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Mammalian Atg8 proteins regulate lysosome and autolysosome biogenesis through SNAREs

Yuexi Gu, Yakubu Princely Abudu, Suresh Kumar, Bhawana Bissa, Seong Won Choi, Jingyue Jia, Michael Lazarou, Eeva-Liisa Eskelinen, Terje Johansen and Vojo Deretic.

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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 3rd May 2019

Thank you for submitting your manuscript entitled "Mammalian Atg8 proteins regulate lysosome and autolysosome biogenesis through SNAREs" to The EMBO Journal. Please accept my apologies for the lengthy review process due to the delayed delivery of one report. Your study has been sent to three referees for evaluation, and their reports are enclosed below for your information.

As you can see, the referees consider the work potentially interesting. However, they also raise several key points that need to be addressed before they can support publication in The EMBO Journal. In particular, referee #1 requests you to 1. identify Syntaxin 16 (STX16) partners in control and serum-starved conditions, 2. test the effect of STX16 knockout on Golgi, TGN and TGN to lysosome transport, and 3. rescue STX16 phenotype using a LIR-dead mutant. Furthermore, referee #2 and #3 are concerned that the results are mainly descriptive and ask you to provide mechanistic insight into the role of STX16 in autophagy, lysosomal biogenesis and mTOR signaling.

Given the overall interest of your study, I would like to invite you to submit a revised version that addresses the above-mentioned points. Note that solving these issues as suggested by the referees is essential to warrant publication of your manuscript in The EMBO Journal.

REFEREE REPORTS

Referee #1:

-In this article, the authors identified several SNAREs as interactors of Atg8 proteins and found the LC3-interacting regions (LIR) using peptide arrays and GST-GABARAP. They found only one R-/v-SNARE: VAMP7 and several Q-/t-SNAREs including GOS1 (which was already known to

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interact with GABARAP), Syntaxin 17 (which has an already known function in autophagy and had been connected to GABARAP via BRUCE), vti1a (which had previously been involved in autophagy), and several syntaxins with no known function in autophagy: 3, 4, 16, 19. This seminal finding is clearly very important in both the fields of autophagy and membrane trafficking. The authors then went on to characterize in detail the function of syntaxin 16, which had been previously involved mainly in retrograde transport to the TGN. They found that autophagic flux is blocked in the absence of both syntaxins 16 and 17, that both are required for several autophagic mechanisms (mitophagy, pexophagy, xenophagy), and that they cooperate in ribophagy. They then focused on syntaxin 16 and found a defect in lysosomal homeostasis, distribution of acidic compartments in the absence of syntaxin 16 and defect in syntaxin 16 localization in Atg8s-KO cells.

This is an important study with rigorous experiments and clear data. The results are fairly discussed.

-There are 3 important outstanding issues that should be easily addressed:
1/what are the partners of Syntaxin 16 in control and starved conditions? answering this by co-immunoprecipitation experiments would be important to understand the role of Syntaxin 16 at the molecular level and position it in the context of membrane fusion reactions
2/what is the effect on the Golgi and TGN, and on TGN to lysosome transport of Syntaxin 16 KO in control and starved conditions? Data in figure 6 unfortunately do not allow to see if TGN46 is or not affected and the EM of figure 2 could also be used to show the status of the Golgi in KO cells. Characterizing the transport of mannose 6-phosphate receptors would be here required to make the point of the authors very clear.
3/what is the result of rescuing syntaxin 16 using a LIR-dead mutant? this data is also required to demonstrate the functional relevance of the novel interaction unraveled here.

Referee #2:

Summary
In this manuscript, Gu et al. examine the relationship of the autophagic LC3/GABARAP ubiquitin-like modifiers and SNARE proteins. Intrigued by their previous observation that the SNARE STX17 binds to LC3/GABARAP proteins, the authors used bioinformatics and peptide arrays to unbiasedly screen for other LC3/GABARAP-binding SNARE family members and identified LIR motifs in the trimeric binding partners STX16, Vti1a and STX6. Biochemical studies confirmed that at least STX16 binds some members of the LC3/GABARAP family in a LIR dependent manner. Next, the authors switched to explore the cellular consequences of STX16 knockout (KO). While deletion of STX16 alone had no overt autophagic phenotype, STX16 KO cells additionally lacking STX17 showed reduced bulk autophagy flux, accumulated immature autophagosome and impaired selective autophagy pathways (i.e. mitophagy, pexophagy, xenophagy and ribophagy). In addition to these changes in the autophagy pathway, the authors observed reduced levels of the lysosomal proteins LAMP1 and LAMP2 in fed conditions and decreased mTOR phosphorylation and altered subcellular distribution of LAMP2 and mTOR in response to starvation in STX16 KO cells. Lastly, the authors showed that LC3/GABARAP proteins are required for the colocalization of STX16 with late endosomes and lysosomes. Together, Gu and colleagues expand the repertoire of SNAREs involved in autophagy. However, the work is mainly descriptive and lack any major mechanistic new insights. Moreover, there are several critical concerns that need to be addressed.

Major points
1) There is no experimental evidence that the interaction between LC3/GABARAP proteins and STX16 is actually required for the observed defective bulk and selective autophagosomal and lysosomal phenotypes. The authors should perform rescue experiments with wild-type and LIR deficient STX16 variants in the context of the main findings in Figure 2, 5 and 6 (e.g. Figure 2C, 5E, 5F, 5G and 6C).

2) What is about the LIR candidates in the STX16 binding partners Vti1 and STX6? Do the three SNARE proteins compete for LC3/GABRAP binding? Does LC3/GABARAP binds STX16 in complex with Vti1 and STX6? What happens to Vti1 and STX6 in STX16 KO cells and in STX16 KO cells reconstituted with a LIR deficient STX16 variant? What is the LC3/GABARAP binding preference of Vti1 and STX6? Does this SNARE complex bridges two different LC3/GABARAP

family members? If so, are these LC3/GABARAP molecules associated with different membranes? Does STX16 (and its SNARE partner) preferentially binds to lipidated LC3/GABARAPs on open or mature autophagosomes? Lastly, the authors should show that the STX16-LC3/GABARAP interaction is persevered at endogenous levels.

3) How does autophagosomal SNARE YKT6 fit into the authors' picture? The authors should at least revisit some of their autophagy assays with cells deleted for STX16 and YKT6.

4) The connection between the defective autophagic pathways and the alterations in the endosomal-lysosomal system is not clear. Are these phenotypes arising independently and in parallel or is one causative of the other? More precisely, are the autophagy defects arising from a prime function of STX16 in facilitating the traffic of lysosomal proteins?

5) Does STX16 actually binds VPS41? Or does the interaction of Vti1 and STX6 with VPS41 occurs in the absence of STX16? Does Vti1 and STX6 bind to the HOPS complex or only to VPS41?

6) Figure 7: Are the phenotypes (in Figure 7A-C) dependent on the LIR docking site (LDS) of LC3/GABARAP proteins? The authors should perform rescue experiments in these settings (Figure 7A-C) with LDS mutants in GAPARAP or LC3C (the seemingly preferred ATG8 binding partners of STX16).

Minor points
7) Please add missing molecular weight markers in Figure 1
8) Please explain what "SM proteins" are (page 5).
9) Please delete the "etc" on page 3 or explain more specifically.

Referee #3:

In the current manuscript, Gu et al dissects the role of SNAREs in autophagy. The authors show that Stx16 and its cognate SNAREs Stx6 and Vti1A bind to specific Atg8-class (LC3 and GABARAP) proteins via LIR-like motifs. Moreover they show that co-deletion of Stx16 with Stx17, previously implicated by the Mizushima group in autophagosome-lysosome fusion, results in impaired autophagic degradation of mitochondria, peroxisomes and intracellular pathogens.
They then show that Stx16 plays a role in regulating lysosomal vesicle biogenesis and mTORC1 signaling, and conclude by providing evidence that Stx16 is recruited to endo-lysosomal compartments in an Atg8-dependent manner.
One problem with the paper is that there seems to be some conceptual confusion between a role of Stx16 in physical association with autophagosomes, and another upstream of it where Stx16 is required for both lysosomal biogenesis and mTOR signaling. Does Stx16 work in concert and redundantly with Stx17 in promoting autophagosome-lysosome fusion? Or does it play a completely distinct role? If so, why would the two synergize in (mildly) compromising autophagic flux?

1. The accumulation of Keima-labeled ribosomes (Fig. 4C-4F) in Stx16/17 DKO cells is interpreted as evidence for defective autophagosome maturation. However, this assay does not allow one to precisely pinpoint at which stage autophagy has arrested. Is autophagosome-lysosome fusion compromised, as shown in Stx17-deleted HeLa cells by Itakura et al (2012)? Assays used in the Mizushima paper, particularly electron microscopy, should be used to better document the autophagy defect due to Stx16/Stx17 loss.

2. The data connecting Stx16 to mTOR make little sense. On the basis of abundant literature, after 6h of continuous starvation mTORC1 should be completely cytoplasmic and thoroughly inactivated. Thus, it is not clear why in Fig. 5G mTOR seems to remain strongly punctate and why significant mTOR signaling remains in Fig. 5F. Given this, the reduction of mTOR puncta seen upon Stx16 deletion is also difficult to interpret.

3. Conceptually, it is difficult to separate the effects of Stx16 on mTORC1 activation (which should promote autophagy) from overall loss of lysosomal compartments (which should compromise autophagy). Which one is more important? Can the authorsw provide experiments to separate the

two effects?

4. Regarding Stx16 localization, the low-mag imaging data in Fig. 7 do not allow one to make any conclusions about where Stx16 resides. Evidence of Stx16 localization to LE/Lys should be gathered via immunofluorescence, live cell microscopy and/or immuno-EM.

5. Related to the previous point, if Atg8 proteins are required to recruit Stx16 to LE/Lys (presumably to enable its autophagy-related functions) how come that the 6X Atg8-deleted cells have no defects in LAMP2 vesicle number? Shouldn't they phenocopy the Stx16-deleted cells?

6. Given the previous points, it is unclear what the significance of the Stx16 LIR motif is. At what stage does Stx16 become recruited to LC3-containing autophagosomes, if ever? Does a LIR-deleted Stx16 rescue the autophagic flux, lysosome biogenesis and/or mTOR signaling defects of Stx16-deleted cells?

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POINT-FOR_POINT RESPONSE EMBO-2019-101994

Mammalian Atg8 proteins regulate lysosome and autolysosome biogenesis through SNAREs

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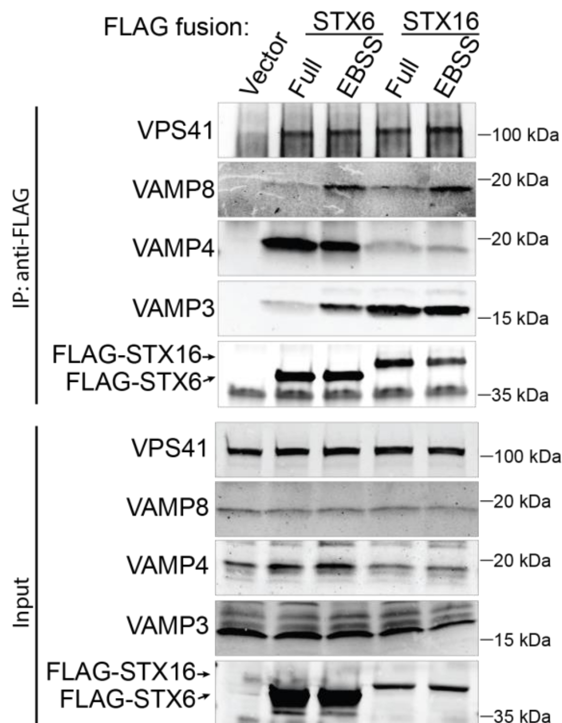
1/what are the partners of Syntaxin 16 in control and starved conditions? answering this by co-immunoprecipitation experiments would be important to understand the role of Syntaxin 16 at the molecular level and position it in the context of membrane fusion reactions.

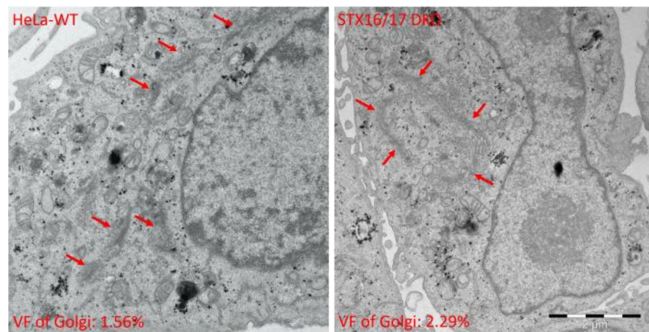


New Data 1, Fig. EV4C. Stx16 and its cognate SNARE Stx6 interact with R-SNAREs VAMP3, VAMP4 and VAMP8.

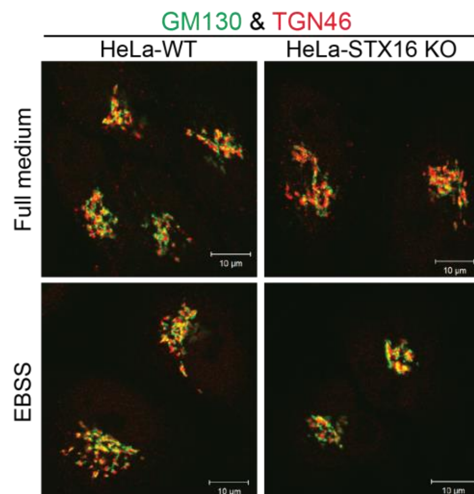
***We thank the reviewer for the suggestion to test the partner SNAREs of Stx16. As requested by the reviewer, we carried out Co-IP studies (New Data 1; Fig. EV4C. and found that Stx16 interacts with VAMP3 and VAMP4, as expected based on the known literature. VAMP3 and VAMP4 act as Stx16 cognate R-SNAREs during retrograde transport from endosomes to TGN (Mallard et al, 2002; Ganley et al, 2008), Importantly, upon starvation another R-SNARE, VAMP8, increases its presence in Stx16 SNARE complexes. The lysosomal SNARE VAMP8 (Jahn and Scheller, 2006) has not been previously implicated in Stx16-dependent trafficking, and represents a new relationship observed only under starvation conditions. This is consistent with our model that the Stx16 SNARE complex plays a role in

anterograde trafficking (as evidenced by LAMP1/2 reduction in *STX16* KO, *VTI1A* KO, and *STX6* KO cells) to the lysosomes. Similarly, Stx6, a Qc-SNARE working together with Stx16, showed increased interactions with VAMP8 in starved cells (New Data 1). We also noticed an increased Stx6-VAMP3 interaction in starved cells, and interpret this as overall increase in trafficking between TGN and lysosomal/endosomal pathway. These results (as well as data with knockouts of *STX6* and *VTI1A*[1]) are now described in the text, p. 10 bottom, new subsection: *"We found that Stx16 interacts with VAMP3 and VAMP4 (Fig. EV4C), consistent with the known literature regarding VAMP3 and VAMP4 as cognate R-SNAREs during retrograde transport from endosomes to TGN* (Ganley et al., 2008; Mallard et al., 2002)*. However, upon starvation another R-SNARE, VAMP8, increased its presence in Stx16 complexes (Fig. EV4C). The lysosomal SNARE VAMP8* (Jahn and Scheller, 2006) *has not been previously implicated in Stx16-dependent trafficking, and our findings suggest a new relationship detected only under starvation conditions. Similarly, Stx6, a Qc-SNARE working together with Stx16, showed increased interactions with VAMP8 in starved cells (Fig. EV4C). Thus, similar relationship to VAMP8 was observed with both Stx16 and Stx6. We also noticed increased Stx6-VAMP3 interactions in starved cells (Fig. EV4C), and interpret this as an overall increase in trafficking between TGN and the lysosomal/endosomal pathway".*

2/what is the effect on the Golgi and TGN, and on TGN to lysosome transport of Syntaxin 16 KO in control and starved conditions? Data in figure 6 unfortunately do not allow to see if TGN46 is or not affected and the EM of figure 2 could also be used to show the status of the Golgi in KO cells. Characterizing the transport of mannose 6-phosphate receptors would be here required to make the point of the authors very clear.



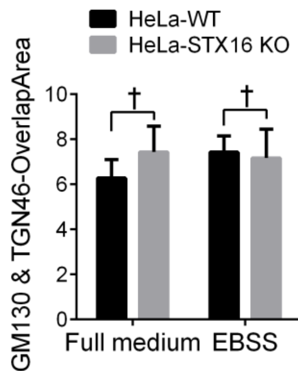
New Data 2, Fig. 6E. Morphology of Golgi does not change in *STX16/STX17* DKO cells; but the volume fraction is increased in the DKO cells.



New Data 3, Fig. EV5A. Confocal images showing morphologically similar overlap between GM130 and TGN46 in WT and *STX16*-KO HeLa cells.

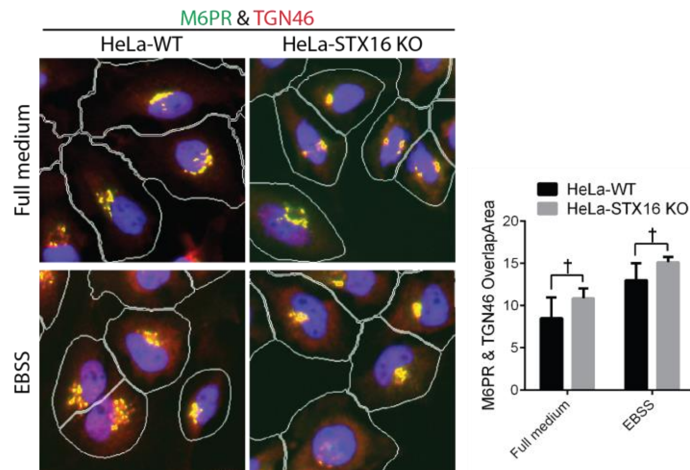
***As suggested by the reviewer regarding the status of the Golgi and TGN in KO cells, we carried out a number of analyses and new experiments: (A) As recommended by the reviewer, we re-examined the EM images already acquired (used for analysis of AVi and AVd profiles in Figure 2E) and found: 1) that the morphology of the Golgi stacks looks similar in WT and mutant cells; and 2) an increase in the size (volume fraction) of the Golgi compartment in mutant cells (1.6% in WT vs 2.3% in *STX16/STX17* DKO cells) (New Data 2, Fig. 6E). (B) We used GM130 to test whether the relationship of TGN46 has changed relative to this Golgi marker. We found by confocal microscopy (New Data 3, Fig. EV5A) and quantification by high content microscopy (HCM) (New Data 4, Fig. EV5B) that Golgi/TGN46 was not perturbed. In sum, all sets of data (ultrastructural, confocal microscopy and HCM) indicate that the Golgi structure is

[1] We also generated CRISPR knockouts in Vti1a and Stx6, and found reduced levels of LAMP2 in *VTI1A*^{KO} and *STX6*^{KO} HeLa cells (New Data 13, on p. 8 of this rebuttal. Fig. 5F, described on p. 10 of the text, section "Stx16 plays a role in lysosomal biogenesis"). Thus, Stx16 and its cognate Qb- and Qc- SNAREs affect cellular LAMP levels.



New Data 4, Fig. EV5B. HCM quantification of the GM130 and TGN46 overlap in WT and STX16-KO HeLa cells. Note: no differences.

not substantially changed. We describe this in the text on p. 12: *"To ensure that the Golgi apparatus was not perturbed, we re-examined the ultrastructural images in Fig. 2E, and found that the overall morphology of the Golgi stacks was similar in WT and mutant cells, but that the volume fraction of the Golgi compartment was increased (Fig. 6E). Furthermore, TGN46 did not show major changes in distribution relative to the Golgi marker GM130 (Fig. EV5A and B)."* (C) As requested, we checked M6PR distribution relative to TGN46, and using HCM quantifications did not detect any major differences (New Data 5, below). We now include the new M6PR localization analyses (Fig. EV5C and D), on p. 12: *"TGN46 did not show major changes in distribution relative to … and another factor in TGN-endolysosomal trafficking, mannose 6-phosphate receptor/M6PR (Fig. EV5C and D)."* We believe that M6PR transport is beyond the scope of our present study, since different lysosomal constituents may traffic through a variety of pathways (Luzio et al., 2014; Saftig and Klumperman, 2009). We hope that this addresses satisfactorily reviewer's concerns.



New Data 5, Fig. EV5C and D. HCM analysis of the overlap between M6PR and TGN46 in WT or STX16-KO HeLa cells. Note: no differences.

3/what is the result of rescuing syntaxin 16 using a LIR-dead mutant? this data is also required to demonstrate the functional relevance of the novel interaction unraveled here.

***As requested, we have complemented *STX16/STX17* DKO cells with WT FLAG-STX16, and this recovered the flux defect (New Data 6; Fig. EV2E; next page). However, due to the small size effects (statistical term) we could not employ this assay to study the contribution of the LIR domain. Instead, we tested the effects of Stx16's LIR on the ability to complement LAMP2 defect, and found that LIR matters (New Data 7, next page; Appendix Fig. S1A and B; described on p. 10). In addition, we also came up with an alternative to test the role of Stx16 LIRs by examining whether absence of mAtg8s (which would be equivalent at some level to the absence of LIRs) affects Stx16-Vti1a-Stx6 complexes. This is shown in New Data 9, for Fig. EV6D, presented on p. 5 of this rebuttal in response to Reviewer 2, Point 2. We hope that these multiple assays will satisfy the reviewer.

Referee #2:

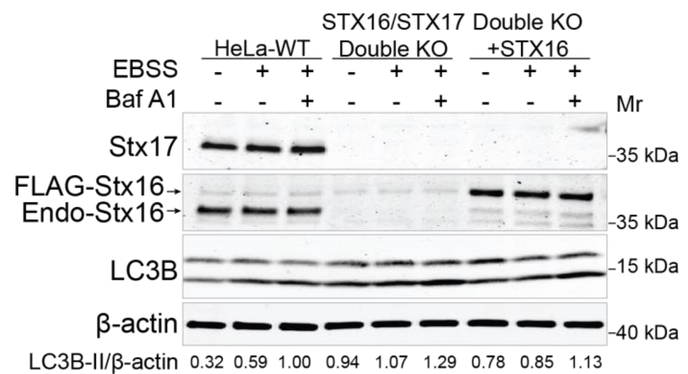
Summary

In this manuscript, Gu et al. examine the relationship of the autophagic LC3/GABARAP ubiquitin-like modifiers and SNARE proteins. Intrigued by their previous observation that the SNARE STX17 binds to LC3/GABARAP proteins, the authors used bioinformatics and peptide arrays to unbiasedly screen for other LC3/GABARAP-binding SNARE family members and identified LIR motifs in the trimeric binding partners STX16, Vti1a and STX6. Biochemical studies confirmed that at least STX16 binds some members of the LC3/GABARAP family in a LIR dependent manner. Next, the authors switched to explore the cellular consequences of STX16 knockout (KO). While deletion of STX16 alone had no overt autophagic phenotype, STX16 KO cells additionally lacking STX17 showed reduced bulk autophagy flux, accumulated immature autophagosome and impaired selective autophagy pathways (i.e. mitophagy, pexophagy, xenophagy and ribophagy). In addition to these changes in the autophagy pathway, the authors observed reduced levels of the lysosomal proteins LAMP1 and LAMP2 in fed conditions and decreased mTOR phosphorylation and altered subcellular distribution of LAMP2 and mTOR in response to starvation in STX16 KO cells. Lastly, the authors showed that LC3/GABARAP proteins are required for the colocalization of STX16 with late endosomes and lysosomes. Together, Gu and colleagues expand the repertoire of SNAREs involved in autophagy. However, the work is mainly descriptive and lack any major mechanistic new insights. Moreover, there are several critical concerns that need to be addressed.

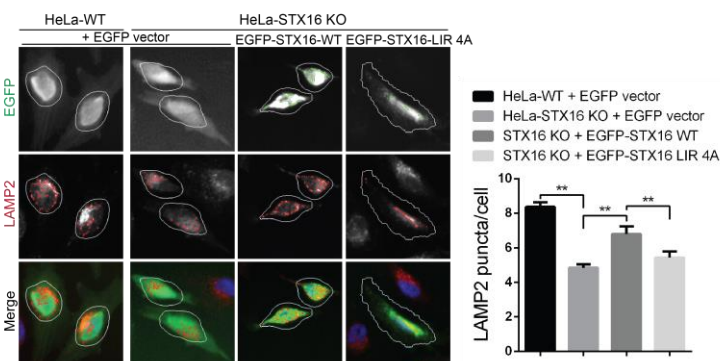
Major points

1) There is no experimental evidence that the interaction between LC3/GABARAP proteins and STX16 is actually required for the observed defective bulk and selective autophagosomal and lysosomal phenotypes. The authors should perform rescue experiments with wild-type and LIR deficient STX16 variants in the context of the main findings in Figure 2, 5 and 6 (e.g. Figure 2C, 5E, 5F, 5G and 6C).

*** As requested, we have complemented *STX16/STX17*^{DKO} cells with WT FLAG-Stx16, and this recovered the flux defect (New Data 6, Fig. EV2E; described on p. 7). We also complemented the requested LAMP2 defect using HCM with LAMP2 puncta (a more robust assay) (New Data 7, Appendix Fig. S1A and B). Furthermore, a LIR mutant of Stx16 had diminished complementation effect relative to WT (New Data 7, Appendix Fig. S1A and B; described on p. 10).



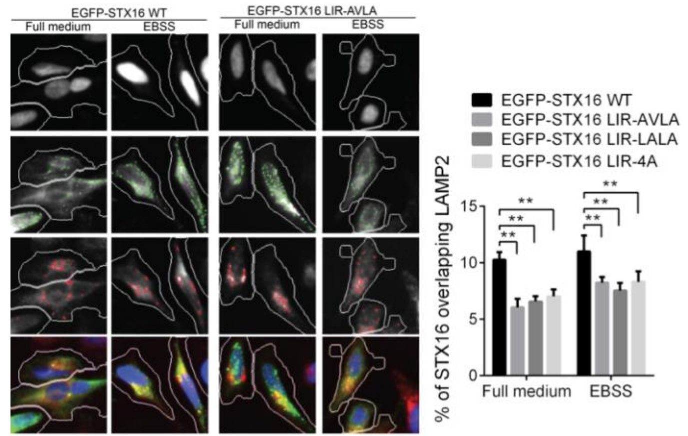
New Data 6, Fig. EV2E. Complementation of FLAG-Stx16 WT in *STX16/STX17*^{DKO} HeLa cells restores the LC3 flux.



New Data 7, Appendix Fig. S1A and B. Effects of overexpression of WT or LIR-mutant EGFP-tagged STX16 in HeLa-STX16 KO cells on LAMP2 puncta.

Due to limitations of the effect size, discerning differences of WT vs LIR mutants is difficult in all requested

figures. Importantly and related to this, as already shown in the initial submission, absence of all 6 mAtg8s does not alter LAMP phenotype under normal conditions. Conversely, *STX16* KO does not have an effect on LysoTracker staining, whereas mAtg8s do. The only phenotype where mAtg8s matter regarding Stx16 is Stx16 localization. Thus, we compared STX16 WT vs. STX16 LIR mutants (STX16 LIR-AVLA, LIR-LALA, LIR-4A) for their localization with LAMP2 (New Data 8, Fig. EV6B and C; described on p. 12). These data show clear, statistically significant reduction in LIR mutant Stx16 localization to LAMP2 profiles relative to WT Stx16. In addition, we are thankful to the reviewer for the idea expressed in question 2, which we believe can be used as a different way of testing whether absence of mAtg8s (a surrogate for the absence of LIRs in this context) affects Stx16-Vti1a-Stx6 complexes: the interactions between the components of this complex are reduced in mAtg8 mutant cells (New Data 9, Fig. EV6D). We hope this is satisfactory[2].

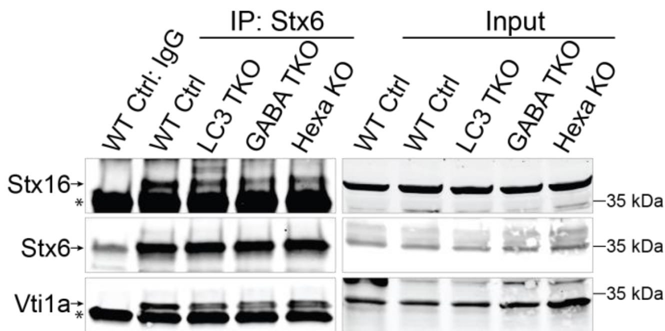


New Data 8, Fig. EV6B and C. HCM analysis of overlaps between WT or LIR-mutant EGFP-STX16 and LAMP2. Note: all the 3 types of LIR-mutant of STX16 had reduced co-localizations with LAMP2 compared to WT STX16.

2) The reviewer's point #2 contains multiple questions that we tried to address in 4 subsets (a-d) as follows:

(a) What is about the LIR candidates in the STX16 binding partners Vti1 and STX6? Do the three SNARE proteins compete for LC3/GABRAP binding? Does LC3/GABARAP binds STX16 in complex with Vti1 and STX6? What happens to Vti1 and STX6 in STX16 KO cells and in STX16 KO cells reconstituted with a LIR deficient STX16 variant?

***As suggested, we have carried out additional experiments. We focused on the aspect of what happens with the Stx16-Vti1a-Stx6 complex in mAtg8 mutants, which we think addresses most of the above questions collectively. New data generated in response to reviewer's questions show reduced Stx16-Vti1a-Stx6 complexes in LC3 TKO (knockout for LC3A,B,C), GABARAP TKO (knockout for GABARAP, -L1, -L2) and Hexa KO (knockout of all 6 mATG8s) cells. We show this in New Data 9, Fig. EV6D and discuss on p. 13.



New Data 9, Fig. EV6D. Co-IP analysis of interactions between components of the Stx16 SNARE complex. Note: reduced amounts of Stx16 co-IPed with Stx6 in GABARAP TKO and Hexa KO cells.

(b) What is the LC3/GABARAP binding preference of Vti1 and STX6? Does this SNARE complex bridges two different LC3/GABARAP family members? If so, are these LC3/GABARAP molecules associated with different membranes?

[2] We hope that the reviewer agrees that complementing a key subset of phenotypes is sufficient.

***As shown above in the new experiments under (a) (New Data 9, Fig. EV6D), triple LC3 or triple GABARAP KO cells have similar phenotypes in terms of SNARE complexes, thus suggesting that there is no preference with respect to the efficiency of SNARE complex formation. This is discussed on p. 13.

(c) Does STX16 (and its SNARE partner) preferentially binds to lipidated LC3/GABARAPs on open or mature autophagosomes?
***We apologize for not being clear on this – that Stx16 is acting on TGN-Lysosome organelles not on autophagosomes. To clarify this, we now provide a summary model (new Fig. 7F) and further emphasize this in the text on p. 14: "*Our findings with Stx16 indicate a function for mAtg8s in the maintenance of lysosomal compartments and in autolysosome biogenesis (Fig. 7F)*".

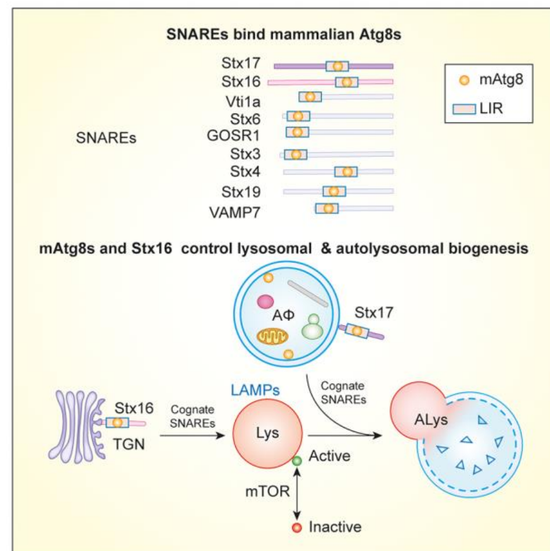
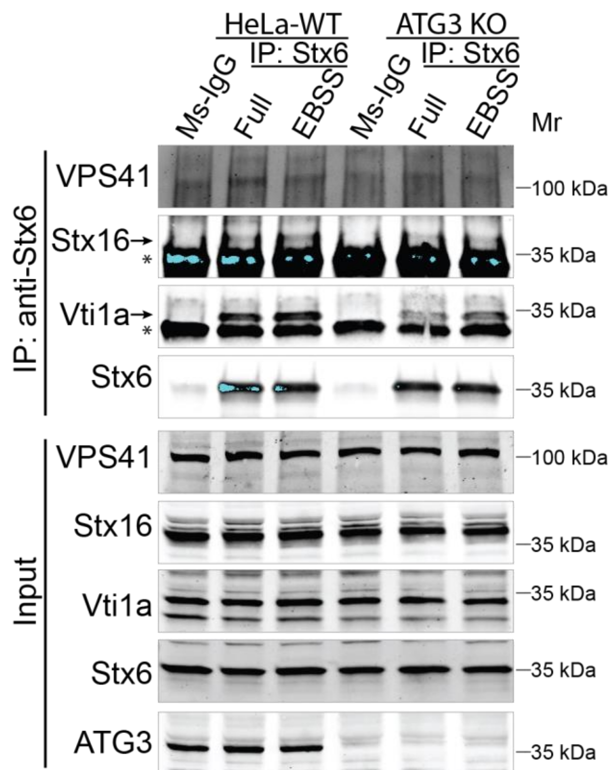


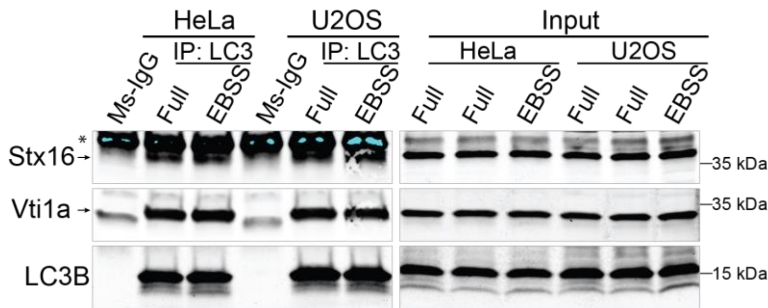
Fig. 7F, overall model of this study. Under autophagy-inducing conditions, Stx16 SNARE complex controls lysosome biogenesis, which in turn regulates mTOR localization and activity.



New Data 10, Fig. EV6E. ATG3 knockout reduces the stability of the Stx16/Vti1a/Stx6 SNARE complex.

Nevertheless, we tested whether there is a change in mAtg8 binding regarding their lipidation state using an ATG3 knockout generated specifically to answer this reviewer's important question. We found that the interactions between Stx16/Vti1a/Stx6 SNARE proteins were reduced in ATG3-KO cells, suggesting again that lipidation of mAtg8s may play a role in regulating these SNARE complexes. This can be interpreted as either that mAtg8s act on these SNAREs when on membranes, and alternatively may suggest a new role for mAtg8s lipidation in regulating SNARE functions. Of course, this opens interesting questions that are beyond the scope of this work (it was not meant to study effects of lipidation, which may be a topic of future investigations). This result is shown in New Data 10, Fig. EV6E, and discussed on p. 13.

(d) Lastly, the authors should show that the STX16-LC3/GABARAP interaction is persevered at endogenous levels.
***As requested, we provide new data with endogenous Stx16 and LC3 interactions. Moreover, we have performed these in two different cells (HeLa and U2OS), and also show this for Vti1a (New Data 11, shown on the next page, Fig. EV1B and discussed on p. 6).



New Data 11, Fig. EV1B. Co-IP analysis of the interaction between LC3 and Stx16/Vti1a at the endogenous level.

3) How does autophagosomal SNARE YKT6 fit into the authors' picture? The authors should at least revisit some of their autophagy assays with cells deleted for STX16 and YKT6.

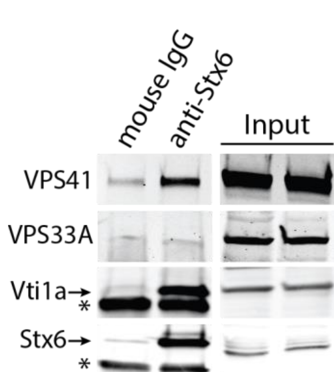
***We thank the reviewer for bringing this up. According to Professor Mizushima's JCB paper on Ykt6, cells couldn't survive attempts to knockout Ykt6. Consequently, we could not generate Ykt6 deletion that the reviewer requested. We have already discussed Ykt6's role in autolysosome formation and referenced relevant papers and pointed out a lesser known fact that Ykt6 also functions in one of the "*retrograde trafficking routes from endosomes to TGN that includes GOS-28/GOSR1 (Tai et al., 2004), an mAtg8-binding SNARE*". We have slightly modified and rearranged the text (p. 16) to emphasize this point.

4) The connection between the defective autophagic pathways and the alterations in the endosomal-lysosomal system is not clear. Are these phenotypes arising independently and in parallel or is one causative of the other? More precisely, are the autophagy defects arising from a prime function of STX16 in facilitating the traffic of lysosomal proteins?

*** The answer is yes. The reviewer is correct. We now clarify this and apologize for not being more explicit concerning the fact that Stx16 acts on the TGN-Lysosome organelles and not on autophagosomes. To clarify this, we now provide a summary model (Fig. 7F, shown on p. 6 of this rebuttal) and further emphasize this in the text (p. 14).

5) Does STX16 actually binds VPS41? Or does the interaction of Vti1 and STX6 with VPS41 occurs in the absence of STX16? Does Vti1 and STX6 bind to the HOPS complex or only to VPS41?

***Pulldowns with FLAG-tagged Stx6 or Stx16 show their interaction with Vps41, which was slightly increased upon starvation (please see New Data 1 on p. 1 of this rebuttal; Fig. EV4C). In addition, we compared interactions of VPS41 and a HOPS component, VPS33A, with the Stx16 SNARE complex (represented by Stx6 and Vti1a) by Co-IPs of endogenous Stx6. The data show interactions between VPS41 and the Stx6 complex. Stx6 pulldowns did not show significant levels of VPS33A (Modified Data 12, Appendix Fig. S1C). Thus, we conclude that it is predominantly the VPS41 component that is involved here, similarly to the findings by Klumperman and colleagues (Pols et al, 2013) who reported that VPS41 functions in delivery of lysosomal proteins independently of the HOPS complex as defined in other trafficking processes. This was already discussed in the manuscript, and now further emphasized on p. 10.



Modified Data 12, Appendix Fig. S1C. Endogenous Co-IP analysis of precipitated Stx6 showed the interaction between Stx6 and VPS41, but not VPS33A.

6) Figure 7: Are the phenotypes (in Figure 7A-C) dependent on the LIR docking site (LDS) of LC3/GABARAP proteins? The authors should perform rescue experiments in these settings (Figure 7A-C) with LDS mutants in GAPARAP or LC3C (the seemingly preferred ATG8 binding partners of STX16).

***Triple KO and Hexa KO mutants have multiple mAtg8s deleted, so complementation is technically not feasible since we do not know which of the 3 GABARAPs is responsible in this context. If we understand correctly, the reviewer suggests to perform "complementation" experiments using WT and LDS (LIR docking sites) mutants of GABARAP and LC3C to rescue Stx16 trafficking. Instead, we carried out experiments with LIR mutant Stx16 to check its localization and found that the LIR-mutant STX16 (LIR-AVLA, LIR-LALA and LIR-4A) all had reduced colocalizations with lysosomes (New Data 8, for reviewer's question 1 on p. 5 of this rebuttal, Fig. EV6B and C). We hope that this is acceptable.

Minor points
7) Please add missing molecular weight markers in Figure 1
***We now have added molecular weight markers missing in the figures as requested.

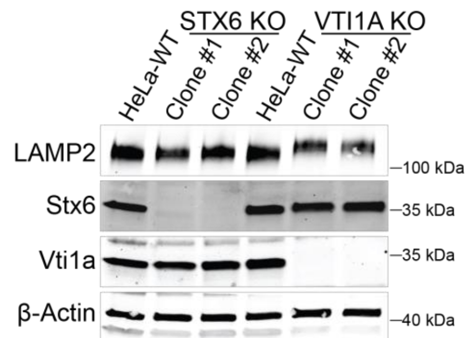
8) Please explain what "SM proteins" are (page 5).
***We now define the SM acronym, Sec1/Munc18, as requested in the text.

9) Please delete the "etc" on page 3 or explain more specifically.
***The word "etc" is now deleted as requested; Thank you!

Referee #3:

In the current manuscript, Gu et al dissects the role of SNAREs in autophagy. The authors show that Stx16 and its cognate SNAREs Stx6 and Vti1A bind to specific Atg8-class (LC3 and GABARAP) proteins via LIR-like motifs. Moreover, they show that co-deletion of Stx16 with Stx17, previously implicated by the Mizushima group in autophagosome-lysosome fusion, results in impaired autophagic degradation of mitochondria, peroxisomes and intracellular pathogens.
They then show that Stx16 plays a role in regulating lysosomal vesicle biogenesis and mTORC1 signaling, and conclude by providing evidence that Stx16 is recruited to endo-lysosomal compartments in an Atg8-dependent manner.
One problem with the paper is that there seems to be some conceptual confusion between a role of Stx16 in physical association with autophagosomes, and another upstream of it where Stx16 is required for both lysosomal biogenesis and mTOR signaling. Does Stx16 work in concert and redundantly with Stx17 in promoting autophagosome-lysosome fusion? Or does it play a completely distinct role? If so, why would the two synergize in (mildly) compromising autophagic flux?

***We thank the reviewer for bringing this up. We apologize if our main conceptual conclusion was not clear, possibly due to the absence of a summary graphical model. An overall model is now provided in the manuscript (Fig. 7F on next page; and described on p. 14: "*Our findings with Stx16 indicate a function for mAtg8s in the maintenance of lysosomal compartments (Fig. 7F)*").



New Data 13, Fig. 5F. Knockout of *STX6* or *VTI1A* reduces LAMP2 protein levels.

The short answer (as above) is that Stx16 plays a distinct role in lysosomal biogenesis. Stx16, and its cognate Qb- and Qc-SNARE partners (please see the newly generated CRISPR KO of *VTI1A* and *STX6*; New Data 13, Fig. 5F) function in lysosome biogenesis, i.e.

specifically LAMP1/2 transport and overall cellular levels (text p. 10: "*We also generated CRISPR knockouts in Vti1a and Stx6, and found reduced levels of LAMP2 in VTI1A^{KO} and STX6^{KO} HeLa cells (Fig. 5F). Thus, Stx16 and its cognate Qb- and Qc-SNAREs affect cellular LAMP levels*". This also explains the mTOR phenotype (since mTOR is on the lysosomes); please see the model in Fig. 7F.

1. The accumulation of Keima-labeled ribosomes (Fig. 4C-4F) in Stx16/17 DKO cells is interpreted as evidence for defective autophagosome maturation. However, this assay does not allow one to precisely pinpoint at which stage autophagy has arrested. Is autophagosome-lysosome fusion compromised, as shown in Stx17-deleted HeLa cells by Itakura et al (2012)? Assays used in the Mizushima paper, particularly electron microscopy, should be used to better document the autophagy defect due to Stx16/Stx17 loss.

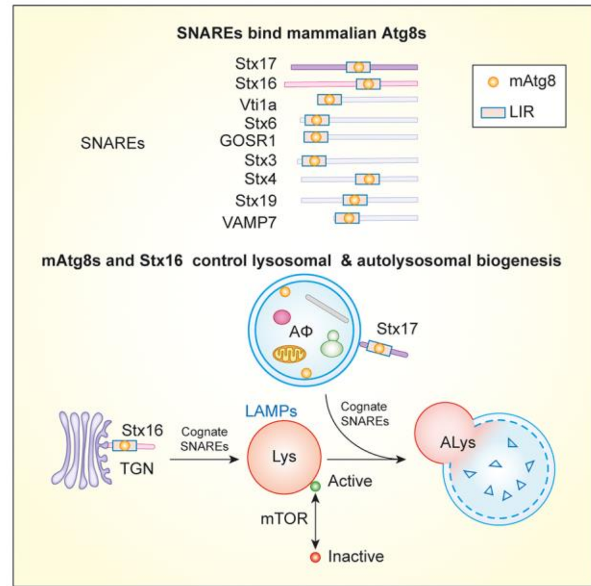
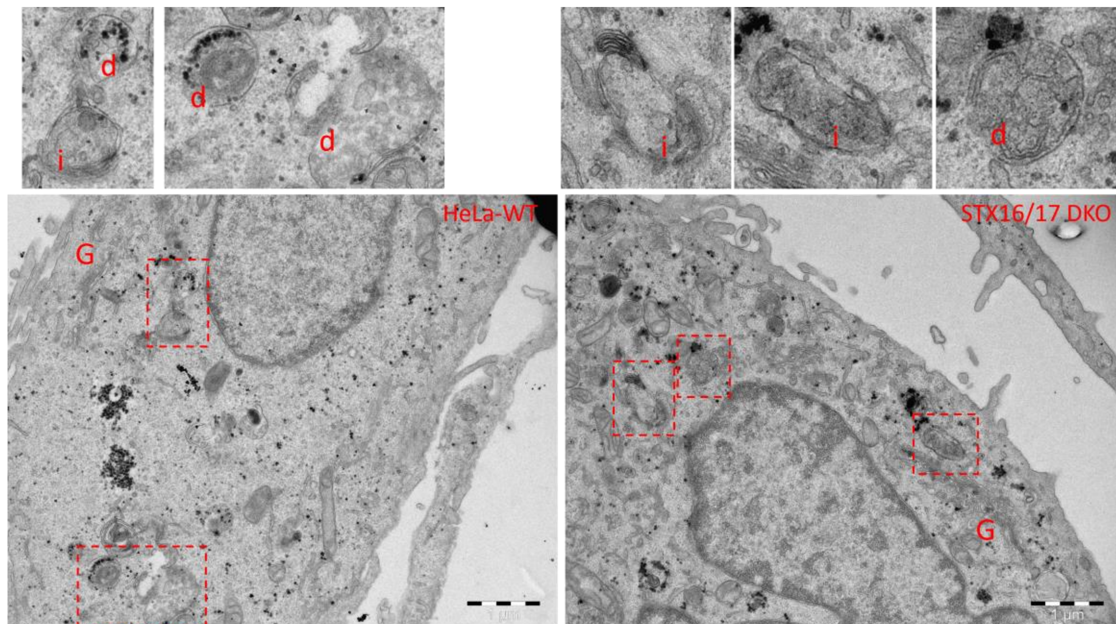


Fig. 7F, overall model of this study. Under autophagy-inducing conditions, Stx16 SNARE complex controls lysosome biogenesis, which in turn regulates mTOR localization and activity.

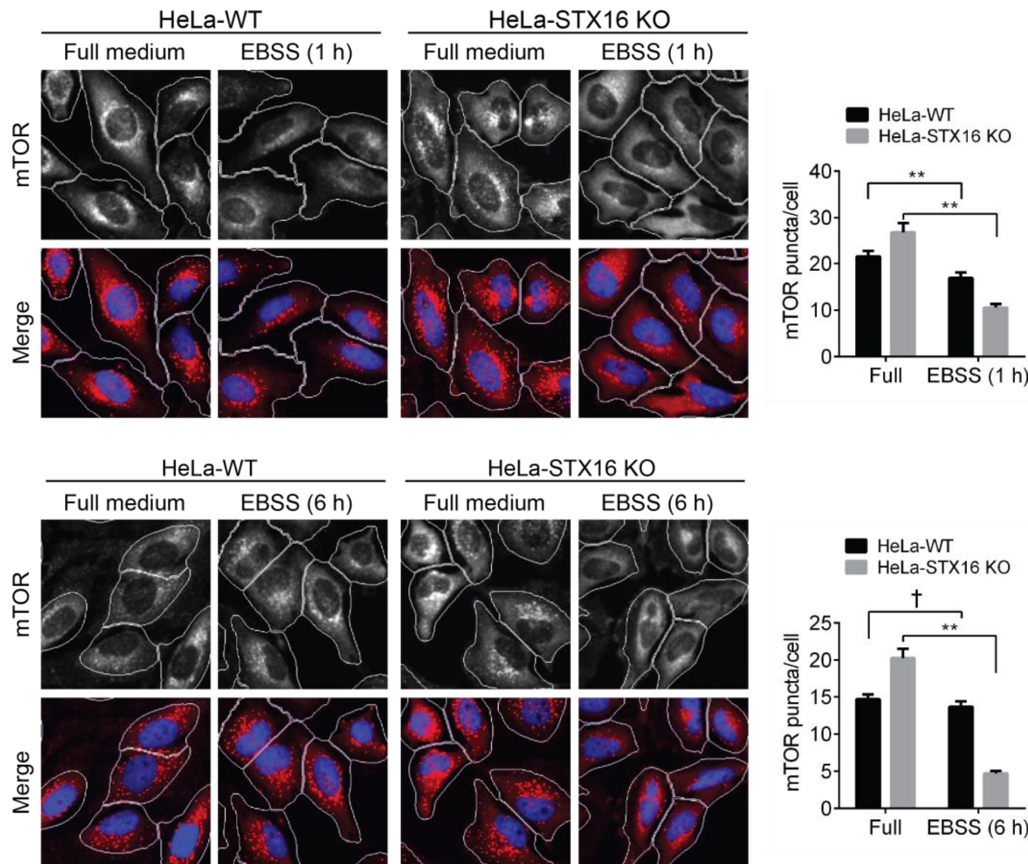
***We provide the EM insets with enlarged sections better illustrating the types of AVi (corresponding to early autophagic structures) and AVd (corresponding to degradative, autolysosomal structures) profiles (Modified Fig. 2E). We thank the reviewer for the suggestion, and hope that this (along with quantifications in Fig. 2F provides enough support the conclusion that autophagosomal maturation is compromised in *STX16/STX17*^{DKO} cells (described on p. 8).



Modified Fig. 2E, EM images showing defective autolysosomes in *STX16/STX17* DKO cells. i: initial autophagic vacuoles (AVi); d: degradative autophagic vacuoles (AVd).

2. The data connecting Stx16 to mTOR make little sense. On the basis of abundant literature, after 6h of continuous starvation mTORC1 should be completely cytoplasmic and thoroughly inactivated. Thus, it is not clear why in Fig. 5G mTOR seems to remain strongly punctate and why significant mTOR signaling remains in Fig. 5F. Given this, the reduction of mTOR puncta seen upon Stx16 deletion is also difficult to interpret.

***To address this important comment by the reviewer, we have now carried out additional experiments for mTOR after only 1 h of starvation (in addition to the previously shown 6 h starvation), and found a reduction in mTOR puncta, as expected and as the reviewer predicted. Furthermore, at 1 h starvation we also see a stronger reduction in mTOR puncta in *STX16*^{KO} cells (New Data 14; Fig. 5H and I). These relationships also hold at 6 h (previously shown data, now moved to Appendix Fig. S2A and B). These data are described on p. 11: "*One hour starvation reduced mTOR puncta, as quantified by HCM (Fig. 5H and I), reflecting mTOR inactivation. This effect was more pronounced in STX16*^{KO} *cells relative to WT cells (Fig. 5H and I). When the cells were starved for 6 h, a time point which coincided with mTOR persistent inactivation assessed by phosphorylation of its targets (Fig. 5G), we found an even stronger reduction in total mTOR puncta (Appendix Fig. S2A and B). Localization of mTOR to LAMP2 profiles was reduced by starvation (examined at 6 h; Fig. EV4D and E), an effect that was more pronounced in STX16*^{KO} *relative to WT cells.*"



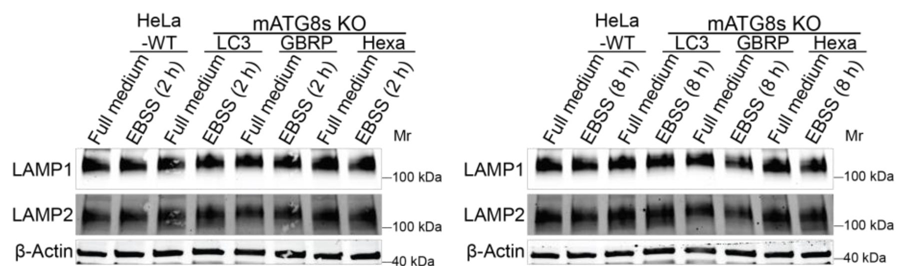
New Data 14, Fig. 5H and I (upper panel), and Appendix Fig. S2A and B (lower panel). HCM analysis of mTOR puncta in WT and *STX16*^{KO} cells after starvation for 1 h or 6 h.

As to the point regarding mTOR being punctate at 6 h of starvation, we refer the reviewer to the study by Rubinsztein and colleagues (Korolchuk et al., 2011) whereby mTOR repositions itself on lysosomes after 5 h of starvation. This is likely due to lysosome reformation processes (Yu et al., 2010).

3. Conceptually, it is difficult to separate the effects of Stx16 on mTORC1 activation (which should promote autophagy) from overall loss of lysosomal compartments (which should compromise autophagy). Which one is more important? Can the authors provide experiments to separate the two effects?
***Loss of Stx16 reduces protein levels of LAMP1/2 and number of LAMP2⁺ profiles. Regardless, the total number of mTOR dots remains unchanged, which suggests that mTOR occupies whatever lysosomes are left and remains efficient in fed cells (as already presented in the manuscript by Western blotting measurement of mTOR targets phosphorylation status (Fig. 5G) and by HCM quantification of mTOR puncta (Fig. 5H and I)). As a result, there is no obvious autophagy induction per se in *STX16*^{KO} cells (without additional stimulation, like starvation). However, under starvation conditions mTOR seems to be far more prone to inhibition in *STX16*^{KO} cells (New Data 14 on previous page of this rebuttal, HCM at 1 h of EBSS; and previously shown data for 6 h EBSS starvation, Fig. 5, now moved to Appendix Fig. S2A and B). We interpret this as a possible lack of some other components of mTOR regulatory machinery on lysosomes, which we have not studied here; we believe that this is beyond the scope of the current study and hope that the reviewer will agree. This is now discussed on p. 11: "*Alternatively, Stx16 may be important in transport to lysosomes of additional mTOR regulatory components important for its reactivation*" in continuation of the previously provided explanations. We apologize if this was not clear.

4. Regarding Stx16 localization, the low-mag imaging data in Fig. 7 do not allow one to make any conclusions about where Stx16 resides. Evidence of Stx16 localization to LE/Lys should be gathered via immunofluorescence, live cell microscopy and/or immuno-EM.
***We performed additional localization experiments using EGFP-tagged proteins (New Data 8, on p. 6 of this rebuttal, Fig. EV6E and F; and described on p. 12 of the manuscript). We hope that these additional localization studies provide the necessary confirmation as requested by the reviewer.

5. Related to the previous point, if Atg8 proteins are required to recruit Stx16 to LE/Lys (presumably to enable its autophagy-related functions) how come that the 6X Atg8-deleted cells have no defects in LAMP2 vesicle number? Shouldn't they phenocopy the Stx16-deleted cells?
***LAMP1 and LAMP2 protein levels are not reduced in Hexa KO cells, which is a surprising phenotype to us as well. However, it is clear that not all Stx16 phenotypes copy mAtg8 phenotypes and vice versa. The only clear/measurable effects that mAtg8s have are on Stx16 itself, i.e. the efficiency of its localization to lysosomes. We nevertheless carried out additional experiments and found that after 8 h of starvation, LAMP1 and LAMP2 proteins seem to be "exhausted" in GABARAP TKO and Hexa KO cells, but not in LC3 TKO cells (New data 15; Appendix Fig. S4C and D). This is described on p. 12: "*We nevertheless observed a minor LAMP reduction, possibly reflecting "exhaustion" during long-term (8 h) starvation, in Tri-GBRP*^{KO} *and Hexa*^{KO} *cells (Appendix Fig. S4D).*"



New Data 15, Appendix Fig. S4C and D. Long-term (8 h) starvation reduced LAMP1/2 levels in GABARAP TKO and Hexa KO cells.

6. Given the previous points, it is unclear what the significance of the Stx16 LIR motif is. At what stage

does Stx16 become recruited to LC3-containing autophagosomes, if ever? Does a LIR-deleted Stx16 rescue the autophagic flux, lysosome biogenesis and/or mTOR signaling defects of Stx16-deleted cells?
***(i) First, we clarify that Stx16 in our model works in lysosomal biogenesis (Fig. 7F), an answer provided under reviewer's point 1. (ii) Second, we carried additional experiments addressing the role of the LIR motif in Stx16 in recruitment to lysosomes: we compared Stx16 WT vs. Stx16 LIR mutants (STX16 LIR-AVLA, LIR-LALA, LIR-4A) for their localization with LAMP2 (New Data 8, Fig. EV6B and C; described on p. 12). These data show reduction in LIR mutant Stx16 localization to LAMP2 profiles relative to WT Stx16. (iii) Third, we checked the Stx16-Vti1a-Stx6 complexes in mATG8s knockout cells in different combinations (triple KO, hexa KO) and found reduced interactions between the components of this complex in in GABARAP TKO and Hexa KO (New Data 9, Fig. EV6D; p. 13). We hope this is satisfactory.

We thank the reviewers for their important and incisive comments.

Thank you for your consideration.

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University of New Mexico School of Medicine
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2nd Editorial Decision 27th August 2019

Thank you for submitting a revised version of your manuscript. It has now been seen by the original referees whose comments are shown below.

As you will see, referee #1 and #2 find that their criticisms have been sufficiently addressed. However, referee #3 feels that there is a logical disconnection between the two main findings of the study, i.e. LIR-mediated interaction between Stx16 and Atg8 family proteins, and the role of Stx16 in endo-lysosomal maturation. She/He thus suggests to remove the analysis of the LIR interaction from the manuscript. While referee #3's point is per se well taken, we find that the recommended change is not mandatory and does not preclude publication of your study in The EMBO Journal. Therefore, we let you decide whether to keep the manuscript as is or, for example, to present these data in the supplementary/appendix section.

REFEREE REPORTS

Referee #1:

The authors have very statisfactorily answered the reviewers' comments.

Referee #2:

The authors adequately addressed all my concerns by performing additional compelling experiments and improving the clarity of the manuscript text. I have no further objections to recommend this work for publication. Well done!

Referee #3:

In their revised manuscript, Gu et al have clarified some of the main concerns raised in the initial submission. In particular, the role of Stx16 in endo-lysosomal maturation, autophagosomal maturation (in cooperation with Stx17) and its (likely indirect) effects on mTOR signaling are more strongly supported by new experiments and additional discussion.

A remaining major weakness is the logical disconnect between part 1: the LIR-mediated interaction between Stx16 and Atg8 family proteins and part 2: role of Stx16 in endolysosomal maturation. The two parts simply are not logically or functionally connected and, if anything, the new experiments provided by the authors make this point even more obvious.
For instance, ablation of Atg8 proteins has different effects on endolysosomes than the lack of Stx16. Overall, the LIR-mediated interaction of Stx16 with Atg8 proteins seems to enable Stx16 localization to Lamp2 vesicles, but with little effect on lysosomal biogenesis, mTOR signaling or autophagic flux, which are also not impacted by Atg8 protein deletion.
Confirming these impressions, the new graphical model provided by the authors fails to conceptually integrate the interaction of Stx16 with Atg8 proteins with its role in endolysosomal maturation: the two remain separate in the top and bottom part of the figure, respectively.

In summary, two distinct stories that have little to do with each other are lumped together into a single manuscript that will, without a doubt, be confusing to the reader instead of sending a clear, unifying message. It is in the authors' best interest to send a simple, linear message that is conceptually solid, so that the EMBO journal readership may understand it and appreciate its relevance to the field.
Thus, I strongly suggest that the authors remove the LIR part of the story, which should be developed as a separate paper, and refocus the manuscript on the stronger aspects, namely, the role of Stx16 in endolysosome maturation and its overall requirement for autophagy.

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2nd Revision - authors' response 7th September 2019

Here is the point by point response to reviewers' comments:

Referee #1:

The authors have very statisfactorily answered the reviewers' comments.

We thank the reviewer for his/her kind assessment.

Referee #2:

The authors adequately addressed all my concerns by performing additional compelling experiments and improving the clarity of the manuscript text. I have no further objections to recommend this work for publication. Well done!

We thank the reviewer for his/her support to publish this work.

Referee #3:

In their revised manuscript, Gu et al have clarified some of the main concerns raised in the initial submission. In particular, the role of Stx16 in endo-lysosomal maturation, autophagosomal maturation (in cooperation with Stx17) and its (likely indirect) effects on mTOR signaling are more strongly supported by new experiments and additional discussion.

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Confirming these impressions, the new graphical model provided by the authors fails to conceptually integrate the interaction of Stx16 with Atg8 proteins with its role in endolysosomal maturation: the two remain separate in the top and bottom part of the figure, respectively.

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Thus, I strongly suggest that the authors remove the LIR part of the story, which should be developed as a separate paper, and refocus the manuscript on the stronger aspects, namely, the role of Stx16 in endolysosome maturation and its overall requirement for autophagy.

We thank the reviewer for recognizing that our new experiments carried out in response to the suggestions by this and other reviewers have strengthened the study. We also appreciate the point regarding the issue that Stx16 KKO and Hexa (all mATg8s) KO do not entirely phenocopy each other. However, the experiments with the LIR mutant Stx16 and with Hexa and GABARAP-triple KO cells show that Stx16 loclization to lysosomes is reduced (showing the role of mAtg8s in this) and effects on mTOR (on lysosomes) indicate connections between the two systems. It is realistic in our view that absence of all mAtg8s must have many more consequences that what Stx16 does. In acknowledging this issue and reviewer's point, we now emphasize in discussion this point:
"Although mAtg8s do not phenocopy in full the Stx16 phenotype, they do affect Stx16 distribution and mTOR activity. Thus, mAtg8s and Stx16 have only partially overlapping effects on the endolysosomal system most likely due to mAtg8 action exceeding the reach of the Stx16 function."

Accepted 13th September 2019

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ➔

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Vojo Deretic
Journal Submitted to: EMBO J
Manuscript Number: EMBO-2019-101994

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(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 χ2 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 values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

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http://www.consort-statement.org/checklists/view/32-consort/66-title
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http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ➔ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	This was based on power analysis performed by a statistician. For high content microscopy the determination of sample size was based on prior published work, effect size and standard deviations.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	This is precisely the reason for using high-content microscopydata acquisition and analysis (Cellomics), which ensures investigator-independent, machine driven unbiased data acquisition and analysis.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Blinding of investigators was not necessary due to utilization of investigator-independent unbiased methods of imaging data collection and analysis as described in point 2. For immunoblots and quantification the samples were not blinded.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	For experiments involving multiplet groups, ANOVA and post-hoc tests were used. When two groups only were compared, t-test was applied.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Priro published work.
Is there an estimate of variation within each group of data?	Yes, we used standard errors of the mean (SEM) to evalue the variation within each group of data.

Is the variance similar between the groups that are being statistically compared?	Based on multiple studies, variance is similar between the groupsusing methodologies employed.

C- Reagents

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), 1DegreeBio (see link list at top right).	In formation regarding antibodies used in this study, including the sources and catalog numbers, is provided in Materials and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All the cell lines used in this study were originally from ATCC.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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 WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. 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 e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized 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 JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No