THE
EMBO
JOURNAL

ER-to-lysosome-associated degradation of proteasome-resistant ATZ polymers occurs via receptor-mediated vesicular transport

Ilaria Fregno, Elisa Fasana, Timothy J. Bergmann, Andrea Raimondi, Marisa Loi, Tatiana Soldà, Carmela Galli, Rocco D'Antuono, Diego Morone, Alberto Danieli, Paolo Paganetti, Eelco van Anken and Maurizio Molinari.

Review timeline:

Submission date:	19th Febuary 2018.
Editorial Decision:	3rd April 2018
Revision received:	22nd May 2018
Editorial Decision:	19th June 2018
Revision received:	22nd June 2018
Accepted:	30th June 2018

Editor: Elisabetta Argenzio

Transaction Report:

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

1st Editorial Decision 3rd April 2018

Thank you for submitting your manuscript on the ER-to-Lysosome clearance of ATZ polymers to The EMBO Journal. We have now received three referee reports on your study, which are enclosed below for your information.

As you can see, while all referees consider the findings novel and interesting, they also raise some critical points that need to be addressed before they can support publication here.
In particular, referees #1 finds that the ER-derived, ATZ-laden vesicles need to be better characterized; referee #2 and #3 point out that the strength of the study will greatly increase if you further investigate the role of FAM134B-Calnexin complex and ATG8 proteins in ATZ sorting to degradative organelles. Addressing these issues through additional data as suggested by the referees would be essential to warrant publication in The EMBO Journal. Given the overall interest of your study, I would thus like to invite you to revise the manuscript in response to the referee reports.

REFEREE REPORTS

Referee #1:

In this manuscript Fregno and co-workers report that proteasome-resistant polymers of alpha1-antitrypsin Z (ATZ) are cleared by a mechanism that requires Calnexin, LC3 lipidation machinery (ATG4B and ATG7) and the autophagy receptor FAM134B. Surprisingly, translocation of ATZ from the ER lumen to lysosomes was found to be independent of autophagy and instead involved vesicles budding from the ER and fusing with lysosomes by a Syntaxin-17 and VAMP8 dependent

mechanism.
This paper is of potential conceptual interest since it involves a novel degradation pathway for a pathology-associated aggregated protein. The experimental data are of excellent quality, with extensive use of knockout cell lines, high-quality light and electron microscopy, and adequate quantifications.

Specific comments:

The ER-derived vesicles that contain ATZ should be better characterized.

- Can we be sure that they are indeed vesicles and not continuous with the ER?

- Are these vesicles positive for COP-II?

- Electron microscopy is required to assess whether ER-derived vesicles are truly docked onto lysosomes in Syntaxin-17 and VAMP8 KO cells (Fig. 7).

Referee #2:

GENERAL COMMENTS
In this manuscript, Frengo et al., delineated the molecular mechanism of delivery of proteasome resistant ATZ to lysosome for clearance. Authors show that a chaperon protein calnexin and ER-phagy receptor FAM134b are required for delivery of ATZ to degradative organelles ("DO", a bit fuzzy in definition - perhaps this can be defined a bit better?) and that binding of FAM134b to LC3 is important for delivery of ATZ to DO. Additionally, using CRISPR KO against various ATG proteins, authors show that LC3 lipidation machinery (ATG4B and ATG7) but not autophagy initiation machinery (FIP200, ATG13, ULK and ATG9) is required for delivery of ATZ to "DO". Furthermore, autophagic SNAREs Stx17 and VAMP8 are required for delivery of ATZ to "DO". This work, thus mechanistically explains that the later stages of autophagy machinery are important for delivery of ATZ for degradation.
In most cases the conclusions are supported by the data and overall quality of the data is good. However, in certain cases the manuscript lacks strong evidence and control experiments, and therefore additional experiments are needed to strengthen the study.

SPECIFIC COMMENTS:

1. Figs 1G-K: Were the cells treated with BafA1? Figure legend reads as if the experiment was done similar to Fig 1A where BafA1 was not used. If the cells were not treated with BafA1 there should no accumulation of degradative organelles.

2. Fig 1H: does ATZ mislocalize from ER to other organelles in CST-treated cells? Is ATZ exclusively localized to ER and is ER localization important to deliver it to "DO"?

3. Fig 1M: Authors show that ATZ enriches LC3-II in immune complexes with FAM134B; authors should test here weather ATZ interacts with LC3. Authors should also quantify the enrichment of FAM134B and LC3-II complexes (shown in Fig 1M lane 8,9) in presence of ATZ in three independent experiments.

4. Fig 2D: Does FAM134B KO affect the overall level of ATZ? The overall fluorescence of ATZ seems to be lower in FAM134B KO cells.

5. It seems that ATG8 members may be important for delivery of ATZ to "DO". Fig 3 shows that FAM134 binding to LIR is important for delivery, while Fig 4 shows that lipidation machinery is important for delivery of ATZ to "DO". But his is all circumstial. Therefore, authors should test weather Atg8s (at least LC3B and GABARAP) are important for delivery of ATZ to "DO".

6. Fig EV2B: authors should include LC3 blots as control. Also, please include blot showing ATG9

and FIP200 KO in Figs 4 and EV2.

7. Fig 7: Authors should also test SNAP29, a Qbc SNARE, which regulates autophagosomal maturation together with Stx17 and VAMP8 (Itakura et al., 2012; Diao et al., 2015).

8. Must define "DO" better.

Minor:

Abstract: FAM134b is an autophagy/ER-phagy not LC3-II receptor.

Does LAMP1 always form from these empty rings in BafA1 treated cells or these "DOs" are formed when co-stained with ATZ?

Referee #3:

The pathway for exit of ATZ fibrils from the ER to the lysosome for degradation has been explored. It has already been reported that ATZ fibrils are degraded by the lysosome and the focus of this story is definition of the mechanism for sorting of ATZ from the contents of the ER-lumen into the lysosome. The story is interesting because the data suggest a previously unappreciated mechanism for sorting of ERAD resistant proteins from the ER lumen into single membrane vesicles that bud from the ER and fuse to lysosomes. DO vesicle formation requires the interaction of calnexin with the ER-phagy factor FAM134 and autophagy components required for LC3 lipid conjugation, but is not dependent upon the regulatory kinase ULK1. Fusion of DO's with lysosomes requires Syntaxin 17, so information on early and late states of ATZ fibril degradation is provided.

The authors propose the existence of a novel mechanism for delivery of ERAD resistant oligomers of misfolded proteins that accumulate in the ER lumen to lysosomes.

The story important and is of broad general interest, but there are some questions that should be addressed to help refine the proposed model.

A complex between FAM134B and calnexin is proposed to select ATZ for sorting to DOs. Yet, it is not clear how FAM134B and LC3 mediate the formation of DOs. This is the most novel aspect of the story, but is a bit of a black box?

The sentence below from in the discussion does not do an adequate job to explain how DO's are formed and liberated from the ER. How FAM134B would drive DO formation is not explained in sufficient detail.

"Apparently, the condensation of ATZ aggregates and the ensuing concentration of FAM134B in ER subdomains activates FAM134B to drive ER fragmentation, which likely occurs by virtue of the reticulon domain of FAM134B and depends on LC3II binding as has been demonstrated previously (Khaminets et al., 2015)".

Are components of the COPII export machinery required for DO formation of is it just FAM134B and calnexin and LC3? How would binding of LC3 to FAM134B help drive DO formation in the absence of additional co-factors?

ULK1 does not appear to be required for ATZ degradation, but the LC3 conjugation machinery is required for this process. Yet, the authors do not explain how flux through ERLAD is regulated?

Is FAM134B induced in response to ATZ? Is the PI3 kinase complex that contains Beclin-1 and VPS34 required to regulate ERLAD.

Author's Point by Point response.

Referee #1:

In this manuscript Fregno and co-workers report that proteasome-resistant polymers of alpha1-antitrypsin Z (ATZ) are cleared by a mechanism that requires Calnexin, LC3 lipidation machinery (ATG4B and ATG7) and the autophagy receptor FAM134B. Surprisingly, translocation of ATZ from the ER lumen to lysosomes was found to be independent of autophagy and instead involved vesicles budding from the ER and fusing with lysosomes by a Syntaxin-17 and VAMP8 dependent mechanism.

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Specific comments:

The ER-derived vesicles that contain ATZ should be better characterized.

1- Can we be sure that they are indeed vesicles and not continuous with the ER?

Our submission contains a movie showing the 3D model of the distribution of ATZ in ER subdomains obtained from an electron microscopy tomographic reconstruction of tilted series acquisition of serial 250 nm thick sections. This movie refers to panel F, Figure 5 and shows that ER-derived vesicles (EV) are not continuous with the ER and are indeed vesicles. In panel H, Fig 2, we now added evidences showing that overexpression of FAM134BLIR that cannot bind lipidated LC3 is sufficient to generate ATZ-containing ER-derived vesicles.

2- Are these vesicles positive for COP-II?

These vesicles are negative for COP-II. For reviewer convenience, we show here (but we do not include in the manuscript) that both in WT MEF and in cells where ER-derived vesicles accumulate (e.g., in cells ablated of STX17, see Fig 7) the ATZ-containing vesicles are not stained with an antibody to COP-II and ATZ does not accumulate in the COP-II compartment.

(Figures for Referees not shown)

3- Electron microscopy is required to assess whether ER-derived vesicles are truly docked onto lysosomes in Syntaxin-17 and VAMP8 KO cells (Fig. 7).

Figure 7 (new panels G, H, I) now shows a comparison in Immuno Electron Microscopy between WT CRISPR (panel G, ATZ (immunogold) is delivered within endolysosomes (EL)) and cells lacking STX17 (panel H) or VAMP8

(panel I), where ATZ accumulates in ER-derived vesicles in close proximity (docked) to the EL that do not release their content within the EL (please also refer to Fig 7D-F and J).

Referee #2:

GENERAL COMMENTS

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1. Figs 1G-K: Were the cells treated with BafA1? Figure legend reads as if the experiment was done similar to Fig 1A where BafA1 was not used. If the cells were not treated with BafA1 there should no accumulation of degradative organelles.

We apologize for the mistake. The text has been corrected to “similar to Figure 1B”

2. Fig 1H: does ATZ mislocalize from ER to other organelles in CST-treated cells? Is ATZ exclusively localized to ER and is ER localization important to deliver it to "DO"?

For the convenience of the reviewer, we show here immunofluorescence panels showing that ATZ co-localizes with the luminal ER marker GFP-KDEL in WT cells (panel A), as well as in cells exposed to CST (B) and in cells lacking CNX (C). The panels also show insets that confirm lack of ATZ delivery to endolysosomes on exposure to CST or on deletion of CNX (as shown in Fig 1G-L). The co-localization of ATZ with the luminal ER marker GFP-KDEL has been quantified (panel D).

(Figures for Referees not shown)

In the new version of the manuscript we add a flow cytometric analysis showing that incubation with CST and ablation of CNX do not affect formation of ATZ polymers (new Fig EV2A, fourth and fifth panels, respectively). Thus, ATZ polymers are normally formed in cells exposed to CST or lacking CNX. However, they are not delivered to the DOs.

Finally, the ER-resident chaperone CNX is required for ATZ delivery to the endolysosomes (Fig. 1I, L and panel C shown above) and the luminal ER marker sfGFP-KDEL is co-delivered within endolysosomes (Fig 6A, B, EV5B). Thus, ER localization is required for delivery.

3. Fig 1M: Authors show that ATZ enriches LC3-II in immune complexes with FAM134B; authors should test here weather ATZ interacts with LC3. Authors should also quantify the enrichment of FAM134B and LC3-II complexes (shown in Fig 1M lane 8,9) in presence of ATZ in three independent experiments.

This is now shown in Fig 2A, B. ATZ does not directly interact with LC3. In fact, both CNX:FAM134B (Figure 2A, lane 9) and CNX:FAM134BLIR (lane 10) complexes contain ATZ, but the latter complex does not contain LC3 (FAM134BLIR has a mutation in the LIR that prevents LC3 binding). The presence of LC3 in this complex is mediated by FAM134B.

The enrichment of FAM134B and LC3-II complexes (shown in Fig 2A lanes 8,9) is now quantified in the new panel 2B.

4. Fig 2D: Does FAM134B KO affect the overall level of ATZ? The overall fluorescence of ATZ seems to be lower in FAM134B KO cells.

This is now Fig 3D. We would not compare the overall ATZ level by looking at a single cell in two different cell lines as shown in IF. FAM134B ablation (Fig 3D, F) or inactivation (Fig 2D) significantly reduce delivery of ATZ polymers to the endolysosomes and significantly delay polymers clearance (Fig 2I, J) thus enhancing the intraluminal levels of both total (Figure 2A, lanes 4-5) and polymeric ATZ (Fig 2K).

5. It seems that ATG8 members may be important for delivery of ATZ to "DO". Fig 3 shows that FAM134 binding to LIR is important for delivery, while Fig 4 shows that lipidation machinery is important for delivery of ATZ to "DO". But his is all circumstantial. Therefore, authors should test weather Atg8s (at least LC3B and GABARAP) are important for delivery of ATZ to "DO".

To directly assess requirement of LC3 and GABARAP, ATZ delivery to the endolysosomes was monitored in HeLa cells lacking all six LC3 family members (LC3A, B, C and GABARAP, GABARAPL1 and L2) (Nguyen et al JCB 2016). In this cell line, ATZ polymers are not delivered to the endolysosomes. These results are shown here for the convenience of the referee and are not included in the manuscript.

(Figures for Referees not shown)

6. Fig EV2B: authors should include LC3 blots as control. Also, please include blot showing ATG9 and FIP200 KO in Figs 4 and EV2.

As requested by the referee, for all cell lines, we have included LC3 blots as control in the new EV3B. We also have the WB to control all the KO generated/used in this work (FAM134B, SEC62 (Figure 2A, B), STX17, VAMP8 (Figure 7A, B), ATG4B, ATG7, ULK1, ATG13, ATG9 (Figure EV3A). Unfortunately, for FIP200, we could not find a suitable antibody. However, and as expected on FIP200 ablation, in this cell line there is a clear defect in LC3 lipidation (new EV3B).

In the new EV3A, we added the control for MEF ablated of RUBICON (Martinez et al Nature Cell Biol 17, 893-906 (2015)). The normal delivery of ATZ polymers to the endolysosomes in cells lacking RUBICON is now shown in Fig. 4H-4I. Dispensability of RUBICON distinguishes ERLAD from LAP (please also refer to referee 3, response 2). This is now commented.

7. Fig 7: Authors should also test SNAP29, a Qbc SNARE, which regulates autophagosomal maturation together with Stx17 and VAMP8 (Itakura et al., 2012; Diao et al., 2015).

For assessing the involvement of the STX17:VAMP8:SNAP29 complex in delivery of ATZ polymers in the endolysosomes, we prepared and tested the CRISPR/Cas9 edited for STX17 and VAMP8 (the corresponding immunogold electron microscopy images have been added in the new submission, new panels G-I in Fig 7). Individual deletion of the two SNAREs abolishes delivery. Unfortunately, we have not been able to generate, so far, cells lacking SNAP29.

8. Must define "DO" better.

We have added a new EV1 to show that DOs (now defined as endolysosomes, EL) also display the small GTPase RAB7 at their limiting membrane. Based on their characteristics (degradative organelles displaying LAMP1/RAB7, but not RAB5 at their limiting membrane), the presence of intraluminal vesicles (ILVs) and the VAMP8-mediated fusion with ER-derived vesicles, the degradative organelles are now defined as endolysosomes (as per definition in (Huotari and Helenius, 2011)). We specify this in the manuscript.

Minor:

Abstract: FAM134b is an autophagy/ER-phagy not LC3-II receptor.

We have changed this, thank you.

Does LAMP1 always form from these empty rings in BafA1 treated cells or these "DOs" are formed when co-stained with ATZ?

The new EV5D shows that LAMP1 rings also form in cells expressing NHK, which is not polymerogenic, is a proteasomal substrate and is not delivered within endolysosomes. LAMP1 "rings" also forms in all cell lines tested in this work, where ATZ is not delivered (Fig. 1H, I, 2D, 3D, 4B, C, 7D, E, J), as well as in mock-transfected (Fig EV5C) and in non-transfected cells (e.g., the two cells in the upper right and left corners, Fig 1B, panels LAMP1, Merge (HA) and Merge (2C1)).

Referee #3:

The pathway for exit of ATZ fibrils from the ER to the lysosome for degradation has been explored. It has already been reported that ATZ fibrils are degraded by the lysosome and the focus of this story is definition of the mechanism for sorting of ATZ from the contents of the ER-lumen into the lysosome. The story is interesting because the data suggest a previously unappreciated mechanism for sorting of ERAD resistant proteins from the ER lumen into single membrane vesicles that bud from the ER and fuse to lysosomes. DO vesicle formation requires the interaction of calnexin with the ER-phagy factor FAM134 and autophagy components required for LC3 lipid conjugation, but is not dependent upon the regulatory kinase ULK1. Fusion of DO's with lysosomes requires Syntaxin 17, so information on early and late states of ATZ fibril degradation is provided.

The authors propose the existence of a novel mechanism for delivery of ERAD resistant oligomers of misfolded proteins that accumulate in the ER lumen to lysosomes.

The story is important and is of broad general interest, but there are some questions that should be addressed to help refine the proposed model.

1. A complex between FAM134B and calnexin is proposed to select ATZ for sorting to DOs. Yet, it is not clear how FAM134B and LC3 mediate the formation of DOs. This is the most novel aspect of the story but is a bit of a black box. The sentence below from the discussion does not do an adequate job to explain how DO's are formed and liberated from the ER. How FAM134B would drive DO formation is not explained in sufficient detail. "Apparently, the condensation of ATZ aggregates and the ensuing concentration of FAM134B in ER subdomains activates FAM134B to drive ER fragmentation, which likely occurs by virtue of the reticulon domain of FAM134B and depends on LC3II binding as has been demonstrated previously (Khaminets et al., 2015)". Are components of the COPII export machinery required for DO formation or is it just FAM134B and calnexin and LC3? How would binding of LC3 to FAM134B help drive DO formation in the absence of additional co-factors?

DO (for degradative organelle) has been replaced with EL for endolysosomes throughout the text and figures, please refer to Response 8, reviewer 2. We have better characterized this compartment and now show that it also displays the small GTPase RAB7 at the limiting membrane (new Fig EV1). Based on their characteristics (LAMP1/RAB7-positive, RAB5-negative (unpublished), the presence of intraluminal vesicles and the VAMP8-mediated fusion with

ER-derived vesicles), the degradative organelles can be defined as endolysosomes (Huotari and Helenius, 2011). We specify this in the manuscript.

DO (EL in the new submission) are not "formed and liberated from the ER". Rather, they are pre-existing RAB7/LAMP1-positive endolysosomal compartments visible in cells where ATZ is not delivered within them (Fig. 1H, I, 2D, 3D, 4B, C, 7D, E, J), as well as in mock-transfected (Fig EV5C) and in non-transfected cells (e.g., the two cells in the upper right and left corners, Fig 1B, panels LAMP1, Merge (HA) and Merge (2C1)).

We think that the question of the referee does not refer to the formation of the DOs, as written, but to the formation of the ER-derived vesicles (EV in our manuscript).

EV deliver luminal ER material to endolysosomes. Their formation is triggered, or strongly enhanced, by intraluminal accumulation of proteasome-resistant ATZ. This can be inferred by the observation that at steady state (Fig EV5C) and in cells expressing NHK (Fig EV5D) delivery of the luminal ER marker GFP-KDEL within endolysosomes remains below detection level, whereas it is strongly induced in cells expressing ATZ polymers as symptom of induced ER to endolysosomal transport (Fig 6A, B, EV5B).

We now add immunoelectron microscopy images showing that the expression of FAM134BLIR (it cannot bind LC3) is sufficient to generate ATZ-containing EV (Fig. 2F, G, H). The EV generated under these conditions remain dispersed in the cytosol and do not release their content into the EL (Fig 2D, F, G). Thus, the LC3-binding function of FAM134B is dispensable for formation of a complex with CNX and ATZ (Fig 2A, lanes 8-10) and for generation of EV. The LC3-binding function of FAM134B is required for docking of EV to the endolysosomal membrane (Fig 7J, K, EV5H) that precedes STX17/VAMP8-regulated fusion (Fig. 7C-7F and new panels 7G-7I). The results and discussion sections have been modified accordingly.

We failed to detect a co-localization of COP-II with ATZ (see also response 2, referee 1).

Budding and fission of the EV from the ER membrane could rely on the activity of dynamin or septin family members. It could also rely on a COPII proteins-independent SAR1 activity (as described in Long KR et al JCB 2010). Work to characterize the machinery regulating these early events is ongoing in the lab.

2. ULK1 does not appear to be required for ATZ degradation, but the LC3 conjugation machinery is required for this process. Yet, the authors do not explain how flux through ERLAD is regulated.

This is an interesting issue. Dispensability of ULK1/ULK2 (with requirement of the LC3 conjugation machinery) has previously been reported, for example, for LC3-associated phagocytosis (LAP, Martinez et al Nature Cell Biol 17, 893-906 (2015)). Like ATZ clearance, LAP does not require the activity of the pre-initiation complex and autophagosome biogenesis (ULK1/2, ATG13, FIP200 are dispensable for both pathways, the LC3 conjugation complex is required

for both pathways). Dispensability of Rubicon (new panel 4H, quantification in 4I and control in EV3A) distinguishes ERLAD from LAP. Further studies are needed to understand in detail how processes like LAP and ERLAD that require LC3 lipidation but not biogenesis and involvement of double membrane autophagosomes are regulated. This is now mentioned in the comment of Figure 4 and in the Discussion.

3. Is FAM134B induced in response to ATZ?

We do not observe FAM134B induction on ATZ expression as shown in the WB added here for the convenience of the reviewer. Rather, ATZ expression substantially enhances the fraction of FAM134B associated with CNX and LC3-II (Fig 2A, lanes 8, 9, B).

(Figures for Referees not shown)

4. Is the PI3 kinase complex that contains Beclin-1 and VPS34 required to regulate ERLAD.

To address this, we monitored ATZ delivery to the LAMP1-positive EL in cells exposed to the specific VPS34 inhibitor SAR405, which prevents LC3 lipidation (Ronan B et al Nature Chem Biol 2014). This treatment fully prevents ATZ delivery to the DO (new EV4B). Panels C and D are controls showing that Sar405 inhibits formation of LC3 puncta on conventional, starvation induced macroautophagy. Panel E shows quantification in n=5 cells/condition.

We would like to thank the three referees for the insightful comments and suggestions. We hope that our manuscript will be considered of interest for the EMBO J readership.

2nd Editorial Decision 19th June 2018

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

As you will see they all find that the original criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can go on to officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address.

REFEREE REPORTS

Referee #1:

The authors have successfully addressed the comments I raised, and I am happy to recommend publication of this revised manuscript.

Referee #2:

This is an excellent study. It is ready for publication. The substantive revisions as well as now a proper definition and explicit naming of the degradative compartments make this an attractive and a solid study.

Referee #3:

The authors have done an excellent job addressing the issues raised in the previous round of review. The work presented addresses all of my concerns and provides an advance to our understanding of ERQC as is defines a pathway for degradation of protein polymers that accumulate in the ER lumen and are resistant to ERAD.

This is a very nice story and will be of broad general interest to the EMBO J. readership.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Maurizio Molinari
Journal Submitted to:	EMBO journal
Manuscript Number:	EMBOJ-2018-99259

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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?	In main figures experiments were performed at least three times and in many cases repeated with different techniques to confirm the data. For example, the effect of FAM134B on ATZ delivery and degradation was shown by CLSM, flow cytometry and CHX chase.
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?	Samples were excluded from quantification in case of technical issues and not included in the shown results.
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.	Key experiments were performed independently by at least two different scientists.
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.	Image analysis and quantification was performed after blinded randomization.
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, please refer to the figure captions.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical tests were performed only when n ≥ 3.
Is there an estimate of variation within each group of data?	S.E.M. is shown in Fig. 1F, 1L, 2B, 2F, 2G, 6G and EV5A.
Is the variance similar between the groups that are being statistically compared?	Yes.

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).	Antibodies used for WB and IF: Alpha-1-antitrypsin, Human, mAb 2C1, HycultBiotech Catalog : HM2289 Lot. #22104M0517-A Anti Cnx (anti C-terminus) was a kind gift from A. Helenius, anti SEC62 from R. Zimmermann and anti FAM134B of I. Kurth. Lamp1 1D4B was deposited to the Developmental Studies Hybridoma Bank (DSHB) by August, J.T., H4A3 by August, J.T. and Hildreth, J.E.K. HA Sigma H 6908, Lot. 031M4849. HA-probe (F7), Santa Cruz, sc. 7392. Actin Santa Cruz Biotechnologies, I19sc-1616. LC3B Sigma, APG8C, SAB1301290. LC3B Novus, NB100-2220. GAPDH Merk Millipore, clone 6c5, Catalog # MAB374 GFP Abcam ab290 GR240324-1 p62 MBL, lot. 017. V5 Tag monoclonal antibody, Invitrogen Catalog # R960-25, Lot. #1831141 CLIMP63, CKAP4 polyclonal antibody, proteintech 16686-1-AP Syntaxin17 Sigma HPA001204, Lot. # C91833 Vamp8 Abcam, EP2629Y ATG4B Sigma, A2981, Lot. # 045M4855V ATG7 Sigma, A2856 Lot. # 125M4854V

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

	ATG1/ULK1 Sigma, A7481 Lot. # 016M4824V ATG13 Sigma, SAB4200100 Lot. # 052M4832 ATG9a Catalog # GTX128427 Rubicon, Abcam ab156052, Lot # GR206965-1 Anti-V5 Agarose Affinity Gel antibody produced in mouse, Sigma A7345, clone V5-10 II Antibodies used for WB: anti-rabbit IgG-HRP, Biorad #170-6515. anti-goat-HRP Southern Biotech cat.#1060.01. HRP-ProteinA Invitrogen 101023 lot# 758960A. II Antibodies used for IF: Mouse (Alexa Fluor® 488 conjugated) Jackson Immunoresearch 115-545-166 124083 Mouse (Alexa Fluor® 568 conjugated) ThermoFisher A-11031 1736975 Rat (Alexa Fluor® 568 conjugated) ThermoFisher A-11077 1692966 Rat (Alexa Fluor® 647 conjugated) ThermoFisher A-21247 Rabbit (Alexa Fluor® 488 conjugated) Invitrogen A-11008 51385A Rabbit (Alexa Fluor® 568 conjugated) Thermofisher A-11036 Rabbit (Alexa Fluor® 647 conjugated) Jackson Immunoresearch 111-605-144 107714 Nanogold-
	IgG goat anti mouse IgG #2001 Nanogold-IgG goat anti rabbit IgG #2003
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Atg7 (WT and KO) MEF are kind gifts of M. Komatsu. ATG4BKO of G. Marino. FIP200KO of J.-L. Guan. RubiconKO of D. Green. ULK1/2DKO of S. Tooze. ATG9AKO of T. Saitoh. ATG13KO of X. Wang. CNX KO and CRT KO are kind kift of M. Michalack. ERp57KO of N. Garcia-Garbi. MEF CRISPR WT, FAM134B, SEC62, STX17 and VAMP8 were generated in the lab as described in material and methods section. HEK293 were purchased from ATCC. Cells are tested for mycoplasma contamination periodically.

* 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.	n/a
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	n/a
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.	n/a

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n/a
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.	n/a
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.	n/a
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 a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	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. 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

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	