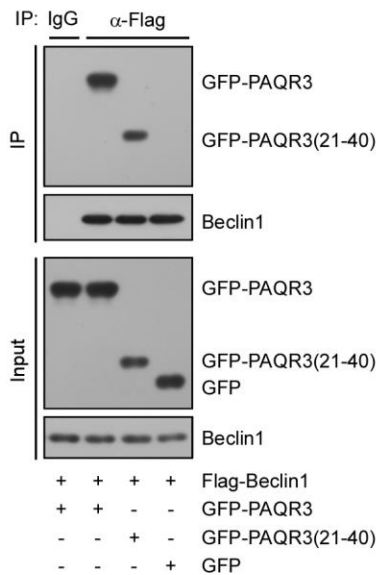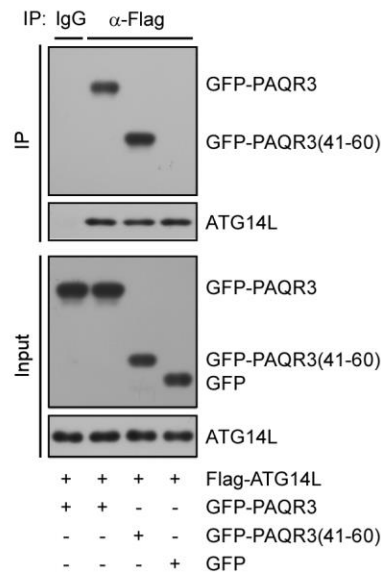
Figure 4C and D. Different truncations of ATG14L(C) or Beclin1(D) were co-transfected with Myc-tagged PAQR3 into HEK293T cells as indicated, followed by IB and IP assays.

Figure 4E. Purified GST or GST-fused PAQR3 (1-71) (GST fused NH$_2$-terminal 71 aa of PAQR3) on glutathione agarose beads was mixed with purified His tagged proteins as indicated. After incubation at 4 °C for 3 h, the samples were washed extensively and subjected to IB analysis with the indicated antibodies.

(2) For the second concern, although we cannot provide the crystal structure evidence showing that PAQR3 could associate with ATG14L and Beclin1 in a 40 aa motif, we addressed this issue by following three experimental settings.

(a) As shown in Figure 4F and 4G of the revised manuscript, the 21~40 aa and 41~60 aa of PAQR3 were critical for its interaction with Beclin1 and ATG14L respectively. Furthermore, we found that the 21~40 aa and 41~60 aa of PAQR3 were sufficient to associate with Beclin1 and ATG14L respectively (Fig EV3A and EV3B). These data, therefore, suggested that PAQR3 interacts with Beclin1 and ATG14L via these two short regions respectively.

Figure 4F

+	+	+	+	+	+	+	+	Flag-Beclin1
+	+	-	-	-	-	-	-	Myc-PAQR3
-	-	+	-	-	-	-	-	Myc-PAQR3(Δ1-20)
-	-	-	+	-	-	-	-	Myc-PAQR3(Δ1-40)
-	-	-	-	+	-	-	-	Myc-PAQR3(Δ1-60)
-	-	-	-	-	+	-	-	Myc-PAQR3(Δ41-60)
-	-	-	-	-	-	+	-	Myc-PAQR3(Δ61-70)
-	-	-	-	-	-	-	+	Myc-PAQR3(Δ299-311)

Figure 4G

+	+	+	+	+	+	+	+	GFP-ATG14L
+	+	-	-	-	-	-	-	Myc-PAQR3
-	-	+	-	-	-	-	-	Myc-PAQR3(Δ1-20)
-	-	-	+	-	-	-	-	Myc-PAQR3(Δ1-40)
-	-	-	-	+	-	-	-	Myc-PAQR3(Δ1-60)
-	-	-	-	-	+	-	-	Myc-PAQR3(Δ41-60)
-	-	-	-	-	-	+	-	Myc-PAQR3(Δ61-70)
-	-	-	-	-	-	-	+	Myc-PAQR3(Δ299-311)

Figure EV3A

+	+	+	+	Flag-Beclin1
+	+	-	-	GFP-PAQR3
-	-	+	-	GFP-PAQR3(21-40)
-	-	-	+	GFP

Figure EV3B

+	+	+	+	Flag-ATG14L
+	+	-	-	GFP-PAQR3
-	-	+	-	GFP-PAQR3(41-60)
-	-	-	+	GFP

Figure 4F and 4G. Different PAQR3 deletion constructs were co-transfected with Flag-tagged Beclin1 (F) or GFP-fused ATG14L (G) into HEK293T cells. The cells were then subjected to immunoblotting (IB) and immunoprecipitation (IP).

Figure EV3A and EV3B. HEK293T cells were transfected with different plasmids as indicated. At 24 h after the transfection, the cells were subjected to immunoprecipitation (IP) and immunoblotting (IB) analyses.

   (b) To further emphasize that the NH$_2$-terminal 21~60 aa of PAQR3 is the binding motif for Beclin1 and ATG14L, we designed synthetic peptides (P21~40 and P41~60) that covered PAQR3 NH$_2$-terminal 21~40 and 41~60 aa respectively. A TAT sequence (RKKRRQRRR) was added in the NH2 terminus of the peptide to facilitate membrane penetrance. As expected, P21~40 dramatically neutralized the interaction between PAQR3 and Beclin1, but not the interaction between PAQR3 and ATG14L, demonstrating the NH$_2$-terminal 21~40 aa of PAQR3 is critical for Beclin1 interaction

(Fig EV3C). In contrast, the association between PAQR3 and ATG14L was specifically ameliorated by P41~60, suggesting the 41~60 aa of PAQR3 is the binding motif for ATG14L (Fig EV3C). These new data have been included in Figure EV3C of the revised manuscript.

Figure EV3C
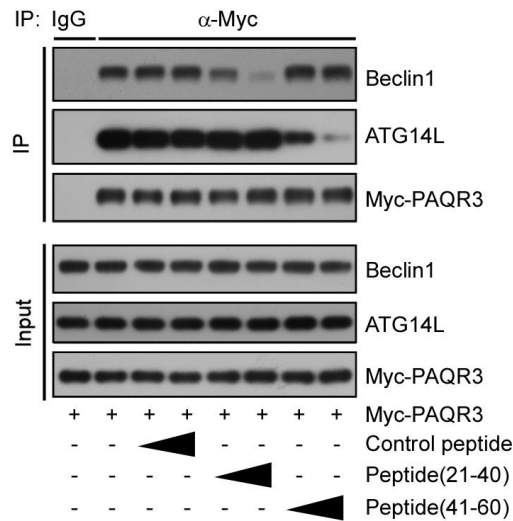
Figure EV3C. HEK293T cells were transfected with Myc-tagged PAQR3. At 24 h after transfection, the cells were treated with a control peptide, P21~40 or P41~60 (4 or 20 ng/ μl) for 12 h respectively and the cell lysates were used for immunoblotting (IB) and immunoprecipitation (IP) with the indicated antibodies.

(c) We performed a two-step co-immunoprecipitation assay with coexpression of Flag-tagged Beclin1, Myc-tagged ATG14L and GFP-fused PAQR3 NH_2 terminal 21~60 aa. We found that the 40 aa of PAQR3 could interact with Beclin1 and ATG14L simultaneously (Fig EV3D). These new data have been included in Figure EV3D of the revised manuscript.
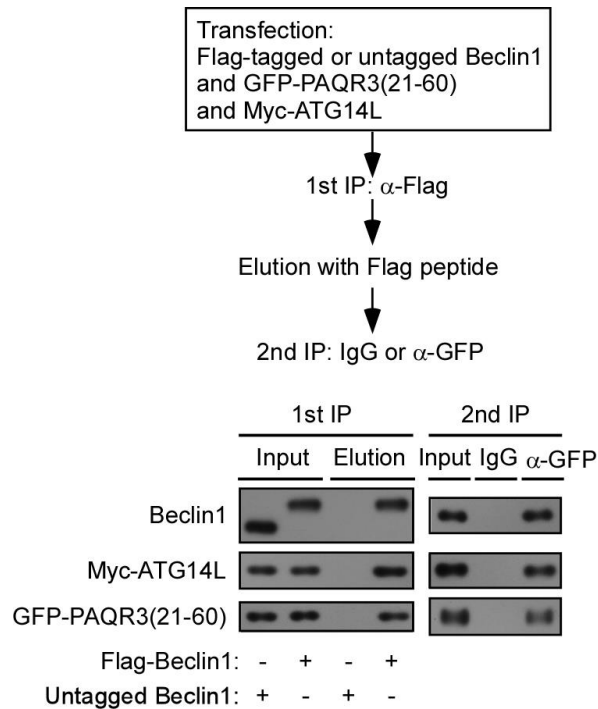
Figure EV3D. A two-step co-immunoprecipitation assay to determine the complex formation among PAQR3 NH$_2$ terminal 21~60 aa, Beclin1 and ATG14L. The procedures of the assay are outlined in the top panel. HEK293T cells were transfected with the plasmids as indicated, followed by immunoblotting (IB) and immunoprecipitation (IP) with the indicated antibodies.

4. There is no introduction of PAQR3 KO mice (e.g. literature) at all. If it is the first time to characterize the KO mice, necessary information is required for the confirmation of a true KO and any phenotypes (if there is, such as developmental or adult) should also be mentioned.

Response: Thank you for your suggestion. The PAQR3 knockout mice have been described in detail previously by our group (*Proc Natl Acad Sci U S A. 2007. PMID:17724343*). We have added the citation in the revised manuscript (page 6, last line; page 26, line 7; page 35, line 9).

5. There are numerous places that have awkward expression, such as "capacitance", "PI3P machine", etc. The authors are recommended to consult native English speakers for appropriate expression.

Response: Thanks for the suggestion. In this part, we originally intended to give the readers a more vivid understanding about how PAQR3 regulates autophagy using physical and electrical metaphors. However, in order to avoid these probably inappropriate metaphors that may confuse the readers, we deleted this paragraph in the revised manuscript.

Reviewer #2:

Xu et al. showed that a Golgi-localized transmembrane protein PAQR3 functions as a scaffold protein that facilitates formation of ATG14 but not UVRAG-linked VPS34 complex, leading to enhanced PI3P production and autophagy activation. In addition, AMPK phosphorylates PAQR3 at T32 and switches on PI3P production after glucose starvation. Deletion of PAQR3 in mice leads to reduction of exercise-induced autophagy. The study is potentially interesting given the novelty and specific regulation of autophagy specific PI3K activity. The data quality is good in general. However, several concerns need to to be addressed before publication.

1.   The localization of PAQR3 is not clear. The authors showed that GFP-PAQR3 almost perfectly colocalizes with LC3 in Fig. 1A, but very limited colocalization of GFP-PAQR3 with LC3 in Fig. S1F. How to explain these discrepancies? Does PAQR3 normally localize to Golgi? These staining should be shown in Fig. 1A as controls. If the translocation of PAQR3 from Golgi to autophagosomes upon glucose starvation is clear, that will become a strong supporting evidence for their hypothesis.

Response: Thanks for raising this issue. We would like to address these issues by following discussions.

(1) In Figure 1A, we used GFP-fused PAQR3, so that the fluorescence signals could reflect the cellular localization of PAQR3. In contrast, in Figure EV1H of the revised manuscript (i.e., Figure S1F in the previous manuscript), HeLa cells were infected by PAQR3-expressing lentivirus. The lentivirus-based vector used for stable expression of PAQR3 in this experiment was pHAGE-fullEF1a-MCS-IZsGreen (clone ID EvNO00061604, Harvard Medical School, USA). There is an IRES sequence between PAQR3 and ZsGreen, so that PAQR3 and ZsGreen are not forming a fusion protein. Therefore, in this experiment, the fluorescence signal is only a marker for cells infected with the lentivirus, and it cannot provide any information about the cellular localization of PAQR3 itself.

(2) PAQR3 is localized in the Golgi apparatus under normal medium conditions (Fig 1A). In the revised manuscript, we have included the fluorescence staining in Figure 1A as controls. Upon glucose starvation, punctiform PAQR3 was no longer co-localized with Golgi (Fig EV1A), but co-localized well with the autophagosome marker, LC3 (Fig 1A). This piece of data supported our conclusion that PAQR3 is translocated from Golgi to autophagosomes upon glucose starvation. Thanks again for the insightful advice.

Figure 1A
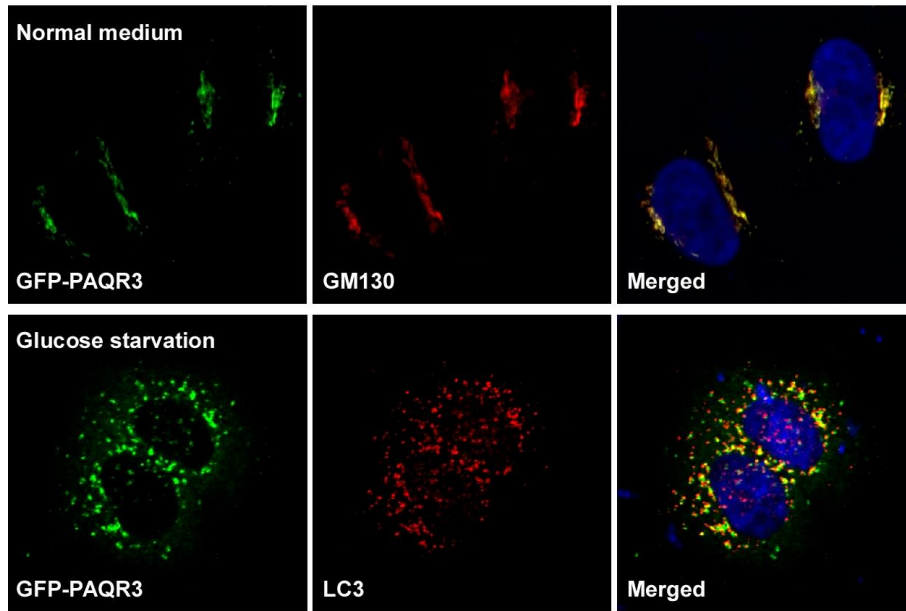
Figure EV1A
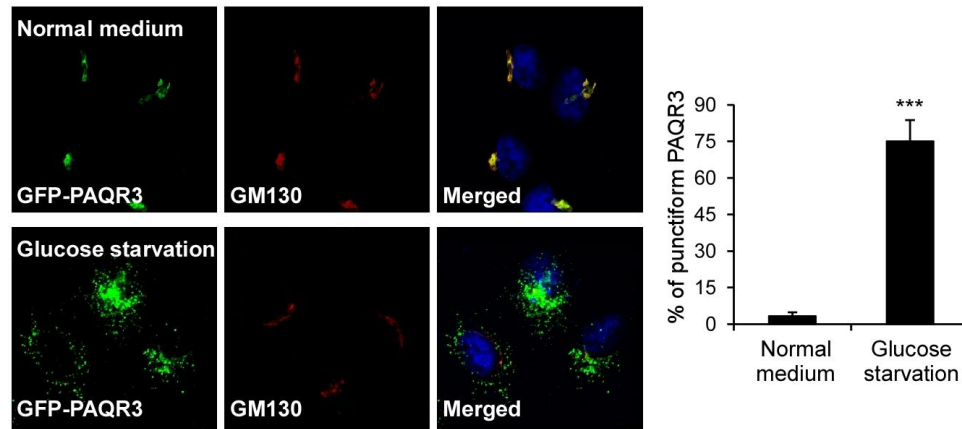
Figure 1A. GFP-PAQR3 transfected HeLa cells were fixed for immunofluorescence staining with indicated antibodies before or after glucose starvation (GS) for 4 h. The nuclei were stained with Hoechst 33342.

Figure EV1A. GFP-PAQR3 transfected HeLa cells were fixed for immunofluorescence staining with indicated antibodies before or after glucose starvation (GS) for 4 h. At least 100 cells were counted per experiment and the data on the right represent the percentage of punctiform PAQR3 with or without glucose starvation (GS), the results were obtained from three independent experiments (*** for $p < 0.001$).

2. The authors concluded that PAQR3 is a constitutive component of the ATG14/Beclin1/VPS34 complex, mutually exclusive with UVRAG, mainly based on co-IP experiments. However, PAQR3 is not presented as a stoichiometric subunit of PI3KC3 in published literatures (Sun PNAS 2008, Matsunaga NCB 2009, Zhong NCB 2009). The conclusion could be strengthened if the authors are able to detect

ATG14/Beclin1/VPS34 as stoichiometric subunits of PAQR3 cellular complex. Also, in vitro pull-down assay using recombinant proteins will help to elucidate if these interactions are direct.

Response: Thanks for the great question. We would like to address these issues by following experiments.

(1) To figure out whether ATG14L-linked VPS34 complex was the stoichiometric subunits of PAQR3 cellular complex, we stably transfected Myc-tagged PAQR3 into PAQR3-deficient HeLa cells, in which the expression level of the exogenous PAQR3 was comparable to that in WT cells (shown in Fig 6F). PAQR3 immunoprecipitated from these "PAQR3-rescued" HeLa cells was subjected to SDS/PAGE and silver staining (Appendix Fig S1A). As shown in Appendix Figure S1B-E, the indicated bands were excised and identified as Beclin1, ATG14L, VPS34 and VPS15 by mass spectrometry respectively. Therefore, these data indicated that ATG14L-linked VPS34 complex was stoichiometric subunits of PAQR3 cellular complex. Thanks for the great advice.
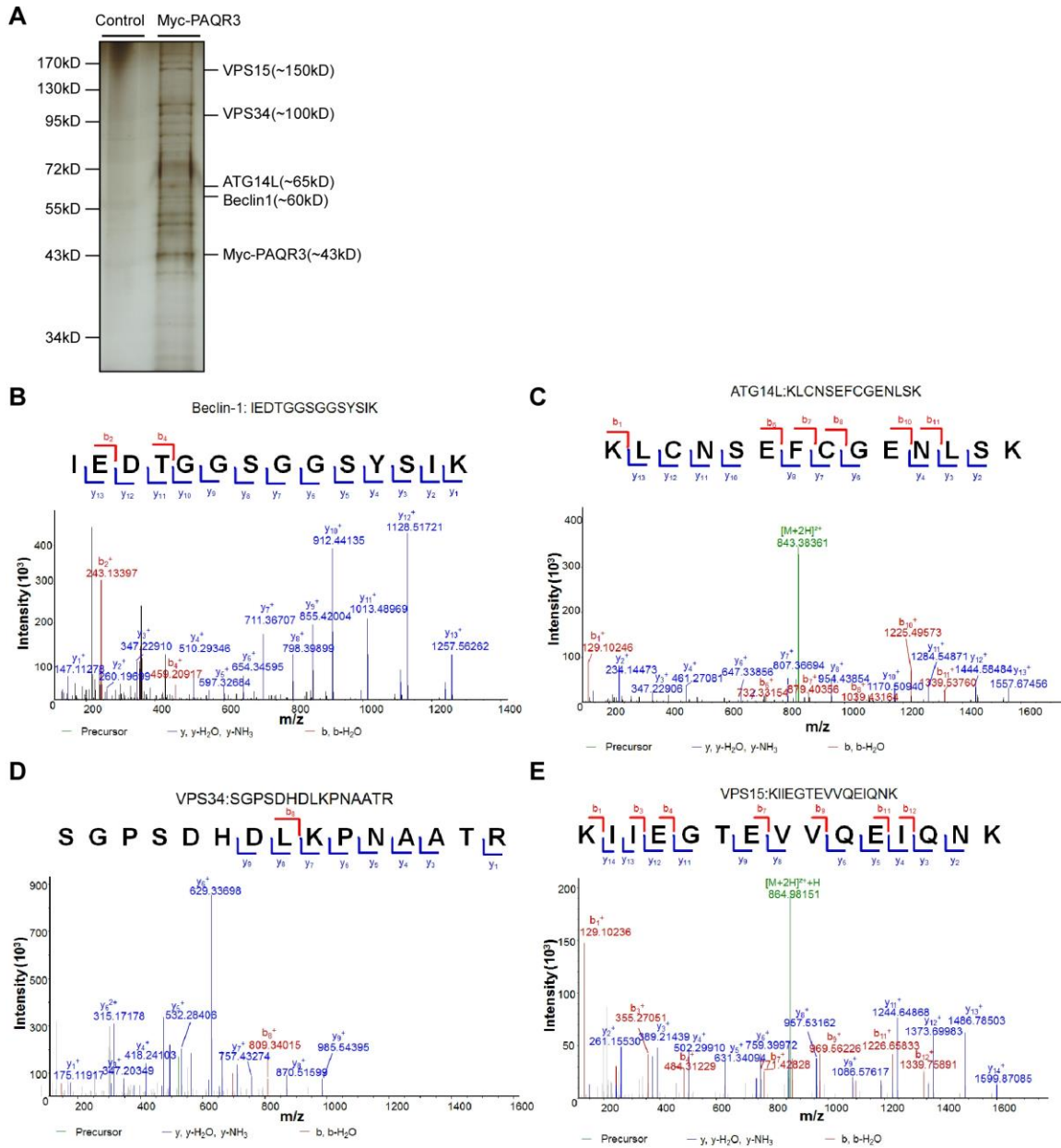
Appendix Fig S1. ATG14L-linked VPS34 complex is the stoichiometric binding partner of PAQR3.

(A) PAQR3-deficient HeLa cells were infected with lentivirus expressing Myc-tagged PAQR3, which was immunoprecipitated by an anti-Myc antibody. The associated proteins were eluted by a Myc peptide, and subjected to silver staining.

(B~E) Proteins in the gel bands were extracted and identified by mass spectrometry as indicated.

(2) For your second concern, we agree with the reviewer that *in vitro* GST pull down assay is one of the best ways to investigate whether PAQR3 directly interacts with

ATG14L-associated VPS34 complex. However, we could hardly purify full-length Beclin1 and ATG14L in bacterial expression system, likely due to the instability or poor solution behavior of these two proteins (as mentioned in reported papers, such as *Cell. 2013. PMID:23332761; Cell Res. 2012. PMID:22310240; Proc Natl Acad Sci U S A. 2011. PMID:21518905*). Also, we have not obtained the purified full-length PAQR3 in bacterial expression system until now, perhaps owing to the hydrophobic surfaces, flexibility and instability of membrane proteins (*Nat Methods. 2010. PMID: 20508636; Curr Opin Struct Biol. 2008. PMID: 18674618*). Alternatively, based on our findings shown in Figure 4C and 4D for mapping the interacting domains, we purified following proteins including GST-tagged 1-71 aa of PAQR3, His-tagged 181~492 aa of ATG14L and His-tagged 1~88 aa of Beclin1 respectively. Our new data revealed that the GST-fused PAQR3 NH_2-terminal 71 aa domain, but not GST alone, could pull down His-tagged 181~492 aa of ATG14L (Fig 4E), indicating a direct interaction between PAQR3 and ATG14L. In contrast, PAQR3 could not associate with Beclin1 directly. These data have been included in the revised manuscript in Figure 4E.
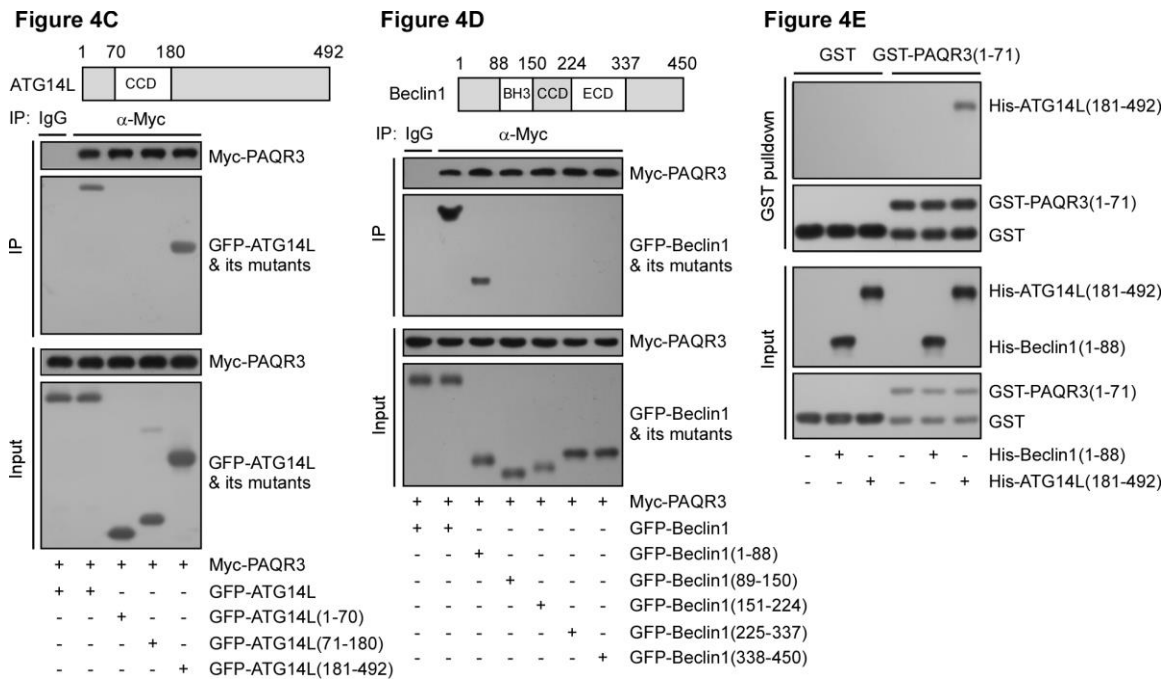
Figure 4C and 4D. Different truncations of ATG14L(C) or Beclin1(D) were co-transfected with Myc-tagged PAQR3 into HEK293T cells as indicated, followed by IB and IP assays with the indicated antibodies.

Figure 4E. Purified GST or GST-fused PAQR3(1-71) (GST fused NH_2-terminal 71 aa of PAQR3) on glutathione agarose beads was mixed with purified His tagged proteins as indicated. After incubation at 4 °C for 3 h, the samples were washed extensively and subjected to IB analysis.

Similarly, in the revised manuscript, we added new experiments to map the interaction domains of VPS15 and VPS34 with PAQR3 (Appendix Fig S2A and B). Based on the mapping results, we purified the C2 and catalytic domain of VPS34, as well

as the catalytic domain and WD repeats of VPS15 *in vitro* respectively. As shown in the GST-pulldown assay of Appendix Figure S2C, PAQR3 directly interacts with VPS15, but not with VPS34. Thanks again for the thoughtful advice.
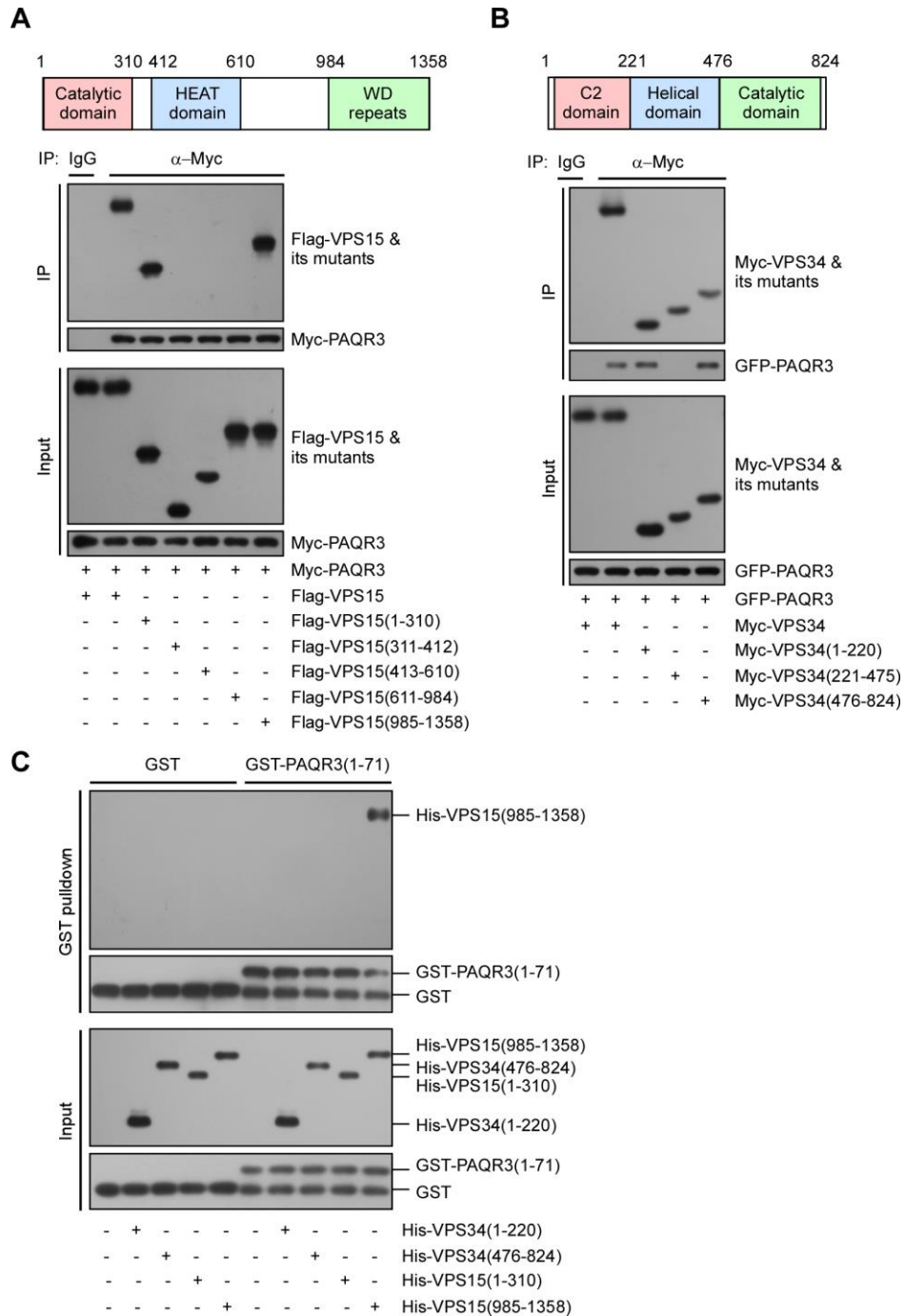
Appendix Fig S2. PAQR3 directly interacts with VPS15, but not VPS34.

(A) Different truncations of VPS15 were co-transfected with Myc-tagged PAQR3 into HEK239T cells as indicated, followed by immunoblotting (IB) and immunoprecipitation (IP) analysis.

(B) GFP-fused PAQR3 was co-transfected with different truncations of VPS34 into HEK239T cells as indicated, followed by IB and IP analysis.

(C) GST and GST-PAQR3 N71 (GST fused NH$_2$-terminal 71 aa of PAQR3 on glutathione agarose beads was mixed with purified His-tagged proteins as indicated. After incubation at 4 °C for 3 h, the samples were washed extensively and subjected to IB analysis.

3. If PAQR3 is a constitutive component of the ATG14/Beclin1/VPS34 complex even in the absence of autophagic stress, can the authors detect the Golgi localization of ATG14/Beclin1/VPS34 complex? How is PAQR3 attracted to autophagosomes/ phagophores upon glucose starvation?

Response: Thank you for the great question. We would like to address these issues by following experiments.

(1) For your first concern: The data in Figure 3 indicated that PAQR3 is a constitutive component of the ATG14/Beclin1/VPS34 complex. To further confirm this conclusion, we purified the Golgi complexes from mouse livers to analyze the localization of endogenous VPS34 complex using a biochemical approach as previously reported (please refer to *Diabetes. 2013. PMID:23086038*). As shown in Figure 3B of the revised manuscript, ATG14L-linked VPS34 complex could be clearly detected in the Golgi fractions of wild type mice, supporting that there indeed exists Golgi localization of ATG14/Beclin1/VPS34 complex. However, PAQR3 knockout led to dramatic reduction in the Golgi compartmentalization of Beclin1, ATG14L, VPS15 and VPS34, but not UVRAG (Fig 3B). These new data demonstrated that PAQR3 is critical for the Golgi localization of ATG14L-linked VPS34 complex. Furthermore, we detected the effect of PAQR3 on the cellular localization of ATG14L-linked VPS34 complex by immunofluorescence staining assay. As shown in Figure EV2A, when transfected alone, exogenous Beclin1, ATG14L, VPS15, VPS34 are all diffusely distributed in the cytoplasm with minimal co-localization with the Golgi marker GM130. Remarkably, when co-expressed with PAQR3, these proteins were all co-localized well with the Golgi marker (with Pearson's correlation coefficient > 0.7, Fig EV2B and C). These data further indicated that the ATG14L-linked VPS34 complex could co-exist in the Golgi apparatus with PAQR3. Consistently, upon glucose starvation, PAQR3 was released from the Golgi complex into puncta and no longer co-localized with the Golgi marker GM130 (Figs EV1A and EV2D). However, the punctiform PAQR3 was still basically co-localized with ATG14L-associated VPS34 complex (Fig EV2D), further supporting our major conclusion that PAQR3 is a constitutive binding partner of ATG14L-linked class III PI3K regardless of glucose starvation.
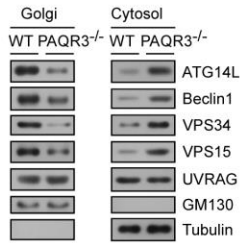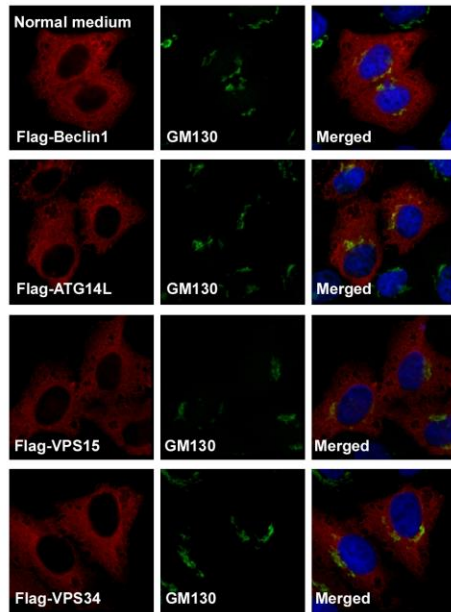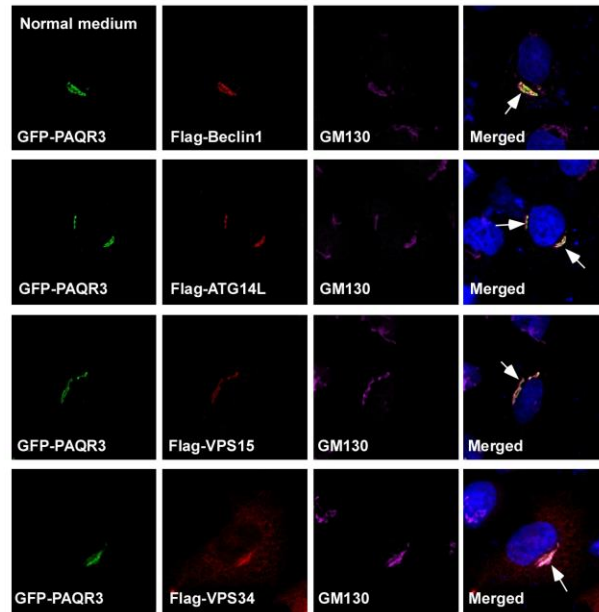
Figure 3B

Figure EV2A

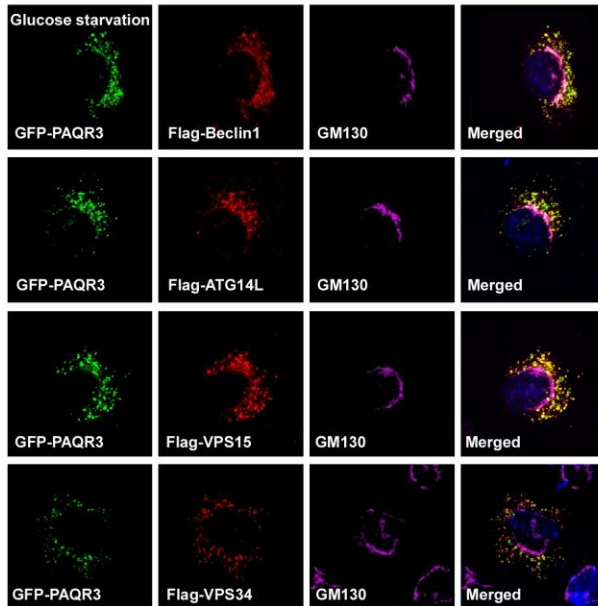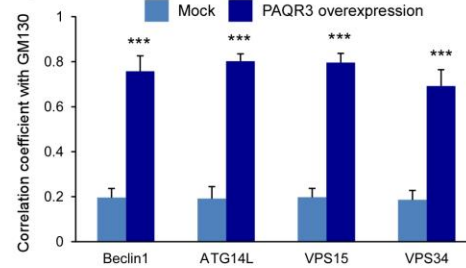**Figure EV2B**

Figure EV2D

Figure EV2C

Figure 3B: Cytosol and Golgi fractionations were isolated from the livers of PAQR3 knockout mice and their littermate controls. Equal protein amounts of cytosol and Golgi fractions were subjected to immunoblotting analysis with the indicated antibodies.

Figure EV2A and EV2B: HeLa cells were transfected with the plasmids as indicated, followed by immunofluorescence staining with the indicated antibodies. The arrow indicates co-localization of PAQR3 with the indicated proteins at the Golgi apparatus.

Figure EV2C: Co-localization coefficient between the indicated proteins and GM130 in the presence or absence of PAQR3 overexpression.

Figure EV2D: HeLa cells were transfected with the plasmids as indicated. After glucose starvation for 4 h, the cells were fixed and used in immunofluorescence staining and confocal analysis.

(2) For your second concern: As PAQR3 was redistributed into autophagosomes upon glucose starvation, during which T32 of PAQR3 was phosphorylated by active AMPK. Therefore, we suspected that PAQR3 T32 phosphorylation is implicated in its punctiform distribution. To test this hypothesis, we investigated the cellular localization of PAQR3 T32A and T32E upon glucose starvation respectively. As shown in Appendix Figure S5A, PAQR3 T32A was always localized in the Golgi apparatus regardless of glucose starvation, different from the punctiform localization of wild type PAQR3 after glucose starvation (Fig EV1A). However, PAQR3 T32E, which mimicked its phosphorylation by AMPK, exhibited as punctiform structures under both normal medium and glucose starvation (Appendix Fig S5B and C). These data suggested that T32 phosphorylation is a switch to initiate the punctiform distribution of PAQR3 upon glucose starvation.
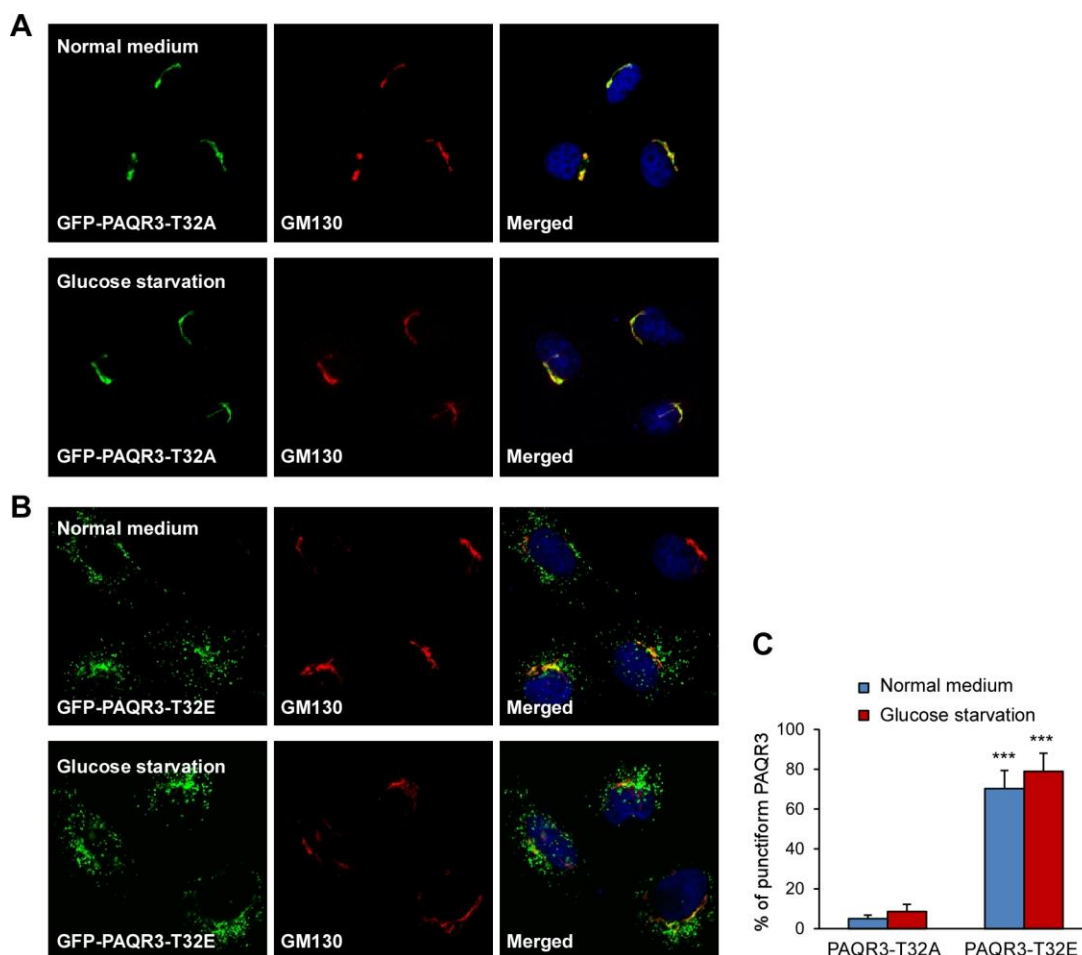
Appendix Fig S5. PAQR3 T32 phosphorylation is required for its punctiform distribution upon glucose starvation

(A, B) PAQR3 T32A or T32E transfected HeLa cells were treated with or without glucose starvation (GS) for 4 h. The cells were then fixed for immunofluorescence staining with GM130 antibody.

(C) At least 100 cells were counted per experiment and the data represented the percentage of punctiform PAQR3 with or without glucose starvation from three independent experiments (*** for $p < 0.001$).

4. PAQR3 T32 phosphorylation by AMPK is potentially important. However, how this modification affect PI3K lipid kinase activity is not elaborated in this study. The detailed mechanism will be helpful to clarify the significance of this phosphorylation event.

Response: Thanks for the great question. It was reported that two different regulatory mechanisms controls the activity of class III PI3K upon glucose starvation: (1) formation of different VPS34 complexes or (2) post-translational modification of its core component (*Cell. 2013. PMID:23332761*). As T32 phosphorylation of PAQR3 could not

alter its interaction with ATG14L-linked VPS34 complex (Fig 6D), we focused on the second possibility. It was worth noting that human Beclin1 phosphorylation at S93/S96 (S91/S94 in mouse) is required for ATG14L-associated VPS34 complex activation in response to glucose starvation (*Cell. 2013. PMID:23332761*). Therefore, we investigated whether T32 phosphorylation of PAQR3 is required for human Beclin1 S93 phosphorylation. As shown in Appendix Figure S4B, in PAQR3-deficient cells, phosphorylation of Beclin1 at S93 was markedly reduced no matter treated with or without glucose starvation and AMPK activator 2-DG. However, reconstitution of wild type PAQR3, rather than PAQR3 T32A mutant, could obviously rescue the impaired Beclin1 phosphorylation (Appendix Fig S4B). These data provided one possible mechanism that PAQR3 T32 phosphorylation might regulate ATG14L-linked class III PI3K activity through affecting Beclin1 S93 phosphorylation. Thanks again for raising this important issue.
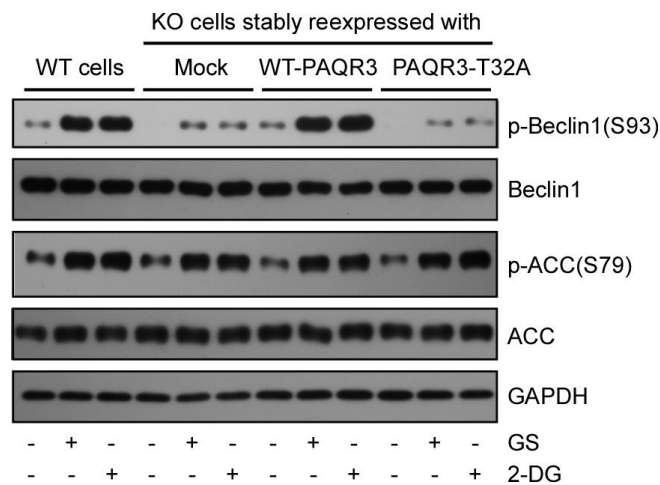

Appendix Fig S4B: PAQR3-deficient HeLa cells were infected with lentivirus expressing WT or T32A PAQR3 respectively. After glucose starvation (GS) or 2-DG (25 mM) treatment for 1 h, the cell lysates were analyzed by IB with the indicated antibodies.

5. The authors showed that PAQR3 is required for exercise-induced autophagy. How about the neurological phenotype of PAQR3 knockout mice? The neurodegenerative phenotypes are well documented in autophagy deficient mice.
**Response:** Thanks for the great suggestion. We evaluated the motor and behavioral characteristics of PAQR3 knockout mice by examining their stability on the accelerating rotarod, limb clasping, paw print intensity, and grip strength. There was no difference in the body weight between PAQR3 knockout mice and the wild type littermates at the age of 11 months (unpublished observations) However, the PAQR3 knockout mice displayed more limb clasping and were less capable on the rotating rod than the wild type mice (Fig 7E and F). Furthermore, abnormal paw print intensity and dispersion also indicated an ataxic walking pattern in PAQR3 knockout mice (Fig EV5A). Consistent with these results, the limb grip strength of PAQR3 knockout mice was significantly weaker than those of wild type mice (Fig EV5B). Thus, PAQR3 knockout is indeed involved in the progression of motor and behavioral abnormalities in the aged mice.

In fact, our group previously focused on metabolism study of PAQR3 knockout mice, in which young adult mice before 10 weeks old were commonly used. However, we did not notice any obvious abnormal behavioral phenotypes of these PAQR3 knockout mice before10 weeks old in appearance. Unfortunately, we cannot figure out the exact age that the PAQR3 knockout mice began to show neurodegenerative disorders right now. We will track and identify their neurodegenerative phenotype termly in our future work.

Again, thanks for the great suggestion.
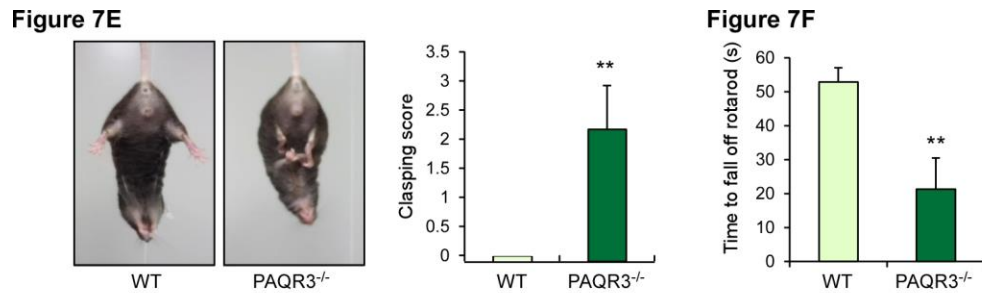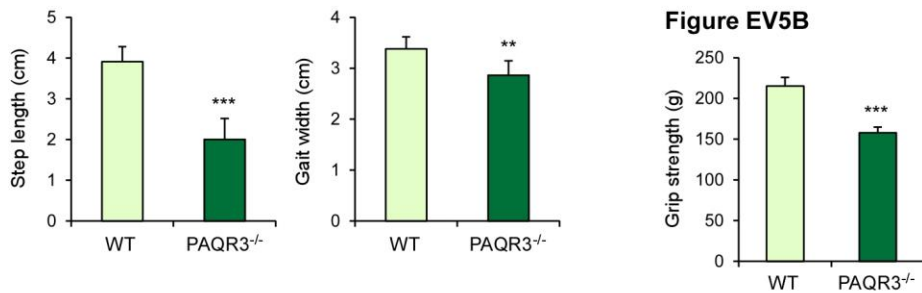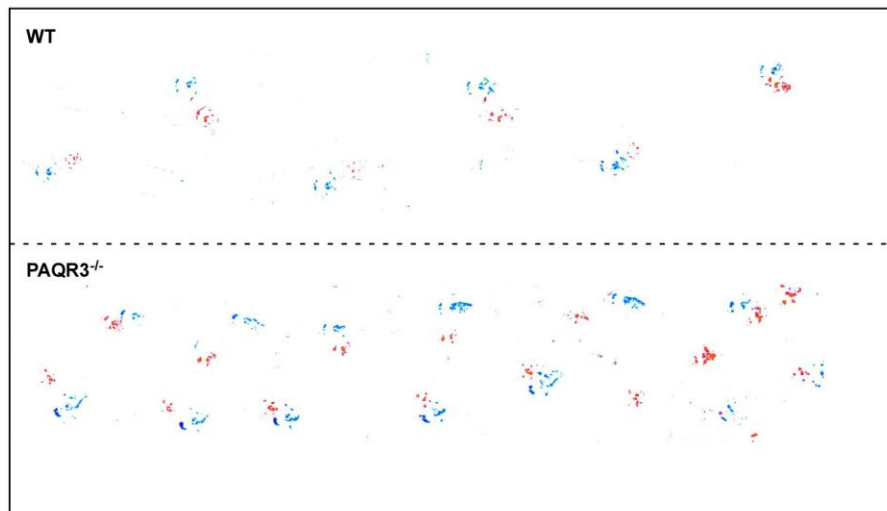
Figure 7E：Abnormal limb-clasping in PAQR3 knockout mice when suspended by its tail. Quantification scoring of the limb clasping phenotype is shown on the right (n = 7 for each group; ** $p < 0.01$).

Figure 7F: Motor performance was measured using an accelerating rotarod apparatus, and the time of each mouse spent on the rotating rod until it fell off was recorded. Average time on the rod for each group is shown ($n = 7$ for each group; ** $p < 0.01$).

Figure EV5A: Gait abnormalities in the PAQR3 knockout mice were evaluated by footprint analysis during walking (red, fore-limbs; blue, hind-limbs). Quantification of the stride length and gait width of paws print is shown in the bottom panel (n=7; * *p < 0.01; *** $p < 0.001$).

Figure EV5B: Grip strength analysis of all limbs in PAQR3 knockout mice and their WT littermates (n=7; *** $p < 0.001$).

Minor points:

6. In Figure 1F, it is striking to observe essentially no LC3 puncta in PAQR3 KO cells upon GS, since residual LC3 lipidation could still be observed (Fig. 1B). Is this image representative?

Response: Thank you for raising this issue. The images of LC3 immunostaining were representative in our data. In fact, the LC3 puncta indeed existed in the immunostaining image of PAQR3-deficient MEF cells. However, as we intended to show more cells in one picture, the autophagosome puncta in the PAQR3-deficient MEFs were too small to be clearly observed. In order to see the autophagosome puncta more clearly, a small number of cells in these two pictures were chosen randomly and enlarged as shown in Figure 1F (pictures in the right panel of the revised manuscript. Remarkably, the density and fluorescence intensity of LC3 puncta (indicated as arrows) in PAQR3-deficient cells were obviously less than that of WT cells upon glucose starvation. To better illustrate the LC3 punta, we included both the original (more cells included) and the enlarged pictures in the revised manuscript. Thanks again for raising this question.
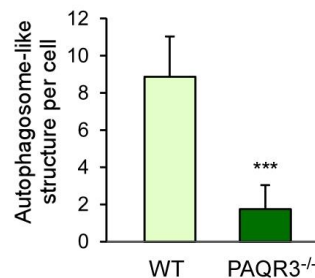
Figure 1F
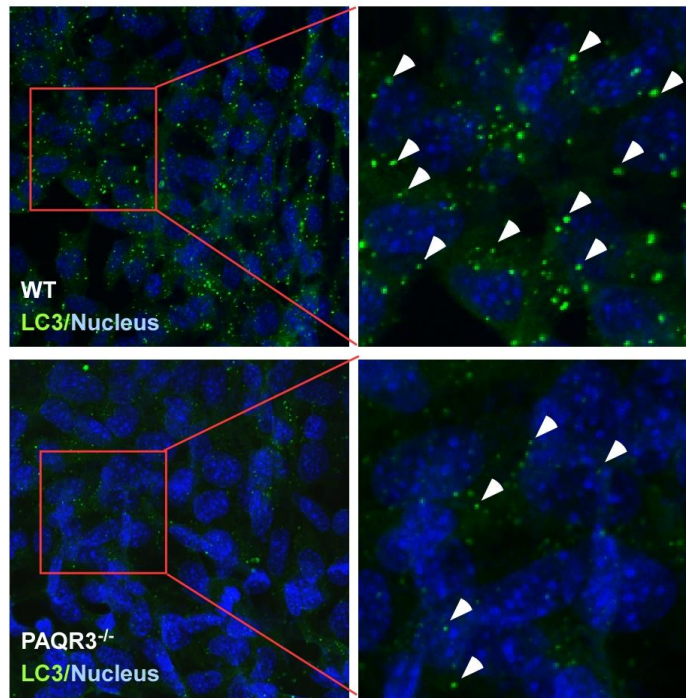
Figure 1F: Immunostaining of LC3 in WT or PAQR3-deficient MEFs after GS for 4 h. The arrowhead indicates representative LC3-positive puncta.

7. In Fig. 1D, quantitative analysis of autophagic vacuoles in EM images should be performed.

Response: Thank you for raising this issue. Quantitative analysis of the autophagic vacuoles in transmission electron microscopy images is now shown in Figure 1E of the revised manuscript.
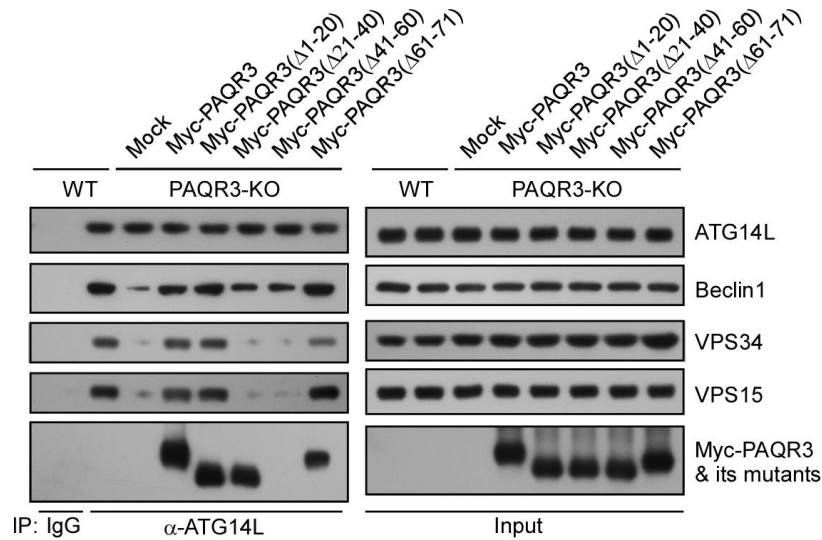
Figure 1E

Figure 1E. Quantitative analysis of autophagic vacuoles in transmission electron microscopy images. Thirty cells were quantified from each independent experiment which was repeated for three times with similar results. Values are presented as mean $\pm$ SD, ***$p < 0.001$.

8. In Fig. 2H, left bottom panel, one mutant (del41-60) is missing. Is this mutant protein expressed?

Response: Thanks for raising the question. In Figure 2H, the PAQR3 mutant with deletion of NH$_2$-terminal 41~60 aa was expressed, as revealed by immunoblotting analysis in the **INPUT** samples (the bottom panel on the **RIGHT**). The non-detected band in the left bottom panel shows results of immunoprecipitation samples, indicating PAQR3(Δ41~60) mutant could not interact with ATG14L. In other words, PAQR3(Δ41~60) mutant was expressed but could not interact with ATG14L.

Figure 2H: PAQR3-knockout HeLa cells were transfected with plasmids of various PAQR3 deletion mutants. At 24 h after the transfection, the cell lysates were used to immunoprecipitate VPS34 complex by ATG14L antibody, followed by immunoblotting (IB) with the antibodies as indicated.

9. In Fig. 3F, this experiment lacks a negative control, seems all presented proteins are reduced upon depletion. This could be a non-specific artifact caused by antibody depletion.

Response: Thanks for the suggestion. According to your advice, we added a negative control in this immunodepletion assay. As shown in the new Figure 3G of the revised manuscript, the amount of a housekeep protein "GAPDH" was not reduced after immunodepletion by using increasing Beclin-1 or ATG14L antibodies. This piece of data ruled out non-specific artifact caused by immunodepletion.
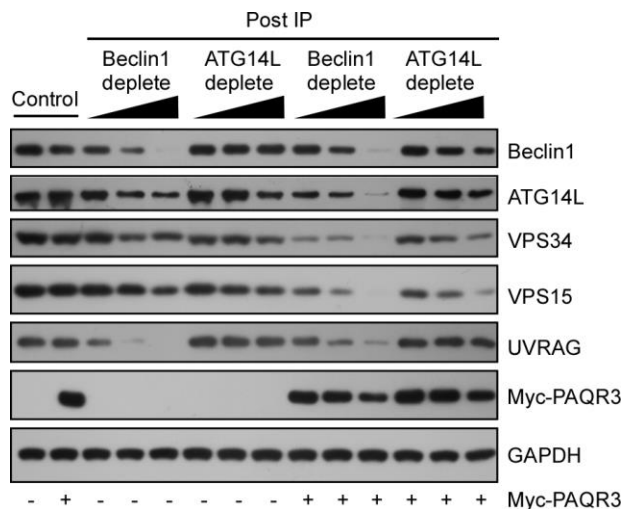
Figure 3G: MEFs were infected with control or PAQR3-expressing lentivirus. A quantitative immunodepletion assay was performed with increasing amounts of the indicated antibodies. Supernatants of MEF lysates after immunodepletion (Post-IP) were examined to determine the level of VPS34 complex proteins.

10. In Fig. 7F, this data is not convincing. Fraction 1 and 2 could be protein aggregates rather then real protein complexes. Can the authors repeat the fractionation assay in ATG14 or Beclin1 knockdown cells? It is not well documented if the complex could be disrupted by missing certain subunits.

Response: Thank you for raising this issue.

(1) In the gel filtration assay, our experimental procedure was the same as previously reported (*Nat Cell Biol. 2009. PMID:19270696; Cell Res. 2014. PubMed ID 24980960*). In fact, in each gel filtration assay, a total of 80 fractions were collected after starting the runs at a rate of 1 fraction/min (0.5 ml/min), and the 24th fraction was indicated as 670 KDa after calibration. We are sorry that our previous fraction number labels may mislead the readers, so we have changed them into the real collected fraction numbers in the gel filtration assay (Fig 7H and Appendix Fig S6).As it was reported that the "peak" of the core components in VPS34 complex was mainly distributed in a molecular weight of ~670KDa (*Nat Cell Biol. 2009. PMID:19270696; Nat Cell Biol. 2009. PMID:19270693*), the first 15 fractions were discarded to avoid the disturbance of the possible protein aggregates. Although the molecular weight of proteins in fraction 18 and 20 (previous label: fraction 1 and 2) were greater than ~670KDa, it can also be regarded as the real VPS34 complex, rather than protein aggregates, according to previously published papers (*Nat Cell Biol. 2009. PMID:19270696; Nat Cell Biol. 2009. PMID:19270693; Cell Res. 2014. PMID:24980960*). Thank you again for raising this issue.

(2) According to your advice, we repeated the gel filtration assay in Beclin1-knockdown HeLa cells. In this experiment, we could hardly detect endogenous ATG14L in Beclin1-knockdown cells (Fig R1A), as Beclin-1 is necessary for the stability of ATG14L (please refer to *Mol Biol Cell. 2008. PMID:18843052*). Therefore, we mainly investigated VPS15 and VPS34 distribution in the gel filtration assay of

Beclin1-knockdown cells (Fig R1B). Remarkably, VPS34 and VPS15 were dramatically shifted to fractions with lower molecular weight upon Beclin1 knockdown (Fig R1C). Meanwhile, Both VPS15 and VPS34 with molecular weight higher than 670 KDa (Fractions 18&20) were hardly detected in Beclin1-knockdown cells, which resembled the effect of PAQR3 on ATG14L-linked VPS34 complex integration. These data, together with our data shown in Figure 7H, indicated that PAQR3 and Beclin-1 were both critical for sufficient assembly of the VPS34 complex. Also, these results suggested that the ATG14L-linked VPS34 complex could be disrupted by missing certain subunits such as Beclin1. Interestingly, these results are similar to a research about Dapper1, another protein that promotes the assembly of ATG14L-associated VPS34 complex (please refer to *Cell Res. 2014.PMID:24980960*). In Dapper1 knockout cells, the components of this complex also tended to form a smaller complex as shown by gel filtration analysis. As these data in Figure R1 were not directly related to the function of PAQR3, we prefer not to include these data in the revised manuscript. We can also include these data into the Appendix Figures if requested by the reviewers. Thanks again for your understanding.
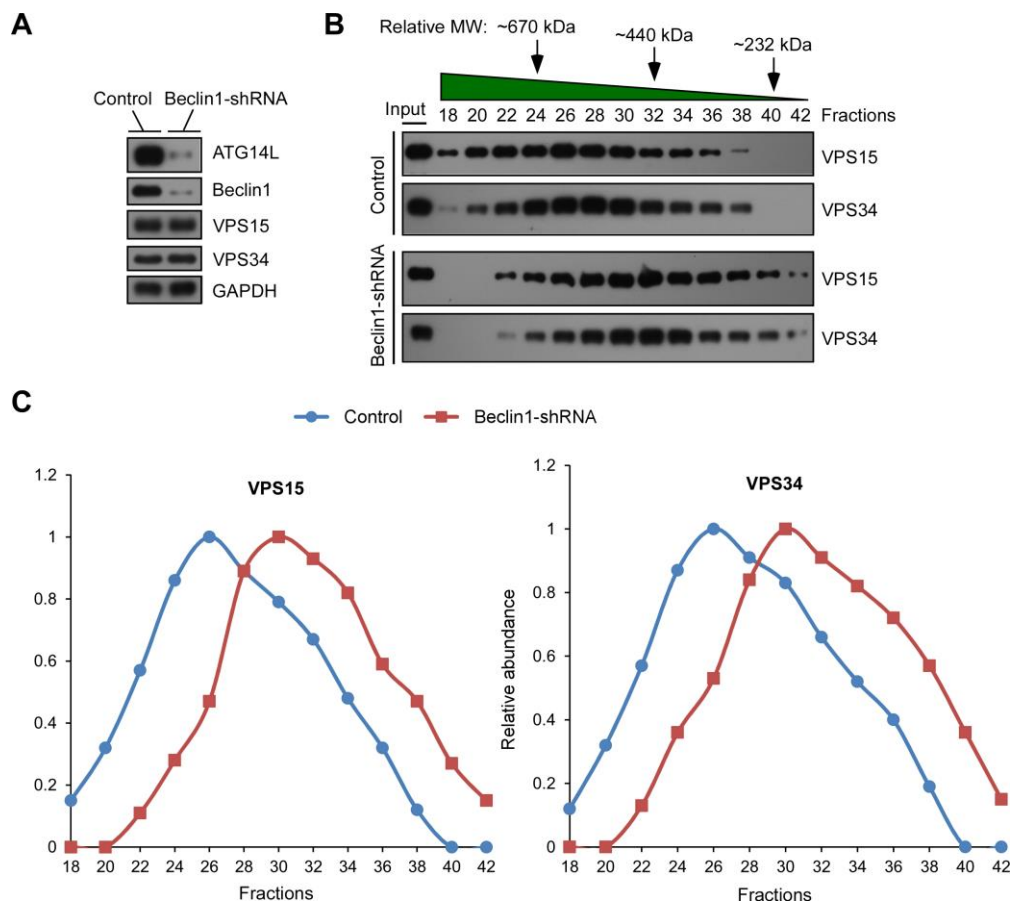
Figure R1 (for response to reviewers only): Beclin-1 is critical for sufficient assembly of the VPS34 complex.

(A) The whole cell lysates of WT or Beclin1-knockdown HeLa were harvested for IB with indicated antibodies.

(B) The supernatant fractions of WT or Beclin1-knockdown HeLa cells were subjected to gel fraction analysis, followed by IB using the antibodies as indicated.

(C) The relative distribution of each protein in different fractions after densitometry analysis of the blots in B.

Reviewer #3:

The manuscript reports that the protein PAQR3 has a pro-macroautophagic role upstream of PI3P synthesis. PI3P is a signaling lipid essential for autophagosome formation. The authors went on to show that the transmembrane protein PAQR3 is an interaction partner of the class III PI3K complex 1. The class III PI3K complex 1 contains the VPS34, VPS15, Beclin1 and ATG14 subunits. The authors show that PAQR3 interacts via two adjacent regions in its N-terminus with Beclin1 and ATG14, respectively. The interactions appear to promote the assembly of the complex and to stimulate PI3K activity. In contrast, assembly of the class III PI3K complex 2 which contain the UVRAG subunit instead of ATG14 is not promoted by PAQR3. PAQR3 is phosphorylated by AMPK on threonine 32. This phosphorylation is not required for the PI3K complex assembly activity but for the lipid kinase stimulatory activity of PAQR3. Phosphorylation of T32 is also shown to be required for the autophagy promoting effect of PAQR3 as measured by LC3-II and p62 accumulation. Finally, the authors show that PAQR3 is required for exercise-induced autophagy in skeletal muscle and liver tissue (again as measured by LC3-II and p62 accumulation) and that in the absence of PAQR3 the subunits of the class III PI3K complex 1 are shifted to lower molecular weight fraction.

In summary the manuscript by Xu et al. is a very solid and well-conducted study. The data are of very high quality. The paper will be interesting for people working on autophagy and the PI3K in particular. I have only a few comments:

1. The authors write "In contrast, the autophagic activity of a PAQR3-stably transfected cell line was profoundly augmented compared to the wild type cells (Figure S1E)." In a recent study it was shown that PAQR3 overexpression causes disruption of the Golgi (Hewavitharana, Cell Signal, 2015 PMID: 26327583). Could the elevated level of autophagy seen in PAQR3 overexpressing cells be the results of Golgi fragmentation and ER stress caused by overexpression of a transmembrane protein? Is the Golgi apparatus intact the cells the authors used?

Response: Thank you for raising the question. We would like to answer this question in following two aspects.

(1) In our experiment system, no obvious Golgi disruption/fragmentation was observed in the PAQR3-overexpressed cells. As shown in Figure R2A and R2B, in complete medium, the Golgi marker GM130 was always intact no matter whether PAQR3 was overexpressed or not. Upon glucose starvation, although PAQR3 exhibited as punctiform distribution, the Golgi was still intact (Fig R2A and R2B), suggesting that

the elevated level of autophagy in PAQR3-overexpressed cells was not caused by Golgi fragmentation. Due to space limitation of the manuscript, we only provided the organelle morphology of Golgi maker GM130 with PAQR3 overexpression in Figure EV1A of the revised manuscript. If requested by the reviewers, we can also include the remaining data into the Appendix Figures. Thanks for your understanding.
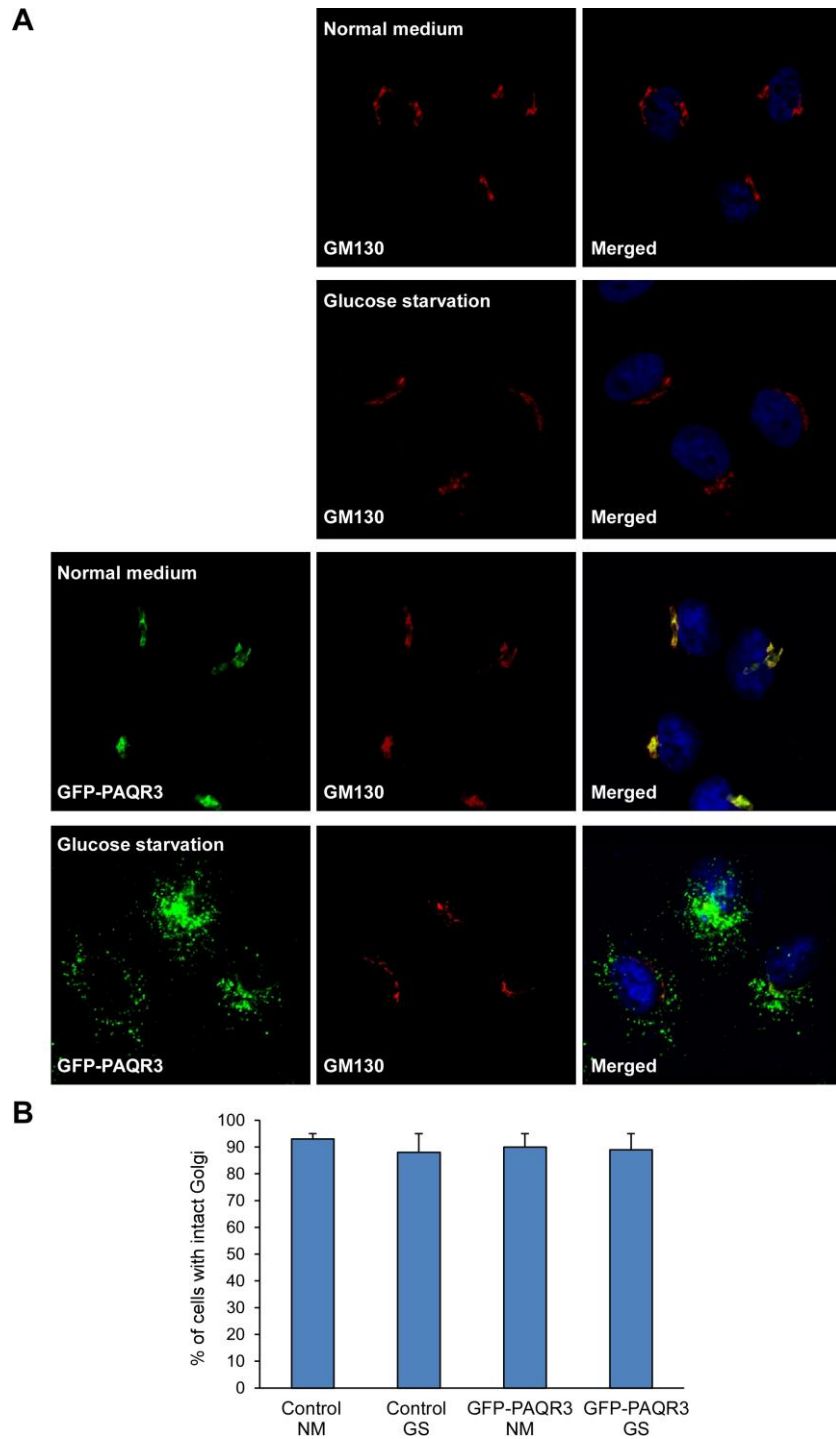
Figure R2 (for response to reviewers only): The punctiform distribution of PAQR3 upon glucose starvation is not caused by Golgi fragmentation.

(A) HeLa cells with or without GFP-PAQR3 transfection were fixed for immunostaining with indicated antibodies before or after glucose starvation for 4 h.

(B) At least 100 cells were counted per experiment for Figure R2A and the data represent the percentage of cells with intact Golgi apparatus with or without PAQR3 transfection from three independent experiments.

(2) Furthermore, the autophagy induced by glucose starvation was specifically augmented by stable transfection of PAQR3, but not by another Golgi-localized membrane protein PAQR11 (*Cell Res. 2012.PMID: 21968647*) (Fig EV1G).   Therefore, these data indicated that the enhanced autophagic activity of PAQR3 transfected cells was not caused by the artificial effect of a transmembrane protein overexpression. The new data are shown in Figure EV1G.
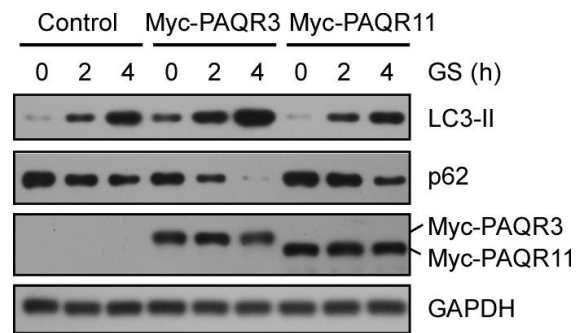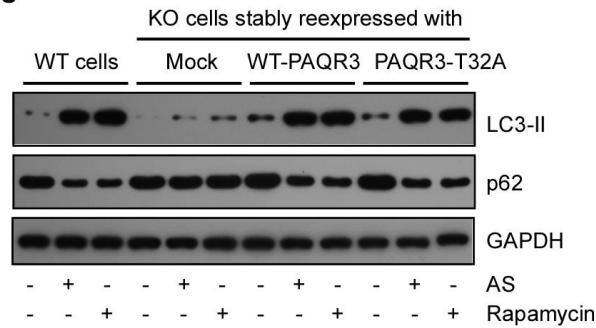

Figure EV1G: Hela cells were infected with lentivirus expressing PAQR3 or PAQR11 respectively. After glucose starvation (GS) for 2 h or 4 h, the whole cell lysates were harvested for immunoblotting with the antibodies as indicated.
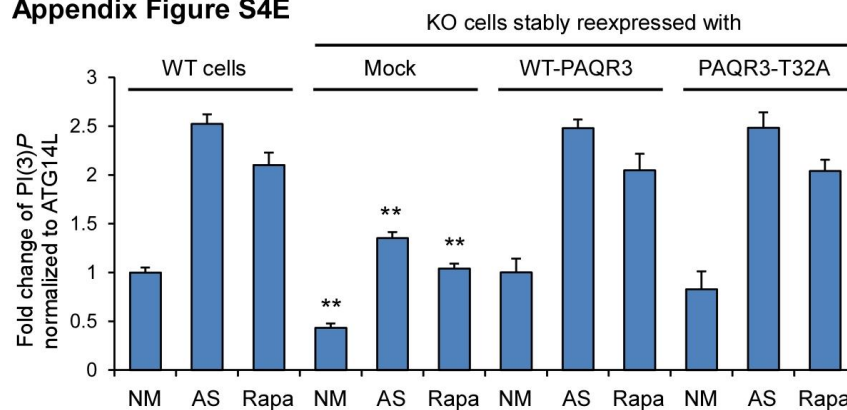
2. Most of the experiments are done with glucose starvation whereas amino acid starvation is the autophagic inducer most commonly used in the autophagy field. In fact the authors show that PAQR3 is required for full amino-acid and rapamycin-induced autophagy (Figure S1). Is T32A phosphorylation also important for amino-acid starvation-induced autophagy and increased PI3K activity?

Response: Thanks for the great question. According to your advice, we investigated whether PAQR3 T32 phosphorylation also played an important role in autophagy induced by other autophagy-stimulating conditions, such as amino acid depletion or rapamycin treatment. As shown in Appendix Figure S4D, both amino acid depletion- and rapamycin-induced autophagy was significantly ameliorated in PAQR3 knockout cells. However, the reduced autophagic activity could be rescued by reconstitution of both WT PAQR3 and its T32A mutant (Appendix Fig S4D). Similarly, upon amino acid depletion or rapamycin treatment, both WT and T32A PAQR3 could significantly augment the impaired ATG14L-linked VPS34 activity in PAQR3-knockout cells (Appendix Fig S4E). In summary, these data, together with the results shown in Figure 6E and 6G, indicated that PAQR3 T32 phosphorylation is specifically involved in autophagy initiation in response to glucose starvation, but not in autophagy induced by amino acid depletion or rapamycin.

Appendix Figure S4D

KO cells stably reexpressed with

| WT cells | Mock | WT-PAQR3 | PAQR3-T32A |

LC3-II
p62
GAPDH

| - | + | - | - | + | - | - | + | - | - | + | - | AS |
| - | - | + | - | - | + | - | - | + | - | - | + | Rapamycin |

Appendix Figure S4E

KO cells stably reexpressed with

Fold change of PI(3)P normalized to ATG14L

NM AS Rapa NM AS Rapa NM AS Rapa NM AS Rapa

Appendix Fig S4D: PAQR3 knockout HeLa cells were infected with lentivirus expressing WT or T32A PAQR3. After amino acid starvation (AS) or rapamycin (50 nM) treatment for 4 h, the whole cell lysates were harvested for immunoblotting with indicated antibodies.

Appendix Fig S4E: PAQR3-deficient HeLa cells were infected with lentivirus expressing WT or T32A mutant PAQR3 respectively. After amino acid starvation (AS) or rapamycin (50 nM) treatment for 4 h, VPS34 complexes were immunopurified by ATG14L antibody, followed by VPS34 activity detection by a quantitative PI(3)P ELISA assay. The PI(3)P level was normalized to the amount of ATG14L (n = 5; **p < 0.01 as compared to the first group with the same treatment.

3. The authors measure autophagic activity by accumulation of LC3-II (the lipidated form of LC3). However, in their western blots only one LC3 band is visible. How do the authors know that this is LC3-II and not LC3-I (the non-lipidated form).

Response: Thank you for the question. The LC3 antibody we used for immunoblotting was LC3B antibody (#2775) from Cell signaling Technology. This antibody appears to have more affinity for LC3-II, but not LC3-I (as shown in their datasheet), so that LC3-II is much easier to be detected in immunoblotting assay. Actually, after exposure for very long time, both LC3-I and LC3-II could be visible in most of our experiments (For example, please refer to Figure R3, related to Figure 1C and Figure 7C respectively). As the long-exposed LC3-II bands are not the best choice to clearly figure out the differences between different experimental groups, we prefer to show the more easily detected and

shortly-exposed LC3-II bands only. Besides, it is generally acceptable to show only LC3-II as a marker of autophagy in many high-profile papers, such as *Cell. 2013. PMID 23332761; Science. 2012. PMID: 22539723*. If requested by the reviewers, we can also include these data in the manuscript. Thanks again for your understanding.
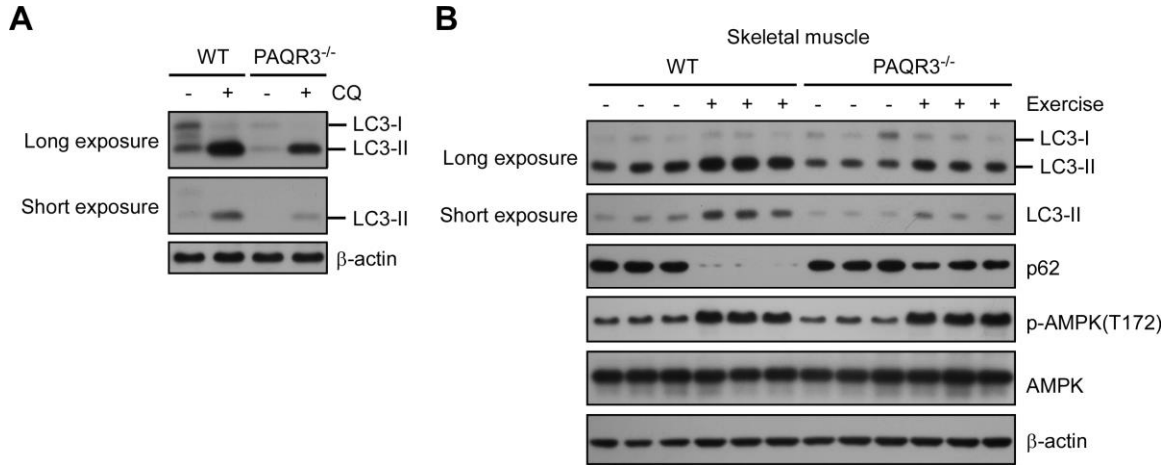
Figure R3 (for response to reviewers only): (A) WT and PAQR3-deficient MEFs were treated with 80 mM chloroquine (CQ) for 2 h to induce LC3-II accumulation. Whole-cell lysates were harvested for immunoblotting analysis of LC3. (B) Immunoblotting analysis of in skeletal muscle from WT or PAQR3 knockout mice at rest or after 80 min exercise.

4. The authors used a N-terminal fragment of PAQR3, combined it with ATG14L and added AMPK to show that AMPK directly phosphorylates PAQR3 (Fig 5E). It is not clear to me how Flag-ATG14L was purified for this experiment (please add to the methods) but the authors should include an additional control where they add Flag-ATG14L and a catalytic dead AMPK (or no AMPK) in order to exclude that the phosphorylation they detect is not mediated by another kinase co-purifying with Flag-ATG14L.

Response: Thank you for the great advice. We have added the method of ATG14L purification in the revised manuscript by referring to a previous report (page 34, lines 14-15). In addition, according to your advice, we added a negative control in Figure 5E of the revised manuscript. As expected, ATG14L enhanced the phosphorylation of PAQR3 only by wild-type AMPK, but not by the dominant negative AMPK (Fig 5E). These data indicated that ATG14L specifically enhanced PAQR3 phosphorylation by AMPK.
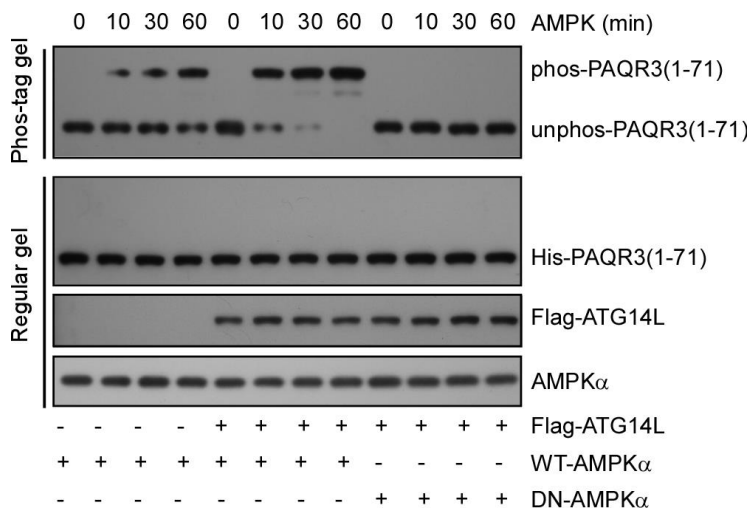
Figure 5E: Bacterial purified His-tagged NH₂-terminal 71 amino acid of PAQR3 was incubated with or without purified Flag-tagged ATG14L. Then the complexes were treated with WT AMPK or DN-AMPK (dominant negative AMPK) for the indicated time *in vitro*. PAQR3 phosphorylation was examined by Phos-tag gel analysis.

5. The experiment shown in Figure 6G in which the authors show the impact of the PAQR3 T32A mutation should be repeated with the inclusion of a lysosomal inhibitor such as chlorquine or bafilomycin.

Response: Thanks for the great suggestion. According to your advice, we repeated this experiment with the inclusion of a lysosomal inhibitor CQ (chloroquine diphosphate salt). As shown in Appendix Figure S4C, WT PAQR3, rather than its T32A mutant, could significantly rescue the ameliorated autophagic activity of PAQR3-deficient cells. These data further supported that PAQR3 T32 phosphorylation is important for autophagy initiation in response to glucose starvation.
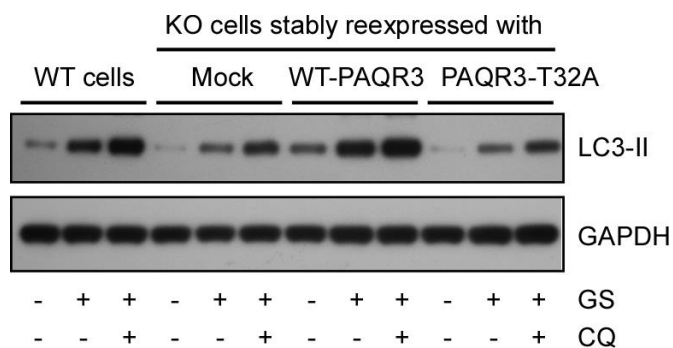
Appendix Fig S4C: PAQR3-deficient HeLa cells were infected with lentivirus expressing WT or T32A PAQR3. After glucose starvation (GS) in the absence or presence of 80 mM chloroquine (CQ) for 4 h, the cell lysates were harvested for immunoblotting with indicated antibodies.

6. In Figure 7F the authors show gel filtration profiles of the PI3K complex subunits using lysates from wild type and PAQR3 deficient livers. The result indicates that PAQR3

is required for the presence of these subunits in fractions 1 and 2. If PAQR3 functions as scaffold, as suggested by the authors the protein should also be present in these fractions. Can the authors provide evidence for this?

Response: Thanks for the great suggestion. We included the analysis of PAQR3 protein in this experiment as suggested by the reviewer. As shown in Figure 7H of the revised manuscript, in the gel filtration assays using the liver tissues of the wild-type mice, PAQR3 was also detected in the 18th & 20th fractions with molecular weight higher than 670KDa (i.e., previous fraction numbers: 1 & 2). We are sorry that our previous fraction number labels may mislead the readers, so we have changed them into the real collected fraction numbers in the gel filtration assay (Fig 7H). In addition, PAQR3 was overlapped with Beclin1, ATG14L, VPS15 and VPS34 in multiple fractions. These data, therefore, provided another hint for the crosstalk between PAQR3 and ATG14L-linked VPS34 complex.
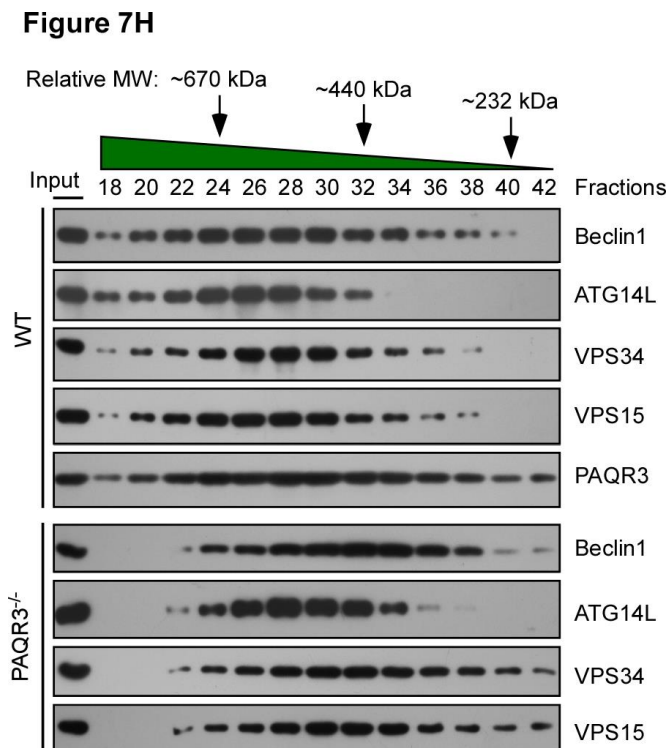
Figure 7H: The supernatants of liver lysates from WT or PAQR3 knockout mice were subjected to gel filtration analysis, followed by immunoblotting using the antibodies as indicated.

7. The authors write on page 22 "PAQR3-deleted mice had an apparent reduction in exercise-induced autophagy as assayed by immunohistochemical staining for LC3 in both skeletal muscle and liver (Fig 7A and B)". An increase in the LC3 level does not directly show enhanced autophagy. Thus the authors should tone down this statement.

Response: Thanks for the suggestion. According to your advice, we have toned down the statement in this part of the revised manuscript (page 26, lines 9-14).

8. The authors should show the expression levels of the PAQR3 T32A and T32E mutants compared to the wild type protein.

Response: Thanks for the great suggestion. We reconstituted exogenous PAQR3 into PAQR3-deficient cells using two methods: plasmid transfection and lentivirus infection.

(1) For the experiment in Figure 6E, WT, T32A and T32E PAQR3 were transfected into PAQR3-deficient cells to rescue the impaired ATG14L-linked VPS34 activity. According to your advice, we detected the expression levels of these transfected PAQR3 and added this piece of data to the revised manuscript (Appendix Fig S4A).

(2) For other experiments (such as Fig 6G and Appendix Fig S4B-E), the PAQR3-deleted HeLa cells were infected with lentivirus expressing WT or T32A PAQR3. Consistently, the expression levels of these exogenous PAQR3 were comparable to that in wild type cells (Fig 6F).

Appendix Figure S4A
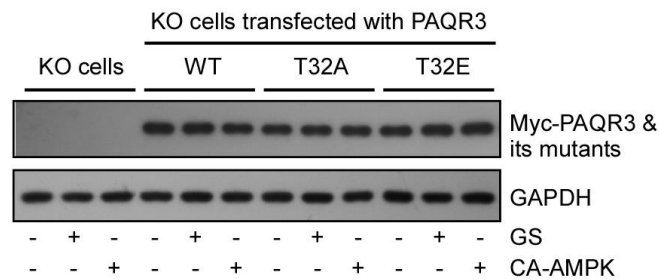
Figure 6F
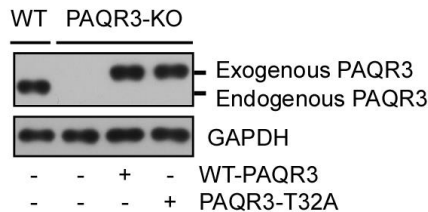
Appendix Fig S4A: WT, T32A or T32E mutants of PAQR3 were transfected into PAQR3-deficient HeLa cells. 10% of the cell lysates were subjected to immunoblotting (IB) analysis of PAQR3 expression.

Figure 6F: PAQR3-deficient HeLa cells were infected with lentivirus expressing WT or T32A PAQR3. Then PAQR3 expression levels were examined by immunoblotting.

9. On page 16, first line S4B must be S2B.

Response: Thank you for reminding us of this mistake. As the figure orders have been rearranged in the revised manuscript, we corrected it to EV3B (i.e. S2B in the previous manuscript) (page 17, lines 13-14).

Enclosure

2nd Editorial Decision   22 December 2015

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three referees are now broadly in favour of publication, pending satisfactory minor revision.

I would therefore like to ask you to address the remaining concerns by slight re-wording as suggested by referee #2. Please note that you should not remove the neuronal data as suggested by referee #1, since these were added to satisfy referee #2. Referee #3 suggests to slightly alter your title. I propose a title along the following lines (please modify if appropriate): PAQR3 controls autophagy by enhancing ATG14L-associated PI3K complex formation and integrates AMPK signaling into enhanced PI3K activity

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we should have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

REFEREE REPORTS

Referee #1:

The authors have performed substantial amount of work to address my concerns and the overall quality of the revised manuscript has significantly improved. I have only one last comment. The revision has added the behavior test results in which the PAQR3 knockout mice display locomotor function deficits. The result by itself is interesting, suggesting CNS abnormality of KO mice. However, without showing further the neuropathological evidence, it becomes a distraction and raises additional questions as to the exact impact of PAQR3 loss of function in nervous system. The role for PAQR3 in nervous system would be an interesting topic, but it goes beyond the main focus of the manuscript. My recommendation is to remove the behavioral analysis (Figure 7 E and F) and the data from mouse exercise and liver is sufficient to address the physiological role for PAQR3 in vivo in this first report of PAQR3 in autophagy regulation.

Referee #2:

The authors have addressed adequately my previous concerns.

One minor issue:
Although the authors have shown that ATG14-Beclin 1 associate with PAQR3 in PAQR3 affinity purification, PAQR3 has not been detected in the Beclin 1 or ATG14 complexes in the published literatures (Sun et.al, PNAS 2008; Zhong et al., NCB 2009; Matsunaga et. al, NCB, 2009). Direct binding is also missing. It is therefore more reasonable to claim that PAQR3 is a binding partner rather than the stoichiometric subunit of the ATG14-Beclin 1 complex.

Referee #3:

The authors have addressed all my comments satisfactorily. However, I would consider changing the title slightly, for example to "PAQR3 controls autophagy upon glucose starvation by enhancing ATG14L-associated PI3K complex formation via AMPK-mediated phosphorylation" since phosphorylation on T32 is not required for amino acid and rapamycin induced autophagy.

2nd Revision - authors' response   24 December 2015

Response to Referees
Referee #1:
The authors have performed substantial amount of work to address my concerns and the overall quality of the revised manuscript has significantly improved. I have only one last comment. The revision has added the behavior test results in which the PAQR3 knockout mice display locomotor

function deficits. The result by itself is interesting, suggesting CNS abnormality of KO mice. However, without showing further the neuropathological evidence, it becomes a distraction and raises additional questions as to the exact impact of PAQR3 loss of function in nervous system. The role for PAQR3 in nervous system would be an interesting topic, but it goes beyond the main focus of the manuscript. My recommendation is to remove the behavioral analysis (Figure 7 E and F) and the data from mouse exercise and liver is sufficient to address the physiological role for PAQR3 in vivo in this first report of PAQR3 in autophagy regulation.

Response: We would like to thank you for the positive comments of our revised manuscript. Referee #1 suggested us to remove the behavioral analysis of mice, however, these data were added to answer the questions of referee #2. Therefore, as suggested by the editor, we prefer not to remove these data. Thanks for understanding.

Referee #2:
The authors have addressed adequately my previous concerns. One minor issue:
Although the authors have shown that ATG14-Beclin 1 associate with PAQR3 in PAQR3 affinity purification, PAQR3 has not been detected in the Beclin 1 or ATG14 complexes in the published literatures (Sun et.al, PNAS 2008; Zhong et al., NCB 2009; Matsunaga et. al, NCB, 2009). Direct binding is also missing. It is therefore more reasonable to claim that PAQR3 is a binding partner rather than the stoichiometric subunit of the ATG14-Beclin 1 complex.

Response: Thanks. Based on your suggestion, we have re-worded our description in the newly revised manuscript (page 12, line16; page 13, line 2; the title of Appendix Fig S1).

Referee #3:
The authors have addressed all my comments satisfactorily. However, I would consider changing the title slightly, for example to "PAQR3 controls autophagy upon glucose starvation by enhancing ATG14L-associated PI3K complex formation via AMPK-mediated phosphorylation" since phosphorylation on T32 is not required for amino acid and rapamycin induced autophagy.

Response: We appreciate your suggestion. We have now changed the title into "PAQR3 controls autophagy by integrating AMPK signaling to enhance ATG14L-associated PI3K activity" in the newly revised manuscript. Due to the strict restriction on the number of character (100 limit) in the title, we cannot add "upon glucose starvation" in the title.

3rd Editorial Decision   04 January 2016

Thank you for sending the revised version of your manuscript. I appreciate the introduced changes and I am pleased to accept your manuscript for publication in The EMBO Journal.

Thank you for your contribution to our journal!