Autophagosome-Lysosome Fusion in Neurons Requires INPP5E, A Protein Associated with Joubert Syndrome

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Review timeline:

Submission date: 25 September 2015
Editorial Decision: 21 October 2015
Revision received: 26 April 2016
Editorial Decision: 18 May 2016
Revision received: 24 May 2016
Accepted: 27 May 2016

Editor: Andrea Leibfried

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 21 October 2015

Thank you for submitting your manuscript to us. I have now received reports of all three referees, which are enclosed below.

As you will see, while the referees consider that your work is well-performed and potentially interesting, they are not fully supportive of publication here. All of them note that some more insight into the mechanism underlying INPP5E mediated autophagosome-lysosome fusion would be required for publication in The EMBO Journal.

Given the interest into the topic and the constructive comments provided by the referees, I can offer to consider a revised version should you be able to substantiate your model along the lines suggested by the referees. This might demand a lot of work, and I will be happy to extend the revision time should that be useful. I am certain that adding more insight into INPP5E mediated fusion will make your paper an outstanding one. However, I would also like to note that it is important to add such further insight in order to consider publication here. I think it might be helpful in this case to discuss the revisions further, and I would be grateful if you could send me a point-by-point response upfront detailing what type of experiments you can undertake to address the concerns raised. Please also note our scooping protection; we will not reject your manuscript based on novelty issues in case similar work is published elsewhere while yours is under consideration here. Thank you for the opportunity to consider your work for publication. I look forward to your revision.
REFEREE REPORTS

Referee #1:

In this manuscript Hasigawa and co-workers present evidence that the inositol polyphosphate-5-phosphatase E (INPP5E) is important for fusion between autophagosomes and lysosomes. They show that depletion of INPP5E inhibits autophagic flux, leads to accumulation of autophagosomes and increased levels of lysosomal PI(3,5)P2. The phosphatase activity and the CAAX motif of INPP5E are required for its function in autophagosome-lysosome fusion. In contrast, endocytosis and lysosomal hydrolase activity seem not affected by INPP5E depletion. Interestingly, the authors found that expression of INPP5E having mutations associated with Joubert syndrome, a rare brain abnormality, caused reduced autophagic flux.

The data in this manuscript are conclusive and nicely presented, and the manuscript is well written. The finding that INPP5E, and its phosphatase activity, is important for autophagosome-lysosome fusion is novel, but a role for PI(3,5)P2 (and the PI3 5-kinase PIKfyve/Fab1) in autophagosome maturation has been described earlier. The data provides no clue to whether increased PI(3,5)P2 or decreased PI3P levels are important for fusion of autophagosomes with lysosomes, as there is no mechanistic understanding of the observed phenotype. In order to meet the high standards set for publication in EMBO J the authors should provide mechanistic insight into why INPP5E-mediated regulation of lysosomal PI(3,5)P levels is required for autophagosome-lysosome fusion.

Specific comments:

- As mentioned above, the authors should aim to provide mechanistic insight into why INPP5E-mediated regulation of lysosomal PI(3,5)P levels is required for autophagosome-lysosome fusion. Is there a difference in localization/recruitment of PI3P or PI(3,5)P2 effector proteins (e.g. WIPIs, FYCO1, ESCRTs,...) in control and siINPP5E cells?

- statistical significance levels should be included in all figures where data has been quantified (e.g. Figure 1 D and F, 2C, 5C)

- Figure 2A; the authors should include as a control blotting against a protein that should not be protected by proteinase K. Data from several experiments should be quantified. One might have expected more membrane-bound p62 in siINPP5E cells than in siControl cells if flux was significantly inhibited (as shown in Fig. 1)? The proteinase K assay is not described in the methods.

- In figure 2B-C they show that colocalization between LC3 and Lamp1 is reduced in siINPP5E cells. Can autophagosomes fuse with earlier endocytic compartments in these cells? EM should be used to look at the morphology of the accumulating LC3 positive vesicles.

- Figure 3D: colocalization should be quantified.

- Figure 4C: as a control they should include siRNA-mediated depletion of a protein that will inhibit (e.g. TSG101) or stimulate (e.g. Rubicon) EGFR degradation

- In figure 5 they show nicely that localization of a PI(3,5)P2 probe to lysosomes is increased in INPP5E depleted cells. How specific is this probe? Depletion of PIKfyve should be included as a control. Can they detect PI(3,5)P2 on autophagosomes as well (colocalization with LC3)?

- The authors discuss that the inhibited autophagosome-lysosome fusion seen in cells lacking INPP5E could be due to increased levels of PI(3,5)P2 or decreased PI3P levels. Are lysosomal PI3P levels decreased (co-stain with 2xFYVE probe)? Are autophagic PI3P levels and recruitment of PI3P effector proteins affected?

- Figure EV3: why are the number of Atg5 spots increased in siINPP5E cells? Shouldn't Atg5 come off autophagosomes prior to their closure?
- How is recruitment of INPP5E to lysosomes regulated?

- Fig. EV5A,B: the authors write that "INPP5E-depleted N1E-115 cells exhibited no decrease in Lysotracker staining of lysosomes relative to control cells...". This is correct, but the authors should also mention, and discuss, why there is a large increase in Lysotracker staining intensity (150% of control) in the INPP5E-depleted cells. Is this due to increased numbers or size of lysotracker-positive vesicles? Is the intensity of Lamp1 vesicles also increased? Is lysosomal fusion or fission affected by INPP5E?

Minor:
- introduction p7: they should also mention previous studies showing a role for Fab1/PIKfyve in autophagy.
- p14 top: change to "...lysosomes (Fig 3D, bottom panel), suggesting that the lysosomal localization of INPP5E is essential for autophagy".
- p15: "Because both of the extracellular cargos, DQ-BSA and EGFR,..." should be corrected to read "Because both of the extracellular cargos, DQ-BSA and EGF,...". EGFR is not extracellular cargo!
- p26: Lysosomal enzyme activity. They should include a brief description of the method.

Referee #2:

Hasegawa et al. identified a new regulator for the autophagosome-lysosome fusion, INPP5E, by screening ten phosphoinositide phosphatases. INPP5E regulates this fusion process through its phosphatase activity by converting PI(3,5)P2 to PI3P on lysosomes. Their data also showed that Joubert syndrome causing mutants block the autophagosome-lysosome fusion which suggests a role of autophagy in Joubert syndrome pathogenesis. The study is interesting and potentially important. However, several questions should be addressed before publication in EMBO.

1. The authors emphasized the function of INPP5E, a phosphatase that converts PI(3,5)P2 to PI3P on lysosomes, in autophagosomal fusion with lysosomes. However, the authors failed to demonstrate the importance of PI3P in this process. For example, if PI3P reduction on lysosomes is critical for the fusion defect in INPP5E depleted cells, will depletion of PIKfyve or VAC14 reverse the fusion defect? Or will reduction of PI3P by depleting PI3P generating enzymes on lysosomes display the similar fusion defects?

2. As the authors mentioned, "INPP5E is a 5-phosphatase that dephosphorylates PI(3,4,5)P3, PI(3,5)P2, and PI(4,5)P2 to PI(3,4)P2, PI(3)P, and PI(4)P". How could the authors exclude the possibilities that PIs other than PI(3)P also play a role in the fusion process. For example, PI(4)P has already been proposed to have functions in the fusion process (Wang et al. PNAS 2015). Or the accumulation of PI(3,5)P2 on lysosomes could also cause the fusion defect.

3. The exact mechanism through which INPP5E and potentially PI3P are involved in the autophagosome-lysosome fusion is not explored in this study. Will PI3P or INPP5E affect the dissociation of Atg proteins, recruitment or complex formation of autophagic SNARE proteins, or any other protein factors involved in this fusion process?

4. How could the conclusion "These observations suggest that not only PI(3)P, but also other PIs, function in autophagy. " be drawn from "For instance, PI(4,5)P2 is mainly localized to the plasma membrane, and is involved in trafficking in the endocytic pathway. PI(4)P is concentrated in the trans-Golgi network and recruits its effectors, such as oxysterol-binding protein, to control the secretory pathway (Di Paolo & De Camilli, 2006)." The connection between these observations and autophagy is not clear. Please explain or rephrase.

5. In Fig. EV3A-C, "siINPP5E significantly increased the number of autophagosomes labeled by
LC3, as well as Atg5-labeled autophagosomes in these cells (Figs 1E, F and EV3A-C).” Why are Atg5 dots also increased, does that mean the phagophore number is also increased? In that case, the authors need to distinguish if INPP5E depletion causes accumulation of complete autophagosomes or phagophores?

6. Again, the proteinase K protection assay doesn't support the authors' conclusion. If complete autophagosomes are accumulated, more p62 and LC3-II should be protected, which is opposite to the authors' observation in F2A.

7. To further support the conclusion that complete autophagosomes are accumulated in INPP5E depleted cells, the authors should present high quality EM results to visualize complete autophagosomes in INPP5E depleted cells to be more persuasive.

8. In Fig. 5B, the quality of the images is not good enough to support the conclusion that mSt-2xML1N dots are increased on lysosomes.

9. In Figure EV5, it will be more sensitive to use lysosensor instead of lysotracker to detect lysosome acidification defect. A FACS analysis here will be helpful to quantify this defect.

Referee #3:

The manuscript reports the intriguing observation that the 5-phosphatase INPP5E can control the autophagic flux. The authors suggest that this role of INPP5E is not dependent on its role in ciliogenesis and might involve instead the ability of INPP5E to control PI(3,5)P2 levels at lysosomes, possibly due to a direct catalytic action of INPP5E on PI(3,5)P2. While the effect of INPP5E depletion/mutation on the autophagic flux are well substantiated, the suggested mechanisms through which INPP5E exerts its effects on autophagy are not fully supported by the data of the manuscript in their present form. Thus there are key issues that should be resolved.

INPP5E & lysosomes

The authors show the colocalization of human mSt-INPP5E stably expressed in N1E-115 mouse cells. What about other cell types? What is the fraction of INPP5E that localizes to lysosomes? Is INPP5E stably associated with lysosomes, or does it associate to/dissociate from lysosomes only under specific conditions? Does INPP5E localize to lysosomes in ciliated cells?

The localization of the endogenous INPP5E protein should be examined in order to conclude that it localizes to lysosomes. There are published anti-INPP5E antibodies that work in immunofluorescence (such as those described in Humbert et al., PNAS 2012 and in Bielas et al., Nat Genet 2009) that could be used to immunolocalize INPP5E.

INPP5E & PI(3,5)P2

1. While it is well established that PI(4,5)P2 and PI(3,4,5)P3 can be substrates for INPP5E, the evidence that INPP5E can act on PI(3,5)P2 is by far less solid. There is only one report showing that an immune-precipitate obtained with the anti-72kd phosphatase (i.e. INPP5E) contains 5-phosphatase activity towards PI(3,5)P2 (Kong AM et al. JBC, 2000). Thus, more solid evidence should be provided using the purified recombinant INPP5E before concluding that it has 5-phosphatase activity towards PI(3,5)P2.

2. It is not clear how the quantification of PI(3,5)P2 reported in Fig 5D was performed. Since the authors used the overexpressed PI(3,5)P2 probe to detect and measure PI(3,5)P2 and since the global level of the overexpressed probe could affect the amount of the probe associated with the lysosomes, a normalization of the lysosomal-associated probe vs. the total levels of the expressed probe should be performed. It would be also very informative to compare the increase in PI(3,5)P2 observed after INPP5E KD with that consequent to the inactivation of the 5-phosphatase acting of PI(3,5)P2, i.e. Sac3/Fig4. In addition, the image in Fig. 5D shows a brighter staining not only with the PI(3,5)P2 probe but also with the LAMP1 antibody: does the depletion of INPP5E cause an
increase in the number/size of lysosomes? Of course, a crucial question is whether the increase in the lysosomal levels of PI(3,5)P2 in INPP5E-depleted cells can be rescued by the wt INPP5E.

3. What is the relationship between INPP5E and the 5-phosphatase acting of PI(3,5)P2, i.e. Vac4/Sac3? Can the overexpression of Sac3 compensate for the depletion of INPP5E and vice versa?

4. What is the relationship between INPP5E and the PI(3)P 5-kinase PIKfyve? Is the accumulation of PI(3,5)P2 (and the defect in autophagic flow) rescued by decreasing PI(3,5)P2?

5. Are the levels of lysosomal PI(3)P affected by the depletion of INPP5E.

INPP5E & autophagy
The authors suggest that the role of INPP5E in autophagy is independent of its role in controlling ciliogenesis: they reach this conclusion by assessing that the depletion of INPP5E does not affect the cilia length in MEFs stably expressing ARL13. To substantiate their conclusion they should show that INPP5E depletion impairs the autophagic flux in the very same cell line and under the very same experimental conditions.

This appear particularly important since the MEFs in general (but what about the MEFs stably expressing ARL13 in particular?), as also the Authors notice (page 10), appear to be less sensitive to INPP5E depletion in terms of impairment of autophagic flux. From the comparison of Fig.1C (N1E-115) and Fig.EV2B (MEFs) it is evident that, in the presence of serum, the autophagic flux is impaired in N1E-115 but not in MEFs knocked down for INPP5E. The MEFs apparently become sensitive to the lack of INPP5E only under starvation conditions (though the representative images of the western blot showing LC3 do not fully match with the quantitative data shown in the graph).

Finally, as INPP5E depletion can also induce complex transcriptional changes, as recently shown in Plotnikova et al. (JCS 2015), the authors should ascertain that the impaired autophagic flux is direct rather than a consequence of such transcriptional changes.
Specific response:
(Reviewer’s comments are in italics.)

Referee #1:

In this manuscript Hasigawa and co-workers present evidence that the inositol polyphosphate-5-phosphatase E (INPP5E) is important for fusion between autophagosomes and lysosomes. They show that depletion of INPP5E inhibits autophagic flux, leads to accumulation of autophagosomes and increased levels of lysosomal PI(3,5)P2. The phosphatase activity and the CAAX motif of INPP5E are required for its function in autophagosome-lysosome fusion. In contrast, endocytosis and lysosomal hydrolase activity seem not affected by INPP5E depletion. Interestingly, the authors found that expression of INPP5E having mutations associated with Joubert syndrome, a rare brain abnormality, caused reduced autophagic flux.

The data in this manuscript are conclusive and nicely presented, and the manuscript is well written. The finding that INPP5E, and its phosphatase activity, is important for autophagosome-lysosome fusion is novel, but a role for PI(3,5)P2 (and the PI3 5-kinase PIKfyve/Fab1) the in autophagosome maturation has been described earlier. The data provides no clue to whether increased PI(3,5)P2 or decreased PI3P levels are important for fusion of autophagosomes with lysosomes, as there is no mechanistic understanding of the observed phenotype. In order to meet the high standards set for publication in EMBO J the authors should provide mechanistic insight into why INPP5E-mediated regulation of lysosomal PI(3,5)P levels is required for autophagosome-lysosome fusion.

Specific comments:
- As mentioned above, the authors should aim to provide mechanistic insight into why INPP5E-mediated regulation of lysosomal PI(3,5)P levels is required for autophagosome-lysosome fusion. Is there a difference in localization/recruitment of PI3P or PI(3,5)P2 effector proteins (e.g. WIPIs, FYCO1, ESCRTs,...) in control and siINPP5E cells?

We thank the reviewer for this important suggestion. We agree that if we could provide the mechanistic insights, our paper would be greatly improved. To address this, we gave our attention to two recent papers from other groups.

First, Hong et al recently reported that PI(3,5)P2 controls the endosomal actin dynamics by regulating cortactin-actin interactions (J Cell Biol 210: 753–769, 2015). They show that PI(3,5)P2 binds to actin filament-binding region of cortactin, causing release of actin filaments from the rab7-positive late endosomes. Therefore, they propose that decrease of PI(3,5)P2 level promotes stabilization of actin filaments on endosomes.

Second, Lee et al demonstrated that cortactin knockdown inhibits autophagosome-lysosome fusion (EMBO J 29: 969–980, 2010). They and other group also showed that the inhibition of actin polymerization suppresses the fusion as well (Lee et al, EMBO J 29: 969–980, 2010; Zhuo et al, Biochem. Biophys. Res. Commun. 437: 482–488, 2013).

Given these results, we envisaged that cortactin and actin filaments also exist on lysosomes and decrease of lysosomal PI(3,5)P2 by INPP5E enhances cortactin-dependent actin filament stabilization on lysosome, followed by fusion between autophagosome and lysosome.

Then, we firstly confirmed colocalization of F-actin representing actin filament with LAMP1 (see Fig A below). Furthermore, as predicted, INPP5E knockdown decreased the intensity of F-actin on lysosomes to 40% compared to control cells (Fig A). Next, we detected cortactin on lysosomes (Fig B). While INPP5E
knockdown did not affect the amount of total lysosomal cortactin, we found activation of cortactin by INPP5E. The activity of cortactin is known to be regulated by its phosphorylation (Oser et al. J Cell Sci. 2010). Tyr421 and Tyr466 phosphorylation activate cortactin. We found that phosphorylated (activated) cortactin localized to lysosome by using the anti-phospho-Tyr421 or Tyr466 cortactin antibodies. INPP5E knockdown apparently reduces both Tyr421 (Fig C) and Tyr466 (data not shown) phosphorylated cortactin intensity on lysosome (ca. 60% reduction). Finally, we demonstrated that treatment of cells with Latrunculin A (Lat A), an inhibitor of actin polymerization showed the accumulation of LC3 dots (Fig D) and the reduction of the colocalization rate between LC3 and LAMP1 (Fig E), indicating that actin polymerization is required for autophagosome-lysosome fusion as reported previously.

Taken together, it is very likely that suppression of PI(3,5)P₂ level on lysosome by INPP5E-dependent conversion of PI(3,5)P₂ to PI(3)P stabilizes actin filaments on the organelle, followed by the autophagosome-lysosome fusion. Intriguingly, PI(3)P binds to Wiskott-Aldrich syndrome protein and Scar homologue (WASH) complex (Jia et al, 2010). Since WASH complex is known to promote actin polymerization through Arp2/3 complex activation, both decrease of PI(3,5)P₂ and increase of PI(3)P by INPP5E possibly contribute the fusion process via actin polymerization. Although what is a role of actin filaments in the fusion is still enigma, our finding provides important novel mechanistic insights into how PI regulates the cellular function.
- statistical significance levels should be included in all figures where data has been quantified (e.g. Figure 1 D and F, 2C, 5C)

Thank you for pointing out it. We have now added the statistical analysis in all figures.

- Figure 2A; the authors should include as a control blotting against a protein that should not be protected by proteinase K. Data from several experiments should be quantified. One might have expected more membrane-bound p62 in siINPP5E cells than in siControl cells if flux was significantly inhibited (as shown in Fig. 1)? The proteinase K assay is not described in the methods.
We thank the reviewer for the constructive comment. We added a control blotting using Atg2a/b siRNAs (please see the below figure), because it has been reported that the unsealed autophagosomes are accumulated in Atg2a/b-suppressed cells (Velikkakath et al. MBC 2012). Also, we replaced the blotting using siINPP5E#1 with new one using siINPP5E#2 because LC3-II form is more increased in siINPP5E#2-treated cells compared to siINPP5E#1-treated cells. We added description of the method on protection assay in detail.

- In figure 2B-C they show that colocalization between LC3 and Lamp1 is reduced in siINPP5E cells. Can autophagosomes fuse with earlier endocytic compartments in these
cells? EM should be used to look at the morphology of the accumulating LC3 positive vesicles.

According to the reviewer’s comment, we performed EM analysis. Although we could not observe earlier endocytic compartments fused with autophagosomes, autophagosomes were increased in INPP5E-depleted cells.

- Figure 3D: colocalization should be quantified.

We have now added the quantified data.

- Figure 4C: as a control they should include siRNA-mediated depletion of a protein that will inhibit (e.g. TSG101) or stimulate (e.g. Rubicon) EGFR degradation

We thank the reviewer for the suggestion. It has been reported that the suppression of CHMP5, which is involved in multivesicular body formation, inhibits EGFR degradation (Ward et al. JBC 2005). Thus, we performed EGFR degradation assay in CHMP5-depleted cells. As expected, EGFR degradation was inhibited in CHMP5-depleted cells.

- In figure 5 they show nicely that localization of a PI(3,5)P2 probe to lysosomes is increased in INPP5E-depleted cells. How specific is this probe? Depletion of PIKfyve should be included as a control. Can they detect PI(3,5)P2 on autophagosomes as well (colocalization with LC3)?

The previous study has shown that recombinant ML1N protein (a PI(3,5)P2 probe we used in this study) binds strongly to PI(3,5)P2-containing liposomes, but not to liposomes containing PI(3)P, PI(5)P, or PI(4,5)P2 (Li et al. PNAS 2013), suggesting
that this probe is highly specific to PI(3,5)P₂. As requested, PIKfyve knockdown experiment is included as a control. We also used YM201636, a PIKfyve inhibitor (please see Fig B and C below). As expected, binding of the probe to lysosomes significantly decreased. In addition, we examined the colocalization of PI(3,5)P₂ with LC3. As a result, little colocalization between them was observed (Fig A).

- The authors discuss that the inhibited autophagosome-lysosome fusion seen in cells lacking INPP5E could be due to increased levels of PI(3,5)P₂ or decreased PI3P levels. Are lysosomal PI3P levels decreased (co-stain with 2xFYVE probe)? Are autophagic PI3P levels and recruitment of PI3P effector proteins affected?
We thank the reviewer for this insightful question. We have now added the quantification data showing that the intensity of PI(3)P on lysosomes (detected by 2xFYVE and normalized by the cytosolic intensity) decreased in INPP5E-depleted cells (please see Fig A and B below). We have already showed that PI(3,5)P₂ level increased in INPP5E-depleted cells, suggesting that INPP5E dephosphorylates PI(3,5)P₂ to PI(3)P on lysosomes. Moreover, we tested whether the recruitment of WIPI2, an autophagic PI(3)P effector, to autophagosome are affected in INPP5E-depleted cells. Almost 100% of WIPI2 were normally recruited to LC3-positive autophagosomes, indicating that INPP5E is not related to autophagic PI3P levels and recruitment of the PI3P effector there (data not shown).

- Figure EV3: why are the number of Atg5 spots increased in siINPP5E cells? Shouldn't Atg5 come off autophagosomes prior to their closure?

Thanks for indicating the important point. We speculate that to compensate inhibition of fusion between autophagosome and lysosome cells enhance
autophagosome biogenesis (=increase of Atg5 dots). In agreement with the idea, Atg5 dots are accumulated under Bafilomycin treatment condition, which inhibits the fusion (please see below figure).

- How is recruitment of INPP5E to lysosomes regulated?

Thanks for the question. We tested if starvation in MEFs or Torin1 treatment in N1E-115 cells, both of which induces autophagy affects recruitment of INPP5E on lysosomes or not. No change was observed. Although we have not studied what molecule recruits INPP5E to lysosome, we demonstrated that the membrane anchor activity of INPP5E could be required for its lysosomal localization. In future, by using the domain, we would like to investigate the recruiting mechanisms.
- Fig. EV5A,B: the authors write that "INPP5E-depleted N1E-115 cells exhibited no decrease in Lysotracker staining of lysosomes relative to control cells...". This is correct, but the authors should also mention, and discuss, why there is a large increase in Lysotracker staining intensity (150% of control) in the INPP5E-depleted cells. Is this due to increased numbers or size of lysotracker-positive vesicles? Is the intensity of Lamp1 vesicles also increased? Is lysosomal fusion or fission affected by INPP5E?

Thanks for the important questions. Indeed, lysosomes increase in the number and the LAMP1 intensity in INPP5E-depleted cells. Possibly, the elevation of PI(3,5)P2 level and/or the reduction of PI(3)P level on lysosome affects the lysosomal biogenesis and activity. We think that this is an interesting phenomenon that should be studied in future. We discuss it in the revised manuscript.

Note: As requested by the other reviewer, we have replaced the result of Lysotracker to one of Lysosensor, which is more sensitive than Lysotracker. Although the intensity of Lysotracker staining increased in the cells, staining of Lysosensor was not changed, suggesting that INPP5E does not affect the lysosomal pH.

Minor:
- introduction p7: they should also mention previous studies showing a role for Fab1/PIKfyve in autophagy.

We have now included the paper on Fab1/PIKfyve in the revised manuscript.

- p14 top: change to "...lysosomes (Fig 3D, bottom panel), suggesting that the lysosomal localization of INPP5E is essential for autophagy".
We changed the sentence.

- p15: "Because both of the extracellular cargos, DQ-BSA and EGFR,..." should be corrected to read "Because both of the extracellular cargos, DQ-BSA and EGF,...". EGFR is not extracellular cargo!

We corrected “EGFR” to “EGF”.

- p26: Lysosomal enzyme activity. They should include a brief description of the method.

We added a description of method on lysosomal enzyme activity.

Referee #2:

Hasegawa et al. identified a new regulator for the autophagosome-lysosome fusion, INPP5E, by screening ten phosphoinositide phosphatases. INPP5E regulates this fusion process through its phosphatase activity by converting PI(3,5)P2 to PI3P on lysosomes. Their data also showed that Joubert syndrome causing mutants block the autophagosome-lysosome fusion which suggests a role of autophagy in Joubert syndrome pathogenesis. The study is interesting and potentially important. However, several questions should be addressed before publication in EMBO.

1. The authors emphasized the function of INPP5E, a phosphatase that converts PI(3.5)P2 to PI3P on lysosomes, in autophagosomal fusion with lysosomes. However, the authors failed to demonstrate the importance of PI3P in this process. For example,
if PI3P reduction on lysosomes is critical for the fusion defect in INPP5E depleted cells, will depletion of PIKfyve or VAC14 reverse the fusion defect? Or will reduction of PI3P by depleting PI3P generating enzymes on lysosomes display the similar fusion defects?

We thank the reviewer for the insightful suggestion. We tested the effect of double knockdown of PIKfyve and INPP5E on fusion process. As a result, interestingly PI(3,5)P₂ staining was almost abolished (please see Fig A below), suggesting that effect of PIKfyve depletion is stronger and dominant to INPP5E depletion. In the double KD cells LC3 dots was accumulated, suggesting inhibition of autophagy after formation of autophagosomes (Fig B). This result consists with the previous study demonstrating that treatment of cells with PIKfyve inhibitor causes defect in autophagy, which is possibly due to suppression of degradation of autophagosomal contents in autolysosomes by loss of lysosomal activity (de Lartigue et al. Traffic. 2009). Therefore, we conclude that both excess and less PI(3,5)P₂ level in lysosomes compromises autophagy at late stages (fusion or lysosomal degradation), i.e., the optimal level of lysosomal PI(3,5)P₂ (or PI(3)P) is essential to the process.

Anyway, still there is no direct evidence showing importance of lysosomal PI(3,5)P₂ or PI(3)P in the autophagic fusion. However, as described below (answer to the comment #3) our additional rigorous analyses demonstrated that INPP5E is essential to cortactin-dependent actin filament stabilization, followed by autophagosome-lysosome fusion. Since binding of PI(3,5)P₂ to cortactin is known to cause release of actin filament from cortactin, the new results strongly support that INPP5E-dependent conversion of PI(3,5)P₂ to PI(3)P in lysosomes is critical to the fusion process.
2. As the authors mentioned, "INPP5E is a 5-phosphatase that dephosphorylates PI(3,4,5)P3, PI(3,5)P2, and PI(4,5)P2 to PI(3,4)P2, PI(3)P, and PI(4)P". How could the authors exclude the possibilities that PIs other than PI(3)P also play a role in the fusion process. For example, PI(4)P has already been proposed to have functions in the fusion process (Wang et al. PNAS 2015). Or the accumulation of PI(3,5)P2 on lysosomes could also cause the fusion defect.
Thanks for the important comment. We have already showed that only PI(3,5)P₂, but not PI(4,5)P₂ and PI(3,4,5)P₃, localizes to lysosomes (Fig. 5). In addition, we have now added the new data showing that the intensity of PI(4)P (using OSBP-PH probe) on lysosomes (normalized by the cytosolic intensity). PI(4)P exists in lysosomes, but its amount was not changed by depletion of INPP5E (please see the following Fig A and B), suggesting that PI(4)P is not involved in the fusion process.

As the reviewer thought, we now believe that the accumulation of PI(3,5)P₂ on lysosomes causes the fusion defect. Why we think so is explained in our answer to the next question.

3. The exact mechanism through which INPP5E and potentially PI3P are involved in the autophagosome-lysosome fusion is not explored in this study. Will PI3P or INPP5E affect the dissociation of Atg proteins, recruitment or complex formation of autophagic SNARE proteins, or any other protein factors involved in this fusion process?
We thank the reviewer for this important question. To provide the mechanistic insights into how INPP5E and PI3P (or PI(3,5)P₂) are involved in the fusion process, we performed substantial additional experiments.

First, we gave our attention to two recent papers from other groups. Hong et al recently reported that PI(3,5)P₂ controls the endosomal actin dynamics by regulating cortactin-actin interactions (J Cell Biol 210: 753–769, 2015). They show that PI(3,5)P₂ binds to actin filament-binding region of cortactin, causing release of actin filaments from the rab7-positive late endosomes. Therefore, they propose that decrease of PI(3,5)P₂ level promotes stabilization of actin filaments on endosomes. In addition, Lee et al demonstrated that cortactin knockdown inhibits autophagosome-lysosome fusion (Lee et al, 2010). They and other group also showed that the inhibition of actin polymerization suppresses the fusion as well (Lee et al, EMBO J 29: 969–980, 2010; Zhuo et al, Biochem. Biophys. Res. Commun. 437: 482–488, 2013). Given these results, we envisaged that cortactin and actin filaments also exist on lysosomes and decrease of lysosomal PI(3,5)P₂ by INPP5E enhances cortactin-dependent actin filament stabilization on lysosome, followed by fusion between autophagosome and lysosome.

We firstly confirmed colocalization of F-actin representing actin filament with LAMP1 (Fig A). Furthermore, as predicted, INPP5E knockdown decreased the intensity of F-actin on lysosomes to 40% compared to control cells (Fig A). Next, we detected cortactin on lysosomes (Fig B). While INPP5E knockdown did not affect the amount of total lysosomal cortactin, we found cortaction activation by INPP5E. The activity of cortactin is known to be regulated by its phosphorylation (Oser et al. J Cell Sci. 2010). Tyr421 and Tyr466 phosphorylation activate cortactin. We found that phosphorylated (activated) cortactin localized to lysosome by using the anti-phospho-Tyr421 or Tyr466 cortactin antibodies. INPP5E knockdown apparently reduces both Tyr421 (Fig C) and Tyr466 (data not shown) phosphorylated cortactin intensity on lysosome (ca. 60% reduction).
Finally, we demonstrated that treatment of cells with Latrunculin A (Lat A), an inhibitor of actin polymerization showed the accumulation of LC3 dots (Fig D) and the reduction of the colocalization rate between LC3 and LAMP1 (Fig E), indicating that actin polymerization is required for autophagosome-lysosome fusion as reported previously.

Taken together, it is very likely that suppression of PI(3,5)P₂ level on lysosome by INPP5E-dependent conversion of PI(3,5)P₂ to PI(3)P stabilizes actin filaments on the organelle, followed by the autophagosome-lysosome fusion. Intriguingly, PI(3)P binds to Wiskott-Aldrich syndrome protein and Scar homologue (WASH) complex (Jia et al, 2010). Since WASH complex is known to promote actin polymerization through Arp2/3 complex activation, both decrease of PI(3,5)P₂ and increase of PI(3)P by INPP5E possibly contribute the fusion process via actin polymerization. Although what is a role of actin filaments in the fusion is still enigma, our finding provides important novel mechanistic insights into how PI regulates the cellular function.
4. How could the conclusion "These observations suggest that not only PI(3)P, but also other PIs, function in autophagy." be drawn from "For instance, PI(4,5)P2 is mainly localized to the plasma membrane, and is involved in trafficking in the endocytic pathway. PI(4)P is concentrated in the trans-Golgi network and recruits its effectors, such as oxysterol-binding protein, to control the secretory pathway (Di Paolo & De Camilli, 2006)." The connection between these observations and autophagy is not clear. Please explain or rephrase.

Thank you for pointing out the wrong description. We replaced the sentence “These observations suggest that not only PI(3)P, but also other PIs, function in autophagy.” to “Therefore, there is a possibility that other PIs than PI(3)P also function in other steps of autophagy.”
5. In Fig. EV3A-C, "siINPP5E significantly increased the number of autophagosomes labeled by LC3, as well as Atg5-labeled autophagosomes in these cells (Figs 1E, F and EV3A-C)." Why are Atg5 dots also increased, does that mean the phagophore number is also increased? In that case, the authors need to distinguish if INPP5E depletion causes accumulation of complete autophagosomes or phagophores?

Yes, the number of phagophore seems to increase. Since bafilomycin A1 which inhibits the fusion also increases Atg5 dots (see Fig below), we speculate that to compensate inhibition of fusion between autophagosome and lysosome cells enhance autophagosome biogenesis. However, accumulated autophagic vesicles in INPP5E-depleted cells are not only phagophores but also complete autophagosomes, which are detected by the proteinase K protection assay (see the answer to the next question).

![Non-treated vs Bafilomycin A1](image)

6. Again, the proteinase K protection assay doesn't support the authors' conclusion. If complete autophagosomes are accumulated, more p62 and LC3-II should be protected, which is opposite to the authors' observation in F2A.
We replaced the blotting using siINPP5E#1 with new one using siINPP5E#2 because the protected LC3-II form is more increased in siINPP5E#2-treated cells compared to siINPP5E#1-treated cells (please see figure below). Since both phagophores and complete autophagosomes increased in INPP5E-depleted cells, the ratio of the protected LC3-II/p62 to the total is similar to the control one.

7. To further support the conclusion that complete autophagosomes are accumulated in INPP5E depleted cells, the authors should present high quality EM results to visualize complete autophagosomes in INPP5E depleted cells to be more persuasive.
Thanks for the suggestion. We performed EM analysis accordingly and observed the significant number of autophagosomes, which seem to be completed in INPP5E-depleted cells. However, we should note that one cannot conclude the complete closure of autophagosomes from the observation of a single thin section. For this purpose, observation of serial sections or electron tomography of whole autophagosome is necessary, which is not easy and requires tremendous works.

8. In Fig. 5B, the quality of the images is not good enough to support the conclusion that mST-2xML1N dots are increased on lysosomes.

We have replaced the images with the better ones.

9. In Figure EV5, it will be more sensitive to use lysosensor instead of lysotracker to detect lysosome acidification defect. A FACS analysis here will be helpful to quantify this defect.

We thank the reviewer for this good suggestion. We have now replaced the Lysotracker data with the new result of lysosensor (please see figure below). There were no differences of the intensity ratio (Yellow / Blue) between control siRNA- and INPP5E siRNA-treated cells, demonstrating that INPP5E knockdown does not affect pH in lysosomes.
Referee #3:

The manuscript reports the intriguing observation that the 5-phosphatase INPP5E can control the autophagic flux. The authors suggest that this role of INPP5E is not dependent on its role in ciliogenesis and might involve instead the ability of INPP5E to control PI(3,5)P2 levels at lysosomes, possibly due to a direct catalytic action of INPP5E on PI(3,5)P2. While the effect of INPP5E depletion/mutation on the autophagic flux are well substantiated, the suggested mechanisms through which INPP5E exerts its effects on autophagy are not fully supported by the data of the manuscript in their present form. Thus there are key issues that should be resolved.

INPP5E & lysosomes

The authors show the colocalization of human mSt-INPP5E stably expressed in N1E-115 mouse cells. What about other cell types?

Thanks for the question. We have now added the data showing that mSt-INPP5E also localizes to lysosomes in MEFs under both nutrient-rich and starved conditions (please see below figure, Fig B).

What is the fraction of INPP5E that localizes to lysosomes? Is INPP5E stably associated with lysosomes, or does it associate to/dissociate from lysosomes only under specific conditions?

To answer the question, we tested effect of Torin1, an inducer of autophagy on association of INPP5E to lysosomes in N1E-115 cells, but there is no change (please
see Fig A below). INPP5E may constitutively localize to lysosomes, although there could be other condition(s) that alters the association.

*Does INPP5E localize to lysosomes in ciliated cells?*

As requested, we tested the localization of INPP5E in MEFs under ciliated conditions. INPP5E was localized at both lysosomes and cilia (please see figure, Fig B below, arrowhead; cilia).
The localization of the endogenous INPP5E protein should be examined in order to conclude that it localizes to lysosomes. There are published anti-INPP5E antibodies that work in immunofluorescence (such as those described in Humbert et al., PNAS 2012 and in Bielas et al., Nat Genet 2009) that could be used to immunolocalize INPP5E.

We thank the reviewer for the important suggestion. We have stained cells with the indicated antibodies, but unfortunately we failed to detect lysosomal localization of endogenous INPP5E. Since the phosphatase-dead mutant of INPP5E was associated with lysosome stronger than the wild type, the association is likely to be weak and transient.

INPP5E & PI(3,5)P2

1. While it is well established that PI(4,5)P2 and PI(3,4,5)P3 can be substrates for INPP5E, the evidence that INPP5E can act on PI(3,5)P2 is by far less solid. There is only one report showing that an immune-precipitate obtained with the anti-72kd phosphatase (i.e. INPP5E) contains 5-phosphatase activity towards PI(3,5)P2 (Kong AM et al. JBC, 2000). Thus, more solid evidence should be provided using the purified recombinant INPP5E before concluding that it has 5-phosphatase activity towards PI(3,5)P2.

According to the reviewer’s suggestion, we tried to purify the GST-tagged recombinant INPP5E (full-length and deletion mutants) from E coli., but we could not obtain the enough amount to measure the phosphatase activity. Therefore, instead of this we purified FLAG-tagged INPP5E (wild-type;WT and phosphatase inactive mutant;D477N) expressed in HEK293A cells by immunoprecipitation. By using them, we could successfully measure the phosphatase activity against
PI(3,5)P$_2$ and PI(4,5)P$_2$ *in vitro* using malachite green reagent which develops the green color if it detects free phosphoric acid. The malachite green assay is known to be standard method to measure the phosphatase activity (Maehama et al. Anal. Biochem. 2000). As a result, the phosphatase activity of INPP5E (WT), but not INPP5E (D477N), against both PI(3,5)P$_2$ and PI(4,5)P$_2$ was detected at similar level, suggesting that INPP5E acts as phosphatase of PI(3,5)P$_2$ (please see below figure).
2. It is not clear how the quantification of PI(3,5)P2 reported in Fig 5D was performed. Since the authors used the overexpressed PI(3,5)P2 probe to detect and measure PI(3,5)P2 and since the global level of the overexpressed probe could affect the amount of the probe associated with the lysosomes, a normalization of the lysosomal-associated probe vs. the total levels of the expressed probe should be performed.

We agree with the reviewer that a normalization of the lysosomal-associated probe vs. the total levels of the expressed probe should be performed. We have now replaced the data with new one normalized by cytosolic (total level) intensity of the probe. Consequently, we could not find the difference between the previous result (before normalization) and the new one (after normalization).

It would be also very informative to compare the increase in PI(3,5P)2 observed after INPP5E KD with that consequent to the inactivation of the 5-phosphatase acting of PI(3,5)P2, i.e. Sac3/Fig4.

As suggested by the reviewer, we performed the knockdown experiment using siRNA against Sac3, but Sac3 knockdown displayed no significant change of the PI(3,5)P2 level on lysosomes. It is possible that Sac3 does not play a role in regulation of lysosomal PI(3,5)P2.

In addition, the image in Fig. 5D shows a brighter staining not only with the PI(3,5)P2 probe but also with the LAMP1 antibody: does the depletion of INPP5E cause an increase in the number/size of lysosomes? Of course, a crucial question is whether the increase in the lysosomal levels of PI(3,5)P2 in INPP5E-depleted cells can be rescued by the wt INPP5E.
Yes, the depletion of INPP5E causes an increase in the number/size of lysosomes. Possibly, the elevation of PI(3,5)P$_2$ level and/or the reduction of PI(3)P level on lysosome affects the lysosomal biogenesis and activity. We think that this is an interesting phenomenon that should be studied in future.

As requested, we carried out rescue experiment to test if INPP5E (WT) expression could decrease in the levels of PI(3,5)P$_2$ on lysosomes in INPP5E-depleted cells. We have now added the data showing that the expression of INPP5E (WT), but not INPP5E (D477N), restored the intensity of ML1N (a PI(3,5)P$_2$ probe) in INPP5E-depleted cells (please see below figure).
3. What is the relationship between INPP5E and the 5-phosphatase acting of PI(3,5)P2, i.e. Vac4/Sac3? Can the overexpression of Sac3 compensate for the depletion of INPP5E and vice versa?

We overexpressed Sac3-GFP in INPP5E-depleted cells, but the accumulation of LC3 dots was not restored. As mentioned above, it is possible that Sac3 cannot affect PI(3,5)P2 level in lysosomes.

4. What is the relationship between INPP5E and the PI(3)P 5-kinase PIKfyve? Is the accumulation of PI(3,5)P2 (and the defect in autophagic flow) rescued by decreasing PI(3,5)P2?

We thank the reviewer for the insightful question. As requested, we tested the effect of double knockdown of PIKfyve and INPP5E on fusion process. Interestingly PI(3,5)P2 staining was almost abolished (see Fig A below), suggesting that effect of PIKfyve depletion is stronger and dominant to INPP5E depletion. In the double KD cells LC3 dots was accumulated, suggesting inhibition of autophagy after formation of autophagosomes (Fig B). This result consists with the previous study demonstrating that treatment of cells with PIKfyve inhibitor causes defect in autophagy, which is possibly due to suppression of degradation of autophagosomal contents in autolysosomes by loss of lysosomal activity (de Lartigue et al. Traffic. 2009). Therefore, we conclude that both excess and less PI(3,5)P2 level in lysosomes compromises autophagy at late stages (fusion or lysosomal degradation), i.e., the optimal level of lysosomal PI(3,5)P2 (or PI(3)P) is essential to the process.
5. Are the levels of lysosomal PI(3)P affected by the depletion of INPP5E.

Thanks for the constructive question. We have now added the data showing that lysosomal PI(3)P level is decreased in INPP5E-depleted cells (Please see Fig A and B below).
INPP5E & autophagy

The authors suggest that the role of INPP5E in autophagy is independent of its role in controlling ciliogenesis: they reach this conclusion by assessing that the depletion of INPP5E does not affect the cilia length in MEFs stably expressing ARL13. To substantiate their conclusion they should show that INPP5E depletion impairs the autophagic flux in the very same cell line and under the very same experimental conditions.

This appear particularly important since the MEFs in general (but what about the MEFs stably expressing ARL13 in particular?), as also the Authors notice (page 10), appear to be less sensitive to INPP5E depletion in terms of impairment of autophagic flux. From the comparison of Fig. 1C (N1E-115) and Fig.EV2B (MEFs) it is evident that, in the presence of serum, the autophagic flux is impaired in N1E-115 but not in MEFs knocked down for INPP5E. The MEFs apparently become sensitive to the lack of INPP5E only under starvation conditions (though the representative images of the western blot showing LC3 do not fully match with the quantitative data shown in the graph).
We thank the reviewer for pointing out this important issue. As suggested by the reviewer, we tried to examine the effect of INPP5E knockdown on ciliogenesis under ciliated condition in N1E-115 stably expressing Arl13b-GFP. However, we have not observed the ciliogenesis in these cells under ciliated condition (starved condition) even without INPP5E knockdown. Although this may imply that INPP5E can function in autophagosome-lysosome fusion without ciliogenesis, we could not exclude that ciliogenesis is related to the effect of INPP5E depletion on the fusion process at this moment. We have now toned down our description regarding the issue in the manuscript.

Finally, as INPP5E depletion can also induce complex transcriptional changes, as recently shown in Plotnikova et al. (JCS 2015), the authors should ascertain that the impaired autophagic flux is direct rather than a consequence of such transcriptional changes.

As requested, we examined the effect of INPP5E knockdown on the transcriptional levels of several autophagy-related factors and SNARE proteins involved in autophagy such as Syntaxin 17 and VAMP-8. INPP5E knockdown did not reduce the transcriptional level of the indicated factors (please see a figure below), suggesting that the effect on autophagic flux is direct rather than a consequence of transcriptional changes of the related proteins expression.
Thank you for submitting your revised manuscript for our consideration. Your manuscript has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are broadly in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address the remaining concerns of referee #1 and #3 by providing a point-by-point response to the issues raised.

Referee #1:

The authors have satisfactorily addressed my comments and concerns, except my comments to Figure 2A. The authors use subcellular fractionation and Proteinase K protection (of p62 and LC3) to conclude that autophagosomes accumulating in INPP5E depleted cells are properly closed, in contrast to those formed in siATG2 cells. This is nice, but they should i) indicate whether the siATG2 data are significantly different from siControl and siINPP5E cells and ii) blot for a membrane-bound protein that is not protected by Proteinase K.

Referee #2:

The authors have addressed my concerns adequately.

Referee #3:

The authors have addressed most of the criticisms raised in my previous review. However, I have one residual concern dealing with a crucial point: the formal proof that INPP5E is an actual PI(3,5)P2 5-phosphatase is still missing. In their reply the authors report data of a phosphatase assay run on an immunoprecipitate of cells transfected with wt or catalytically dead INPP5E. The problem I see is that the difference between the two conditions is tiny, as is the difference between cells transfected with empty vector and with INPP5E. I strongly encourage the authors to make an effort to obtain more convincing data on INPP5E as a PI(3,5)P2 5-phosphatase and to include these data in the manuscript.

Specific response:

(Reviewer’s comments are in italics.)

Referee #1:

The authors have satisfactorily addressed my comments and concerns, except my comments to Figure 2A. The authors use subcellular fractionation and Proteinase K protection (of p62 and LC3) to conclude that autophagosomes accumulating in INPP5E depleted cells are properly closed, in contrast to those formed in siATG2 cells. This is nice, but they should i) indicate whether the siATG2 data are significantly different from siControl and siINPP5E cells and ii) blot for a membrane-bound protein that is not protected by Proteinase K.

We thank positive comments of referee #1. To address the remaining concern, we added the data showing that siAtg2a/b knockdown data are significantly different from siControl and siINPP5E knockdown (Fig 2A).
Although we agree that it is better to include a membrane-bound protein that is not protected by Proteinase K as a control, we believe that both p62 and LC3 can be regarded as this kind of control, since they bind to both of outside and inside of autophagosomes and then the molecules outside should be degraded by Proteinase K. Indeed, they are partially degraded under Proteinase K treatment, whereas most of them are degraded by treatment of Proteinase K and Triton-X, indicating that our protection assay works well.

Referee #2:

The authors have addressed my concerns adequately. We are grateful to Referee #2 effort to evaluate our manuscript.

Referee #3:

The authors have addressed most of the criticisms raised in my previous review. However, I have one residual concern dealing with a crucial point: the formal proof that INPP5E is an actual PI(3,5)P2 5-phosphatase is still missing. In their reply the authors report data of a phosphatase assay run on an immunoprecipitate of cells transfected with wt or catalytically dead INPP5E. The problem I see is that the difference between the two conditions is tiny, as is the difference between cells transfected with empty vector and with INPP5E. I strongly encourage the authors to make an effort to obtain more convincing data on INPP5E as a PI(3,5)P2 5-phosphatase and to include these data in the manuscript.

We thank positive comment of referee #3. We totally agree with referee #3 that in the experiment showing PI(3,5)P2 5-phosphatase activity of INPP5E the difference between the two conditions is tiny, as is the difference between cells transfected with empty vector and with INPP5E. We have already made an effort to obtain stronger data by using the recombinant INPP5E made in E coli. However, unfortunately we failed to purify the enough amount of it to measure the enzymic activity by unknown reason. Then, we overexpressed and purified from HEK cells, and that is the current data (see below). We tried different conditions/protocols but we couldn't improve more than the current data. Although it is tiny, the present data shows the difference and it is statistically significant and standard deviation is very small so that I strongly believe that the data sufficiently, albeit not perfectly, support that INPP5E can act as a PI(3,5)P2 5-phosphatase. Therefore, we include this data in the second revision.

In addition, this conclusion is also supported by the results in Fig. 5 showing that siINPP5E caused both decrease of PI3P and increase of PI(3,5)P2 on lysosomes. This is not directly answering ref #3 but still is quite important data to show the role of INPP5E.
Editorial points:

A few editorial points need to be taken care of at this stage as well:
- please note that we can accommodate only up to five expanded view figures at the moment. Please select the most relevant ones as EV figures and move the remaining figures into an appendix file (see our author guidelines for appropriate call-outs to be used)

We changed EV figures to appendix figures.

- please check whether all figure files are of adequate resolution and quality for production, and upload improved versions if necessary.

We checked the resolution of each figure (300ppi).

- it would be nice if you could provide a simple model figure displaying how INPP5E acts to allow autophagosome-lysosome fusion. We will use this for the synopsis in the HTML version of your manuscript. For this, it would have to be arranged to fit best within the format restrictions of 550 pixels (width) x 150-400 pixels (height).

We uploaded our model of how INPP5E acts to allow autophagosome-lysosome fusion.

- as you might know, we encourage our authors to provide original source data, particularly uncropped/-processed electrophoretic blots for the main figures of your manuscript. If you would...
like to add source data, we would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.

We uploaded the source data files for main figures as each PDF file.

3rd Editorial Decision

Thank you for submitting your revised manuscript. I appreciate the point-by-point response to the remaining concerns and I am happy to accept your manuscript for publication in The EMBO Journal. Congratulations!
Reporting Checklist for Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal’s authorship guidelines in preparing your manuscript.

A- Figures

1. Data
   - The data shown in figures should satisfy the following conditions:
     - The data were obtained and processed according to the field’s best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
     - Figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
     - Graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
     - If n=5, the individual data points from each experiment should be plotted and any statistical test employed should be justified.

2. Captions
   - Each figure caption should contain the following information, for each panel where they are relevant:
     - A description of the experimental system investigated (e.g. cell line, species name).
     - The group(s) and method(s) used to carry out the reported observations and measurements.
     - An explicit mention of the biological and chemical entities(ies) that are being measured.
     - An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
     - The exact sample size (n) for each experimental group/condition, given as a number, not a range.
     - A description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
     - A statement of how many times the experiment shown was independently replicated in the laboratory.
     - Definitions of statistical methods and measures:
       - Common tests, such as t test (please specify whether paired vs. unpaired), simple β tests, Wilcoxon and Mann‐Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section.
       - Are tests one-sided or two-sided?
       - Are there adjustments for multiple comparisons?
       - Exact statistical test results, e.g., P value ≤ 0.05 but P value < 0.01.
       - Definition of “center values” as median or average;
       - Definition of error bars (e.g. s.d. or s.e.m).

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, magnetic resonance imaging studies, animal models and human subjects.

B- Statistics and general methods

<table>
<thead>
<tr>
<th>Question</th>
<th>Answer</th>
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<tr>
<td>1. a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?</td>
<td>NA</td>
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<tr>
<td>1. b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.</td>
<td>NA</td>
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<td>2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?</td>
<td>NA</td>
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<td>3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.</td>
<td>NA</td>
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<td>4. For animal studies, include a statement about randomization even if no randomization was used.</td>
<td>NA</td>
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<td>4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes, please describe.</td>
<td>NA</td>
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<tr>
<td>4.b. For animal studies, include a statement about blinding even if no blinding was done</td>
<td>NA</td>
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<td>5. For every figure, are statistical tests justified as appropriate?</td>
<td>Yes, (99% in 95% in figure legends)</td>
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<tr>
<td>Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess this.</td>
<td>Normal distribution. We used student’s t-test for statistical analysis.</td>
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<td>7. b. Have an estimate of variation within each group of data?</td>
<td>Yes</td>
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<tr>
<td>7. c. Is there variance either between the groups that are being statistiically compared?</td>
<td>Yes</td>
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C- Reagents
D- Animal Models

1. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail husbandry and the source of animals.

2. For experiments involving in vitro cultures, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

3. We recommend consulting the ARRIVE guidelines (PLoS Biol. 8(1), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH Office of Safety Assurance (OSA) guide for Biomedical Research and MRC, UK, guide to right recommendations. Please confirm compliance.

4. For experiments involving in vivo experiments, provide a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

5. Report any restrictions on the availability (and/or on the use) of human data or samples.

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., Antibodypedia (see link list at top right). When possible, standardised format (SBML, CellML) should be used instead of scripts (e.g., MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as BioModels (see link list at top right) or in an equivalent), where applicable.

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail husbandry and the source of animals.

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10. Report any restrictions on the availability (and/or on the use) of human data or samples.

11. Identify the committee(s) approving the study protocol.

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

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16. For experiments involving in vitro cultures, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

17. For publication of patient photos, include a statement confirming that consent to publish was obtained.

E- Human Subjects

1. Identify the committee(s) approving the study protocol.

2. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

3. For publication of patient photos, include a statement confirming that consent to publish was obtained.

4. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

5. Report any restrictions on the availability (and/or on the use) of human data or samples.

6. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

8. For experiments involving in vivo experiments, provide a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

9. Report any restrictions on the availability (and/or on the use) of human data or samples.

10. Identify the committee(s) approving the study protocol.

11. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

14. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

F- Data Accessibility

16. Provide accession codes for deposited data. See author guidelines, under 'Data Depositor'.

17. Data deposition in a public repository is mandatory for:

a. Protein, DNA and RNA sequences
b. Macromolecular structures
c. Crystallographic data for small molecules
d. Functional genomics data

18. For experiments involving in vitro cultures, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

19. We recommend consulting the ARRIVE guidelines (PLoS Biol. 8(1), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH Office of Safety Assurance (OSA) guide for Biomedical Research and MRC, UK, guide to right recommendations. Please confirm compliance.

20. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

21. Report any restrictions on the availability (and/or on the use) of human data or samples.

22. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

23. For experiments involving in vitro cultures, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

24. We recommend consulting the ARRIVE guidelines (PLoS Biol. 8(1), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH Office of Safety Assurance (OSA) guide for Biomedical Research and MRC, UK, guide to right recommendations. Please confirm compliance.

25. Report any restrictions on the availability (and/or on the use) of human data or samples.

26. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

27. For experiments involving in vivo experiments, provide a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the NIH Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

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G- Dual use research of concern

26. Could your study fall under dual use research restrictions? Please check biosecurity guidelines, under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right).

27. According to our biosecurity guidelines, provide a statement only if it could.