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TOLLIP acts as a cargo adaptor to promote lysosomal degradation of aberrant ER membrane proteins

Yuki Hayashi, Sho Takatori, Waleed Warsame, Taisuke Tomita, Takao Fujisawa, and Hidenori Ichijo
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Corresponding author(s): Hidenori Ichijo (ichijo@mol.f.u-tokyo.ac.jp)

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Ichiro,

Thank you again for the submission of your manuscript entitled "TOLLIP acts as a cargo adaptor to promote lysosomal degradation of aberrant ER membrane proteins" (EMBOJ-2023-114272) and for your patience during the review process. We have now received the reports from the referees, which I copy below.

As you can see from their comments, while referee #1 stated clearly that your data are not able to support the scheme of a TOLLIP/PI3P-dependent ER to lysosome transport route for mis-folded ER proteins, all referees appreciated the potential significance of your work.

Therefore, based on the overall interest expressed in the reports, I would like to invite you to address all concerns from the referees in a revised version of the manuscript. Final acceptance of the manuscript will be dependent on a positive evaluation of the study by all three referees. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. I believe that the referees' comments are reasonable and addressable, but represent an ambitious work programme. I therefore think a Zoom call would be sensible at this stage. Please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

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: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

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

Best regards,

William

William Teale, Ph.D.
Editor
The EMBO Journal

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under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

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9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (22nd Aug 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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Referee #1:

Hayashi et al. report that TOLLIP mediates selective lysosomal degradation of misfolded ER membrane proteins. The authors find that misfolded ER proteins are recognized by the IDR and ubiquitin-binding CUE domain of TOLLIP and appear in PI3P-containing endosomes or lysosomes where they are degraded. In contrast to previously characterized ER-phagy pathways, the TOLLIP-dependent pathway appears to selectively clear misfolded ER proteins. Even though TOLLIP has already been shown to be involved in autophagic degradation of ER proteins, the proposed mechanism is novel and potentially interesting. Unfortunately, however, the data are too preliminary to allow firm conclusions to be drawn.

Major points:

1. Conclusions in this manuscript are mainly based on Western blots of cells expressing chimeric fusions with mCherry or Venus and exposed to different treatments. While these experiments show differences in mCherry cleavage that are consistent with the proposed model, they do not pinpoint the mechanisms involved. Electron microscopy is necessary in order to establish that misfolded proteins are transferred from ER to lysosomes via vesicular intermediates.

2. The claim that TOLLIP clears aberrant ER proteins via PI3P-dependent lysosomal trafficking is primarily based on co-localization of mCherry with PI3P-positive profiles, but the presence of PI3P does not necessarily imply that PI3P is involved. The effect of the PI3K inhibitor SAR405 on processing of mCh-A53T-HP is minimal at best (Figure 4A), which argues against a role of PI3P in degradation of ER proteins.

3. The mechanism of TOLLIP-mediated degradation has been poorly characterized. The model in Figure 7D seems to imply that degradation occurs by direct fusion of PI3P- and TOLLIP-containing endosomes with lysosomes, but this has not been demonstrated. The alternative mechanism of microautophagy needs to be explored by depletion/knockdown of ESCRT proteins.

Referee #2:

Toll-interacting protein, TOLLIP was implicated in numerous processes, including aggrephagy receptor, endosomal sorting regulator and formation of mitochondrial-derived vesicles (MDV). In the current manuscript, Hayashi et. al reports a new role for TOLLIP as a specific cargo-adaptor that recognizes misfolded ER membrane proteins and directly routes them for lysosomal degradation in a process that requires further characterization.

The authors initially utilized an experimental system of chimeric protein A53T-HP comprised of Parkinson's disease-linked A53T α-synuclein mutant fused to a hairpin of E-Syt1 (ER membrane protein) to identify factors that mediate its selective lysosomal degradation. Using this system, TOLLIP, but not the other autophagy receptors, is needed for such degradation in its LIR-independent manner. Evidence indicates that the IDR and CUE domains of TOLLIP are responsible for recognizing ER aberrant membrane protein A53T-HP. At the same time, IDR acts as a protein misfolding sensor that recognizes the misfolded substrate, while the CUE domain is responsible for recognizing ubiquitinated A53T-HP. The authors also found that TOLLIP's phosphoinositide-binding C2 domain is needed for the vesicular trafficking of A53T-HP to the lysosomes. Finally, the authors demonstrate that TOLLIP-dependent lysosomal degradation of physiological substrates such as P56S VAPB (ALS-linked) and N88S,S90L Seipin (spastic paraplegia-linked).

Overall, the experiments are well thought out, and the data seem convincing. However, the authors are encouraged to characterize better the PI3P-positive vesicles that serve as the vehicle for the TOLLIP-dependent degradation pathway. This can be achieved by improved fluorescent images, electron microscopy (preferably CLEM), and careful subcellular fractionations. This will significantly improve the quality of this study.

Minor comments
1. Figure 1E - the authors use the fluorescent construct mCh-A53T-HP and measure the cleavage of mCherry by western blot. To support that data, they should also employ mCherry-EGFP-A53T-HT and measure the quenching of EGFP.
2. Figure 4B - the Baf. A1 treatment should be mentioned in that panel (in addition to the legend).
3. Figure 4C - the recovery experiment was performed on siTOLLIP background; the KO used in other experiments may be more convincing.

Referee #3:

In the present manuscript authors characterized a new function for the protein TOLLIP. Authors proposed that TOLLIP, via its IDR domain, can recognize the misfolded ER proteins which are anchored to the ER membrane and present a cytosolic domain that is ubiquitinated. Moreover, TOLLIP can recognize the misfolded proteins and mediates their lysosomal degradation via the PI3P trafficking pathway. The molecular mechanisms regarding the connection between TOLLIP and PI3P are not too clear and needs some extra investigation as the authors commented in the discussion.
TOLLIP function as an autophagy receptor is known. However, its role in degrading ER membrane bounded proteins seems to be autophagy independent. Of note, the recognition of specific degradation of single type proteins, not discrete portion of ER, is new and interesting for its biological and medical applications. Moreover, differently from FAM134B and RTN3, TOLLIP seems to directly recognize the misfolded proteins, without chaperones involvement, and it is selective for membrane proteins that face the cytosolic environment rather than for the ER luminal protein aggregates.
The manuscript has a novelty, and it is potentially interesting for the scientific community. Some experiments would be necessary to finalize the final taking message.

1. Authors used pharmacological approaches to inhibit autophagy (Bafilomycin A) and the PI3P (SAR405). These are widely used reagents. However, it would be appropriate to confirm the major finding also using a genetic approach. siRNA or CRISPR some of the key autophagy and PI3P genes.
2. Authors performed several experiments to show the delivery of misfolded protein to the lysosome. However, it would be appropriate to perform some high-resolution and/or electron microscopy experiments to clearly show that TOLLIP is moving the misfolded protein directly to lysosomes without the presence of any other intermediates (like autophagosomes).

Concerning the proposed experiments

1. Line 138-139 " Among the receptors tested, only overexpression of TOLLIP....." maybe it is more appropriate .... Mostly overexpression of TOLLIP....
2. Fig2B and line 141-142. Not only TOLLIP but also OPTN seems to have an effect (minor but present) so authors should edit this part.
3. Line 150-152 and related EV1. This part is not clear and difficult to follow in the figure. Please edit to make easier to visualize.
4. Authors used mCherry-A53T-HP to measure the rate of degradation looking at the mCherry cleavage. Sometime is not so clear the cleavage ratio so maybe authors should consider repeating some experiment looking to GFP degradation too or performing some additional experiments to improve the quality of the data.
5. Fig 5D. It is a bit difficult to appreciate differences in that WB. Maybe consider adding a lower exposure film to better visualize the data.

Minor Points

1. Appendix Figure could be moved into EV Figures to make the manuscript more compact.
2. The written part, especially in the results session, is a bit lengthily. Some details and comments could be shortened.

Point-by-point response to the referees' comments

Firstly, we would like to thank all the referees for providing insightful feedback and constructive suggestions. We have addressed all concerns from the referees by performing the requested experiments (new figures: **Fig 2F and G, 4A and D–F, 5C, and EV3A–C**). The previous Fig 4 was split into **Fig 4 and 5**, thus the previous Fig 5, 6, and 7 are presented as new **Fig 6, 7, and 8** in the revised manuscript. The previous Fig EV3D and E were moved to **Appendix Fig S2** due to space limitations.

We hope that the referees' concerns are relieved in the revised manuscript.

Referee #1:

Hayashi et al. report that TOLLIP mediates selective lysosomal degradation of misfolded ER membrane proteins. The authors find that misfolded ER proteins are recognized by the IDR and ubiquitin-binding CUE domain of TOLLIP and appear in PI3P-containing endosomes or lysosomes where they are degraded. In contrast to previously characterized ER-phagy pathways, the TOLLIP-dependent pathway appears to selectively clear misfolded ER proteins. Even though TOLLIP has already been shown to be involved in autophagic degradation of ER proteins, the proposed mechanism is novel and potentially interesting. Unfortunately, however, the data are too preliminary to allow firm conclusions to be drawn.

Response:

We highly appreciate the referee's careful assessment of our manuscript and deep understanding of the novelty of our findings. We apologize for lack of strong evidence supporting our conclusions in the initial manuscript. We have addressed the referee's concerns by performing several new experiments or by improving the quality of data in the initial manuscript.

Major points:

1. Conclusions in this manuscript are mainly based on Western blots of cells expressing chimeric fusions with mCherry or Venus and exposed to different treatments. While these experiments show differences in mCherry cleavage that are consistent with the proposed model, they do not pinpoint the mechanisms involved.

Response:

We agree with the referee that our conclusions were relying too much on mCherry cleavage assays in the initial manuscript. To support our conclusions, we have confirmed our important findings, i.e., the requirement of each TOLLIP domain (new **Fig 2G**) and PI3P (new **Fig EV3A and B**) in the TOLLIP-dependent lysosomal trafficking of the artificial model substrate A53T-HP, by assessing the accumulation of A53T-HP in LAMP1-positive lysosomes.

(*continued*)

Electron microscopy is necessary in order to establish that misfolded proteins are transferred from ER to lysosomes via vesicular intermediates.

Response:

We quite agree with the referee's opinion that electron microscopy is necessary to establish our model, which is also suggested by all the other referees. Collaborating with new authors, we have performed CLEM analysis.

First, to facilitate identifying transport intermediates for the TOLLIP-dependent pathway in CLEM analysis, we have adopted BiFC technology to visualize the protein complex of A53T-HP and TOLLIP. For this purpose, the N-terminal of mVenus (VN) was appended to A53T-HP and the C-terminal (VC) to TOLLIP. As shown in the **Figure for referees 1** below, BiFC signal (reconstituted mVenus) was detected only when both VC-TOLLIP and VN-A53T-HP were expressed (please compare i, iii, and iv). The BiFC signal colocalized with PI3P puncta as probed by 2xFYVE (i). When TOLLIP lacked substrate binding domains IDR and CUE, BiFC signal was weak and did not colocalize with PI3P puncta (compare i and ii and see the quantification in the graph), suggesting that the TOLLIP–A53T-HP complex moves toward PI3P puncta after substrate recognition by TOLLIP. For simplicity, only the conditions i and ii are presented as new **Fig 4D**.

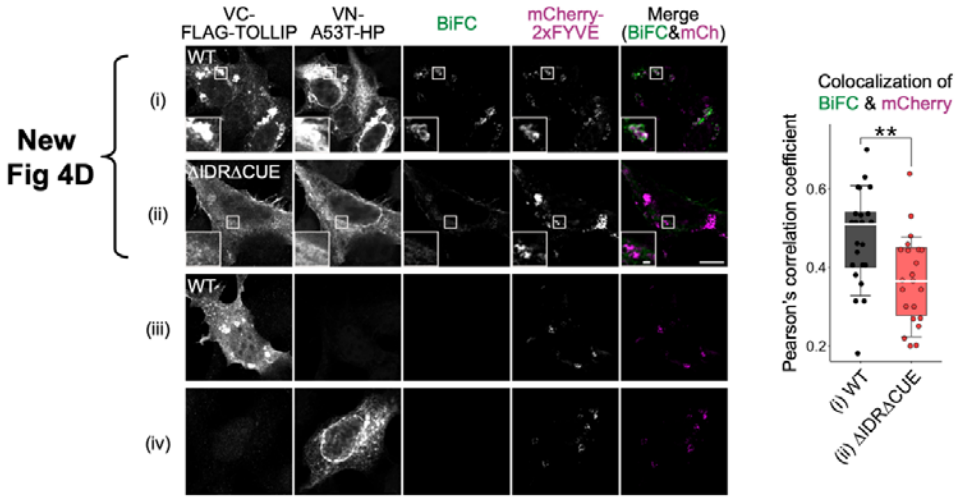

Figure for referees 1. Visualization of TOLLIP–A53T-HP complex on PI3P puncta by BiFC.

Fluorescence microscopy imaging to monitor the trafficking of the TOLLIP–A53T-HP complex as visualized by BiFC to PI3P-enriched puncta. HEK293A cells were transfected with VC (156–239 aa of mVenus)-FLAG-TOLLIP variants, VN (1–155 aa of mVenus)-A53T-HA-HP, and mCherry-2xFYVE, and stained with anti-FLAG and HA antibodies. The graph represents Pearson's correlation coefficient measured between BiFC signal and mCherry-2xFYVE. A total of 23 (WT) or 22 (ΔIDRΔCUE) cells pooled from $n = 2$ experiments were analyzed. Box centers indicate medians, box edges represent first and third quartiles, and whiskers show the 10th to 90th percentile. $^{**}P < 0.01$ by unpaired two-tailed Student's t test. Scale bars, 10 μm and 1 μm (insets).

By combining this BiFC technology and CLEM analysis, we visualized transport intermediates of the TOLLIP-dependent pathway. Two representative CLEM images are presented in our revised manuscript (new **Fig 4E and EV3C**). The PI3P puncta positive for the TOLLIP–A53T-HP complex were vesicles surrounded by single membranes (**Fig 4E and EV3C**, black arrowheads). These vesicles were also partly associated with a smooth ER-like structure (**Fig 4E and EV3C**, white arrowheads), indicating that they were in close proximity to the ER. Since TOLLIP has been reported to constitute contact sites between the ER and intracellular vesicles (Jongsma *et al*, 2016, PMID 27368102), the observed ER like structure–PI3P puncta contacts may have a role in transferring A53T-HP from the ER to vesicular compartments, which will be assessed in our future study. In the new **Fig 4F**, the BiFC-positive PI3P puncta were negative for LysoTracker, suggesting that TOLLIP utilizes these PI3P-enriched vesicles as transport intermediates to deliver its cargo to acidic lysosomal compartments.

2. The claim that TOLLIP clears aberrant ER proteins via PI3P-dependent lysosomal trafficking is primarily based on co-localization of mCherry with PI3P-positive profiles, but the presence of PI3P does not necessarily imply that PI3P is involved. The effect of the PI3K inhibitor SAR405 on processing of mCh-A53T-HP is minimal at best (Figure 4A), which argues against a role of PI3P in degradation of ER proteins.

Response:

We apologize for presenting weak evidence for the role of PI3P. We have repeated the experiment in **Fig 4A** by improving the experimental scheme as shown in the bottom of **Figure for referees 2** and using another specific VPS34 inhibitor VPS34-IN1 in addition to SAR405. The result shows that VPS34 inhibitors suppress TOLLIP overexpression-dependent lysosomal degradation of A53T-HP. As mentioned above, we have also confirmed this result by showing the requirement of VPS34 in the TOLLIP overexpression-dependent lysosomal trafficking of A53T-HP using siVPS34 and the inhibitor SAR405 (new **Fig EV3A and B**).

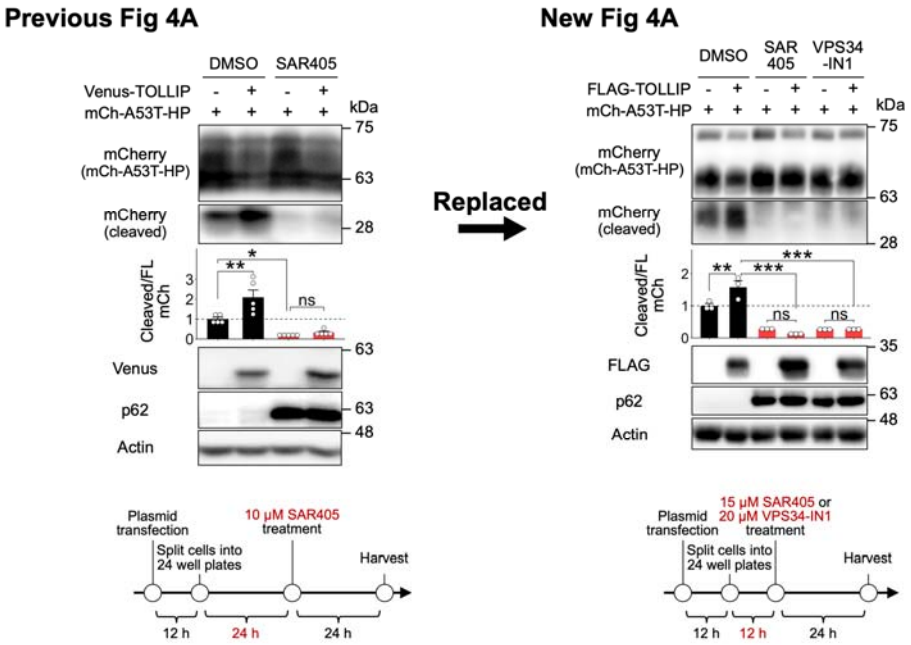

Figure for referees 2. Replaced figure.

We also confirmed the importance of PI3P by using point mutants of TOLLIP, i.e., R78A and R123A, which cannot interact with phosphoinositides. In a previous study, these mutations have been shown to disrupt the binding of the C2 domain to PI3P by in vitro lipid-protein overlay assay (Ankem *et al*,

2011, PMID 21294713). First, we confirmed this result in cells by assessing the colocalization of the TOLLIP variants with PI3P puncta (**Figure for referees 3**). By using the R123A mutant (designated as $C2^{mut\ \#1}$ in the manuscript), we revealed that TOLLIP binding to phosphoinositides is required for the mCherry processing (new **Fig 2F**) and the lysosomal trafficking of A53T-HP (new **Fig 2G**).

Since the statement in the previous Fig EV1G, i.e., dispensability of LIR motifs, is also included in this new **Fig 2G**, Fig EV1G was removed (**Figure for referees 4**). As already presented in the initial manuscript, the C2 mutants are defective in promoting turnover of A53T-HP (Fig EV1E) and transferring A53T-HP to PI3P puncta (Fig 4C).

Overall, these results support our notion that PI3P is important for the TOLLIP-dependent lysosomal degradation of aberrant ER membrane proteins.

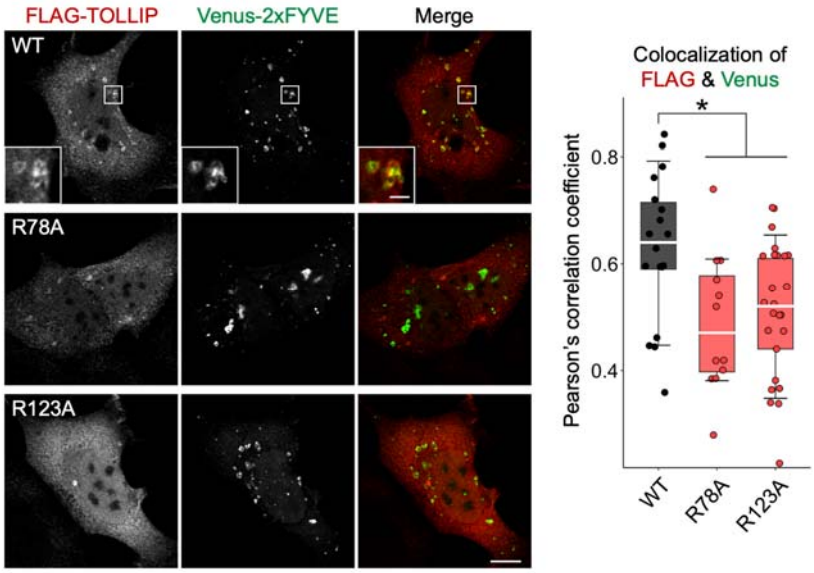

Figure for referees 3. R78A and R123A mutations disrupt binding of TOLLIP with PI3P puncta.

Fluorescence microscopy imaging to monitor the colocalization of TOLLIP variants with PI3P puncta. HEK293A cells were transfected with FLAG-TOLLIP variants and Venus-2xFYVE, and stained with an anti-FLAG antibody. The graph represents Pearson's correlation coefficient measured between FLAG-TOLLIP and Venus-2xFYVE. From 12 to 25 cells pooled from $n = 2$ experiments were analyzed. Box centers indicate medians, box edges represent first and third quartiles, and whiskers show the 10th to 90th percentile. *$P < 0.05$ by one-way ANOVA with Dunnett's multiple comparison test. Scale bars, 10 μm and 2 μm (insets).

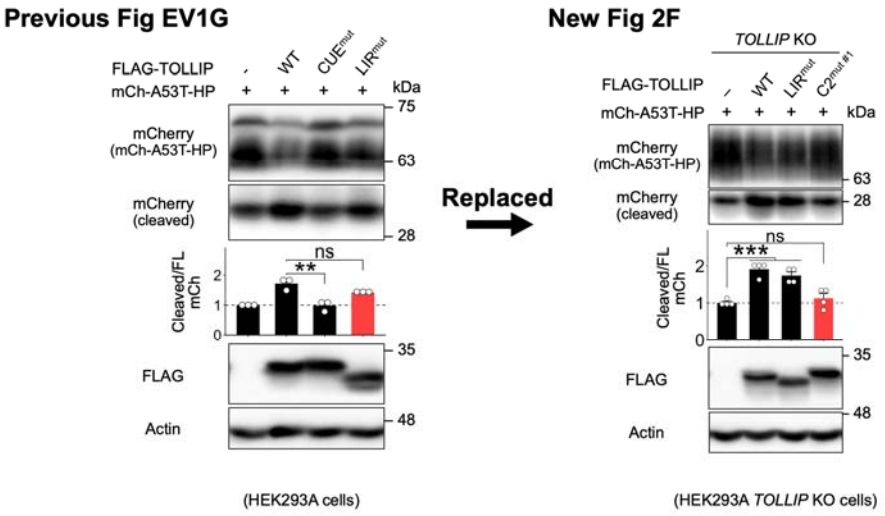

Figure for referees 4. Replaced figure.

3. The mechanism of TOLLIP-mediated degradation has been poorly characterized. The model in Figure 7D seems to imply that degradation occurs by direct fusion of PI3P- and TOLLIP-containing endosomes with lysosomes, but this has not been demonstrated. The alternative mechanism of microautophagy needs to be explored by depletion/knockdown of ESCRT proteins.

Response:

According to the suggestion, we have investigated the involvement of microautophagy by knocking down ESCRT-III components. Among mammalian ESCRT-III components, CHMP4 is especially important for inward membrane invagination at endolysosomes, including micro-ER-phagy-dependent lysosomal trafficking of an ER-phagy receptor SEC62 (Loi *et al*, 2019, PMID 31699981), endosomal microautophagy of autophagy receptors (Mejlvang *et al*, 2018, PMID 30018090), and degradation of EGFR after EGF stimulation (Wenzel *et al*, 2018, PMID 30050131). First, we confirmed the involvement of CHMP4 in endosomal membrane invagination by monitoring EGF-dependent EGFR degradation. As reported by Wenzel *et al*, CHMP4B KD partially impaired EGFR degradation, while the effect of knocking down its paralog CHMP4C was larger (**Figure for referees 5A**). Even in this CHMP4C KD condition, the TOLLIP-dependent lysosomal degradation of A53T-HP was not impaired (**Figure for referees 5B**). Moreover, knockdown of CHMP4 paralogs did not impair the TOLLIP-dependent lysosomal trafficking of A53T-HP (**Figure for referees 5C**). A similar result was obtained when CHMP2 was depleted, which is required for autophagosomal membrane closure (Takahashi *et al*, 2018, PMID 30030437) (**Figure for referees**

5A and C). These results suggest that ESCRT-dependent microautophagy is not involved in the TOLLIP-dependent pathway.

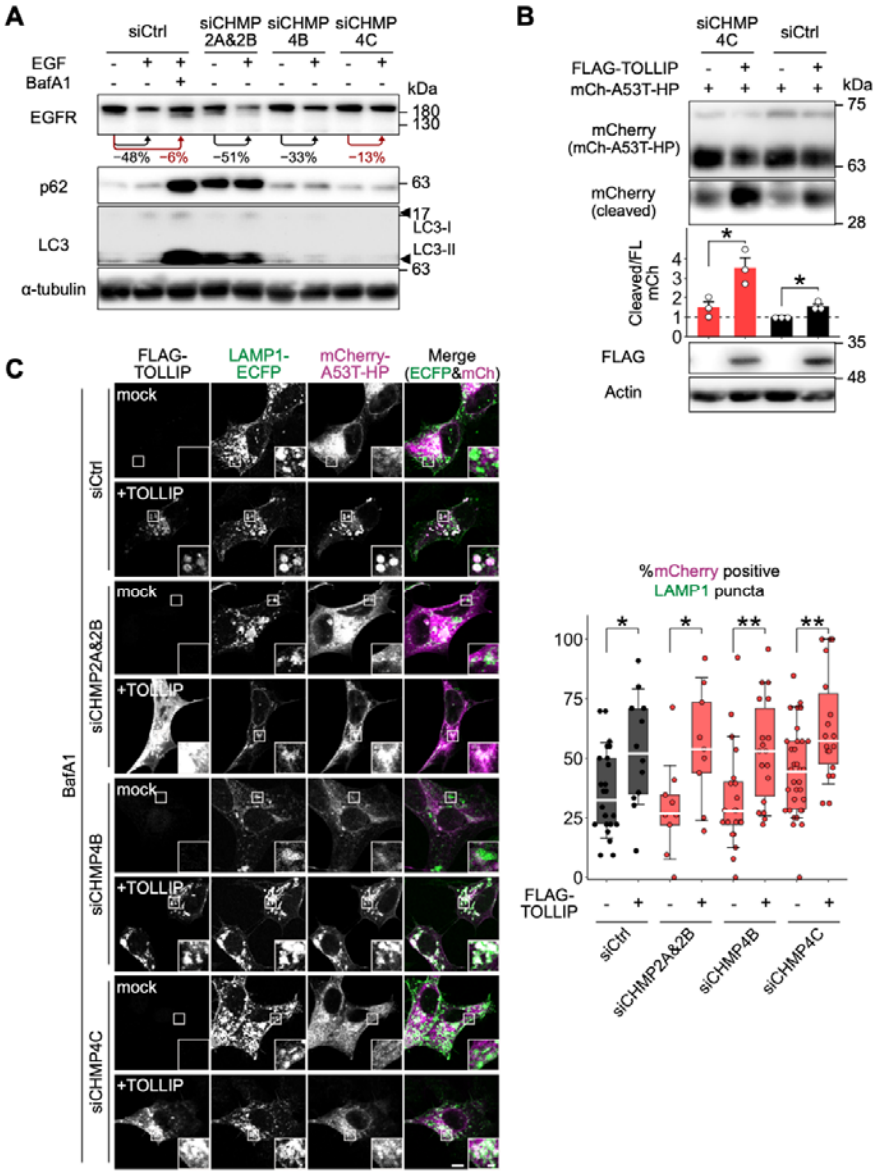

Figure for referees 5. ESCRT-III components CHMP2 and CHMP4 were dispensable for lysosomal degradation of A53T-HP.

A Immunoblot of HEK293A cells transfected with indicated siRNAs, starved in DMEM without serum for 6 h, and then treated with 100 ng/mL EGF and 200 nM BafA1 for 14 h. Cells were harvested 4 days after siRNA transfection. Numerical data represent means of the

EGF-dependent decrease in EGFR level normalized to α-tubulin ($n = 2$).

B Lysosomal degradation of A53T-HP in HEK293A cells transfected with indicated siRNAs in the same conditions as in (A) and with indicated constructs. The graph represents mean ± SEM of mCherry processing ($n = 3$). *$P < 0.05$ by unpaired two-tailed Student's t test.

C Fluorescence microscopy imaging to monitor the lysosomal delivery of A53T-HP. HEK293A cells were transfected with indicated siRNAs in the same conditions as in (A) and with indicated constructs, treated with 100 nM BafA1 for 15 h, and stained with an anti-FLAG antibody. The percentage of LAMP1 puncta containing mCherry-A53T-HP in each cell was quantified. From 9 to 33 cells in each condition pooled from $n = 2$ experiments (siCtrl, siCHMP2A&2B, and siCHMP4C) or $n = 1$ experiment (siCHMP4B) were analyzed. *$P < 0.05$, **$P < 0.01$ by unpaired two-tailed Student's t test. Scale bars, 10 μm and 2 μm (insets).

As pointed out by the referee, whether the PI3P- and TOLLIP-containing vesicles fuse with or are engulfed by lysosomes remains uncertain, considering that membrane invagination at endolysosomes can occur through ESCRT-independent pathways in mammals (Stuffers et al, 2009, PMID 19490536). We completely agree that this point is an important issue that should be elucidated in the future study through extensive microscopic and biochemical analysis. However, we think that it is beyond the scope of this manuscript focusing on revealing a novel type of cargo adaptor for the clearance of specific ER constituents and its significance in ER proteostasis. We have included brief discussions about this point in the revised manuscript (Discussion part, page 14).

Referee #2:

Toll-interacting protein, TOLLIP was implicated in numerous processes, including aggrephagy receptor, endosomal sorting regulator and formation of mitochondrial-derived vesicles (MDV). In the current manuscript, Hayashi et. al reports a new role for TOLLIP as a specific cargo-adaptor that recognizes misfolded ER membrane proteins and directly routes them for lysosomal degradation in a process that requires further characterization.

The authors initially utilized an experimental system of chimeric protein A53T-HP comprised of Parkinson's disease-linked A53T α-synuclein mutant fused to a hairpin of E-Syt1 (ER membrane protein) to identify factors that mediate its selective lysosomal degradation. Using this system, TOLLIP, but not the other autophagy receptors, is needed for such degradation in its LIR-independent manner. Evidence indicates that the IDR and CUE domains of TOLLIP are

responsible for recognizing ER aberrant membrane protein A53T-HP. At the same time, IDR acts as a protein misfolding sensor that recognizes the misfolded substrate, while the CUE domain is responsible for recognizing ubiquitinated A53T-HP. The authors also found that TOLLIP's phosphoinositide-binding C2 domain is needed for the vesicular trafficking of A53T-HP to the lysosomes. Finally, the authors demonstrate that TOLLIP-dependent lysosomal degradation of physiological substrates such as P56S VAPB (ALS-linked) and N88S,S90L Seipin (spastic paraplegia-linked).

Overall, the experiments are well thought out, and the data seem convincing. However, the authors are encouraged to characterize better the PI3P-positive vesicles that serve as the vehicle for the TOLLIP-dependent degradation pathway. This can be achieved by improved fluorescent images, electron microscopy (preferably CLEM), and careful subcellular fractionations. This will significantly improve the quality of this study.

Response:

We highly appreciate the referee's kind and thoughtful comments to our manuscript. We quite agree with the referee's opinion that the TOLLIP-dependent delivery route for aberrant ER membrane proteins should be further characterized through analyzing the PI3P-positive vesicles.

As also suggested by the other referees, we performed CLEM analysis to visualize the PI3P-positive vesicles. First, we visualized the protein complex of the artificial model substrate A53T-HP and TOLLIP by using BiFC technology (new **Fig 4D**). Then, we observed punctate structures positive for both the BiFC signal and 2xFYVE probe, i.e., A53T-HP-, TOLLIP-, and PI3P-positive puncta by CLEM. The result shows that these PI3P puncta are vesicles basically surrounded by single membranes (new **Fig 4E and EV3C**). Since the BiFC-positive PI3P puncta were negative for LysoTracker, TOLLIP seems to utilize these vesicles as transport intermediates to deliver its cargo to acidic lysosomal compartments (new **Fig 4F**). For more detail on the BiFC and CLEM analyses, please also refer to Referee #1 Major point 1.

Furthermore, the signals of normal ER constituents stained by anti-KDEL and BAP31 antibodies were excluded from the A53T-HP-, TOLLIP-, and PI3P-positive puncta (new **Fig 5C**). This result suggests that these vesicles are dedicated to the clearance of aberrant cargoes, which is consistent with the evidence that TOLLIP is dispensable for bulk ER turnover (Fig 5B). Since the pictures in Fig EV3A in the initial manuscript showing the colocalization of A53T-HP, TOLLIP, and PI3P are also included in new **Fig 5C**, Fig EV3A was removed (**Figure for referees 6**).

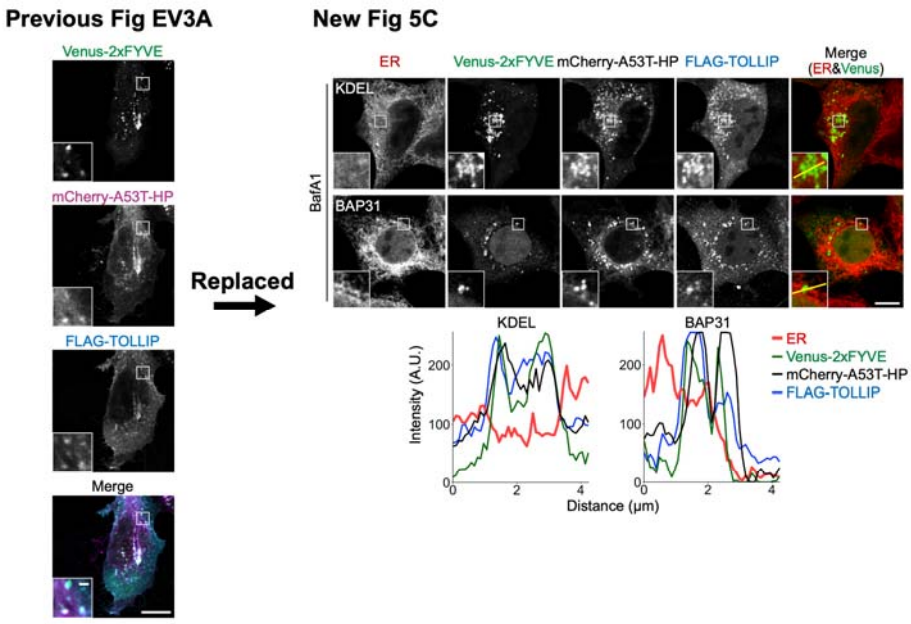

Figure for referees 6. Replaced figure.

Minor comments

1. Figure 1E - the authors use the fluorescent construct mCh-A53T-HP and measure the cleavage of mCherry by western blot. To support that data, they should also employ mCherry-EGFP-A53T-HT and measure the quenching of EGFP.

Response:

We agree with the referee. Unfortunately, due to high cell-to-cell variance in the quantification values (please see Fig 2C), EGFP quenching assay is not so robust to monitor the lysosomal trafficking of A53T-HP, especially when comparing more than two conditions. This is maybe because RFPs including mCherry are not completely resistant to lysosomal environment (Katayama *et al*, 2008, PMID 18256512), and thus several cells without $mCherry^{+}/EGFP^{-}$ puncta are actually observed even in the siCtrl condition (Fig 2C). We instead monitored the lysosomal trafficking by counting LAMP1-positive lysosomes that contain A53T-HP after BafA1 treatment in the revised manuscript. The accumulation of cargos in LAMP1-positive puncta is a more direct measure of lysosomal trafficking compared to EGFP quenching assay and widely used in the autophagy research field, including recent papers (Omari *et al*, 2018, PMID 30287488; Kuchitsu et al, 2023, PMID

36918692). Therefore, we think that it is maybe not important for readers to show the BafA1-dependent accumulation of A53T-HP in lysosomes by repeating Fig 1E by using this assay. Instead, we confirmed our important findings, i.e., the requirement of each TOLLIP domain (new **Fig 2G**) and PI3P (new **Fig EV3A and B**) in the TOLLIP-dependent pathway, which was shown only by mCherry processing assays in the initial manuscript.

2. Figure 4B - the Baf. A1 treatment should be mentioned in that panel (in addition to the legend).

Response:

As suggested, we mentioned the BafA1 treatment in the panel of Fig 4B, as well as in all other fluorescence microscopy experiments where BafA1 was used (Fig 2G, 4C, 5C, and EV3A).

3. Figure 4C - the recovery experiment was performed on siTOLLIP background; the KO used in other experiments may be more convincing.

Response:

We agree with the referee. We reperformed this experiment using *TOLLIP* KO cells and confirmed that the C2, IDR, and CUE domains of TOLLIP are required for transferring A53T-HP to PI3P puncta (**Figure for referees 7**). Since the requirement of TOLLIP for this process was shown by siTOLLIP (Fig 4B), and the knockdown efficiency of the TOLLIP siRNA is fairly high in HEK293A cells (please see TOLLIP blots in Fig 2B, 5B, 6B, 6D, EV1A, EV3F, and EV4A), we think that the recovery experiment in Fig 4C performed on siTOLLIP background is also convincing and have left this figure unchanged.

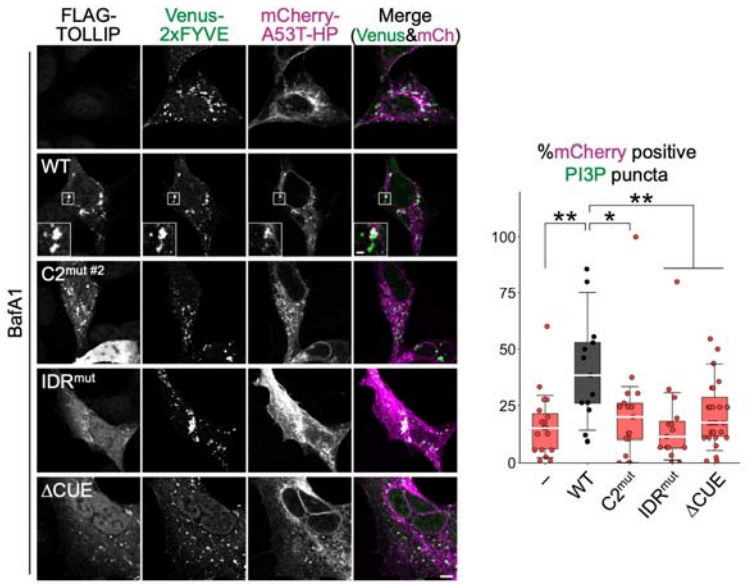

Figure for referees 7. Requirement of TOLLIP domains for trafficking of A53T-HP to PI3P puncta.

Fluorescence microscopy imaging to monitor the trafficking of A53T-HP to PI3P-enriched puncta. HEK293A *TOLLIP* KO cells were transfected with indicated FLAG-TOLLIP variants, Venus-2xFYVE, and mCherry-A53T-HP, and treated with 100 nM BafA1 for 16 h. The percentage of 2xFYVE puncta containing mCherry-A53T-HP in each cell was quantified. From 13 to 27 cells in each condition pooled from $n = 2$ experiments were analyzed. $C2^{mut\ \#2}$, R78A; IDR^{mut}, 181–229>$(GGGGS)_9GGGG$; ΔCUE, Δ231–274. Box centers indicate medians, box edges represent first and third quartiles, and whiskers show the 10th to 90th percentile. $*P < 0.05$, $**P < 0.01$ by one-way ANOVA with Dunnett's multiple comparison test. Scale bars, 10 µm and 2 µm (insets).

Referee #3:

In the present manuscript authors characterized a new function for the protein TOLLIP. Authors proposed that TOLLIP, via its IDR domain, can recognize the misfolded ER proteins which are anchored to the ER membrane and present a cytosolic domain that is ubiquitinated. Moreover, TOLLIP can recognize the misfolded proteins and mediates their lysosomal degradation via the PI3P trafficking pathway. The molecular mechanisms regarding the connection between TOLLIP and PI3P are not too clear and needs some extra investigation as the authors commented in the discussion.

TOLLIP function as an autophagy receptor is known. However, its role in degrading ER membrane bounded proteins seems to be autophagy independent. Of note, the recognition of specific degradation of single type proteins, not discrete portion of ER, is new and interesting for its biological and medical applications. Moreover, differently from FAM134B and RTN3, TOLLIP seems to directly recognize the misfolded proteins, without chaperones involvement, and it is selective for membrane proteins that face the cytosolic environment rather than for the ER luminal protein aggregates.

The manuscript has a novelty, and it is potentially interesting for the scientific community. Some experiments would be necessary to finalize the final taking message.

Response:

We appreciate the referee's detailed comments to our manuscript and deep understanding of the novelty of our findings in the light of recent progress of the ER-phagy research field. We apologize that it was unclear how TOLLIP and PI3P cooperatively promote the lysosomal degradation of aberrant ER membrane proteins in the initial manuscript, as pointed out by the referee. According to the referee's suggestion, we performed several additional experiments to confirm our conclusions.

1. Authors used pharmacological approaches to inhibit autophagy (Bafilomycin A) and the PI3P (SAR405). These are widely used reagents. However, it would be appropriate to confirm the major finding also using a genetic approach. siRNA or CRISPR some of the key autophagy and PI3P genes.

Response:

We agree with the referee's opinion that a genetic approach should be undertaken to confirm our findings. Since we have already revealed that FIP200 and ATG7 were dispensable for the TOLLIP-dependent clearance of the artificial model substrate A53T-HP by using KO cell lines (Fig EV3G–I), we tried to generate additional KO cell lines of other autophagy-related genes *VPS34* and *BECN1*. However, KO cell lines of these genes generated by CRISPR-Cas9 technology were not viable. Therefore, we instead tried transient depletion of these genes and found that siVPS34 clearly inhibited autophagy (new **Fig EV3B**). Both siVPS34 and the VPS34 inhibitor SAR405 impaired the TOLLIP-dependent lysosomal trafficking of A53T-HP, confirming the importance of PI3P in the TOLLIP-dependent pathway (new **Fig EV3A**). We also validated the requirement of VPS34 in the TOLLIP-dependent lysosomal degradation of A53T-HP by using another VPS34-specific inhibitor VPS34-IN1 (new **Fig 4A**).

2. Authors performed several experiments to show the delivery of misfolded protein to the lysosome. However, it would be appropriate to perform some high-resolution and/or electron microscopy experiments to clearly show that TOLLIP is moving the misfolded protein directly to lysosomes without the presence of any other intermediates (like autophagosomes).

Response:

We quite agree with the referee's opinion that electron microscopy is necessary to further characterize the trafficking route for aberrant ER membrane proteins. As also suggested by the other referees, we performed CLEM analysis to visualize and characterize the PI3P-positive vesicles, which are used as the carrier for the TOLLIP-dependent pathway. First, we visualized the protein complex of A53T-HP and TOLLIP by using BiFC technology. The BiFC signal translocated to PI3P puncta in a TOLLIP IDR and CUE domains-dependent manner, suggesting that TOLLIP escorts A53T-HP to PI3P puncta after recognizing it (new **Fig 4D**). Then, we observed punctate structures positive for both the BiFC signal and 2xFYVE probe, i.e., A53T-HP-, TOLLIP-, and PI3P-positive puncta, by CLEM. The result shows that these PI3P puncta are vesicles basically surrounded by single membranes, not double membranes as is the case for autophagosomes (new **Fig 4E and EV3C**). Since the BiFC-positive PI3P puncta were negative for LysoTracker, TOLLIP seems to utilize these vesicles as transport intermediates to deliver its cargo to acidic lysosomal compartments (new **Fig 4F**). For more detail on the BiFC and CLEM analyses, please also refer to Referee #1 Major point 1.

Although it is difficult to completely rule out the potential involvement of other intermediates, at least, dispensability of FIP200 and ATG7 for the clearance of A53T-HP (Fig EV3G–I) and our new CLEM data suggest that autophagosomes are not involved.

Concerning the proposed experiments

1. Line 138-139 " Among the receptors tested, only overexpression of TOLLIP....." maybe it is more appropriate .... Mostly overexpression of TOLLIP....

Response:

We apologize for the unclear description. We have edited this part to a simpler description as below.

(Initial manuscript) "Among the receptors tested, only overexpression of TOLLIP promoted the cleavage of lysosomal degradation-resistant mCherry from mCherry-A53T-HP"

(Revised manuscript) “When overexpressed, TOLLIP most strongly promoted the cleavage of lysosomal degradation-resistant mCherry from mCherry-A53T-HP”

2. Fig2B and line 141-142. Not only TOLLIP but also OPTN seems to have an effect (minor but present) so authors should edit this part.

Response:

Thank you for the insightful suggestion. We have edited this part as below.

(Initial manuscript) “In contrast, siRNA-mediated knockdown of TOLLIP, but not other receptors, impaired mCherry-A53T-HP processing in HEK293A and HeLa cells”

(Revised manuscript) “In contrast, siRNA-mediated knockdown of TOLLIP impaired mCherry-A53T-HP processing in HEK293A and HeLa cells, while the effect of knocking down other receptors was smaller”

3. Line 150-152 and related EV1. This part is not clear and difficult to follow in the figure. Please edit to make easier to visualize.

Response:

We apologize for the unclear description and have edited the manuscript as below.

(Initial manuscript) “TOLLIP overexpression enhanced mCherry cleavage from A53T-HP but not from cytosolic A53T α-synuclein or native E-Syt1, suggesting that TOLLIP selectively promotes the lysosomal degradation of A53T-HP”

(Revised manuscript) “TOLLIP overexpression enhanced the lysosomal degradation of A53T-HP as examined by mCherry processing, but not that of cytosolic A53T α-synuclein or native E-Syt1, suggesting cargo selectivity of TOLLIP”

We have also removed the unintelligible bar graph and instead indicated numerical values of fold change in mCherry processing in new **Fig EV1D** (**Figure for referees 8**).

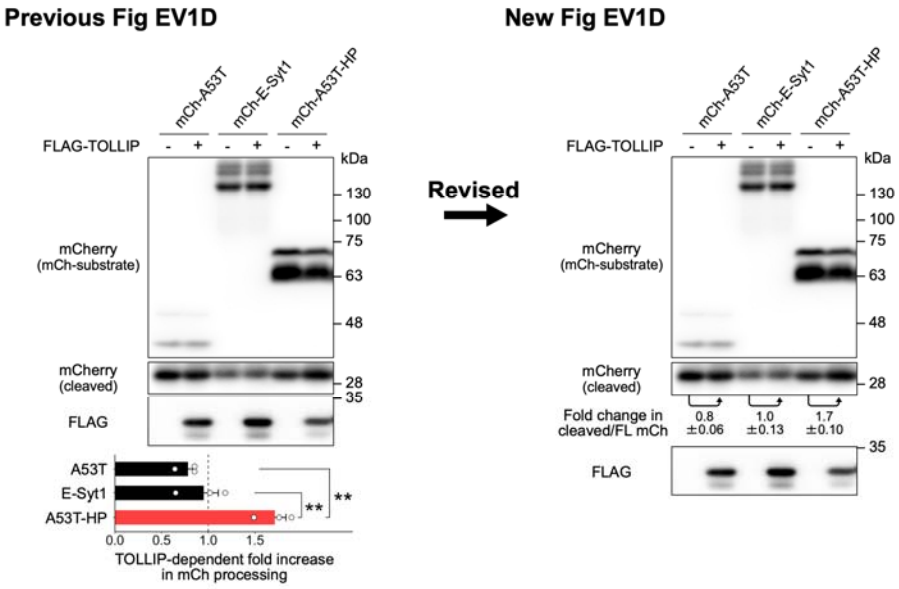

Figure for referees 8. Revised figure.

4. Authors used mCherry-A53T-HP to measure the rate of degradation looking at the mCherry cleavage. Sometime is not so clear the cleavage ratio so maybe authors should consider repeating some experiment looking to GFP degradation too or performing some additional experiments to improve the quality of the data.

Response:

We agree with the referee that our conclusions were relying on the ambiguous results of mCherry cleavage assays in our initial manuscript. In the revised manuscript, we confirmed our important findings, i.e., the requirement of each TOLLIP domain (new **Fig 2G**) and PI3P (new **Fig EV3A and B**) in the TOLLIP-dependent pathway, by assessing the accumulation of A53T-HP in LAMP1-positive lysosomes, which is a more direct measure of lysosomal trafficking than the GFP degradation assay presented in Fig 2C.

5. Fig 5D. It is a bit difficult to appreciate differences in that WB. Maybe consider adding a lower exposure film to better visualize the data.

Response:

We are sorry for presenting the unclear figure. In the revised manuscript, we presented a bit lower exposure image of mCherry-VAPB and indicated numerical values of TOLLIP KD-dependent fold

increase in mCherry-VAPB together with arrows that clearly show which lane is compared to which (**Figure for referees 9**).

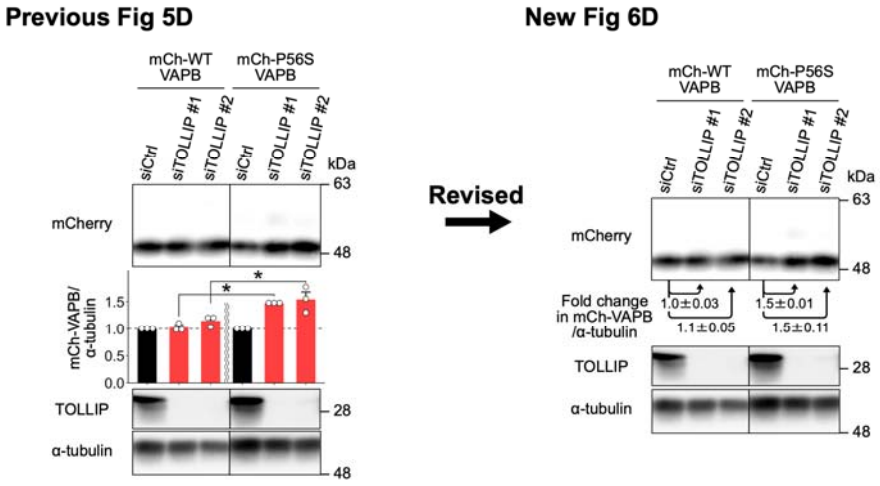

Figure for referees 9. Revised figure.

Minor Points

1. Appendix Figure could be moved into EV Figures to make the manuscript more compact.

Response:

We carefully considered this point. However, because the number of EV figures is limited to five by the journal's policy, and because we have added several new data in the revised manuscript, we could not move Appendix figures to EV figures.

2. The written part, especially in the results session, is a bit lengthily. Some details and comments could be shortened.

Response:

Following the reviewer's advice, we have removed several redundant or unnecessary descriptions. The changes have been recorded in the revised manuscript file of .docx format in a traceable manner.

Dear Dr Ichijo,

Thank you submitting a revised version of your manuscript. It was sent to the same three reviewers that originally appraised your work; their comments are attached to the bottom of this email. As you will see, all three referees are satisfied with the changes you made. Before we can move forwards towards publication of your manuscript, though, there are some remaining editorial points which need to be addressed. In this regard, would you please:

- remove the author credit section from the main manuscript,
- in the Appendix Figure, place legends below the corresponding figures,
- in figure legends, although 'n' is provided, please describe the nature of entity for 'n' in the legends of figures 1d-e; 2a-b, e-f; 4a; 5a-b, d-e; 6b-c; 7a-b; 8a-d; EV1a-d; EV2a, EV3c-d; d, f, h, i; EV4b-f; EV5b-d, and
- ensure all Source Data files are named correctly.

I look forward to receiving these changes. EMBO Press is an editorially independent publishing platform for the development of EMBO scientific publications.

Best wishes,

William Teale

William Teale, PhD
Editor
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (7th Jan 2024). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors have done an impressive job in addressing all my concerns, including the inclusion of substantial new experimental data. I am happy to recommend publication of this revised manuscript.

Referee #2:

The authors successfully addressed the comments raised by all referees.

Referee #3:

Authors addressed all the referee points.

All editorial and formatting issues were resolved by the authors.

2nd Revision - Editorial Decision **11th Oct 2023**

Dear Dr. Ichijo,

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

Congratulations to you and all of your co-workers for this really exciting and insightful work.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

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Yours sincerely,

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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.
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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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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 χ^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;
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Please complete ALL of the questions below.
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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Appendix Table S1

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods and Appendix Table S1

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Appendix Table S1
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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Please detail **housing and husbandry conditions**.	Not Applicable	

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Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends and Materials and Methods

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In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

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Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
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If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
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If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	