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Dedicated SNAREs and specialized TRIM cargo receptors mediate secretory autophagy

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(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 06 July 16

Thank you for submitting your manuscript to us. I have now received three reports on your work, which I enclose below.

As you will see, the referees appreciate your study. However, they also think that your conclusions are currently not supported by the data provided. Alternative explanations for the observed phenotypes for IL1b secretion exist and further insight into cargo localization is required. Furthermore, the referees raise several technical issues. I won't list the individual concerns, as all reports are concise and constructive and consistent with each other.
Given the interest in the topic, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers and, importantly, the points mentioned above. The required revision is rather extensive, so please feel free to contact me to discuss the individual points in more detail with me. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the

conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS

Referee #1:

Kimura et al. delineate the molecular components and vesicular network involved in autophagy-dependent unconventional secretion. To identify novel regulators of this emerging pathway the authors have carried out a siRNA screen of individual tripartite motif-containing (TRIM) proteins, a protein family that regulates precision autophagy, and assessed the impact on the lysosome stress-induced IL1beta secretion. Their screen revealed that a number of TRIMs required for efficient IL1beta secretion, including TRIM16. Molecular characterization of TRIM16 function in this pathway confirms that TRIM16 interacts with IL1beta and that it is necessary for IL1beta secretion in response to diverse lysosomal damage/inflammasome activating agents, in various macrophage cell lines and primary cells.

The authors then reveal additional components of the autophagy-dependent secretory pathway. Subsequent studies uncovered novel TRIM interacting proteins, including Galectin-8 and the R-SNARE Sec22b, which appear involved in its recruitment to damaged lysosomes and LC3-decorated membranes, respectively, and are also required for IL1b secretion. R-SNARE proteins such as Sec22b typically direct vesicular trafficking and fusion via ternary complexes formed with SNAP family proteins and membrane integrated Syntaxins (Q-SNAREs). To determine how secretory autophagy and degradative autophagy are functionally distinct, the authors screen a number of SNAPs and Q-SNAREs for their role in IL1b secretion. Intriguingly, the secretory autophagy pathway appears to require SNAP23 (and possibly SNAP29), as well as Syntaxins 3 and 4; However, Syntaxin17 is dispensable for IL1b secretion, despite being required for degradative autophagy, and suggests that secretory autophagy occurs through a distinct set of R-SNARE, SNAP and Q-SNARE interactions that diverge from the classical autophagy degradation pathway. Finally, the authors use mass spectrometry to identify ferritin as a novel candidate released via secretory autophagy and demonstrate that its efficient secretion requires the same machinery as IL1b.

The discovery that TRIM16 and a unique set of SNARE-SNAP interactions direct capture and secretion of IL1b, the paradigm substrate of secretory autophagy, should be considered significant advancements in the filed. Furthermore, the identification of Ferritin as potential substrate of secretory autophagy helps to extend the biology of this pathway beyond a limited set of cargo. Despite these interesting and important observations, several conclusions in the manuscript require additional experimental support. The biggest deficiency is that the authors have failed to control for the potential impact of various genetic and chemical manipulations on the function of the autophagy degradation pathway as well as on IL1b expression and processing. Furthermore, some aspects of the authors' proposed model for autophagy-dependent secretion appear discrepant with the result within the field and need to be addressed and discussed. These additions would greatly strengthen the manuscript.

Major Issues:

1) The identification of TRIM16, Galectin-8, Sec22b, SNAP23 and Stx3/4 as novel effectors of secretory autophagy is interesting and important. However, the relationship between these proteins and the canonical autophagy pathway, as well as IL1b expression and maturation, is somewhat unclear. Although genetic knockdown of these various effectors may be observed to reduce IL1b secretion, it is unclear whether these phenotypes are due to a specific defect in secretory autophagy. It is also possible that genetic ablation of these regulators may generally impact the autophagy pathway, thus leading to secondary defects in secretion. For example, Sec22 is required for normal Atg9 transport and autophagosome biogenesis in yeast (Nair et al 2011). In light of these

observations, it might be anticipated that Sec22 knockdown would result in a defect in the formation of LC3+ autophagic membranes that are necessary for both secretory and degradative autophagy. Therefore, it is very important to examine how knockdown of these novel effectors impacts autophagic flux in order to have a clearer understanding of how secretory autophagy carriers versus degradative carriers are formed.

2) For similar reasons, it is important to also assess how siRNA knockdown of the various effectors influences IL1b expression and processing. Caspase1 is a Golgi localized enzyme and its activity might be altered by siRNAs that affect secretory pathway proteins. To control for these possibilities, the intracellular levels of pro-IL1b and m-IL1b should monitored in the various knockdown cells. This is an essential missing control.

3) In this study, lysosomotropic agents are commonly used (after LPS treatment) to provide the secondary trigger signal for inflammasome activation and IL1b processing/secretion, but they also will interfere with degradation through the canonical autophagy pathway, which poses a major caveat. Prior work has revealed that in addition to directly promoting IL1b secretion, the autophagy pathway also sequesters and degrades the inflammasome to restrict IL1b release. Therefore, the use of LLOMe and other lysosome damaging agents may actually confound the role of autophagy in IL1b secretion by impairing inflammasome and/or IL1b degradation and generate a pool of cargo-enriched lysosomes with the potential to exocytose their contents into the extracellular space. Although the observation that Stx17 is dispensable for IL1b release and that starvation can induce secretion in a reconstituted system would seem to argue against this possibility, it is important to determine if secretory autophagy has the same genetic requirements under conditions that do not compromise the lysosome. Does secretory autophagy have similar genetic requirements when an inflammasome agonist that does not impact the lysosome such as extracellular ATP is used? Alternatively, analysis of short term LLOMe treatment followed by washout may be a useful strategy.

4) The results do not support the author's conclusions that secretory autophagy can stimulated by depleting specific cargo receptors that control degradation, such as NCOA4 and p62/SQSM1. It is unclear whether the increased Ferritin and/or IL1b detected in conditioned media is actively secreted via autophagy or a secondary to passive release in cells that accumulate increased amounts of these substrates intracellularly due to cargo receptor deficiency.

5) Aspects of authors' proposed model for autophagy-dependent secretion (depicted in Fig 5D) appear somewhat discrepant with the other key results within the field. Notably, Schekman and colleagues recently proposed a model for IL1b secretion using a reconstituted system in which the substrate is unfolded and transported through the membrane in between the autophagosome membranes (Zhang et al. eLife. 2015; 4: e11205). In the author's model shown here, secretory autophagy substrates are shown both between the double autophagosome membrane and in the lumen of the autophagosome itself, but the author's do not provide direct evidence illuminating either or both of these possibilities. Importantly, is secreted ferritin also loaded into the intra-membrane space of the autophagosome for secretion or is it captured in the autophagosome lumen like classical cargo? Biochemical analysis of the 25K membrane fraction with protease and detergent in cells deficient for ATG2 will help clarify this issue.

6) The requirement of Syntaxins 3/4 and dispensability of Stx17 for secretory autophagy suggest that vesicular carriers can directly fuse at the plasma membrane, as the authors have indicated with their depiction in Fig 5D. In contrast, prior work from the Deretic lab indicated that Bafilomycin A1 reduces IL1b secretion, thus suggesting that substrates may transit through an endolysosomal compartment before release. Please clarify the role of the lysosome/ late endosome in this model.

Minor Issues:

1) Why does LC3B knockdown impact IL1b secretion if TRIM16 primarily interacts with GABARAP and Sec22b?

2) The western blot in Fig. EV1 panel I is extremely washed out on the right side of the IL1b blot and not supportive.

3) Why isn't IL1b detected in the mass spec for autophagy-dependent secretion substrates?

Referee #2:

In this manuscript, Kimura et al. report that TRIM16 associates with IL-1b and is required for unconventional secretion of IL-1b upon treatment of lysosome-damaging agents such as LLOMe or starvation in macrophages. TRIM16 is important for translocation of IL-1b to LC3-II(+) membranes. TRIM16 interacts with galectin-8 and the R-SNARE Sec22b, both of which are required for IL-1b secretion. In addition, these factors are needed for secretion of ferritin, another leaderless cytosolic protein. Although the topic is important and timely, the current data are insufficient to conclude that the TRIM16/galecin-8/Sec22b axis is required for autophagosome-mediated unconventional secretion of IL-1b and ferritin.

Major comments:

1. The information on the TRIM16(+) structure is not sufficient. In Figure 1C, TRIM16(+)LC3B(+) structures are formed in cells treated with LLOMe, which causes lysosomal damage and lysophagy. Do these TRIM16(+)LC3B(+) structures represent autophagosomes containing damaged lysosomes (lysophagy-related structures)? Do they also contain IL-1b? These TRIM16 structures should be co-stained with IL-1b, phagophore markers, lysosome markers (as substrates), and lysophagy markers (e.g. galectin). These TRIM16 structures should also be characterized by electron microscopy (CLEM or immuno-electron microscopy).

2. The structure of starvation-induced autophagosomes and LLOMe-induced autophagosomes should be different. As mentioned above, the latter should contain lysosomes. How can the model in Figure 5 be applied to starvation-induced IL-1b secretion? Do TRIM16 and IL-1b colocalize with lysosomes during starvation? How do TRIM16 and IL-1b translocate from the lysosome to autophagosome/phagophore?

3. Related to the above comment, the model in Figure 5 is not fully supported by the present data. Are TRIM16 and IL-1b colocalized with phagophore markers such as DFCP1 and ATG5? When and how is Sec22 recruited to the phagophore? Is it dependent on TRIM16? The genetic hierarchy between TRIM16 and galectin-8 should also be investigated. Is the colocalization between LC3B and TRIM16 reduced in galectin-8-knockdown cells?

4. If STX17 does not affect IL-1b secretion, are Sec22b(+) autophagosomes negative for STX17? In other words, are Sec22b and STX17 indeed mutually exclusive? This is extensively discussed in the Discussion part, but there is no supporting data.

5. The biochemical data in Figure 3A-3C are important and need to be investigated more vigorously. The distribution of TRIM16, IL-1b, and Sec22b in wild-type and TRIM16 KO cells should be analyzed using a density gradient with markers of major organelles. A proteinase protection assay should also be performed to determine whether TRIM16, IL-1b, and Sec22b are present inside membranes. Immunoblotting of both m- and pro-IL-b should be performed using whole cell lysates and culture media of wild-type and TRIM16 KO cells. If TRIM16 is indeed required for secretion but not for maturation of IL-1b, mIL-1b should accumulate.

6. The quality of the immunofluorescence data is generally not high. Why is the pattern of TRIM16 so different between Figure 1C and Figure 2F?

7. Is there reduced colocalization between IL-1b and LC3B in TRIM16 KO cells? This will be another clue to support the authors' hypothesis.

8. Figure 1A: The authors should determine the requirement of other ATG factors such as GABARAP. The requirement of ATGs should also be determined in ferritin secretion in Figure 6.

9. Figure 1B: The authors show that knockdown of not only TRIM16 but also TRIM19 or TRIM10 causes a reduction of IL-1b secretion. Do TRIM10, TRIM16, and TRIM19 function redundantly? This can be tested by double- or triple-knockdown. The interaction of TRIM10 and TRIM19 with

IL-1b and ferritin should also be analyzed.

10. Figure 2B: The function of galaectin-8 in starvation-induced IL-1b secretion is totally unclear. Are lysophagy-related structures (galectin-8-positive structures) formed by starvation? At which step is galectini-8 required?

11. Figures 4 and 5: The involvement of Sec22b in the "unconventional secretion" of IL-1b and ferritin is not directly shown. Does IL-1b accumulate in LC3(+) structures in Sec22b-, SNAP23-, or SNAP29-knockdown cells (due to a defect in fusion with the plasma membrane)? What about the effect of double knockdown of SNAP23 and SNAP29 on IL-1b secretion?

12. This study mostly relies on siRNA but off-target effects are not ruled out. Furthermore, in Figure 3H, the authors should perform a rescue assay using TRIM16-knockdown cells expressing wild-type TRIM16 or a TRIM16 mutant defective in the interaction with Sec22b to show the significance of the TRIM16-Sec22b interaction.

13. Figure 3I: This reviewer does not understand what the authors want to express with the statement "co-clustering of Sec22b and TRIM16 in cells with a characteristic separation of 70 nm". The data are inconsistent with the idea that Sec22b and TRIM16 are present on the same structures as shown in Figure 5D.

14. Figure 6: The intracellular localization of NCOA4 should be determined in wild-type, siSec22b, and siTRIM16 cells.

Minor comments:

1. The LDH data in Figure EV1 are not explained in the text. Why does LLOMe cause LDH release?
2. There are no figures to refer to for the following statement in the last paragraph of the Result section "Similarly, only increased IL-1b secretion in response to inflammasome agonist (Fig EV6E)"
3. Figure 2: The interaction between galectin-8 and TRIM16 should also be tested in vivo.
4. Figure 3: Why did the authors focus on Sec22b? Any specific reason?
5. Figure 5D: LC3 should interact with TRIM (Mandell et al. 2014).

Referee #3:

The proinflammatory cytokine Interleukin 1beta lacks a canonical N-terminal signal sequence, and is secreted by an unconventional pathway that has been intensely investigated. Previous work, including a seminal contribution from the corresponding author's group (Dupont et al:, EMBO J., 2011), has implicated the autophagosome as an intermediate carrier leading to IL-1beta secretion. In the submitted manuscript, the authors attempt to identify the players of this secretory autophagy pathway, identifying TRIM16 as a central component. The inhibition of IL-1beta secretion after TRIM16 knockdown, the interaction of the cytokine with TRIM16, as well as an increase in its secretion upon TRIM16 overexpression, had all been previously reported by another group (Munding et al., Cell Death Diff., 2006). Here, the authors investigate TRIM16 binding partners, and find that it interacts both with galectin 8 and with Sec22b; furthermore, knockdown of Sec22b inhibits IL1-beta secretion, while leaving autophagic flux unaltered. They propose that TRIM16 provides a platform for recruitment of IL-1beta to a Sec22b-containing sequestration compartment; because of its molecular composition the resulting cytokine-containing autophagosome avoids fusion with the lysosomes, and instead engages in exocytosis triggered by the interaction of Sec22b with plasma membrane SNAREs. The authors further identify ferritin as a protein that follows the same TRIM16-dependent pathway for secretion.

The findings of this manuscript are of high interest; however, as detailed below, it is not easy to place them in the context of the myriad players of autophagocytosis; in other words, it was not clear to this reviewer whether the observed effects of knockdown of candidate players on IL-1beta secretion were always due to interference with the specific autophagic secretory pathway or rather to effects on autophagocytosis in general. Several points that appeared unclear to this reviewer would have deserved at least come comment. Importantly, the authors should have more clearly informed

the reader of what new information their study brings versus what has already been published (Munding et al., 2006). Finally, there are a number of technical issues that should be addressed before the article is suitable for publication.

1. The problem of interference with secretory autophagy vs interference with autophagy in general: In Fig. 1, the authors show that knockdown of a number of proteins of the TRIM family lead to decreased IL-1beta secretion. Of these, TRIM16 was previously shown to interact with IL-1beta (and confirmed here). One would like to know whether autophagy in general is affected by these TRIMs (as done in Fig. 4 for Sec22b depletion); in this way it might be possible to distinguish TRIM16's effect from that of the other TRIMs. The same question comes to mind for Galectin-8. The specific effect of Galectin-8 depletion vs no effect with Galectin-3 is interesting. Again, one would to know whether any previous work has shown functional differences for these two galectins, and what effect Galectin-8 depletion has on autophagocytosis in general.

2. Unclear points:

2a. In the knockdown experiments there was at most a 65% inhibition of IL-1beta secretion. TRIM16 knockdown itself lead to about 40% inhibition. Is this incomplete effect due to incomplete silencing? Or can it be explained by the existence of alternative TRIM16-independent pathways for IL-1beta secretion? The extent of silencing was never quantified. Also, no rescue experiments were done to rule out off-target effects; at least, the authors should demonstrate that siRNAs targeting different regions of the same mRNA have the same effect. These controls need to be done.

2b. An interesting finding is that IL-1beta secretion is dependent on Sec22b but not on Syntaxin 17; the latter is a SNARE required for fusion of autophagosomes with lysosomes, an event avoided by IL-1beta secretory autophagosomes. It is, however, surprising that observe an inhibitory effect of SNAP29 knockdown is observed (Fig. 5), as this SNARE is thought to collaborate with Syntaxin 17 in the fusion of autophagosomes with lysosomes. This should be commented.

2c. In Fig. 3F and G, evidence is presented for the presence of Sec22b and TRIM16 in the same macromolecular complexes; in the model of Fig. 5, the two are depicted as directly interacting. In Fig. 3I and J, the authors show that Sec22b and TRIM16-containing structures are close to each other (70 nm distance) but do not co-localize. The two observations are contradictory. If the two proteins interact, they should co-localize. From the images of Fig. 3, it seems they are in distinct structures.

3. Technical issues.

3a. The quality of many of the blots is poor. Number of repetitions of experiments are nowhere specified. Inputs are shown, but what percentage input has been loaded compared to the Ips is not stated.

3b. The Methods and Figure Legends are insufficient for full comprehension of what has been done. On p. 13, the authors state that they give full information on super-resolution fluorescence microscopy analysis in the Supplementary Experimental Procedures, but this file was not available to me. An example of lack of clarity is given by Fig. EV3, panel C. Why are the TRIM16 bands duplicated (upper boxed panel, and middle panel)? Why is A9 shown twice?

3c. The results of the fractionation experiment (Fig. 3A-C) are weak. The quality of the blot of Fig. 3B is so poor that one wonders how the quantitative data of Fig. 3C could be obtained. Also, recoveries of the analyzed molecules in all the fractions are not given. If there is less IL-1beta in the 25k fraction in the TRIM16 KO cells, is there more of it in the other fractions? Or is there a lower level of expression of the cytokine in these cells?

3d. Co-localization of TRIM16 with LC3B (Fig. 1C) and with Galectin-8 (Fig. 2F) in non-stimulated and LLOMe-stimulated cells: the most striking effect of LLOMe seems to be an increase in TRIM16 expression (provided that the non-stimulated and stimulated cells have been acquired with the same microscope settings). The line plots between non-stimulated and stimulated cells are not readily comparable, as in the former condition, lines do not go through LC3 puncta (which are present, although to a lesser extent than in LLOMe-stimulated cells).

3e. On p. 9, the authors state that TRIM16-dependency is not observed for ferritin secretion in cells infected with the ESX-1 mutant of Mycobacterium tuberculosis Erdmann, but this is not apparent from the data presented in Fig. 6D: from Fig. 6D it is clear that there is less ferritin secretion in ESX-1 infected cells silenced for TRIM16, for Sec22b or for Gal-8 compared to the scrambled control.

3f. The manuscript has been prepared without much care. For instance, two sentences on p. 9 ("Mirroring this observation.... agonist") are incomprehensible. In the bibliography, a number of references are incomplete.

1st Revision - authors' response 14 September 2016

Thank you very much for the reviews of our manuscript “Cellular and molecular definition of the mammalian secretory autophagy pathway” and for your decision and editorial recommendations.

We thank the reviewers for their extensive and excellent analysis of our findings and for their suggestions for improvement. We have addressed the majority of reviewers’ comments experimentally, resulting in the following additions: 8 new or revised panels in the main figures, 20 new or revised panels in the Extended View figures, and 17 panels in 4 new Appendix figures. A full inventory of new datasets (45 in total) generated in response to reviewer’s request is appended*** at the end of this rebuttal.

Below is our detailed point-for-point response to reviewers’ criticisms and suggestions.

Referee #1:

Kimura et al. delineate the molecular components and vesicular network involved in autophagy-dependent unconventional secretion. To identify novel regulators of this emerging pathway the authors have carried out a siRNA screen of individual tripartite motif-containing (TRIM) proteins, a protein family that regulates precision autophagy, and assessed the impact on the lysosome stress-induced IL1beta secretion. Their screen revealed that a number of TRIMs required for efficient IL1beta secretion, including TRIM16. Molecular characterization of TRIM16 function in this pathway confirms that TRIM16 interacts with IL1beta and that it is necessary for IL1beta secretion in response to diverse lysosomal damage/inflammasome activating agents, in various macrophage cell lines and primary cells.

The authors then reveal additional components of the autophagy-dependent secretory pathway. Subsequent studies uncovered novel TRIM interacting proteins, including Galectin-8 and the R-SNARE Sec22b, which appear involved in its recruitment to damaged lysosomes and LC3-decorated membranes, respectively, and are also required for IL1b secretion. R-SNARE proteins such as Sec22b typically direct vesicular trafficking and fusion via ternary complexes formed with SNAP family proteins and membrane integrated Syntaxins (Q-SNAREs). To determine how secretory autophagy and degradative autophagy are functionally distinct, the authors screen a number of SNAPs and Q-SNAREs for their role in IL1b secretion. Intriguingly, the secretory autophagy pathway appears to require SNAP23 (and possibly SNAP29), as well as Syntaxins 3 and 4; However, Syntaxin17 is dispensable for IL1b secretion, despite being required for degradative autophagy, and suggests that secretory autophagy occurs through a distinct set of R-SNARE, SNAP and Q-SNARE interactions that diverge from the classical autophagy degradation pathway. Finally, the authors use mass spectrometry to identify ferritin as a novel candidate released via secretory autophagy and demonstrate that its efficient secretion requires the same machinery as IL1b.

The discovery that TRIM16 and a unique set of SNARE-SNAP interactions direct capture and secretion of IL1b, the paradigm substrate of secretory autophagy, should be considered significant advancements in the field. Furthermore, the identification of Ferritin as potential substrate of secretory autophagy helps to extend the biology of this pathway beyond a limited set of cargo.

We thank the reviewer for the generous assessment of our findings, and for the specific comments and criticisms below.

Despite these interesting and important observations, several conclusions in the manuscript require additional experimental support. The biggest deficiency is that the authors have failed to control for the potential impact of various genetic and chemical manipulations on the function of the autophagy degradation pathway as well as on IL1b expression and processing.

We now provide additional controls regarding effects on degradative autophagy and IL-1β expression and processing, as described below under specific comments.

Furthermore, some aspects of the authors' proposed model for autophagy-dependent secretion appear discrepant with the result within the field and need to be addressed and discussed. These additions would greatly strengthen the manuscript.

We thank the reviewer for all suggestions, comments and criticisms and have tried to address them experimentally to the maximum extent possible. We also discuss differences as requested, with amendments summarized under specific comments.

Major Issues:

1) The identification of TRIM16, Galectin-8, Sec22b, SNAP23 and Stx3/4 as novel effectors of secretory autophagy is interesting and important. However, the relationship between these proteins and the canonical autophagy pathway, as well as IL1b expression and maturation, is somewhat unclear. Although genetic knockdown of these various effectors may be observed to reduce IL1b secretion, it is unclear whether these phenotypes are due to a specific defect in secretory autophagy. It is also possible that genetic ablation of these regulators may generally impact the autophagy pathway, thus leading to secondary defects in secretion. For example, Sec22 is required for normal Atg9 transport and autophagosome biogenesis in yeast (Nair et al 2011). In light of these observations, it might be anticipated that Sec22 knockdown would result in a defect in the formation of LC3+ autophagic membranes that are necessary for both secretory and degradative autophagy. Therefore, it is very important to examine how knockdown of these novel effectors impacts autophagic flux in order to have a clearer understanding of how secretory autophagy carriers versus degradative carriers are formed.

We thank the reviewer for this important comment. We have presented data in Figs EV4A and EV4B showing that Sec22b knockdown did not affect LC3 puncta formation whereas it inhibited IL-1β secretion. We now emphasize better these important controls (p. 8), and have expanded the description of these studies to a separate paragraph. Furthermore, our data are consistent with the report by Renna et al., (2011) showing that Sec22b does not affect degradative autophagy in mammalian cells at the stage of LC3-puncta formation. Rena et al have carefully established that Sec22b does not affect LC3-puncta formation but instead affects lysosomal enzyme delivery to autolysosomes at degradative stages. We now expand the discussion of the already referenced study by Renna et al., (p. 8).

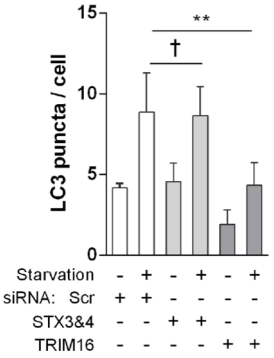
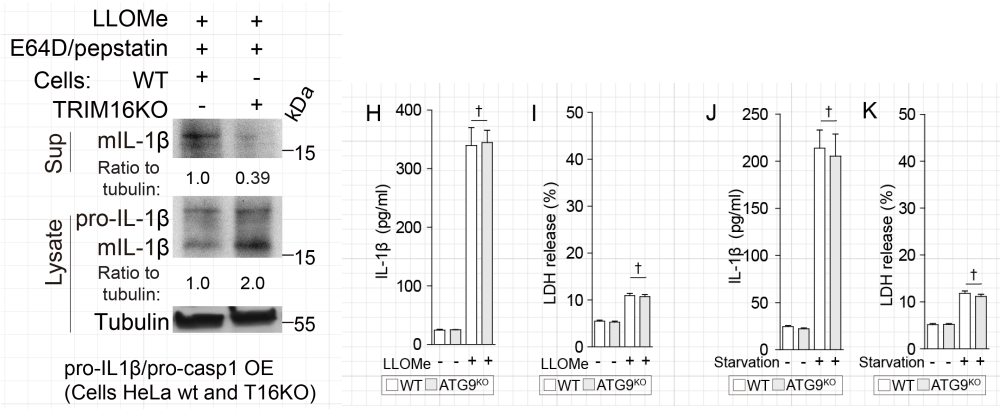


Fig EV5 R (new data). Double Stx3 and Stx4 knockdown does not affect conventional autophagy detected by LC3 puncta formation in response to starvation.

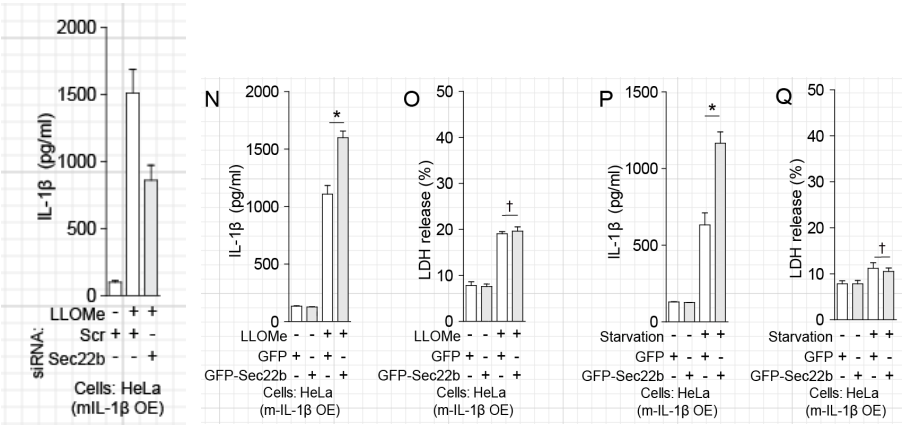
We furthermore now reference the study by Nair et al (2011) in yeast and discuss it in this context. Moreover, in response to reviewer's comment we carried additional experiments. As reviewer states, Nair et al., have shown in yeast that Atg9 trafficking is affected by the yeast exocytic SNAREs Sso1 and Sso2 whereas it is fair to assume that Atg9 may affect unconventional secretion. Thus, we tested whether the exocytic SNAREs used in our study (syntaxins 3 and 4) influenced autophagosome formation. The results shown in new Fig. EV5R and S indicate that Stx3/Stx4 double knockdown did not affect LC3 *puncta formation (p. 10). We also tested directly whether Atg9 affected unconventional secretion of IL-1β using Atg9 KO MEFs. As shown in new Fig. EV4H-K, absence of Atg9 did not affect secretion of IL-1β in response to the two stimuli tested (LLOMe and starvation). These experiments are now described on p. 8 (Atg9 studies).*



Left: Fig EV3D (new data). Undiminished processing of pro-IL1β (cell lysates) contrasting with reduced secretion of IL-1β (culture supernatant). / Right: Fig. EV4 H-K (new data). Absence of Atg9 ($Atg9^{KO}$ MEFs) does not affect IL-1β secretion.

2) For similar reasons, it is important to also assess how siRNA knockdown of the various effectors influences IL1b expression and processing. Caspase1 is a Golgi localized enzyme and its activity might be altered by siRNAs that affect secretory pathway proteins. To control for these possibilities, the intracellular levels of pro-IL1b and m-IL1b should monitored in the various knockdown cells. This is an essential missing control.

We thank the reviewer for asking for these important controls. We have now carried out several experiments to address this important issue: First, we tested (new Fig. EV3D) intracellular levels of pro-IL-1β and mIL-1β in parallel with secreted mIL-1β. Whereas there is less mIL-1β secreted from $TRIM16^{KO}$ CRISPR cells than from parental $TRIM16^{wt}$ cells (expressing pro-IL-1β and pro-Caspase-1), the intracellular levels of caspase-1 processed pro-IL-1β into mIL-1β are not diminished. Thus, caspase-1 activity/action on pro-IL-1β was not reduced and cannot explain reduced secretion of IL-1β. Second, we bypassed potential issues concerning indirect effects of Golgi-localized caspase 1 by expressing mature IL-1β (mIL-1β); in these cells, a Sec22b knockdown reduced mIL-1β secretion (new Fig. EV4L, M). In a complementary set of experiments (new Fig. EV4N-Q), Sec22b overexpression increased mIL-1β secretion in cells transfected with mature IL-1β. Thus, Sec22b downregulation decreases, whereas Sec22b overexpression increases mIL-1β secretion in cells regardless of pro-IL-1β processing. The above experiments collectively demonstrate that TRIM16 and Sec22b requirements for IL-1β secretion are not indirect (i.e. not due to the suggested effects on pro-caspase-1 activation at the Golgi).



Left: Fig EV4 L (new data). HeLa cells were transfected with mature IL-1β (mIL-1β), thereby bypassing the requirement for pro-IL-1β processing by caspase 1, Sec22b knockdown still reduced levels of secreted IL-1β. / Right: Fig EV4 N-Q (new data). Overexpression of Sec22b in cells transfected with mature IL-1β (bypassing a requirement for increases secretion of mIL-1β.

3) In this study, lysosomotropic agents are commonly used (after LPS treatment) to provide the secondary trigger signal for inflammasome activation and IL1b processing/secretion, but they also

will interfere with degradation through the canonical autophagy pathway, which poses a major caveat. Prior work has revealed that in addition to directly promoting IL1b secretion, the autophagy pathway also sequesters and degrades the inflammasome to restrict IL1b release. Therefore, the use of LLOMe and other lysosome damaging agents may actually confound the role of autophagy in IL1b secretion by impairing inflammasome and/or IL1b degradation and generate a pool of cargo-enriched lysosomes with the potential to exocytose their contents into the extracellular space.

This is a key point and we are glad the reviewer brought it up. We do not believe that secretion of IL-1β is normally disconnected from the status of the lysosomal system. In fact, our model is that cells throttle IL-1β secretion in response to the extent of endomembrane damage, and that this is the reason why (a well known fact in the inflammasome field) many endomembrane damaging agents activate IL-1β secretion. Consider the following scenarios: If cells can contain/remove the lysosomal damage/insult there will be no need to secrete IL-1β and as pointed out by the reviewer inflammasome components are removed through autophagy; however, excessive damage will impede this homeostatic removal and IL-1β is secreted to elicit a broader inflammatory response. This is additionally elaborated upon in the footnote below[1] as discussed on p12.
We point out that we used a range of conventional inflammasome activating agents such as silica, alum and uric acid (Fig. 1F). Furthermore, starvation (Figs. 2B; 4B; 5B; EV1C, D; EV1K,L; new Figs. EVJ, K, etc.) yielded results similar to LLOMe.

Although the observation that Stx17 is dispensable for IL1b release and that starvation can induce secretion in a reconstituted system would seem to argue against this possibility, it is important to determine if secretory autophagy has the same genetic requirements under conditions that do not compromise the lysosome. Does secretory autophagy have similar genetic requirements when an inflammasome agonist that does not impact the lysosome such as extracellular ATP is used? Alternatively, analysis of short term LLOMe treatment followed by washout may be a useful strategy.

We used widely utilized inducers of inflammasome (silica, monosodium urate) with important connections to disease. We have used elsewhere and in the present study (e.g. Table 1) the standard inflammasome activator nigericin[2].

4) The results do not support the author's conclusions that secretory autophagy can stimulated by depleting specific cargo receptors that control degradation, such as NCOA4 and p62/SQSM1. It is unclear whether the increased Ferritin and/or IL1b detected in conditioned media is actively secreted via autophagy or a secondary to passive release in cells that accumulate increased amounts of these substrates intracellularly due to cargo receptor deficiency.

We apologize for this unintentional over-statement and lack of clarity. The knockdown of NCOA4 and p62 (receptors for degradative autophagy) was used exclusively for comparison with TRIM16 as a receptor. If these receptors were also utilized for secretory autophagy, their knockdown would have reduced secretion. The results showed no effect (and even went just a bit in the opposite direction, which we overstated). We now restate this on p. 10 to accommodate reviewer's objection.[3,4]

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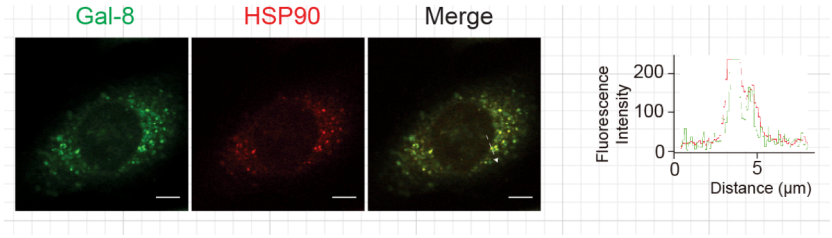
[1] New text (p. 12): *The placement of TRIM16-regulated autophagic processes at the intersection between lysosomal repair Chauhan 2016] and IL-1β secretion (this study) is not coincidental. The central role of lysosomes in control of autophagy by mTOR and TFEB is well known (Napolitano et al, 2016). The new regulatory factors including galectins and TRIM16 as revealed in present study and elsewhere (Chauhan et al, 2016) are also positioned on lysosomes where they can sense damage or signaling changes. From this common station, these factors can direct either autophagic degradation/repair (Maejim et al, 2013; Chauhan et al, 2016) or secretion of inflammatory mediators such as IL-1β (Dupont et al, 2011; Zhang et al, 2015). This is aligned with the known links between lysosomal damage and inflammasome activation (Schroder & 2010) (Hornung et al, 2008)(Martinon et al, 2006)(Ito et al, 2015) and represents a previously unanticipated decision node: On the one hand, autophagy acts to eliminate or repair damaged lysosomal and other endomembranes (Maejima et al, 2013; Chauhan et al, 2016) and thus reduces the endogenous sources of pro-inflammatory stimuli, adding to the repertoire of inflammasome agonists that are downregulated by autophagy (Nakahira et al, 2011; Zhou et al, 2011). On the other hand, when lysosomal repair is not possible or damage exceeds the autophagic capacity to maintain lysosomal homeostasis in the cell, IL-1β secretion facilitated by the same autophagic systems, now in its secretory mode, serves to elicit tissue-level homeostatic responses.*

[2] ATP causes cell death and causes non-specific release. We hope that this is acceptable to the reviewer.

[3] Underlined text: change from "increased" used in the prior version: *"In contrast to TRIM16, a knockdown of NCOA4, which is a ferritin receptor for autophagic degradation of this cargo (Dowdle et al, 2014; Mancias et al, 2014), did not decrease ferritin secretion (Figs 6E and EV6H). Mirroring this observation, knockdown of p62*

5) Aspects of authors' proposed model for autophagy-dependent secretion (depicted in Fig 5D) appear somewhat discrepant with the other key results within the field. Notably, Schekman and colleagues recently proposed a model for IL1b secretion using a reconstituted system in which the substrate is unfolded and transported through the membrane in between the autophagosome membranes (Zhang et al. eLife. 2015; 4: e11205). In the author's model shown here, secretory autophagy substrates are shown both between the double autophagosome membrane and in the lumen of the autophagosome itself, but the author's do not provide direct evidence illuminating either or both of these possibilities. Importantly, is secreted ferritin also loaded into the intra-membrane space of the autophagosome for secretion or is it captured in the autophagosome lumen like classical cargo? Biochemical analysis of the 25K membrane fraction with protease and detergent in cells deficient for ATG2 will help clarify this issue.

We thank the reviewer for the opportunity to address this topic. The following is a direct quote from the paper by Zhang et al., 2015: "...we also found IL-1β localized in the center of the ring structure, where cytoplasmic autophagic cargoes fill, surrounded by LC3 (Figure 6-figure supplement 4). This portion of IL-1β was possibly being engulfed by the autophagosome." The aforementioned study by Zhang et al., includes super-resolution microscopy data showing IL-1β in both the inter-membrane space and in the lumen of autophagic organelles. Thus, our model, as depicted, and our findings do not contradict reports by Zhang et al., 2015. We feel that differentiation between these two modes of capture (if needed, given that our work is compatible with both) are outside the scope of our work and in principle should be studied by the Schekman lab We previously explained these relationships only in a figure legend, for which we apologize, and now discuss this in more detail in the text (p. 13). Prompted by the reviewer's comment, we have nevertheless carried out additional experiments[5], including HSP90, a chaperone shown by Schekman and colleagues to play a role in the processes alluded to by the reviewer, and found colocalization between Galectin-8 and HSP90 in cells treated with LLOMe (new Fig. EV2F p.7), thus showing potential alignment of processes studied by us and by Schekman and colleagues.



Data corresponding to EV2 F. Galectin-8 and HSP90 show similar overall intracellular distribution and colocalize in cells treated with LLOMe. Note: Zhang et al. have recently shown that HSP90 participates in secretion of IL-1β (Zhang et al, 2015).

6) The requirement of Syntaxins 3/4 and dispensability of Stx17 for secretory autophagy suggest that vesicular carriers can directly fuse at the plasma membrane, as the authors have indicated with their depiction in Fig 5D. In contrast, prior work from the Deretic lab indicated that Bafilomycin A1 reduces IL1b secretion, thus suggesting that substrates may transit through an endolysosomal compartment before release. Please clarify the role of the lysosome/ late endosome in this model.

We thank the reviewer for the opportunity to clarify this. Bafilomycin A1 is not a reagent that specifically and exclusively affects autophagosomal maturation. Bafilomycin A1 affects Golgi/TGN function[6], and, as the reviewer pointed out elsewhere, Golgi-localized caspase 1 may be affected.

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previously reported to act as a receptor for degradative autophagy of IL-1ß (Figs EV6I-K)(Shi et al, 2012) did not decrease IL-1ß secretion in response to inflammasome agonist (Fig EV6E)."

[4] Please also note LDH controls, which argue against passive release.

[5] Our repeated attempts with proteinase K analysis of the 25 k membrane fractions, carried out in response to reviewer's request, were inconclusive.

[6] JBC 264 (1989) 18445-18450. Eur J Cell Biol. 74 (1997) 417-23; BBA 1763 (2006) 1017–1023 Glycobiology 11 (2001) 633-644, etc.

However, we have not experimentally studied this further, and instead relied on more specific genetic manipulations available.

Minor Issues:

1) Why does LC3B knockdown impact IL1b secretion if TRIM16 primarily interacts with GABARAP and Sec22b?

LC3B is a key element of early autophagic organelles. Thus, we do not find it surprising that LC3B is important for secretory autophagy. Concerning the second part of the comment re TRIM16, although some TRIMs do not interact directly with LC3B they do colocalize with LC3B (e.g. Fig. 1D), reflecting the role of LC3B in organization of the early autophagic organelles (as reviewed recently in Kimura et al. 2016). In addition, in response to reviewer's comment we have now tested the role of GABARAP and found that GABARAP is important for secretion of IL-1β (Fig 1B and Appendix Figure S1A-D; p. 6).

2) The western blot in Fig. EV1 panel I is extremely washed out on the right side of the IL1b blot and not supportive.

We now provide a less washed image of the same blot (EV1 I, top set), as well as a set of blots from a new experiment (EV1 I, bottom set).

3) Why isn't IL1b detected in the mass spec for autophagy-dependent secretion substrates?

The isobaric tandem mass tag spectrometry depends on peak detection before fragmentation and identification, and lower abundance peaks are missed, thus resulting in many false negatives.

Referee #2:

In this manuscript, Kimura et al. report that TRIM16 associates with IL-1b and is required for unconventional secretion of IL-1b upon treatment of lysosome-damaging agents such as LLOMe or starvation in macrophages. TRIM16 is important for translocation of IL-1b to LC3-II(+) membranes. TRIM16 interacts with galectin-8 and the R-SNARE Sec22b, both of which are required for IL-1b secretion. In addition, these factors are needed for secretion of ferritin, another leaderless cytosolic protein. Although the topic is important and timely, the current data are insufficient to conclude that the TRIM16/galecin-8/Sec22b axis is required for autophagosome-mediated unconventional secretion of IL-1b and ferritin.

We thank the reviewer for exceptionally insightful comments and important suggestions for improvement. We are pleased that the study has elicited so many questions, which we take as a sign of interest. We have tried to address them to the maximum extent possible.

Major comments:

1. The information on the TRIM16(+) structure is not sufficient. In Figure 1C, TRIM16(+)LC3B(+) structures are formed in cells treated with LLOMe, which causes lysosomal damage and lysophagy. Do these TRIM16(+)LC3B(+) structures represent autophagosomes containing damaged lysosomes (lysophagy-related structures)? Do they also contain IL-1b? These TRIM16 structures should be co-stained with IL-1b, phagophore markers, lysosome markers (as substrates), and lysophagy markers (e.g. galectin). These TRIM16 structures should also be characterized by electron microscopy (CLEM or immuno-electron microscopy).

Prompted by the reviewer's request, we now show (new Fig. EV1O, p. 6) that TRIM16 profiles are positive for pro-IL-1β in cells treated with LLOMe, whereas a subset is also positive for LAMP2 (thus overlapping with damaged lysosomes), consistent with the model in Fig. 5F. Additional information on the role of TRIM16 in the context of autophagic repair of damaged lysosomes

(pertinent to other reviewer's questions) is available in a study in press in Developmental Cell that deals with lysosomal homeostasis[7]*. This is now explained on p. 6.*

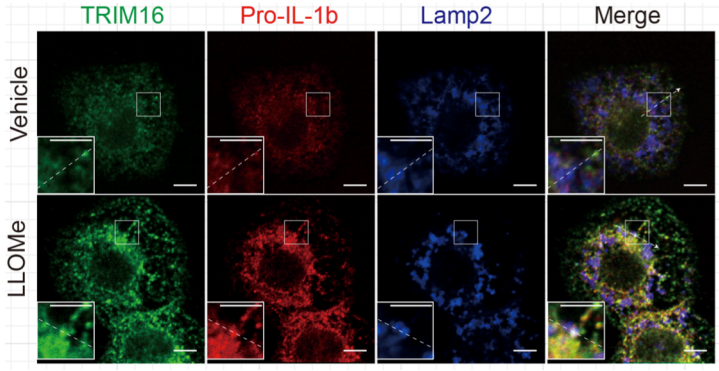


Fig. EV1O. Intracellular colocalization analysis of TRIM16, pro-IL-1β and LAMP2 in THP-1 cells (control and after LLOMe treatment).

2. The structure of starvation-induced autophagosomes and LLOMe-induced autophagosomes should be different. As mentioned above, the latter should contain lysosomes. How can the model in Figure 5 be applied to starvation-induced IL-1b secretion? Do TRIM16 and IL-1b colocalize with lysosomes during starvation? How do TRIM16 and IL-1b translocate from the lysosome to autophagosome/phagophore?

In principle, the reviewer poses an excellent question regarding comparisons between LLOMe and starvation. The key to understanding how starvation may have similar effects to those of lysosomal damage is the convergence of autophagy regulatory systems upon the lysosome, including those that sense and respond to starvation. The Ballabio and Sabbatini laboratories have now established in a series of brilliant publications, that lysosomes are not just degradative organelles but are sensory stations conveying lysosomal and nutritional signals to the autophagy apparatus. In the revised version, this is discussed and referenced more extensively (p. 12&13). Thus, it should not be as surprising that starvation and LLOMe treatment yielded similar results in our work (Figs. 2B; 4B, F; 5B; EV1C,D; EV1K,L), now further confirmed with new data (Figs. 3N, 4F, 5D, EV3E, EV4J,K,P,Q, EV5R, Apendix Fig S1B-D). The transfer of TRIM16-IL-1β from the lysosome to phagophores is a very important question, and we believe is based on sequential TRIM16-Galectin-8 and TRIM16-Sec22b interactions (this is now explained on p. 13).

3. Related to the above comment, the model in Figure 5 is not fully supported by the present data. Are TRIM16 and IL-1b colocalized with phagophore markers such as DFCP1 and ATG5? When and how is Sec22 recruited to the phagophore? Is it dependent on TRIM16? The genetic hierarchy between TRIM16 and galectin-8 should also be investigated. Is the colocalization between LC3B and TRIM16 reduced in galectin-8-knockdown cells?

Sec22b "recruitment" [8] *to LC3-organelles is now further analyzed (please see response to point 5) in new fractionation experiments using TRIM16^{wt} wt and TRIM16^{KO} cells (new Fig. 3F,G; p. 8). Considering the posed question of Sec22b "recruitment" to the phagophore, this has already been indirectly shown (albeit not stated explicitly) in functional studies by the Schekman group (Ge at al 2013, 2014; Zhang et al., 2013)* [9]*. Both the conventional autophagosomal membranes earmarked for fusion with the lysosomes and those that are destined for secretion may be derived from a subset of Sec22b-positive ERGIC-derived autophagosomal precursors (Ge et al, 2014), with the defining*

[7] We note that process of lysophagy has been studied and characterized by the Yoshimori group – Fujita et al., 2013 and Maejima et al 2013 – and is not the focal topic of the present study. However, further details on this role of TRIM16 in autophagic responses and endomembrane homeostasis elicited by lysosomal damage are available in Chauhan et al., 2016. *TRIMs and Galectins globally cooperate and TRIM16 and Galectin-3 co-direct autophagy in endomembrane damage homeostasis*. Dev. Cell In Press.

[8] We think it is the other way around, that Sec22b^{+} membranes acquire LC3.

[9] Citing from Ge et al 2013: "The lipidation activity *(of LC3)* was distributed in fractions two through four which co-distributed with SEC22B …(Figure 7),"

events separating the downstream organelles being syntaxin-17-dependent differentiation. We might have not explained this in full, and now additionally elaborate on this in Discussion (p. 13).

Furthermore, our new data (Appendix Figure S3B,C), generated as additional controls in response to other reviewers' requests, indicate dependence on TRIM16 for Sec22b's action in IL-1β secretion (p. 9). Although this was not the focus of our study, prompted by the reviewer we have tested whether GFP-Atg5 (construct form Dr. Mizushima) and endogenous TRIM16 colocalize in 293T cells and found no evidence of colocalization. Similarly, GFP-DFCP1 and endogenous TRIM16 did not colocalize in these standard time-point experiments used with other markers. However, we cannot fully exclude that at an earlier time point these markers may be colocalized or juxtaposed. Atg5 plays a very early role in autophagic response to membrane damage, being recruited at 15 min as per the excellent studies by Yoshimori and colleagues (Fujita JCB 2013). At that time point, while likely active, TRIM16 does not make profiles large enough to be detected (similarly, at 30 min, cells are negative for discernible TRIM16 puncta) and shows visible profiles only at 45 min of treatment with LLOMe. The difficulties in visualizing TRIM16 structures early in the process preclude us from making firm conclusions related to Atg5 and DFCP1. The colocalization between TRIM16 and LC3B does not depend on Gal8 (Appendix Figure S2C,D), and it is likely that the TRIM16-Gal3 interactions (Chauhan et al., 2016; please see response to point 1) act redundantly in these processes.

4. If STX17 does not affect IL-1b secretion, are Sec22b(+) autophagosomes negative for STX17? In other words, are Sec22b and STX17 indeed mutually exclusive? This is extensively discussed in the Discussion part, but there is no supporting data.

We apologize for overstating this, and have modified the discussion accordingly (p. 12) [10].

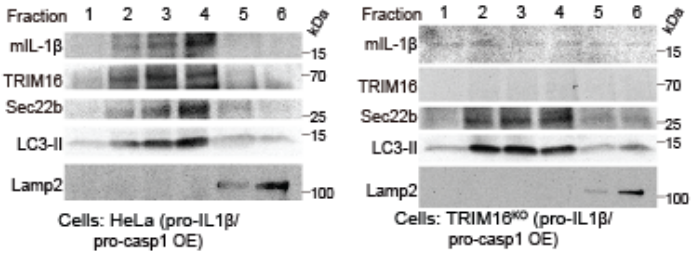


Fig. 3 F,G (new data). Sucrose density gradient analysis of 25k membranes. Note that mature IL-1β (mIL-1β) is recruited to and co-fractionates with TRIM16^{+}, Sec22b^{+}, and LC3-II^{+} membranes in TRIM16^{wt} HeLa cells reconstituted with pro-IL-1βand pro-caspase 1, but not in TRIM16^{KO} mutant cells. Input control (whole cell lysate) is in panel E of Fig 3 (not shown here).

5. The biochemical data in Figure 3A-3C are important and need to be investigated more vigorously. The distribution of TRIM16, IL-1b, and Sec22b in wild-type and TRIM16 KO cells should be analyzed using a density gradient with markers of major organelles. A proteinase protection assay should also be performed to determine whether TRIM16, IL-1b, and Sec22b are present inside membranes. Immunoblotting of both m- and pro-IL-b should be performed using whole cell lysates and culture media of wild-type and TRIM16 KO cells. If TRIM16 is indeed required for secretion but not for maturation of IL-1b, mIL-1b should accumulate.

As requested, we have now performed density gradient separation of membranous organelles in TRIM16^{wt} and TRIM16^{KO} cells. The data (new Fig. 3F,G, p. 7-8) indicate that in TRIM16^{wt} cells, mIL-1β, TRIM16 and Sec22b co-fractionate with LC3/autophagosomes and are separated from lysosomes (LAMP2). In the absence of TRIM16 (TRIM16^{KO} cells), mIL-1β is no longer enriched in Sec22b^{+} LC3^{+} fractions.

[10] The sorting step between LC3^{+} Sec22b^{+} IL-1β secretory carriers and ERGIC-derived (Ge et al, 2014) autophagosomal membranes destined for degradative autophagosomes could involve differential syntaxin-17 insertion (Itakura et al, 2012) or control of its subsequent fusion steps with lysosomes (Diao et al, 2015; Hamasaki et al, 2013; Itakura et al, 2012; Takats et al, 2014) if it is present on IL-1β secretory carriers, which has not been excluded in our studies.

As to the question of whether TRIM16, IL-1β and Sec22b are in the inter-membrane space or in the inner lumen of autophagosomes, we emphasize that this is neither a goal of our studies nor do our conclusions depend on these issues[11]. This interesting process has been reported and studied by Schekman and colleagues. In our attempts to satisfy the reviewer, we have carried out proteinase K protection assays but the results were inconclusive. Since our study is not focused on this phenomenon, we hope that the reviewer will agree that while very intriguing these phenomena are not directly relevant for the specific relationships studied in our work, and neither is contradicting each other. Nevertheless, we carried out new experiments with Galectin-8 and HSP90 (HSP90 being implicated in the transport process studied by Zhang et al., 2015) and found that they colocalized in cells treated with LLOMe (new Fig. EV2F), indicative of a possible convergence between the two processes (with outcomes illustrated in Fig. 5F). We now discuss this more extensively (p. 13).

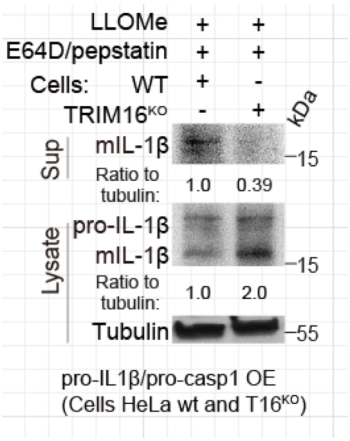


Fig. EV3 E (new data). As anticipated by Reviewer 2, mIL-1β does accumulate in the cytoplasm of TRIM16 KO cells.

As requested, we have carried out immunoblotting analysis of cell lysates and culture media with TRIM16^{wt} and TRIM16^{KO} cells. We found absence of mIL-1β in secreted fractions from TRIM16^{KO} cells and accumulation of mIL-1β in TRIM16^{KO} cell-lysates (new Fig. EV3D, p. 7).

6. The quality of the immunofluorescence data is generally not high. Why is the pattern of TRIM16 so different between Figure 1C and Figure 2F?

In Fig. 2F the background is higher than in Fig. 1C due to different antibody pairing and staining, but the TRIM16 pattern is not much different. Additional analyses of TRIM16 patterns in LLOMe-treated cells can be seen in Chauhan et al. Dev. Cell in press[4].

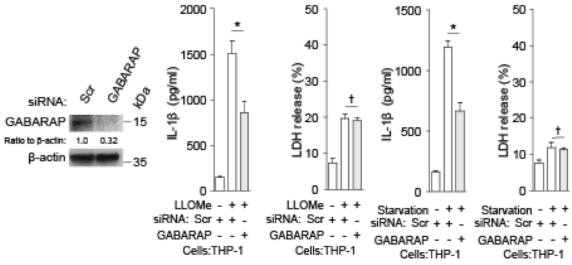
7. Is there reduced colocalization between IL-1b and LC3B in TRIM16 KO cells? This will be another clue to support the authors' hypothesis.

As requested, we carried out high content analysis of IL-1β-LC3 colocalization in TRIM16^{wt} and TRIM16^{KO} cells and found that TRIM16 is required for colocalization of IL-1β and LC3 (Appendix Fig. S3A, p. 7). Furthermore, new biochemical data with subcellular fractionation of intracellular membranes, generated in response to reviewer's comment 5, show that in TRIM16^{KO} cells IL-1β is no longer enriched/focused on LC3-II^{+} membranes (Fig. 3F,G, p. 8).

8. Figure 1A: The authors should determine the requirement of other ATG factors such as GABARAP.

As requested, we tested GABARAP for its effects on IL-1β secretion. As shown in new Fig. 1B and Appendix Figure S1A-D, knocking down GABARAP reduces IL-1β secretion, in keeping with the previously reported TRIM16-GAPARAP interactions (Mandell et al, 2014). This is now described on p. 6.

[11] We nevertheless thank the reviewer for the opportunity to address this topic. The following direct quote from the paper by Zhang et al., 2015 indicates that IL-1β was both in the intermembrane and in the inner lumen of autophagosomes: "…*we also found IL-1β localized in the center of the ring structure, where cytoplasmic autophagic cargoes fill, surrounded by LC3 (Figure 6-figure supplement 4). This portion of IL-1β was possibly being engulfed by the autophagosome*."



Figs 1B and Appendix Figure S1A-D (new data). GABARAP is required for optimal IL-1β secretion.

The requirement of ATGs should also be determined in ferritin secretion in Figure 6.

We already tested the requirement for LC3B (the most commonly used ATG) in ferritin secretion (Fig. 6C, last bar in the panel). We now underscore this further in the text (p. 10) as it was not mentioned in the text, for which we apologize. Additionally, secretory autophagy of ferritin was discovered in this work by using Atg5 KO cells (Table 1).

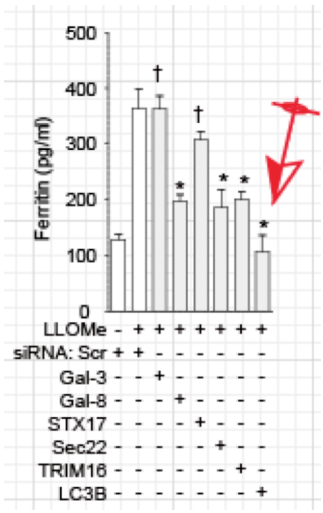
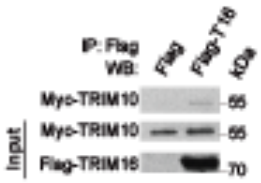


Fig. 6C. Note that ferritin secretion depends on LC3B. This is now emphasized in the text. Please also see Table 1 for ferritin secretion dependence on Atg5.

9. Figure 1B: The authors show that knockdown of not only TRIM16 but also TRIM19 or TRIM10 causes a reduction of IL-1b secretion. Do TRIM10, TRIM16, and TRIM19 function redundantly? This can be tested by double- or triple-knockdown. The interaction of TRIM10 and TRIM19 with IL-1b and ferritin should also be analyzed.

This study is focused on identification of the first receptor (which also functions as a regulator) for secretory autophagy, TRIM16. We hope that the reviewer will agree on the significance of this alone, and that the amount of work requested on other TRIMs requires many months (perhaps a year) of study to properly examine their roles and interplay. We nevertheless include new data on the interaction between TRIM16 and TRIM10, new Appendix Figure S1G, and discuss this in the text (p. 6-7).



New Appendix Figure S1 showing potential interactions between TRIM16 and TRIM10.

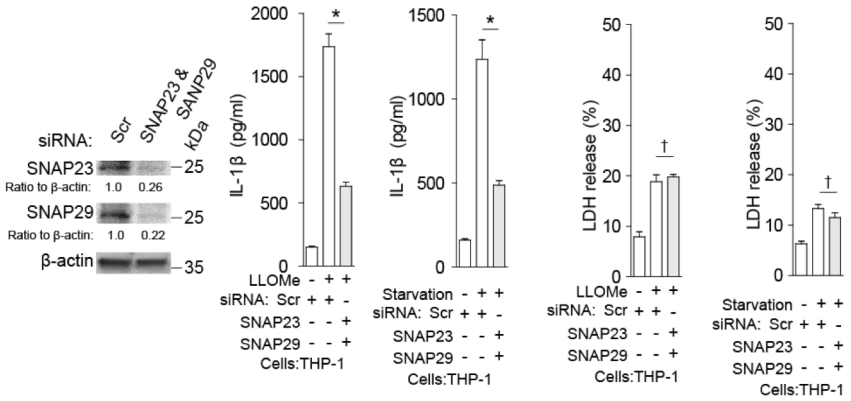
10. Figure 2B: The function of galaectin-8 in starvation-induced IL-1b secretion is totally unclear. Are lysophagy-related structures (galectin-8-positive structures) formed by starvation? At which step is galectini-8 required?

"Lysophagy-related structures" (the reviewer most likely refers to the lysophagy work by Yoshimori and colleagues) are formed by Galectin-3 (Chauhan et al., Dev Cell in press). Galectin-8 specializes in secretion. This is now further experimentally addressed using bone marrow derived macrophages from Galectin-3 and Galectin-8 transgenic mice (new Appendix Figure S2A, B) and described in the text (p. 7)[12].

[12] We agree with the reviewer, and believe that the role of galectins in starvation induced autophagy poses a fascinating question, and could open new avenues in understanding how the lysosomally localized systems, such as mTOR, TEFB, etc., act in the sensing of lysosomal functionality in starvation (calcineurin currently being one known mediator) including its potential membrane integrity. Understanding these processes requires at least a year or more of appropriate work to understand whether and how the lysosomal integrity is affected during starvation and whether this is relayed via galectins or some other systems to regulators of autophagy.

11. Figures 4 and 5: The involvement of Sec22b in the "unconventional secretion" of IL-1b and ferritin is not directly shown. Does IL-1b accumulate in LC3(+) structures in Sec22b-, SNAP23-, or SNAP29-knockdown cells (due to a defect in fusion with the plasma membrane)? What about the effect of double knockdown of SNAP23 and SNAP29 on IL-1b secretion?

As requested by the reviewer, we carried out SNAP23/SNAP29 double KD. These data are shown in new Fig. 5C,D and Appendix Figure 3A-C and are discussed in the text (p. 9).



Elements of Fig. 5C,D and Appendix Figure 3A-C (new data) showing results with the requested double knockdown of SNAP23 and SNAP29 and their effects on IL-1β secretion.

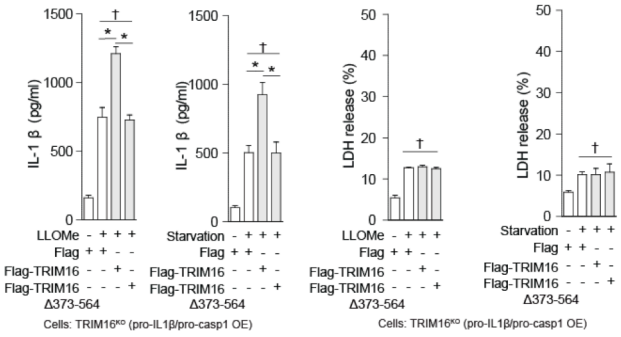
The question regarding accumulation of IL-1β in suggested knockdown cells is compounded in principle by the competition between secretory and degradative autophagy. Downregulating a secretory autophagy SNARE may not necessarily cause accumulation but rather accelerate degradation of the cargo (both IL-1β and ferritin were initially reported as cargo for degradative autophagy). While the interplay between degradative and secretory branches of IL-1β biology poses an interesting series of questions, they are in our opinion outside of the scope of the present study and represent a separate line of inquiry.

12. This study mostly relies on siRNA but off-target effects are not ruled out. Furthermore, in Figure 3H, the authors should perform a rescue assay using TRIM16-knockdown cells expressing wild-type TRIM16 or a TRIM16 mutant defective in the interaction with Sec22b to show the significance of the TRIM16-Sec22b interaction.

Following the request by the reviewer to carry out complementation, we have complemented the TRIM16 KO with TRIM16 (new Fig. 3M,N and Fig. EV3H,I).

Findings with additional knockout mutants (and not just siRNA knockdowns) are now available using bone marrow derived macrophages from Galectin 8 and Galectin 3 knockout mice (new data, Appendix Figure S2A, B).

13. Figure 3I: This reviewer does not understand what the authors want to express with the statement "co-clustering of Sec22b and TRIM16 in cells with a characteristic separation of 70 nm".



Complementation of TRIM16^{KO} mutant (new; Fig. 3, EV3).

The data are inconsistent with the idea that Sec22b and TRIM16 are present on the same structures as shown in Figure 5D.

We apologize for having used this confusing term[13]. This simply reflects the physical measurements using super-resolution for colocalization studies. The 70 nm characteristic proximity (to use that word instead of separation) is very much consistent with close colocalization and presence of Sec22b and TRIM16 on the same structures. We now corrected the statement "co-clustering" to colocalization.

14. Figure 6: The intracellular localization of NCOA4 should be determined in wild-type, siSec22b, and siTRIM16 cells.

NCOA4 is a published receptor for autophagic degradation of ferritin. Two papers in Nature and Nature Cell Biology (Dowdle et al., 2014; Mancias et al., 2014) have already extensively dealt with the functions and localization/distribution of NCOA4 as a degradative autophagy receptor. Our data show that NCOA4 is not involved in secretory autophagy of ferritin. Thus, the purpose of gathering data on NCOA4 intracellular localization in cells knocked down for Sec22b and TRIM16 would appear unjustified when the finding show that NCOA4 is not a part of this pathway. This is now clarified in the text (p. 10).

Minor comments:

1. The LDH data in Figure EV1 are not explained in the text. Why does LLOMe cause LDH release?

LDH is used as a control for nonspecific release of cytosolic components. This is now explained on p. 6 in association with Figs 1 and EV1 where its use is first mentioned.

2. There are no figures to refer to for the following statement in the last paragraph of the Result section "Similarly, only increased IL-1b secretion in response to inflammasome agonist (Fig EV6E)."

We apologize for the unintentional mistake during editing – this is a beginning the sentence that should have been together with the (now modified) statement immediately following it. This is now corrected (p. 10). Thank you.

3. Figure 2: The interaction between galectin-8 and TRIM16 should also be tested in vivo.

We are sorry if this was not clear, but we did carry out co-IPs (in cell extracts, i.e. by analyzing the complexes existing in vivo) using both overexpressed proteins in vivo and endogenous proteins in vivo (Fig. 2D and E). On the off chance that the reviewer meant "in vitro" (i.e. in GST pull-down assays) we now moved a previously shown GST pulldown from its former location in Fig EV2 to the main figure (Fig. 2G).

4. Figure 3: Why did the authors focus on Sec22b? Any specific reason?

We apologize if this was not clear, although we did provide a rationale and references in the subsection "R-SNARE Sec22b interacts with TRIM16 and is required for IL-1β secretion". We now elaborate our rationale more extensively as described in individual points above (see also footnote #8) and now additionally clarified in the text (p. 8).

5. Figure 5D: LC3 should interact with TRIM (Mandell et al. 2014).

TRIM16 is on the same membranes as LC3B, but direct interaction has been demonstrated only for GABARAP, as per Mandell et al., 2014, and this is why Fig. 5D (now Fig. 5F) is composed as presented.

[13] We apologize for too precise of a statement, which indicates that TRIM16 and Sec22 colocalize at a distances of 70 nm. See the Gaussian distribution, g(r) 1.5, 1.5, 2.2 when r(nm) is 60, 70, 80. Thus, the distribution is not a random one and these two proteins are always at the distance of 70 nm.

Referee #3:

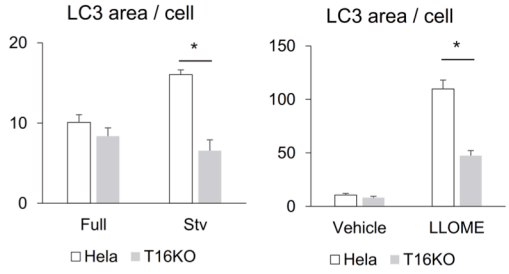
The proinflammatory cytokine Interleukin 1beta lacks a canonical N-terminal signal sequence, and is secreted by an unconventional pathway that has been intensely investigated. Previous work, including a seminal contribution from the corresponding author's group (Dupont et al:, EMBO J., 2011), has implicated the autophagosome as an intermediate carrier leading to IL-1beta secretion. In the submitted manuscript, the authors attempt to identify the players of this secretory autophagy pathway, identifying TRIM16 as a central component. The inhibition of IL-1beta secretion after TRIM16 knockdown, the interaction of the cytokine with TRIM16, as well as an increase in its secretion upon TRIM16 overexpression, had all been previously reported by another group (Munding et al., Cell Death Diff., 2006). Here, the authors investigate TRIM16 binding partners, and find that it interacts both with galectin 8 and with Sec22b; furthermore, knockdown of Sec22b inhibits IL1-beta secretion, while leaving autophagic flux unaltered. They propose that TRIM16 provides a platform for recruitment of IL-1beta to a Sec22b-containing sequestration compartment; because of its molecular composition the resulting cytokine-containing autophagosome avoids fusion with the lysosomes, and instead engages in exocytosis triggered by the interaction of Sec22b with plasma membrane SNAREs. The authors further identify ferritin as a protein that follows the same TRIM16-dependent pathway for secretion.

The findings of this manuscript are of high interest; however, as detailed below, it is not easy to place them in the context of the myriad players of autophagocytosis; in other words, it was not clear to this reviewer whether the observed effects of knockdown of candidate players on IL-1beta secretion were always due to interference with the specific autophagic secretory pathway or rather to effects on autophagocytosis in general. Several points that appeared unclear to this reviewer would have deserved at least come comment. Importantly, the authors should have more clearly informed the reader of what new information their study brings versus what has already been published (Munding et al., 2006).

We thank the reviewer for the overall assessment, and for the time and thought that went into the review. As requested, we now elaborate more clearly on the advance relative to the work of Munding et al. Although Munding et al., (Munding et al, 2006) observed that TRIM16 binds IL-1β, their report did not address the process of secretion. Our findings show that in addition to binding to IL-1β, TRIM16 delivers IL-1β to the secretory autophagy pathway and together with specific SNARE apparatus carries out the process of secretion of IL-1β. This is now explained (p. 13).

Finally, there are a number of technical issues that should be addressed before the article is suitable for publication.

1. The problem of interference with secretory autophagy vs interference with autophagy in general: In Fig. 1, the authors show that knockdown of a number of proteins of the TRIM family lead to decreased IL-1beta secretion. Of these, TRIM16 was previously shown to interact with IL-1beta (and confirmed here). One would like to know whether autophagy in general is affected by these TRIMs (as done in Fig. 4 for Sec22b depletion); in this way it might be possible to distinguish TRIM16's effect from that of the other TRIMs. The same question comes to mind for Galectin-8. The specific effect of Galectin-8 depletion vs no effect with Galectin-3 is interesting. Again, one would to know whether any previous work has shown functional differences for these two galectins, and what effect Galectin-8 depletion has on autophagocytosis in general.



The role of TRIM16 in general autophagic response in collaboration with Galectin 3 is in press in Developmental Cell, with an excerpt shown in the panels to the left. That study shows that TRIM16 in combination with Galectin 3 controls lysophagy/ autophagic lysosomal repair and homeostasis, and in more general terms, controls TFEB activation state by mTOR and calcineurin leading up to nuclear

translocation of TFEB. However, as shown in the present work, TRIM16 in combination with Galectin 8 controls a different process – secretory autophagy. We now obtained additional data (new Appendix Figure S2A,B) in primary macrophages from Galectin 3 and Galectin 8 knockout mice. This is discussed on p. 7.

2. Unclear points:

2a. In the knockdown experiments, there was at most a 65% inhibition of IL-1beta secretion. TRIM16 knockdown itself leads to about 40% inhibition. Is this incomplete effect due to incomplete silencing? Or can it be explained by the existence of alternative TRIM16-independent pathways for IL-1beta secretion? The extent of silencing was never quantified.

The contributing causes may be incomplete knockdowns, contribution of nonspecific release from cells that is independent of TRIM16, and finally alternative TRIM16-independent pathways as suggested by the reviewer. As requested, we now show extent of silencing in all figures, throughout.

Also, no rescue experiments were done to rule out off-target effects; at least, the authors should demonstrate that siRNAs targeting different regions of the same mRNA have the same effect. These controls need to be done.

We point out that the study does not rely entirely on siRNA knockdowns and that we have also generated TRIM16 CRISPR knockouts, confirming siRNA results. Nevertheless, to comply with reviewer's request for genetic complementation, we have carried such experiments by complementing the TRIM16 CRISPR knockout (new Fig. 3M,N; p.8).

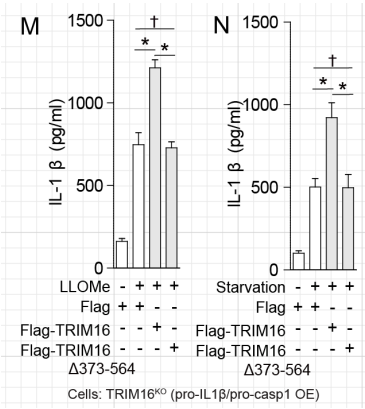


Figure 3M,N (new data). Complementation of TRIM16^{KO} cells with wild type TRIM16 and absence of complementation with TRIM16 mutant that cannot bind Sec22b.

2b. An interesting finding is that IL-1beta secretion is dependent on Sec22b but not on Syntaxin 17; the latter is a SNARE required for fusion of autophagosomes with lysosomes, an event avoided by IL-1beta secretory autophagosomes. It is, however, surprising that observe an inhibitory effect of SNAP29 knockdown is observed (Fig. 5), as this SNARE is thought to collaborate with Syntaxin 17 in the fusion of autophagosomes with lysosomes. This should be commented.

We thank the reviewer for the opportunity to clarify. SNAP29 is a Qbc SNARE that can combine with various R- and Qa-SNAREs, and is not exclusively used in combination with syntaxin-17. It's more classical function in fusion is in association with syntaxins other than syntaxin-17, including those at the plasma membrane (syntaxin-3, syntaxin-1)[14], known long before the description of its more recently uncovered function in combination with syntaxin-17 during autolysosome formation[15].

[14] J Biol Chem. 1998 Dec 18;273(51):34171-9. Steegmaier M1, Yang B, Yoo JS, Huang B, Shen M, Yu S, Luo Y, Scheller RH.

[15] Although SNAP29 interacts with plasma membrane syntaxins, thus far it has been noted for its regulatory (and even inhibitory role (Su, 2001; Morelli, 2014). These complications could apply to all its roles including in autophagosome-lysosome fusion. However, in all cases it is also likely to play a direct or regulatory role in fusion events (Xu, 2014). To make it even more complicated, SNAP29 activity is controlled by recently described O-Clc-NAc-modifications, which are modulated by starvation (Guo, 2014). Nonetheless, SNAP29 is

We thank the reviewer for calling for further discussion on SNAP29, which is now expanded to a whole paragraph in Discussion (p.13). Additionally, we have addressed this with more experiments, performing double SNAP23 and SNAP29 knockdowns, showing that a combined SNAP23+SNAP29 knockdown has additive effects on secretion of IL-1β (Fig. 5C,D, p9).

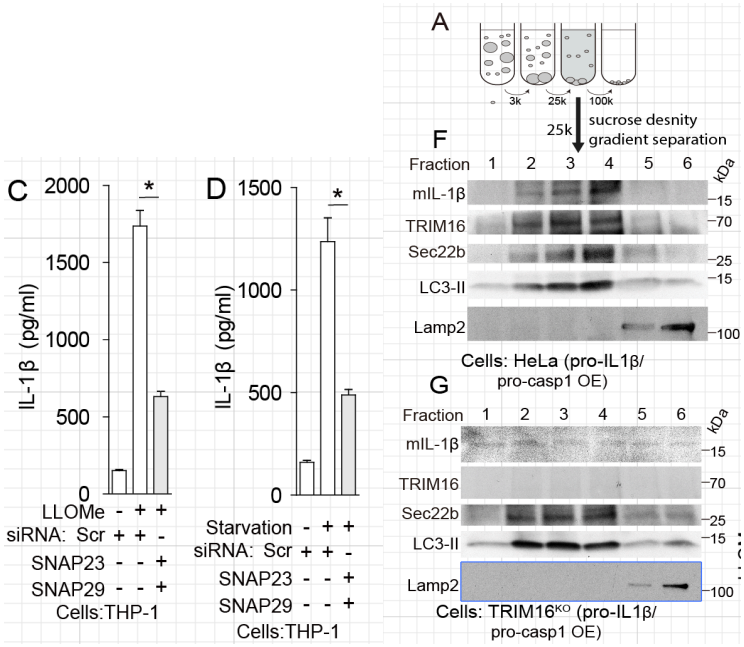
2c. In Fig. 3F and G, evidence is presented for the presence of Sec22b and TRIM16 in the same macromolecular complexes; in the model of Fig. 5, the two are depicted as directly interacting. In Fig. 3I and J, the authors show that Sec22b and TRIM16-containing structures are close to each other (70 nm distance) but do not co-localize. The two observations are contradictory. If the two proteins interact, they should co-localize. From the images of Fig. 3, it seems they are in distinct structures.

Super-resolution at 70 nm is consistent with direct interaction or close proximity in common macromolecular complexes. In principle, two proteins/objects cannot be in the same place at the same time, and thus can never show a complete overlap by super-resolution microscopy. This is now clarified in the text (p. 8).

3. Technical issues.

3a. The quality of many of the blots is poor. Number of repetitions of experiments is nowhere specified. Inputs are shown, but at what percentage input has been loaded compared to the LPS is not stated.

We thank the reviewer for asking for clarification. We have already provided the number of repetitions in Statistical analyses as follows: n≥5, except for immunoblot quantifications where n≥3. We now restate this in figure legends for easier access by the reader. In immunoprecipitation analyses, 10% of extract was used for were loaded as inputs, which is now clarified in the Supplementary Materials and Method section.



Left: Fig 5C,D.(new data). Additional analysis of IL-1β secretion using double SNAP23 and SNAP29 knockdowns.

Right: Fig. 3F,G (new data). Membrane separation in density gradients. Note that mIL-1β cofractionates with Sec22b and LC3-II only in wild type TRIM16 cells but not it TRIM16KO cells. For equal expression of pro-IL-1β in these cells, see Fig. 3E in response to the next question by the reviewer.

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required in *C. elegans* for transport of the apical- and basolateral-directed cargos and when absent accumulates cargo-containing vesicles in the cytoplasm (Sato, 2011), whereas in humans its deficiency has secretory defects (Sprecher, 2005).

3b. The Methods and Figure Legends are insufficient for full comprehension of what has been done. On p. 13, the authors state that they give full information on super-resolution fluorescence microscopy analysis in the Supplementary Experimental Procedures, but this file was not available to me. An example of lack of clarity is given by Fig. EV3, panel C. Why are the TRIM16 bands duplicated (upper boxed panel, and middle panel)? Why is A9 shown twice?

We apologize for the absence of supplementary information (it was uploaded separately). We have uploaded all parts, and hopefully this detailed information will be available to the reviewer in the Appendix, as per the EMBO J rules. We have removed duplication of blots, objected to by the reviewer.

3c. The results of the fractionation experiment (Fig. 3A-C) are weak. The quality of the blot of Fig. 3B is so poor that one wonders how the quantitative data of Fig. 3C could be obtained. Also, recoveries of the analyzed molecules in all the fractions are not given. If there is less IL-1beta in the 25k fraction in the TRIM16 KO cells, is there more of it in the other fractions? Or is there a lower level of expression of the cytokine in these cells?

To address reviewer's comments more comprehensively, we have performed additional density gradient separations of membranous organelles in TRIM16^{wt} and TRIM16^{KO} cells. The results (new Fig. 3F,G, p. 8) show that in TRIM16^{wt} cells mIL-1β is focused on and co-fractionates with Sec22b^{+} LC3-II^{+} membranes (where TRIM16 also partitions). In the absence of TRIM16 (TRIM16^{KO} cells), mIL-1β is no longer enriched in Sec22b^{+} LC3^{+} fractions, with traces of it diffusely (and likely nonspecifically) spread over the gradient. The cytokine (pro-IL-1β) expression was equal in these experiments, as now shown in Fig. 3E. This is now stated in the text (p. 8).

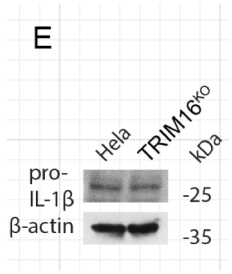


Fig. 3E (new). Equal expression of pro-IL-1β in wt and TRIM16^{KO} cells used in fractionation experiments.

3d. Co-localization of TRIM16 with LC3B (Fig. 1C) and with Galectin-8 (Fig. 2F) in non-stimulated and LLOMe-stimulated cells: the most striking effect of LLOMe seems to be an increase in TRIM16 expression (provided that the non-stimulated and stimulated cells have been acquired with the same microscope settings). The line plots between non-stimulated and stimulated cells are not readily comparable, as in the former condition, lines do not go through LC3 puncta (which are present, although to a lesser extent than in LLOMe-stimulated cells).

The expression of TRIM16 does not change, but rather it forms puncta in response to lysosomal injury, as in the case of LLOMe treatment; in resting cells, the majority of TRIM16 is diffuse in the cytosol, and thus in confocal images appears like there is less of it. We have now modified presentation to reflect some of the comments by the reviewer, and included additional images in Appendix Fig. S1E, F. Clearly, TRIM16 is in a dynamic relationship relative to damaged lysosomes where it assists lysophagy or lysosomal repair (described in detail in a study in press in Developmental Cell), whereas its second function –the topic of the present study— is to load activated mIL-1β on Sec22b^{+} LC3-II^{+} membranes. We also hope that the further intracellular membrane separation experiments, showing that TRIM16 and LC3-II membranes co-fractionate (in response to point 3c above), will convince the reviewer of these relationships.

3e. On p. 9, the authors state that TRIM16-dependency is not observed for ferritin secretion in cells infected with the ESX-1 mutant of Mycobacterium tuberculosis Erdmann, but this is not apparent from the data presented in Fig. 6D: from Fig. 6D it is clear that there is less ferritin secretion in ESX-1 infected cells silenced for TRIM16, for Sec22b or for Gal-8 compared to the scrambled control.

We thank reviewer for this observation (which reflects that in all likelihood M. tuberculosis affects phagosomal membrane in several ways). We have re-worded the statement accordingly (p. 10): "This pattern of dependence for ferritin secretion on TRIM16, galectin-8, and Sec22b, was furthermore observed in response to a physiological endomembrane injury during infection of

macrophages with Mycobacterium tuberculosis Erdman (Figs. 6D and EV6C-G). As expected, ferritin secretion was reduced when M. tuberculosis Esx-1 mutant, which permeabilizes phagosomal membrane less efficiently (Manzanillo et al, 2012), was used instead of the parental wild type Erdman strain (Figs. 6D and EV6C,F,G)." Again, whereas Esx-1 system is the best studied one in M. tuberculosis, this microbe possesses multiple related Esx systems, and the continuing dependence even in the Esx-1 mutant upon TRIM16, galectin-8, and Sec22b most likely reflect the fact that M. tuberculosis can affect the phagosomal membrane in several ways.

3f. The manuscript has been prepared without much care. For instance, two sentences on p. 9 ("Mirroring this observation.... agonist") are incomprehensible. In the bibliography, a number of references are incomplete.

We thank the reviewer and apologize for the incomplete sentence that occurred during editing. This is now corrected. We have also updated the bibliography.

***INVENTORY OF NEW DATASETS ADDED IN REVISION:

Grand total: **45** datasets

Main figures
Fig. 1 (1 panel) B: IL-1β in GABARAP KD ; total: 1
Fig. 2 none
Fig. 3 (1 panel) E: Pro-IL-1β from whole cell for fractionation; (2 panels) F,G: Sucrose gradient fractionation for 25 fractions from wild type and TRIM16 knockout; (2 panels) M,N Complementation analyses with Sec22b nonbinding TRIM16 mutant in TRIM16 KO cells. ; total: 5
Fig. 4 none
Fig. 5 (2 panels) C,D IL-1β in SNAP23 and SNAP29 double KD ; total: 2
Fig. 6 none

Extended view figures
EV1(1 panel) A: LC3-II KD blot was replaced; (1 panel) I mIL-1β in supernatants from T16KD (1 blot was replaced, another set of experiment was also added); (1 panel) O: Confocal images of T16/pro-IL1β colocalization, with Lamp2 (Lamp2:Blue was additionally merged) ; total: 3
EV2 (1 panel) F Confocal microscopy of Gal8/HSP90 colocalization; total: 1
EV3 (1 panel) D: IL-1β in lysates/processing; (2 panels) H,I LDH release for new Fig 3M,N; total: 3;
EV4 (4 panels) H-K: Atg9 MEFs; (2 panels) L,M Sec22bKD mIL-1β; (4 panels)N-Q Sec22b OE-mIL-1β; total: 10;
EV5 (2 panels) R,S: Stx3,4 effect on LC3; total: 2
EV6(1 panel) H LDH release for Fig6E (This was not asked by the reviewer, but I noticed to have forgotten to add this in the original submission.) ; total: 1

Appendix supplementary figures
Appendix S1 (4 panels) A-D: IL-1β, LDH release and KD efficacy in GABARAP KD (Fig 1B); (2 panels) E,F: Confocal images for TRIM16/LC3; (1 panel) TRIM16 and TRIM10 interaction; total: 7
Appendix S2(2 panels) A,B: IL-1β in Gal3 and Gal8 knockout BMM; (2 panel) C,D: TRIM16/LC3 colocalization upon gal8KD; total: 4
Appendix S3(1 panel) A: IL-1b and LC3 colocalization by high content analysis (2 panels) IL-1β in wild type or TRIM16 KO cells complimented with Sec22b; total: 3
Appendix S4 (2 panel) A,B: LDH release for Fig 5C,D; (1 panel) C: Double KD efficacy for Fig 5C,D; total: 3

We close by thanking the reviewers for posing exceptionally probing questions resulting in 45 datasets generated in direct response to reviewers' criticisms. We hope that the results of these experimental efforts will be perceived as constructively answering all comments posed by the reviewers. Thank you for your consideration.

2nd Editorial Decision 10 October 2016

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen again by the three original referees whose comments are enclosed.

As you will see, a few issues still have to be addressed before we move forward towards publication. Most concerns mentioned by referee #2 and #3 can be addressed in a point-by-point response and by text changes in your manuscript, a few need inclusion of additional data. It would be good to add the EM data referee #2 requests (comment #1). You already might have some data at hand to address this point. It would also be good to provide additional insight in support of a dedicated pathway for secretory autophagy versus degradative autophagy (Referee #3, 'previous point 1').

I would thus like to invite you to provide a final version of your manuscript and a point-by-point response addressing the remaining concerns of the referees.

Please let me know in case you have questions regarding this revision. I am looking forward to receiving the final version of the manuscript!

REFEREE REPORTS

Referee #1:

I have read the revised manuscript and the authors' point-by-point response. My previous concerns have been largely addressed and I remain enthusiastic about the work overall. The manuscript provides important new insights into our understanding of secretory autophagy.

Referee #2:

Previous comment #1

In the previous review, this reviewer suggested that "these TRIM16 structures should also be characterized by electron microscopy (CLEM or immuno-electron microscopy)". The authors' response is "additional information on the role of TRIM16 in the context of autophagic repair of damaged lysosomes (pertinent to other reviewer's questions) is available in a study in press in Developmental Cell that deals with lysosomal homeostasis." However, this reviewer could not find any additional information about the ultrastructure of TRIM16-IL1b-LC3B(+) structures in the Developmental Cell paper. The ultrastructural information is important to prove whether TRIM16-IL1b-LC3B(+) structures are indeed autophagosomes.

Previous comment #2

This study still heavily relies on the use of the lysosomotropic agent LLOMe. This reviewer continues to have a concern that the mechanism reported here could be specific to lysosome damage-induced IL-1b secretion as shown in Fig. 5F (rather than general IL-1b secretion). This concern is also raised by Reviewer #1. This Reviewer suggests that the title and abstract, which are currently too general, should contain the information that this study mostly analyzes lysosome damage-induced unconventional secretion.

Previous comment #3

The authors show that the colocalization between TRIM16 and LC3B is not inhibited in galectin-8 knockdown cells. The authors argue in the rebuttal letter that this may be mediated by galectin-3. However, depletion of galectin-3 does not affect IL-1b secretion. How can the IL-1b secretion be reduced by knockdown of galectin-8 (Fig. 2BC) despite the normal recruitment of TRIM16? This discrepancy should be explained.

Others:

After reading the paper by Chauhan et al. that has just been published by the same group in Dev Cell, this reviewer realizes that there are significant overlaps and inconsistencies between the Dev Cell and present papers.

1. The present manuscript shows that Galectin-8 interacts with TRIM16 (Fig. 2G), whereas Chauhan et al. state that TRIM16 does NOT bind Galectin-8 (Fig. 1A). This discrepancy needs to be explained.

2. Colocalization of TRIM16 with LC3 (Fig. 1D) and Lamp2 (Fig. EV1O) have been shown in Chauhan et al. These should be mentioned in the present manuscript to avoid giving an impression that this is the first report.

3. TRIM16 knockout HeLa cells were described in Chauhan et al. Is the same clone used in the present study?

Referee #3:

The revised version of the manuscript by Kimura et al. contains much additional work, and many of my initial concerns have been addressed. Nevertheless, some modification of the manuscript is required before publication.

Citation of Munding et al: the authors have added a sentence in the discussion (p. 13) to give more credit to this previous study. Their sentence ("Although Munding and colleagues have shown that TRIM-16 binds IL-1beta and is co-secreted with this cytokine, their observations lacked an overarching mechanism") does not give full credit to the work of Munding et al., as these authors also showed that knockdown of TRIM16 in COS1 cells expressing proIL-beta and procaspase1 is required for optimal secretion of IL1-beta. Therefore, the sentence of the Discussion, p. 13, should read something like this: "although a role for TRIM16 in IL-beta secretion was described in a previous study, the underlying mechanism had not been uncovered".

Previous point 1. One of my major concerns was the distinction of the roles of the players in secretory autophagy identified in this study from their function in degradative autophagy. With regards to TRIM16, the authors point out that they now have a paper in press that characterizes the role of this protein in lysophagy; indeed, the authors now show in in Fig. EV5, panel R, that TRIM16 deletion affects the generation of LC3 puncta. Thus, the sorting between lysophagy and secretory autophagocytosis would be accomplished by the binding partners of TRIM16: galectin 3 in the case of lysophagy and galectin 8 in the case of secretory autophagy. In Appendix Fig. S2, D, we are shown that silencing of Gal-8 has no effect on the co-localization of LC3 with TRIM16, implying that the lysophagy pathway is active in the absence of Gal-8? This might be an important observation, which could deserve major emphasis, complementing it with the effects of Gal-3 depletion in the same assay. Additionally, what is the effect of gal3 vs gal8 depletion on p62 levels? Apart from Fig. S2,D, there is no information in the manuscript as to how sorting between degradative and secretory autophagy occurs, and the claim that a dedicated pathway for secretory autophagy has been delineated in this study is not supported.

Previous point 2b. In my original report, I had asked for a clarification of the role of SNAP29 at the exocytosis step; this is now provided by the authors in the Discussion of the revised manuscript. They also have included new data on the effects of the combined silencing of SNAP23 and 29. From the new Fig. 5, it is, however, apparent that there actually is not much difference between Snap23 + 29 and Snap23 alone; the small difference is probably not statistically significant. Indeed, the comparison should be between the double KDs and the single ones (as in panel E for Syntaxins 3 and 4). If the right comparison is not made, the two new panels do not add much (or actually raise questions as to the interpretation), and should be removed.

Previous point 2c: my criticism here was that the 70 nm separation demonstrated by super-resolution microscopy is incompatible with the direct interaction illustrated in Fig. 5F. The author replies that

super-resolution at 70 nm is consistent with direct interaction or close proximity in common macromolecular complexes. While I agree that this distance is compatible with the interaction between two rod-shaped proteins, it is equally compatible with the two proteins being in a common macromolecular complex but not directly interacting with each other. Such an indirect interaction is compatible also with the co-immunoprecipitation results of Fig. 3. Analysis of the direct interaction between the two proteins via GST pulldowns, as was done for the TRIM16-galectin 8 interaction (Fig. 2G), has not been carried out. In conclusion, there is no evidence in this study for a direct interaction between Sec22b and TRIM16. Therefore, the text should be modified to include the possibility of indirect interaction, and this possibility should also be indicated in Fig. 5F.

Previous point 3a: here, among other criticisms, I asked the authors to indicate the amount of input on the immunoprecipitation blots relative to the immunoprecipitated samples. The author informs me that the amount of input is 10% and that this is clarified in the Supplementary Materials and Methods section. The value of 10% should be indicated in the figures next to the input; this will help the reader assess the efficiency with which the various interacting proteins are pulled down.

Previous point 3b: Although the duplicated bands have been removed from Fig. EV3, the legend has not been modified to adjust to this alteration.

Previous point 3e: with regards to Fig. 6 and the effect of the various silencing treatments on Mycobacterium tuberculosis Erdman and its ESX-1 mutant-induced secretion of ferritin. In the original manuscript, I noted that the silencing treatments had effect both on the wild-type and mutant Mtb-Erdman-induced secretion, even if in the text it was stated otherwise. The text has now been amended, however, I have difficulty in understanding the indications of statistical significance in the Figure (Fig. 6D). The asterisk is said to indicate statistical significance vs scrambled control, wild-type Mtb Erdman (column 2), while the cross indicates non significant differences: how is it possible that the values from cells infected with Mtb-Esx-1 (columns 7, 9, and 13), which are lower than their wild-type infected counterparts, are not significantly different from the Mtb Erdman-infected scr control (column 2)?

2nd Revision - authors' response 25 October 2016

Thank you very much for the reviews of our manuscript “Cellular and molecular definition of the mammalian secretory autophagy pathway” and for your editorial recommendations.
We thank the reviewers for their extensive and insightful analyses of our findings and for their continuing suggestions for improvement, including those in the previous round of comments, prompting us to include 45 additional datasets in the last revision of the study, and now include one new dataset with experimental results carried out in response to new reviewers’ comments. The remaining criticisms brought up in this round of revisions have been addressed as follows:

Editor:

As you will see, a few issues still have to be addressed before we move forward towards publication. Most concerns mentioned by referee #2 and #3 can be addressed in a point-by-point response and by text changes in your manuscript, a few need inclusion of additional data. It would be good to add the EM data referee #2 requests (comment #1). You already might have some data at hand to address this point.

In this study we have characterized the pathway functionally and biochemically, as well as at the fluorescence microscopy level. We believe that morphological identification of secretory autophagy cargo carriers and their distinction from degradative autophagic organelles is a complicated and highly responsible task that will require a separate in-depth study. We did not carry out ultra-structural analyses, and now explain the limitations of our current study in Discussion (p. 12)[16]*. Further details are provided in our point-for-point rebuttal to reviewers’ comments below.*

[16] “In our study we have characterized the above pathway functionally and biochemically, as well as at the fluorescence microscopy level. A full morphological identification of secretory autophagy cargo carriers and their distinction from degradative autophagic organelles at the ultrastructural level is a task that will require separate in-depth analyses and defines a limitation of our present work.”

It would also be good to provide additional insight in support of a dedicated pathway for secretory autophagy versus degradative autophagy (Referee #3, 'previous point 1').

As recommended, we have now addressed the remaining issues raised in Referee #3 "previous point 1", by including new data Appendix Figure S2E,F.

Referee #1:

I have read the revised manuscript and the authors' point-by-point response. My previous concerns have been largely addressed and I remain enthusiastic about the work overall. The manuscript provides important new insights into our understanding of secretory autophagy.

We thank the reviewer for the overall positive assessment of our study and revisions made.

Referee #2:

Previous comment #1

In the previous review, this reviewer suggested that "these TRIM16 structures should also be characterized by electron microscopy (CLEM or immuno-electron microscopy)". The authors' response is "additional information on the role of TRIM16 in the context of autophagic repair of damaged lysosomes (pertinent to other reviewer's questions) is available in a study in press in Developmental Cell that deals with lysosomal homeostasis." However, this reviewer could not find any additional information about the ultrastructure of TRIM16-IL1b-LC3B(+) structures in the Developmental Cell paper. The ultrastructural information is important to prove whether TRIM16-IL1b-LC3B(+) structures are indeed autophagosomes.

We apologize. The response referring to the study in Developmental Cell was not directly concerning the "CLEM or immuno-EM request" but referred to many of the multiplex questions posed by this reviewer. We believe that morphological identification of secretory autophagy cargo carriers and their distinction from degradative autophagic organelles is a complicated and highly responsible task that will require a separate in-depth study. We did not carry out ultra-structural analyses, and now explain the limitations of our current study in Discussion (p. 12).

Previous comment #2

This study still heavily relies on the use of the lysosomotropic agent LLOMe. This reviewer continues to have a concern that the mechanism reported here could be specific to lysosome damage-induced IL-1b secretion as shown in Fig. 5F (rather than general IL-1b secretion). This concern is also raised by Reviewer #1. This Reviewer suggests that the title and abstract, which are currently too general, should contain the information that this study mostly analyzes lysosome damage-induced unconventional secretion.

As requested, we have modified the title. The new title reads: "Cellular and molecular definition of the mammalian secretory autophagy pathway activated in response to lysosomal damage."

Previous comment #3

The authors show that the colocalization between TRIM16 and LC3B is not inhibited in galectin-8 knockdown cells. The authors argue in the rebuttal letter that this may be mediated by galectin-3. However, depletion of galectin-3 does not affect IL-1b secretion. How can the IL-1b secretion be reduced by knockdown of galectin-8 (Fig. 2BC) despite the normal recruitment of TRIM16? This discrepancy should be explained.

We appreciate this question by the reviewer. The best explanation is that either Galectin-3 or Galectin-8 (acting redundantly in this context, for visualization of TRIM16-colocalization) are sufficient to recruit TRIM16 to damaged lysosomes (which then end up in LC3-positive profiles).

Galectin 3 plays a role in autophagic homeostasis of damaged lysosomes and not in secretion, whereas Galectin 8 plays a role in secretion. Hence, Galecin-8 affects secretion but not (at least detectably) localization between TRIM16 and LC3. This is now additionally experimentally addressed (new Appendix Figure S2,EF) and described in Results as follows: "A question arose whether galectin-3 and galectin-8 could influence the TRIM16-LC3 colocalization detected in response to LLOMe illustrated in Fig. 1D. Neither galectin-8 knockdown alone (Appendix Figure S2C,D) nor galectin-3 knockdown alone (Appendix Figure S2E,F) affected colocalization between TRIM16 and LC3B elicited in cells by LLOMe. However, a combined knockdown of galectin-3 and galectin-8 reduced the % of TRIM16 profiles that were also positive for LC3B (Appendix Figure S2E,F). Thus, galectin-3 and galectin-8 showed redundant effects on bulk (i.e. not differentiated for function) TRIM16-LC3B profiles formed in response to lysosomal damage." This is further explained in Discussion on p.13 as follows: "When compared to galectin-3, which showed no effects on secretion in the present study and is important for autophagic homeostasis of damaged lysosomes (Chauhan et al., 2016) involving the process of lysophagy (Fujita et al., 2013, Maejima et al., 2013), galectin-8 may appear as a galectin specializing in secretory autophagy and as a point of divergence between secretory and degradative autophagy. Galectin-8 contributes (as opposed to galectin-3) to these sorting steps most likely by helping separate the TRIM16 pools participating in secretory autophagy studied in this work from the TRIM16 pools participating in lysosomal homeostasis/lysophagy (Chauhan et al., 2016). However, galectin-8 also has the acknowledged functions in degradative autophagy. For example, galectin-8 is known to play a role in control of intracellular Salmonella (Thurston, Wandel et al., 2012), which occurs through a process termed xenophagy. Thus, the main point of divergence between degradative and secretory autophagy may not be galectins, despite their contributions, but rather it is based on the observed differential utilization of SNAREs: Syntaxin-17 for degradative autophagy, vs. Sec22b/Syntaxins-3 and -4/SNAP23 or SNAP29 for secretory autophagy."

Others:

After reading the paper by Chauhan et al. that has just been published by the same group in Dev Cell, this reviewer realizes that there are significant overlaps and inconsistencies between the Dev Cell and present papers.

1. The present manuscript shows that Galectin-8 interacts with TRIM16 (Fig. 2G), whereas Chauhan et al. state that TRIM16 does NOT bind Galectin-8 (Fig. 1A). This discrepancy needs to be explained.

Binding of galectins to TRIM16 is enhanced by the presence of ULK1 (as shown in Chauhan et al, 2016). This can also be observed in Figure 2G. This is now further emphasized in Results: "TRIM16 associated with galectin-8 in GST-pulldown assays (Figs 2G and EV2G,H), and as described for other galectins, i.e. galectin-3 (Chauhan et al., 2016), this association was enhanced in the presence of ULK1 (Fig 2G)."

2. Colocalization of TRIM16 with LC3 (Fig. 1D) and Lamp2 (Fig. EV1O) have been shown in Chauhan et al. These should be mentioned in the present manuscript to avoid giving an impression that this is the first report.

Although this was already stated in the text[17], we now additionally re-state this specifically in the later context of LAMP2: "A subset of TRIM16$^+$ profiles overlapped with the lysosomal marker LAMP2 (Fig EV1O), in keeping with the recently described role of TRIM16 in autophagic homeostasis of damaged lysosomes (Chauhan, Kumar et al., 2016)."

3. TRIM16 knockout HeLa cells were described in Chauhan et al. Is the same clone used in the present study?

This is the same clone as in Chauhan et al. 2016. This is now referenced (Chauhan, Kumar et al., 2016) in the Results on p. 8.

[17] p. 6: "TRIM16 colocalized with LC3B upon LLOMe treatment (Fig 1D and Appendix Figure S1E,F), in keeping with its recently described role in autophagic response to and repair of endomembrane and lysosomal damage (Chauhan, Kumar et al., 2016)."

Referee #3:

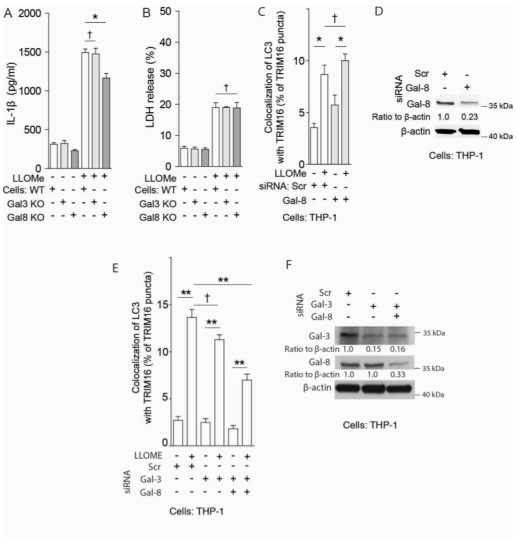
The revised version of the manuscript by Kimura et al. contains much additional work, and many of my initial concerns have been addressed. Nevertheless, some modification of the manuscript is required before publication.

We thank the reviewer for the overall assessment.

Citation of Munding et al: the authors have added a sentence in the discussion (p. 13) to give more credit to this previous study. Their sentence ("Although Munding and colleagues have shown that TRIM-16 binds IL-1beta and is co-secreted with this cytokine, their observations lacked an overarching mechanism") does not give full credit to the work of Munding et al., as these authors also showed that knockdown of TRIM16 in COS1 cells expressing proIL-beta and procaspase1 is required for optimal secretion of IL1-beta. Therefore, the sentence of the Discussion, p. 13, should read something like this: "although a role for TRIM16 in IL-beta secretion was described in a previous study, the underlying mechanism had not been uncovered".

We have now replaced the sentence and used verbatim the reviewer's statement, as requested: "although a role for TRIM16 in IL-beta secretion was described in a previous study, the underlying mechanism had not been uncovered."

Previous point 1. One of my major concerns was the distinction of the roles of the players in secretory autophagy identified in this study from their function in degradative autophagy. With regards to TRIM16, the authors point out that they now have a paper in press that characterizes the role of this protein in lysophagy; indeed, the authors now show in in Fig. EV5, panel R, that TRIM16 deletion affects the generation of LC3 puncta. Thus, the sorting between lysophagy and secretory autophagocytosis would be accomplished by the binding partners of TRIM16: galectin 3 in the case of lysophagy and galectin 8 in the case of secretory autophagy. In Appendix Fig. S2, D, we are shown that silencing of Gal-8 has no effect on the co-localization of LC3 with TRIM16, implying that the lysophagy pathway is active in the absence of Gal-8? This might be an important observation, which could deserve major emphasis, complementing it with the effects of Gal-3 depletion in the same assay.



We thank the reviewer for her/his continuing interest and important suggestions. We appreciate this question by the reviewer. This is now experimentally addressed as requested (new Appendix Figure S2,EF) and described in Results: "A question arose whether galectin-3 and galectin-8 could influence the TRIM16-LC3 colocalization detected in response to LLOMe illustrated in Fig. 1D. Neither galectin-8 knockdown alone (Appendix Figure S2C,D) nor galectin-3 knockdown alone (Appendix Figure S2E,F) affected colocalization between TRIM16 and LC3B elicited in cells by LLOMe. However, a combined knockdown of galectin-3 and galectin-8 reduced the % of TRIM16 profiles that were also positive for LC3B (Appendix Figure S2E,F). Thus, galectin-3 and galectin-8 showed redundant effects on bulk (i.e. not differentiated for function) TRIM16-LC3B profiles formed in response to lysosomal damage." This is further explained in Discussion on p.13: "When compared to galectin-3, which showed no effects on secretion in the present study and is important for autophagic homeostasis of damaged lysosomes (Chauhan et al., 2016) involving the process of lysophagy (Fujita et al., 2013, Maejima et al., 2013), galectin-8 may appear as a galectin specializing in secretory autophagy and as a point of

divergence between secretory and degradative autophagy. Galectin-8 contributes (as opposed to galectin-3) to these sorting steps most likely by helping separate the TRIM16 pools participating in secretory autophagy studied in this work from the TRIM16 pools participating in lysosomal homeostasis/lysophagy (Chauhan et al., 2016). However, galectin-8 also has the acknowledged functions in degradative autophagy. For example, galectin-8 is known to play a role in control of intracellular Salmonella (Thurston, Wandel et al., 2012), which occurs through a process termed xenophagy. Thus, the main point of divergence between degradative and secretory autophagy may not be galectins, despite their contributions, but rather it is based on the observed differential utilization of SNAREs: Syntaxin-17 for degradative autophagy, vs. Sec22b/Syntaxins-3 and -4/SNAP23 or SNAP29 for secretory autophagy."

Additionally, what is the effect of gal3 vs gal8 depletion on p62 levels?

Whether galectins impact autophagic turnover of p62 is of course an interesting standalone question but is beyond the scope of our work. We utilized p62 only as a control for syntaxin-17 depletion effects. We have stated in Discussion, that TRIM16 does not interact with p62 (Mandell et al, 2014), and now further emphasize this point (p. 15)[18]*. Thus, to study the role of galectins on p62 or other degradative autophagy receptors in our opinion is not relevant to the present study, underscored by the absence of p62 effects on secretory autophagy (figure EV6K), similarly to no role for NCOA4 in ferritin secretion (Figure 6E).*

Apart from Fig. S2,D, there is no information in the manuscript as to how sorting between degradative and secretory autophagy occurs, and the claim that a dedicated pathway for secretory autophagy has been delineated in this study is not supported.

We believe that the main point of divergence between degradative and secretory autophagy is differential utilization of SNAREs: Stx17 for degradative autophagy, vs. Sec22b/Stx3,4/SNAP23 for secretory autophagy. This is supported by the data in Figs. 4-6. The sorting step includes transfer of cargo (IL-1β) via a receptor (TRIM16) to autophagosomal precursors that contain Sec22b, with this sorting step being based on TRIM16-Sec22b interactions (note that TRIM16 contains a SNC1/longin domain, and that Sec22b is a longin). Galectin-8 may contribute (as opposed to Galectin 3) by separating the TRIM16 pools participating in lysosomal homeostasis/lysophagy vs secretory autophagy. We now additionally emphasize this throughout the Discussion.

Previous point 2b. In my original report, I had asked for a clarification of the role of SNAP29 at the exocytosis step; this is now provided by the authors in the Discussion of the revised manuscript. They also have included new data on the effects of the combined silencing of SNAP23 and 29. From the new Fig. 5, it is, however, apparent that there actually is not much difference between Snap23 + 29 and Snap23 alone; the small difference is probably not statistically significant. Indeed, the comparison should be between the double KDs and the single ones (as in panel E for Syntaxins 3 and 4). If the right comparison is not made, the two new panels do not add much (or actually raise questions as to the interpretation), and should be removed.

We have removed the data in question from the main figure and show them in Appendix Fig. S4A-D. We agree with reviewer's comments, but the results may reflect the complex nature of SNAP29 action, as extensively covered in Discussion in response to the prior rounds of review (p. 15).

Previous point 2c: my criticism here was that the 70 nm separation demonstrated by super-resolution microscopy is incompatible with the direct interaction illustrated in Fig. 5F. The author replies that super-resolution at 70 nm is consistent with direct interaction or close proximity in common macromolecular complexes. While I agree that this distance is compatible with the interaction between two rod-shaped proteins, it is equally compatible with the two proteins being in a common macromolecular complex but not directly interacting with each other. Such an indirect interaction is compatible also with the co-immunoprecipitation results of Fig. 3. Analysis of the direct interaction between the two proteins via GST pulldowns, as was done for the TRIM16-galectin 8 interaction (Fig. 2G), has not been carried out. In conclusion, there is no evidence in this study for a direct

[18] "Of note, TRIM16 does not interact with Sequestosome-1/p62 (Mandell et al., 2014), a classical degradative autophagy receptor (Bjorkoy et al., 2005), whereas many other TRIMs do (Mandell et al., 2014). The absence of p62 interaction may indicate a special position of TRIM16 among TRIMs to guide autophagic intermediates to secretion".

interaction between Sec22b and TRIM16. Therefore, the text should be modified to include the possibility of indirect interaction, and this possibility should also be indicated in Fig. 5F.

We have modified the text as requested, and indicated that biochemical evidence of direct interaction is not available at this point. It would however be difficult to depict two possibilities in the model. Whether there is a direct interaction or indirect interaction superimposed on their presence in the same macromolecular complexes, the net result is the same and we are afraid that making such nuanced distinctions in the cartoon would confuse the readers. Thus, the figure is retained in its present form but the text is modified as requested[19]*. Furthermore, we included a statement in the figure legend that a direct interaction between Sec22b and TRIM16 remains to be determined*[20]*.*

Previous point 3a: here, among other criticisms, I asked the authors to indicate the amount of input on the immunoprecipitation blots relative to the immunoprecipitated samples. The author informs me that the amount of input is 10% and that this is clarified in the Supplementary Materials and Methods section. The value of 10% should be indicated in the figures next to the input; this will help the reader assess the efficiency with which the various interacting proteins are pulled down.

We now modified all pertinent figures and indicate input amount directly in the figures.

Previous point 3b: Although the duplicated bands have been removed from Fig. EV3, the legend has not been modified to adjust to this alteration.

We have modified Figure EV3 legend as requested.

Previous point 3e: with regards to Fig. 6 and the effect of the various silencing treatments on Mycobacterium tuberculosis Erdman and its ESX-1 mutant-induced secretion of ferritin. In the original manuscript, I noted that the silencing treatments had effect both on the wild-type and mutant Mtb-Erdman-induced secretion, even if in the text it was stated otherwise. The text has now been amended, however, I have difficulty in understanding the indications of statistical significance in the Figure (Fig. 6D). The asterisk is said to indicate statistical significance vs scrambled control, wild-type Mtb Erdman (column 2), while the cross indicates non significant differences: how is it possible that the values from cells infected with Mtb-Esx-1 (columns 7, 9, and 13), which are lower than their wild-type infected counterparts, are not significantly different from the Mtb Erdman-infected scr control (column 2)?

We apologize for the complex labeling. All statistical symbols are given relative to either Erdman-infected scramble control [and †] or to uninfected scramble control [(*) and (†)]. There is no statistical labeling relative to ESX-1 mutant infected cells (which would make the whole legend even more difficult to understand. Thus, the answer to the reviewer's comment is that the statistical signs above bars #7,9 and 13 are relative to bar #1 (uninfected scr control) and not bar #2 (infected, wt Mtb, scramble). The difference between bars (column) 7,9 and 13 vs. bar (column) #2 are obvious and would require an additional statistical symbol. This may not be necessary, as the main point is that in bars (columns) 7,8 and 13, ferritin levels are not different form uninfected controls, which is a more important comparison. We again apologize for the complexity and have now enlarged the font to allow easier grasp of the symbols associated with the analysis.*

Note: The discussion, through the process of accretion of statements in trying to answer various reviewers' questions at different stages, has become somewhat difficult to read. We have made an effort to consolidate several aspects without changing the meaning or references.

Again, we are grateful to the reviewers for their very thoughtful, thorough, and highly constructive comments. Thank you for your consideration.

[19] Discussion, p.13: "Note however that although TRIM16 contains an SNC1/longin-like domain, that Sec22b is a longin, and that super-resolution microscopy and co-IP analyses indicate that TRIM16 and Sec22b are in close proximity and form macromolecular complexes, biochemical evidence for their direct interaction is not available at present."

[20] Figure 5 legend: "TRIM16 forms complexes with Sec22b to transfer the cargo to the autophagy-induced LC3-II$^+$ membrane 25k carriers (a direct interaction between Sec22b and TRIM16 remains to be determined)."

Additional Correspondence Editor 27 October 2016

Many thanks for submitting your manuscript to us. I appreciate the introduced changes, and I am happy to accept your manuscript in principle for publication in The EMBO Journal.
Before sending you an official acceptance letter, I still need some input from you:
- please provide an author contribution statement
- please provide 2-5 short sentences recapitulating your main findings
- I think title and abstract need to be shorter / more precise. I know that you already changed the title in response to the referees, but I think that a shorter title is OK as well - as long as the abstract mentions the lysosomal damage context.
How about:

SNAREs and TRIM16 mediate secretory autophagy

Autophagy is a process delivering cytoplasmic components to lysosomes for degradation. Autophagy may however play a role in unconventional secretion of leaderless cytosolic proteins. How secretory autophagy diverges from degradative autophagy remains unclear. Here we show that in response to lysosomal damage, the prototypical cytosolic secretory autophagy cargo IL-1β is recognized by specialized secretory autophagy cargo receptor TRIM16, and that this receptor interacts with the R-SNARE Sec22b to recruit cargo to the LC3-II+ sequestration membranes. Cargo secretion is unaffected by downregulation of syntaxin-17, a SNARE promoting autophagosome-lysosome fusion and cargo degradation. Instead, Sec22b in combination with plasma membrane syntaxin3 and syntaxin4 as well as SNAP23 and SNAP29 completes cargo secretion. Thus, secretory autophagy utilizes a specialized cytosolic cargo receptor and a dedicated SNARE system. Other cargo, such as ferritin, is secreted via the same pathway.

You can provide the abovementioned statements via response email. If you are OK with changing the title and abstract as outlined, we can do the changes from here.

Additional Correspondence Author 28 October 2016

Thank you very much for your email, decision, and for your input/recommendations (highly appreciated!) re the title and the abstract.

I am attaching as a word file a copy of your original e-mail with embedded point-for-point responses (with some additional explanations re the title, etc.) and the requested additional items.

3rd Editorial Decision 01 November 2016

Many thanks for your message and the input and for sending all the information I requested.
I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Vojo Deretic
Journal Submitted to: EMBO j
Manuscript Number: EMBOJ-2016-95081

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
- 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.

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, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was based on data in prior publications subjected to statistical tesst by t-test and ANOVA.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. The distribution was confirmed graphically.
Is there an estimate of variation within each group of data?	SD and SE were determined and reported in graphs.
Is the variance similar between the groups that are being statistically compared?	Not determined.

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).	Already described in full in the text
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No but the cell lines were obtained directly 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.	Mus musculus was used to obtain bone marrows and derove macrophage. Strains (both genders; 4-6 weeks old): 1) C57Bl Atg5fl/fl LysM-Cre mice and their Cre-negative littermates (both genders; 4-6 weeks old) Castillo EF, Dekonenko A, Arko-Mensah J, Mandell MA, Dupont N, Jiang S, Delgado-Vargas M, Timmins GS, Bhattacharya D, Yang H, Hutt J, Lyons CR, Dobos KM, Deretic V (2012) Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. Proceedings of the National Academy of Sciences of the United States of America 109: E3168-76 Dupont N, Jiang S, Pilli M, Ornatowski W, Bhattacharya D, Deretic V (2011) Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta. EMBO J 30: 4701-11 Zhao Z, Fux B, Goodwin M, Dunay IR, Strong D, Miller BC, Cadwell K, Delgado MA, Ponpuak M, Green KG, Schmidt RE, Mizushima N, Deretic V, Sibley LD, Virgin HW (2008) Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. Cell Host Microbe 4: 458-69. 2) C57BL mice and their galectin-3 (B6.Cg-Lgals3m1Poi/J. Jackson Laboratory) and galectin-8 knockout derivatives (B6;129S5-galt8-Gt(reo)406Lex/Mmucd; Mutant Mouse Resource & Research Centers). All of the above is described in methods in Appx. Housing and husmbandry: UNM HSC inst. animal research program is accredited by AAALAC. We have a robust veterinary and animal care program with committed and experienced staff. The animal facility physical plant supports standard environmental requirements that support housing and research with animal models to include: individual room temperature control between 68-78°F (20-25.5°C), 15 air changes per hour with 100% supply and exhaust and no air recirculation, controlled lighting and remote environmental monitoring and alarming capabilities
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Prior approval of animal protocols by the institutional IACUC and compliance with NIH guidelines and AALAC. The UNM Health Sciences Center maintains an Animal Welfare Assurance on file with the Office for Protection from Research Risks. The Assurance Number is A3350-01. All animal work is strictly regulated and monitored
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.	Prior approval of animal protocols by the institutional IACUC and compliance with NIH guidelines and AAALAC. The UNM Health Sciences Center maintains an Animal Welfare Assurance on file with the Office for Protection from Research Risks. The Assurance Number is A3350-01. All animal work is strictly regulated and monitored.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Human peripheral blood monocytes were either from StemCell Technologies or from healthy individual donors and cultured as previously described (Kimura, Jain et al., 2015), as approved by the Human Research Review Committee (IRB)) at the University of New Mexico
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.	Informed consent was obtained from all subjects and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and Department of Health and Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	No identifier available. All subjects were healthy subjects. All samples were used up and no archiving of tissues or cells was performed.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	N/A
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).	N/A
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).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4026 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. 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.	N/A

G- Dual use research of concern

23. 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.