JCB

TBK1 phosphorylation activates LIR-dependent degradation of the inflammation repressor TNIP1

Jianwen Zhou, Nikoline Rasmussen, Hallvard Olsvik, Vyacheslav Akimov, Zehan Hu, Gry Evjen, Stephanie Kaeser-Pebernard, Devanarayanan Siva Sankar, Carole ROUBATY, Pauline Verlhac, Nicole van de Beck, Fulvio Reggiori, Yakubu Princely Abudu, Blagoy Blagoev, Trond Lamark, Terje Johansen, and Jörn Dengjel

Corresponding Author(s): Jörn Dengjel, University of Fribourg and Terje Johansen, University of Tromsø - The Arctic University of Norway

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November 5, 2021

Re: JCB manuscript #202108144

Prof. Jörn Dengjel
University of Fribourg
Chemin du Musee 10
Fribourg, FR 1700
Switzerland

Dear Prof. Dengjel,

Thank you for submitting your manuscript entitled "TBK1 phosphorylation activates LIR-dependent degradation of the inflammation repressor TNIP1." Your manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that the reviewers feel the subject of your work is interesting and potentially suitable for JCB but also raise significant concerns regarding the major conclusion of the study, that TNIP1 is an autophagy substrate and is selectively degraded under inflammatory conditions.

Although your manuscript is intriguing, we feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic we would be willing to consider a significantly revised and extended manuscript that fully addresses all of the reviewers' concerns and is subject to further peer-review. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Tamotsu Yoshimori, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In the manuscript by Zhou et al., the authors investigate the autophagic degradation of the anti-inflammatory protein TNIP1. Autophagy is generally considered to be an anti-inflammatory pathway mediating the degradation of pro-inflammatory protein complexes. Here the authors provide evidence that autophagy can also have the opposite effect by degrading the anti-inflammatory factor TNIP1. In particular, they suggest that TBK1 phosphorylates the LIR motif of TNIP1, which results in higher affinity binding to ATG8 family proteins and its efficient tethering to autophagic membranes and subsequent degradation. The anti-inflammatory effect of autophagy is a relatively novel concept and will be of interest for a wide research community. However, a few points listed below should be addressed to make this study more convincing.

1. To strengthen the link between poly(I:C)-induced autophagy, TNIP1 phosphorylation by TBK1 and the expression of pro-inflammatory genes, the authors should mutate the TBK1 phosphorylation site in TNIP1 and follow its autophagic degradation as well as the expression of proinflammatory genes after poly(I:C) treatment (but not starvation or mTOR inhibition). These experiments should be relatively straightforward as the respective mutant could be expressed in the TNIP1 KO cells. If the

authors' model is correct, then mutation of the TBK1 phosphorylation site should result in TNIP1 stabilization and reduced expression of pro-inflammatory genes. In addition, the authors should assess the contribution of proteasomal degradation to the reduced expression/degradation of TNIP1 after poly(I:C) treatment.

2. The data resented in Fig. 6A and B should be presented better. First, the signals on the blot are so different between the time points and conditions, that it is questionable that they can be quantified in a meaningful manner. Second, the quantification seems a little at odds with the data shown in the blot. For example, for the Penta KO cells there is a large decrease in the TNIP1 signal from 0h to 2h but the quantification suggest that it is only about twofold. Third, it should be better explained what the stars refer to. Last, it should be tested to which extent inhibition of the proteasome affects TNIP1 expression in their time course.

3. Fig 4D: the recruitment of LC3s between WT and LIR-mutants is calculated based on relative ratios versus input. Here it would be good to repeat this experiment with the complementary set-up to the one in the manuscript, showing for the strongest interactors in a side-by-side comparison (on the same gel) between WT and LIR-mutants.

4. Fig. 7E: Same comment as above, to determine the relative enrichment of the phospho-mimicking variants of TNIP1, it would be better if the WT versus phosphomutants are compared side-by-side on the same gel.

5. Fig 6C: Quantification of the number of TNIP1-puncta/cell would be required to be able to assess if there is a difference between the ATG7-KO and penta-KO condition.

6. Fig. 1D: what is the ratio of ubiquitinated versus non-ubiquitinated TNIP1? From the input fraction it seems only a very small proportion of TNIP1 is ubiquitinated. In addition, the authors conclude that there are multiple ubiquitinated sites on TNIP1, in addition to the two that were mutated in suppl. Fig1. It would be good to show the coverage of TNIP1 in the mass spec experiment, to see where on the protein these additional ubiquitinated-sites could be located as these peptides are supposedly not detected by mass spec. Furthermore, Suppl. Figure 1D is not entirely clear to me. The authors perform a pull-down for TNIP1 and then probe for Ub at much higher molecular weights. From the TNIP1 detection, the protein appears to run at 90kDa. If TNIP1 would be ubiquitinated, you would expect to see a ladder pattern (perhaps upon high exposure only) with the TNIP1 antibody. To me the Ub-antibody does not prove that these bands on the gel are truly TNIP1, these could equally well be other proteins with a ubiquitin tag, which interact with TNIP1.

7. Suppl. Fig 1a: please also provide a shorter exposure of the TNIP1 western blot. The pattern of protein levels (in particular the increase after blocking lysosomal degradation), is not clearly visible in this blot. Panel B does however convincingly show the accumulation TNIP1 under some of the tested conditions.

8. Suppl. Fig 3: the ISG15 levels in the WT versus KO1 and KO2 do not correlate with the fold change presented in Fig. 5G (when comparing the basal/untreated wells from suppl Fig 3).

Minor comments:
9. Fig. 3C: the lower panel shows the EV next to the transfected wells of the gel, on the same membrane. The upper panel has supposedly the EV on the same gel as the transfected lanes but possibly not loaded next to each other, therefore requiring the split of the gel. It would be good if the authors could show the full-blot of the upper panel in supplementary (marking the cropped lanes) to prove the EV was truly loaded on the same gel as the transfected lanes.

10. The authors do not elaborate on other TNIP-family members. Are the identified LIR-motifs conserved? Could these receptors act in a similar way and potentially compensating for the loss of TNIP1?

Reviewer #2 (Comments to the Authors (Required)):

The authors examine the autophagic degradation of the inflammatory mediator TNIP1/ABIN1. The authors propose that selective autophagy receptors (SLRs) such as p62 are needed to degrade TNIP1 during basal conditions. They also propose that TLR3 activation using poly-I:C results in the TBK1-mediated phosphorylation of a LIR in TNIP1 which enhances the selective autophagy of TNIP1 via a direct LC3 interaction. The autophagic degradation of TNIP1 Overall, the data do not robustly support the conclusions that have been drawn and the paper suffers from an abundance of conceptual gaps in the model proposed.

1) In Figure 2C, the experiment with the tandem reporter should be repeated in ATGKO cell lines.

2) For Figure 3, based on the interactions, the authors propose that SLRs target TNIP1/ABIN1 for degradation. However, no functional evidence is provided. The effects of loss-of-function or one or more SLRs on the turnover of TNIP1/ABIN1 should be analyzed.

3) The cellular analysis of the LIR mutant (mLIR1+2) data for ABIN1 in Fig 4D and Fig 4F doesn't seem very convincing. For the most part, a 50% reduction in binding to LC3 family members is observed suggesting there may be another critical LIR. Also, I'm not sure what is the basis of the conclusion that ABIN1 weakly binds to LC3C and GABARRAPL2 compared to other ATG8 family members.

4) The data with poly-IC treatment on autophagic degradation of TNIP1 is difficult to understand, which is a serious weakness because this is the main conclusion of the paper. The degradation in the first few hours following poly-IC is modest and at 6 hours there is a huge increase in WT, ATG7 KO and Penta-KO. The conclusion that poly-IC results in autophagic degradation of TNIIP1/ABIN1 is not strongly supported by the data provided as it is confined to a single timepoint following poly-IC treatment.

5) In Fig 6A, the effects on loss-of-function of an additional ATG not in the LC3 conjugation pathway should be analyzed for its effects on ISG15 levels. Loss of ATGs that mediate LAP can results in the transcriptional upregulation of inflammatory genes.

6) If TNIP1 accumulation drives the activation of inflammatory gene programs in autophagy-deficient cells, they the authors should analyze the effects of TNIP1 loss on reversing the pro-inflammatory transcriptional programs in ATG deficient cells. For these studies, multiple ATGs should be examined including ATGs (e.g., ATG14, FIP200) that are not in the LAP pathway.

7) The author's model is that during basal autophagy conditions, TNIP1 degradation is SLR dependent with little requirement for the LIR. In Figure 8, they propose p62 as a major regulator of that turnover. However, in Fig 6F, there is very little control TNIP1 and no detectable LIR mutant TNIP binding to LC3 in basal conditions. This is in spite of an abundance of p62 in these very same pulldowns. The lack of TNIP1-LC3 interaction in these assays casts doubt that p62 and other SLRs mediate LC3 binding and selective autophagy of TNIP1 in basal conditions.

8) In the S35 labeling studies in Fig 7E, the effects of phosphomimetic TNIP1 binding to LC3 family members appear quite modest.

9) Many of the blots are extremely overexposed and poor in quality.

Reviewer #3 (Comments to the Authors (Required)):

Comments on the manuscript by Jianwen Zhou et al.

Accumulating evidence has shown that autophagy, an intracellular degradation system, is greatly contributed to the regulation of inflammatory responses. In the present study, Jianwen Zhou and colleagues identified TNIP1, a negative regulator of the TLR signaling pathway, as an autophagy substrate and revealed a molecular mechanism underlying the autophagic degradation of TNIP1. The authors showed that TNIP1 interacts with LC3 and GABARAP proteins via the LIR motif, resulting in its degradation in autolysosomes. The authors also showed that TBK1 phosphorylates TNIP1 and induces TNIP1 degradation by autophagy after TLR3 stimulation. This reviewer feels the present study is interesting. However, the present study lacks important data that support the author's conclusion. Major concerns are shown below.

In Fig 2A and Fig 3C, why did rapamycin fail to reduce the level of p62?

In Fig 2B-C, what does FM mean?

In Fig 2C, the authors should show fluorescent images with both BafA1-treated and untreated cells

In Fig 3C upper panels, the authors should load the samples on the same gel and membrane.

In Fig 3C, the authors should examine if endogenous TNIP1 interacts with endogenous p62 independently of autophagy induction.

In Fig 3D, statistical analysis should be done.

In Fig 5G-H, the authors should examine if transcription of the indicated genes is altered by TNIP1 or not.

In Fig 6A, the authors should show the level of TLR3 in three types of cells.

In Fig 6A-B, why did TNIP1 levels NOT increase in untreated ATG7 KO cells compared to untreated WT cells? TNIP1 levels increased in untreated Penta KO cells compared to untreated WT cells. The authors mentioned in the result section regarding Fig 2 that "TNIP1 is degraded by autophagy under basal conditions". Do they mean that ATG7-independent autophagy induces degradation of TNIP1 under basal conditions?

In Fig 6D, what types of KO cells did the authors use?

In Fig 6D, F, experiments using EV-transduced wild-type cells should also be performed under the same condition.

In Fig 6E, each sample should be compared to sample 5 (KO plus TNIP1-WT 0h). Why were the levels of TNIP1-LIR lower than that of TNIP1-WT at 0h? Why did TNIP1-LIR fail to suppress ISG15 expression at 0h? In Fig 5H, TNIP1-LIR suppressed ISG15 expression under basal conditions.

In Fig 7F, what types of KO cells did the authors use?

In Fig 7G, the authors should provide genetic evidence that TBK1 induces phosphorylation and subsequent autophagic degradation of endogenous TNIP1 in polyIC stimulated cells.

In Fig 7 and Fig S1, the authors should examine if phosphorylation of TNIP1 alters its ubiquitination in polyIC-stimulated cells.

In Fig S3, the authors should examine if autophagic degradation of TNIP1 supports the production of inflammatory mediators, such as cytokines, interferons, and chemokines. A20, ISG15, and GBP1-5 are regulators of immune signaling pathways, but not direct mediators of inflammation.

Given interest in the topic we would be willing to consider a significantly revised and extended manuscript that fully addresses all of the reviewers' concerns and is subject to further peer-review. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

We are very grateful to the editors for giving us the opportunity to hand in a revised version of our manuscript. We addressed all raised concerns in detail in the following paragraphs.

Reviewer #1 (Comments to the Authors (Required)):

In the manuscript by Zhou et al., the authors investigate the autophagic degradation of the anti-inflammatory protein TNIP1. Autophagy is generally considered to be an anti-inflammatory pathway mediating the degradation of pro-inflammatory protein complexes. Here the authors provide evidence that autophagy can also have the opposite effect by degrading the anti-inflammatory factor TNIP1. In particular, they suggest that TBK1 phosphorylates the LIR motif of TNIP1, which results in higher affinity binding to ATG8 family proteins and its efficient tethering to autophagic membranes and subsequent degradation. The anti-inflammatory effect of autophagy is a relatively novel concept and will be of interest for a wide research community. However, a few points listed below should be addressed to make this study more convincing.

We thank the reviewer for her/his interest in our study and for the critical feedback!

1. To strengthen the link between poly(I:C)-induced autophagy, TNIP1 phosphorylation by TBK1 and the expression of pro-inflammatory genes, the authors should mutate the TBK1 phosphorylation site in TNIP1 and follow its autophagic degradation as well as the expression of proinflammatory genes after poly(I:C) treatment (but not starvation or mTOR inhibition). These experiments should be relatively straightforward as the respective mutant could be expressed in the TNIP1 KO cells. If the authors' model is correct, then mutation of the TBK1 phosphorylation site should result in TNIP1 stabilization and reduced expression of pro-inflammatory genes. In addition, the authors should assess the contribution of proteasomal degradation to the reduced expression/degradation of TNIP1 after poly(I:C) treatment.

We thank the reviewer for suggesting these additional experiments to further support the proposed TBK1-dependent mechanism of TNIP1 degradation. Several attempts failed to establish stable cell lines ectopically expressing sufficiently similar levels of TNIP1 variants, which makes interpretation of respective results difficult. Hence, as an alternative strategy we studied abundance differences of endogenous TNIP1 after poly(I:C) treatment of TBK1 KO compared to WT cells. As predicted by our model, we did observe poly(I:C)-dependent degradation of TNIP1 in wildtype HeLa (WT) cells, but not in two independent TBK1 KO lines. We added the new data as **new Figure 7H**:

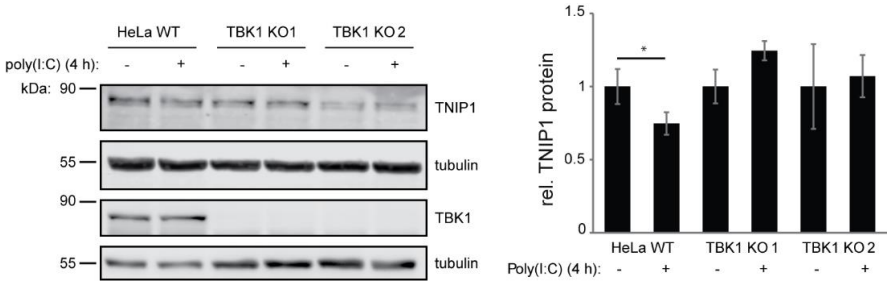
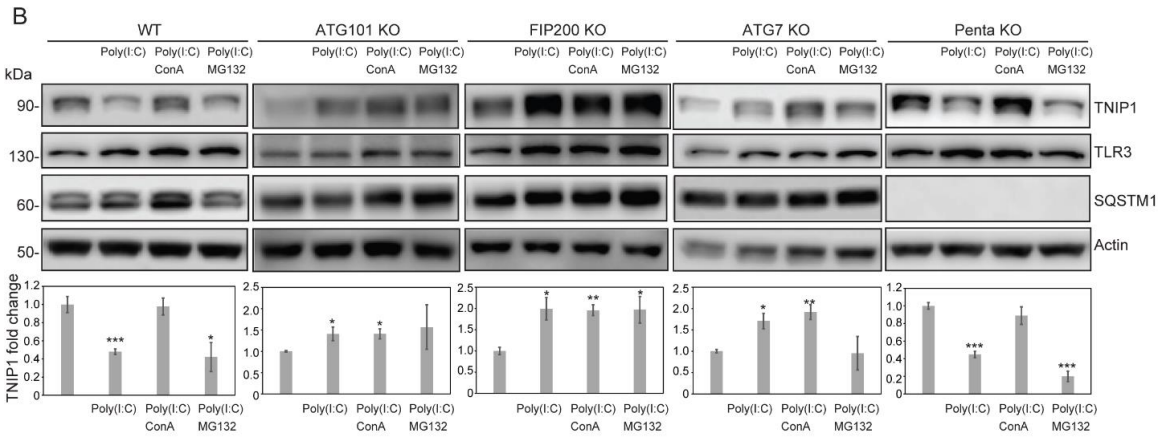


Figure 7H: Poly(I:C)-treatment of TBK KO cells does not induce TNIP1 degradation. One representative blot of three biological replicates is shown. Error bars, SEM ; * : p<0.05, t test.

We also addressed the contribution of proteasomal degradation to the reduced abundance of TNIP1 after poly(I:C) treatment as suggested and added the data as **new supplemental Figure S4B**. Whereas inhibition of the lysosome by ConA blocks poly(I:C)- and autophagy-dependent degradation of TNIP1, inhibition of the proteasome has no effect.

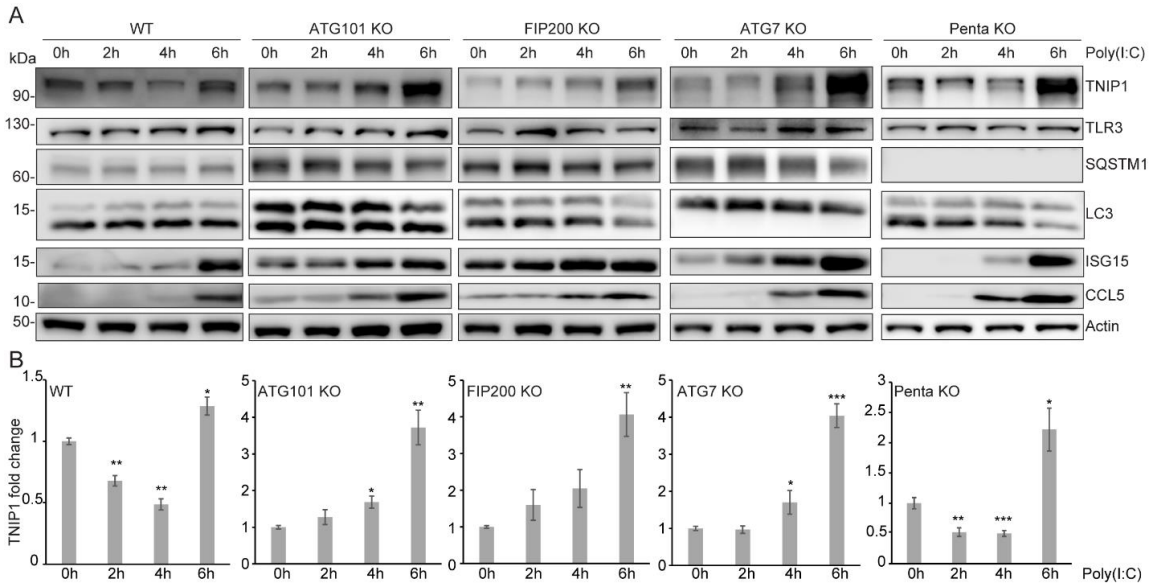


Supplemental Figure S4B: Poly(I:C) treatment leads to an autophagy-dependent and SLR-independent lysosomal degradation of TNIP1. Whereas lysosomal inhibition by ConA treatment leads to a significant block of TNIP1 degradation in WT and pentaKO cells, proteasomal inhibition by MG132 treatment has no effect. In ATG101 KO, FIP200 KO and ATG7 KO, autophagy incompetent cell lines poly(I:C) does not lead to TNIP1 degradation. TLR3 and SQSTM1 are monitored as positive controls, actin as loading control. Shown are representative blots of three biological replicates each. Bar diagram shows quantification, error bars: SEM. *: p<0.05, **: p<0.01, ***: p<0.001, T test.

2. The data resented in Fig. 6A and B should be presented better. First, the signals on the blot are so different between the time points and conditions, that it is questionable that they can be quantified in a meaningful manner. Second, the quantification seems a little at odds with the data shown in the blot. For example, for the Penta KO cells there is a large decrease in the TNIP1 signal from 0h to 2h but the quantification suggest that it is only about twofold. Third, it should be better explained what the stars refer to. Last, it should be tested to which extent inhibition of the proteasome affects TNIP1 expression in their time course.

As suggested, we repeated the analyses and analyzed additional autophagy-non-competent cell lines. As cell lines differ in their expression levels, we analyzed each cell line on separate blots to ensure optimal exposure of the blots. The new analyses were added as **new Figures 6A-B** and confirm our previous results that poly(I:C) treatment induces an autophagy-dependent degradation of TNIP1 (see figure below).

With respect to the role of the proteasome on TNIP1 protein abundance we would like to refer the reviewer to the data presented above.



A Poly(I:C) treatment leads to time dependent changes in TNIP1 abundance. Poly(I:C) stimulation leads to an autophagy dependent and SLR independent decrease of TNIP1 abundance within the first 4 h as indicated by a block of degradation in ATG101, FIP200 and ATG7 KO cells. Autophagy receptors appear to have a minor influence as degradation still occurs in pentaKO cells. **B** Quantification of blots shown in (A) (n=3). Error bars indicate standard deviation. * = p<0.05, ** = p<0.01, *** = p<0.001 unpaired T test compared to 0 h values of respective cell lines.

3. Fig 4D: the recruitment of LC3s between WT and LIR-mutants is calculated based on relative ratios versus input. Here it would be good to repeat this experiment with the complementary set-up to the one in the manuscript, showing for the strongest interactors in a side-by-side comparison (on the same gel) between WT and LIR-mutants.

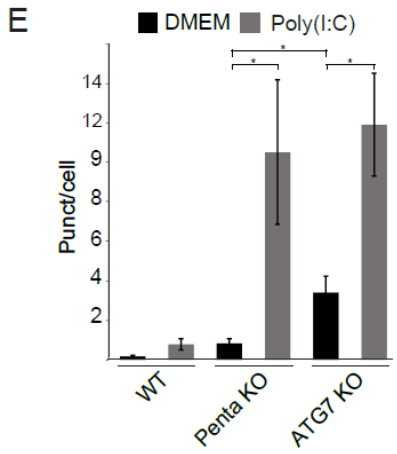
As the reviewer states, the amount of TNIP1 protein bound to the ATG8s is related to the amount of TNIP1 protein in the input for each variant of TNIP1 analyzed. Running the different TNIP1 variants side-by-side on the same gel, as suggested, will not give a "better" readout. This GST pulldown assay is a very reliable assay (used in our lab since 1992) where the readout in the end is the radioactive signal from ^{35}S-labeled Met incorporated into the *in vitro* translated TNIP1 variants analyzed. It does not suffer from the semiquantitative nature of western blot-based readouts. All the gels have been developed simultaneously to obtain the radioactive signals used in the quantifications and are therefore directly comparable.

4. Fig. 7E: Same comment as above, to determine the relative enrichment of the phospho-mimicking variants of TNIP1, it would be better if the WT versus phosphomutants are compared side-by-side on the same gel.

See the answer above to comment 3.

5. Fig 6C: Quantification of the number of TNIP1-puncta/cell would be required to be able to assess if there is a difference between the ATG7-KO and penta-KO condition.

We agree and have quantified the TNIP1 puncta in **new Figure 6E**. The results show an elevated basal level of TNIP1 puncta in the ATG7 KO cells relative to the WT and pentaKO cells. The number of puncta increase with poly(I:C) at the 4h time point in both ATG7 KO and pentaKO cells. Hence, neither ATG7 nor SLRs are required for poly(I:C)-induced TNIP1 aggregation into dots. From our western blot results it is clear that ATG7, but not SLRs, are necessary for TNIP1 degradation upon poly(I:C) stimulation.

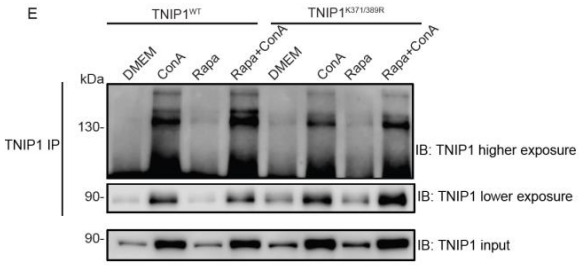


E: Quantification of images shown in (D), error bars indicate SEM. * = p<0.05, unpaired T test.

6. Fig. 1D: what is the ratio of ubiquitinated versus non-ubiquitinated TNIP1? From the input fraction it seems only a very small proportion of TNIP1 is ubiquitinated. In addition, the authors conclude that there are multiple ubiquitinated sites on TNIP1, in addition to the two that were mutated in suppl. Fig1. It would be good to show the coverage of TNIP1 in the mass spec experiment, to see where on the protein these additional ubiquitinated-sites could be located as these peptides are supposedly not detected by mass spec. Furthermore, Suppl. Figure 1D is not entirely clear to me. The authors perform a pull-down for TNIP1 and then probe for Ub at much higher molecular weights. From the TNIP1 detection, the protein appears to run at 90kDa. If TNIP1 would be ubiquitinated, you would expect to see a ladder pattern (perhaps upon high exposure only) with the TNIP1 antibody. To me the Ub-antibody does not prove that these bands on the gel are truly TNIP1, these could equally well be other proteins with a ubiquitin tag, which interact with TNIP1.

This is an important question which is difficult to answer. Indeed, from our affinity purification western blot data only a minor fraction of TNIP1 appears to be ubiquitinated under the tested experimental conditions. We **added a new supplementary figure 1E** highlighting that we do detect TNIP1 bands in the upper gel area of anti-TNIP1 IPs. The TNIP1 bands appear at a similar size as the ubiquitin bands shown in supplementary Figure 1D, indicating that this might indeed be ubiquitinated TNIP1. Together with the presented mass spec data, we are confident that TNIP1 is being ubiquitinated and that the ubiquitinated variant can be stabilized by lysosomal inhibition. However, due to the limited dynamic range of the blots, we cannot accurately quantify the ratio

as we do detect the upper bands only after extended exposure. We also added a respective sentence to the manuscript stating that only a minor fraction of TNIP1 appears to be ubiquitinated and that both non-modified and ubiquitinated TNIP1 get degraded in the lysosome.

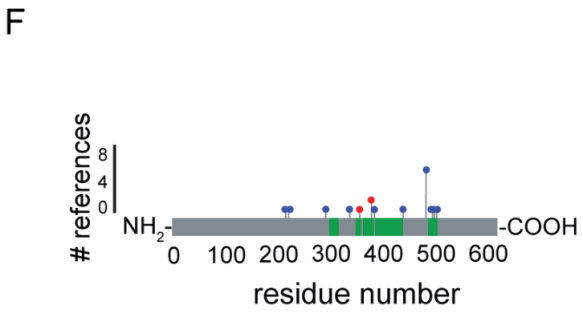


(D, E) Mutated TNIP1K371/389R is still getting ubiquitinated as indicated by anti-TNIP1 IP followed by anti-ubiquitin (D) and anti-TNIP1 (E) western blot. The addition of ConA leads in all cases to a stabilization of non-ubiquitinated and polyubiquitinated protein variants.

New text:

"As only a fraction of TNIP1 appeared to be ubiquitinated, we investigated if ubiquitination of Lys389 is necessary for lysosomal targeting of TNIP1. We performed site-directed mutagenesis and analyzed the stability and ubiquitination of wildtype ($TNIP1^{WT}$) and respective TNIP1 variants. Next to Lys389 we also mutated the neighboring residue Lys371, which we identified in two out of the three SILAC experiments. Blockage of lysosomal degradation led to the accumulation of modified and non-modified variants of TNIP1, indicating that ubiquitination is not decisive for lysosomal degradation (Fig S1B). In addition, arginine-variants did neither exhibit alterations in their global ubiquitination pattern, nor in their stability, indicating the presence of additional ubiquitination sites, which agrees with database entries (suppl. Fig S1B-F). Together, these results indicate that non-ubiquitinated and multi-ubiquitinated variants of TNIP1 accumulate upon the blockage of lysosomal acidification, and that TNIP1 may be an autophagy substrate. Due to its importance in inflammation, we decided to study the regulation of TNIP1 protein abundance in more detail."

Concerning the sequence coverage of TNIP1 and the detected ubiquitination site, we added a **new supplemental figure 1F** highlighting all known ubiquitination sites listed in the database PhosphoSitePlus and marking the tryptic peptides that were detected in the current study. As common for shot-gun proteomics type of experiments, we only cover few potential sites, including K389 which is highlighted in the paper. Thus, we cannot exclude the existence of additional ubiquitination sites which are missed by our study. The existence of such sites is also supported by the blots shown above, which indicate ubiquitinated TNIP1 in K371R and K389R variants. Thus, we provide evidence that K389 is being ubiquitinated without claiming to have comprehensively covered all potential ubiquitination sites. We added a respective statement to the manuscript.

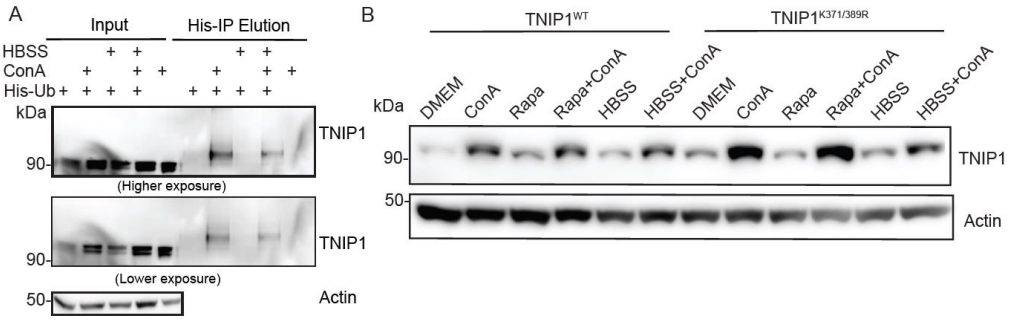


(F) Identified ubiquitination sites according to PhosphoSitePlus database and this study. Grey bar depicts the amino acid sequence of TNIP1. Sections in green mark tryptic peptides identified in this study, i.e. sequence coverage of TNIP1. Amino acids marked in blue highlight published ubiquitination sites, number of references shown on y-axis. Amino acids marked in red were identified in this study.

7. Suppl. Fig 1a: please also provide a shorter exposure of the TNIP1 western blot. The pattern of protein levels (in particular the increase after blocking lysosomal degradation), is not clearly visible in this blot. Panel B does however convincingly show the accumulation TNIP1 under some of the tested conditions.

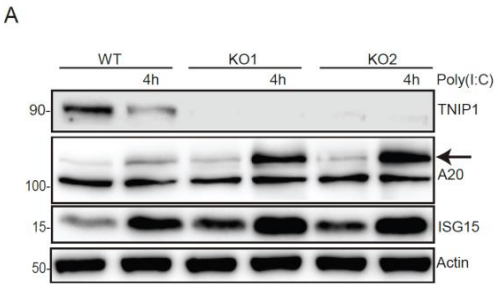
As suggested we now show a longer and a shorter exposure of the blot. As outlined by the reviewer, in combination with suppl. Figure 1B, the conA effect on protein abundance is very clear.

New supplementary figure 1A-B:



8. Suppl. Fig 3: the ISG15 levels in the WT versus KO1 and KO2 do not correlate with the fold change presented in Fig. 5G (when comparing the basal/untreated wells from suppl Fig 3).

We thank the reviewer for noticing this discrepancy. We did not select a representative blot for the old version of this figure. We added now a **new supplemental Figure 4A** in which the changes between WT and KO cells reflects the changes highlighted in the main manuscript. In addition, we added A20 as another marker protein highlighting the differences between WT and KO cells.



Supplemental Figure 4A: Reduction of TNIP1 correlates with an increase of ISG15 and TNFAIP3/A20 in HeLa cells. The increase of the TNIP1 interaction partner TNFAIP3 under poly(I:C) treatment indicates the existence of distinct TNIP1 pools, i.e. free and bound to TNFAIP3. Arrow marks A20 band.

Minor comments:

9. Fig. 3C: the lower panel shows the EV next to the transfected wells of the gel, on the same membrane. The upper panel has supposedly the EV on the same gel as the transfected lanes but possibly not loaded next to each other, therefore requiring the split of the gel. It would be good if the authors could show the full-blot of the upper panel in supplementary (marking the cropped lanes) to prove the EV was truly loaded on the same gel as the transfected lanes.

> We rerun the samples next to each other and exchanged Fig. 3C. In addition, we generated a supplementary source data file in which we show all original blots.

10. The authors do not elaborate on other TNIP-family members. Are the identified LIR-motifs conserved? Could these receptors act in a similar way and potentially compensating for the loss of TNIP1?

> We thank the reviewer for this important point. The two other TNIP-family members lack the region containing the LIR motifs. Hence, it is unlikely that the other two TNIP-family members can compensate for the loss of TNIP1. We have inserted the following sentence at the appropriate place in the Results section of the revised manuscript: *The N-terminal region harboring these LIR motifs is missing in the two other TNIP family members, TNIP2 and -3.*

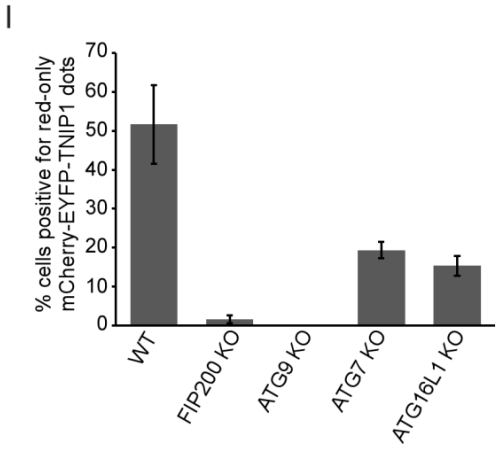
Reviewer #2 (Comments to the Authors (Required)):

The authors examine the autophagic degradation of the inflammatory mediator TNIP1/ABIN1. The authors propose that selective autophagy receptors (SLRs) such as p62 are needed to degrade TNIP1 during basal conditions. They also propose that TLR3 activation using poly-I:C results in the TBK1-mediated phosphorylation of a LIR in TNIP1 which enhances the selective autophagy of TNIP1 via a direct LC3 interaction. The autophagic degradation of TNIP1 Overall, the data do not robustly support the conclusions that have been drawn and the paper suffers from an abundance of conceptual gaps in the model proposed.

We thank the reviewer for her/his interest in our paper!

1) In Figure 2C, the experiment with the tandem reporter should be repeated in ATGKO cell lines.

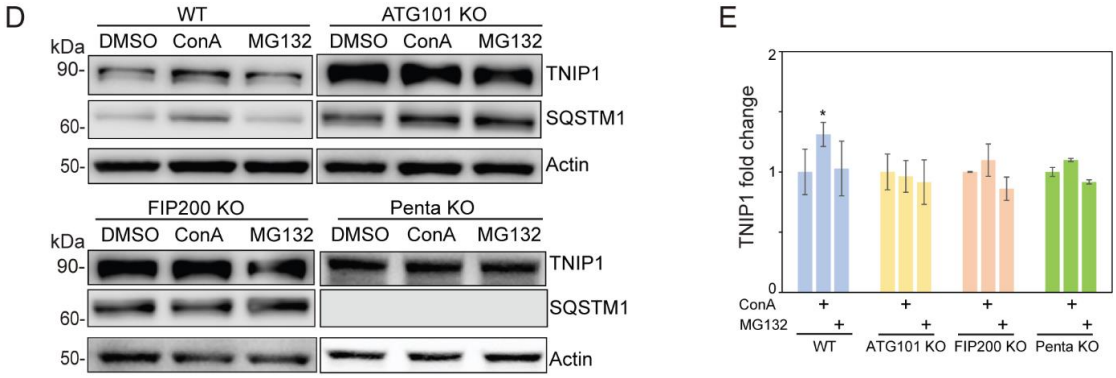
As suggested by the reviewer, we tested the tandem reporter under basal conditions in a selection of KO U2OS cells: FIP200, ATG9, ATG7 and ATG16L1 KO cells. The expression of mCherry-EYFP-TNIP1 in ATG9 and FIP200 KO cells showed little to no red-only dots, consistent with the importance of these proteins in both ATG7-dependent and –independent autophagy. ATG7 and ATG16L1 KOs showed reduced formation of red-only dots, but not a complete loss, suggesting that there may also be ATG7-independent degradation of TNIP1 under basal conditions. Altogether, these results suggest that TNIP1 is indeed degraded by autophagy under basal conditions, and that this turnover may be both ATG7-dependent and –independent. New data was added **as new Figure 3I:**



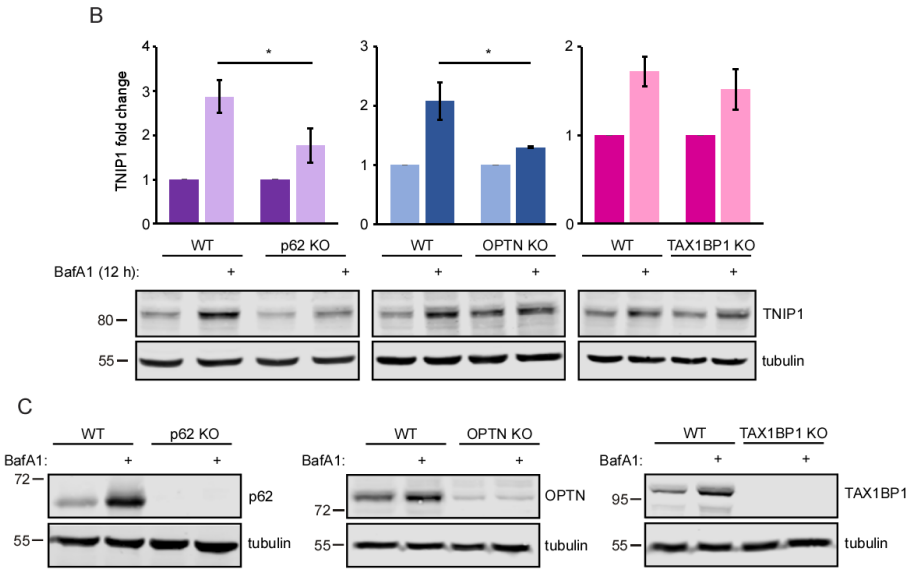
2) For Figure 3, based on the interactions, the authors propose that SLRs target TNIP1/ABIN1 for degradation. However, no functional evidence is provided. The effects of loss-of-function or one or more SLRs on the turnover of TNIP1/ABIN1 should be analyzed.

As suggested we analyzed TNIP1 degradation in SLR KO cells and autophagy-incompetent cells compared to WT cells. We added this new data as **new Figure 3D-E**. In agreement to our model,

only WT cells exhibit a stabilization of TNIP1 by blockage of the lysosome. In Penta KO cells, which do not express p62/SQSTM1, NBR1, NDP52, TAX1BP1 and OPTN, TNIP1 is not stabilized by ConA addition, as in ATG101 and FIP200 KO cells.



We also analyzed TNIP1 stabilization upon lysosomal blockage in cells KO for single SLRs (p62, OPTN and TAX1BP1). Both p62 KO and OPTN KO led to a significant reduction in TNIP1 stabilization upon BafA1-treatment, suggesting that more than one SLR is involved in the basal turnover of TNIP1. The data has been added as a **new supplemental figure S3B-C.**



3) The cellular analysis of the LIR mutant (mLIR1+2) data for ABIN1 in Fig 4D and Fig 4F doesn't seem very convincing. For the most part, a 50% reduction in binding to LC3 family members is observed suggesting there may be another critical LIR. Also, I'm not sure what is the basis of the conclusion that ABIN1 weakly binds to LC3C and GABARRAPL2 compared to other ATG8 family members.

Here we assume that the reviewer means Fig. 4F since Fig. 4D is not a cellular analysis and the reductions in binding are much more than 50% as can be seen from the quantifications of the Fig. 4D autoradiographs shown in Fig. 4E. The point mutations used will in most cases not be able to eliminate binding completely given the sensitivity of the radioactive detection used in our GST pulldown assays. In Fig 4F the quantification shown in Fig. 4G is based on the enzymatic-based chemiluminescence detection used in the Western blot in Fig. 4F which is much less sensitive and Western blots are semiquantitative in nature. Therefore, we do not expect the same reduction in measured binding as we see from the quantitative autoradiography based GST pulldown assay. Also, in the IP from cells in Fig. 4F there are likely also contributions from SLRs that interact with TNIP1 and these have intact LIRs that can contribute to IP LC3.

4) The data with poly-IC treatment on autophagic degradation of TNIP1 is difficult to understand, which is a serious weakness because this is the main conclusion of the paper. The degradation in the first few hours following poly-IC is modest and at 6 hours there is a huge increase in WT, ATG7 KO and Penta-KO. The conclusion that poly-IC results in autophagic degradation of TNIIP1/ABIN1 is not strongly supported by the data provided as it is confined to a single timepoint following poly-IC treatment.

Indeed, as pointed out by the reviewer the regulation of TNIP1 protein abundance in poly(I:C)-treated cells is complex and we might not have explained this properly in the original version of the paper. We changed this in the revised version including more timepoints to better follow TNIP1 protein dynamics and additional analyses. Briefly:

After poly(I:C) stimulation, protein abundance of TNIP1 is regulated by two overlaying processes. Selective, TBK1- and LIR-dependent autophagy leads to a short-term degradation of TNIP1 (2-4 h after poly(I:C) stimulation, **new Figures 6A-B**). The observed increase after 6 h is due to changes on transcriptional level. We added **a new Figure 6C** which highlights that after 4 h of stimulation mRNA of TNIP1 increases significantly, which is mirrored by a respective protein increase after 6 h. As outlined in our model in Figure 8, this increase likely supports the limitation of inflammatory gene activation.

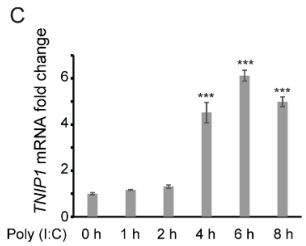


Figure 6C: After 4 hours of poly(I:C) treatment TNIP1 transcription is significantly upregulated Bar diagram shows quantification of three biological replicates, error bars: SEM. *: p<0.05, **: p<0.01, ***: p<0.001, T test.

5) In Fig 6A, the effects on loss-of-function of an additional ATG not in the LC3 conjugation pathway should be analyzed for its effects on ISG15 levels. Loss of ATGs that mediate LAP can results in the transcriptional upregulation of inflammatory genes.

As suggested, we tested additional ATG KO cells: FIP200 and ATG101 KO both block poly(I:C)-dependent degradation of TNIP1 (see Figure above), indicating that TNIP1 degradation is indeed

dependent on canonical macroautophagy. We added a respective statement to the revised manuscript.

6) If TNIP1 accumulation drives the activation of inflammatory gene programs in autophagy-deficient cells, they the authors should analyze the effects of TNIP1 loss on reversing the pro-inflammatory transcriptional programs in ATG deficient cells. For these studies, multiple ATGs should be examined including ATGs (e.g., ATG14, FIP200) that are not in the LAP pathway.

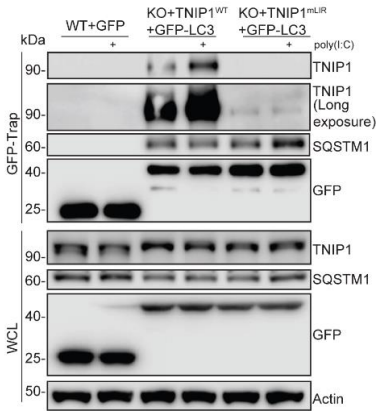
We are not sure how to address this comment. Firstly, it is not TNIP1 accumulation that drives inflammatory gene programs. It is rather the opposite: TNIP1 degradation supports inflammatory gene activation. In addition, it is difficult to study the effects of TNIP1 in ATG KO cells, as many ATG proteins themselves have been shown to be important for inflammation regulation. Thus, regardless of the results, we would not be sure how to interpret these data as the observed phenotype might be indirect.

Nevertheless, we analyzed the effects of FIP200 and ATG101 KO on the abundance of pro-inflammatory proteins ISG15 and CCL5. As predicted by our model, increased TNIP1 protein abundance due to blockage of autophagy led to smaller fold-changes of respective proteins after poly(I:C) stimulation. We added this data as **new Figure 6A**.

7) The author's model is that during basal autophagy conditions, TNIP1 degradation is SLR dependent with little requirement for the LIR. In Figure 8, they propose p62 as a major regulator of that turnover. However, in Fig 6F, there is very little control TNIP1 and no detectable LIR mutant TNIP binding to LC3 in basal conditions. This is in spite of an abundance of p62 in these very same pulldowns. The lack of TNIP1-LC3 interaction in these assays casts doubt that p62 and other SLRs mediate LC3 binding and selective autophagy of TNIP1 in basal conditions.

We thank the reviewer for this critical comment and agree that only little TNIP1 is seen in the anti-GFP-LC3 IPs shown in Figure 6. However, we would like to state that according to our model this would be an indirect interaction mediated by p62/SQSTM1 under basal conditions. Thus, the chosen experimental setup might not be the best to detect such an indirect interaction. In contrast, in Figure 3 we show anti-TNIP1 affinity purifications and detect p62/SQSTM and additional SARs as strong TNIP1 interactors under growth and starvation conditions, in support of our model.

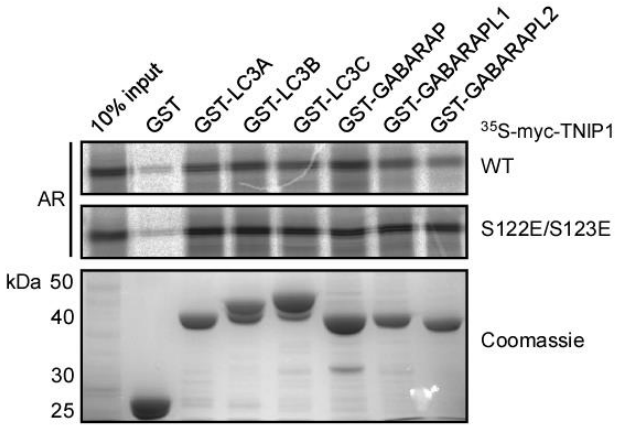
To follow up the comment of the reviewer, we now added a **new Figure 6H** in which we included longer exposures of the anti-GFP purifications and we do indeed detect a LC3-TNIP1 interaction – albeit weak- of mutant TNIP1 under basal conditions as predicted.



Longer exposure highlighting the interaction of mutant TNIP1 with GFP-LC3

8) In the S35 labeling studies in Fig 7E, the effects of phosphomimetic TNIP1 binding to LC3 family members appear quite modest.

In Fig 7E we replaced the autoradiograph with a replicate that has crisper bands, as the previous autoradiograph shown was slightly blurry and potentially difficult to interpret. We have also added a new table displaying the fold increase in binding of the S122E/S123E phosphor-mimicking mutant relative to WT and in the manuscript text we have mentioned that "The binding increase was particularly evident for LC3B (3.4-fold), LC3C (4.4-fold) and GABARAPL2 (5.8-fold) (Fig 7E)." These increases in binding are clearly significant.



	Fold change SS/EE vs WT
GST-LC3A	2.7
GST-LC3B	3.4
GST-LC3C	4.4
GST-GABARAP	1.8
GST-GABARAPL1	3.2
GST-GABARAPL2	5.8

Check the raw data files since the upper panel is about

9) Many of the blots are extremely overexposed and poor in quality.

We changed several blots in the manuscript showing less exposed pictures. In addition, we generated a supplementary source data file in which we show all original blots highlighting data quality. In addition, we added lower exposures of blots to the revised manuscript.

Reviewer #3 (Comments to the Authors (Required)):

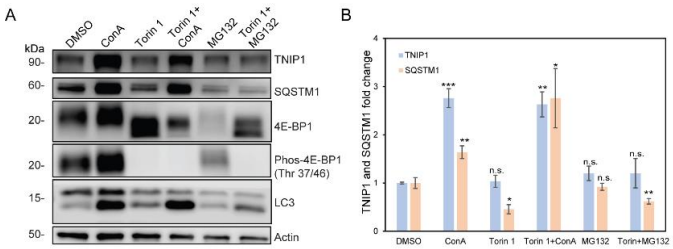
Comments on the manuscript by Jianwen Zhou et al.

Accumulating evidence has shown that autophagy, an intracellular degradation system, is greatly contributed to the regulation of inflammatory responses. In the present study, Jianwen Zhou and colleagues identified TNIP1, a negative regulator of the TLR signaling pathway, as an autophagy substrate and revealed a molecular mechanism underlying the autophagic degradation of TNIP1. The authors showed that TNIP1 interacts with LC3 and GABARAP proteins via the LIR motif, resulting in its degradation in autolysosomes. The authors also showed that TBK1 phosphorylates TNIP1 and induces TNIP1 degradation by autophagy after TLR3 stimulation. This reviewer feels the present study is interesting. However, the present study lacks important data that support the author's conclusion. Major concerns are shown below.

We thank the reviewer for her/his interest in our paper!

In Fig 2A and Fig 3C, why did rapamycin fail to reduce the level of p62?

Rapamycin does inhibit mTORC1, with subsequent induction of autophagy (increased LC3 puncta and LC3-II formation), however it is not a very potent inhibitor of translation (https://doi.org/10.1074/jbc.M900301200 and doi: 10.1038/srep28171). We therefore repeated the experiments using Torin1 instead of Rapamycin and added **new Figures 2A-B**. Indeed, under mTORC1 inhibition by Torin1 we do observe an increased degradation of p62 as predicted.



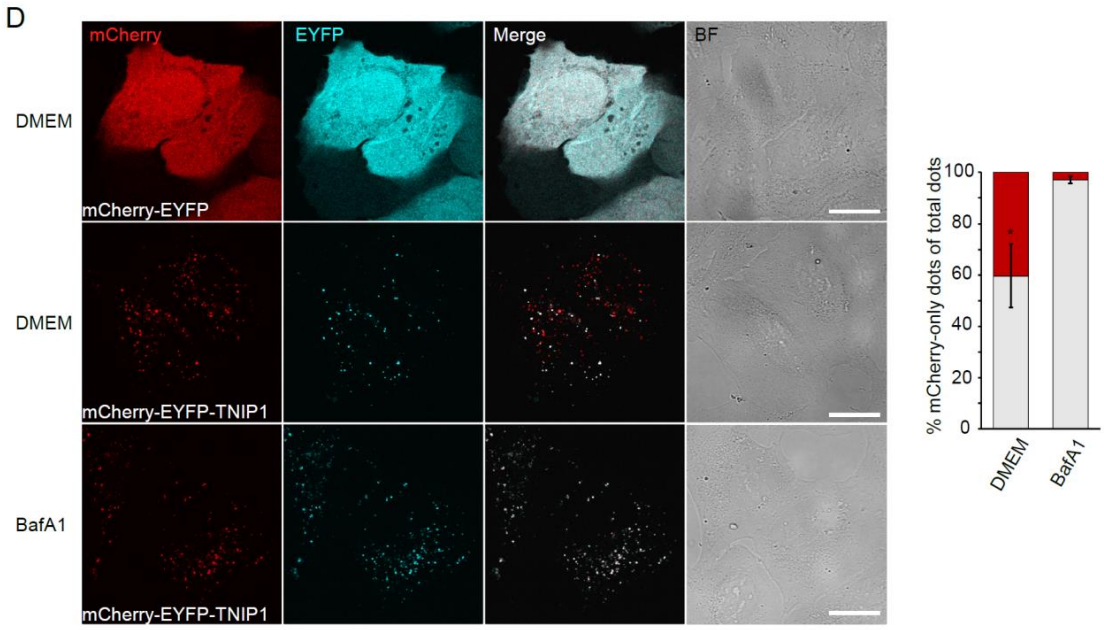
A U2OS cells were treated with 1 µM Torin 1 for 4 h, proteasomal or lysosomal degradation were inhibited by 25 µM MG132 or 2 nM ConA, respectively. Under fed conditions (DMSO) and in Torin 1 treated cells blockage of lysosomal acidification led to a significant increase of TNIP1 protein abundance. Shown are representative blots of three biological replicates. **B** Quantification of blots shown in A (n=3). *: p<0.05, **: p<0.01, ***: p<0.001 T test compared to DMSO treated samples. Error bars indicate SEM.

In Fig 2B-C, what does FM mean?

Please excuse the non-consistent labeling. FM is the abbreviation for “full medium”. We changed this to DMEM throughout the manuscript to be consistent.

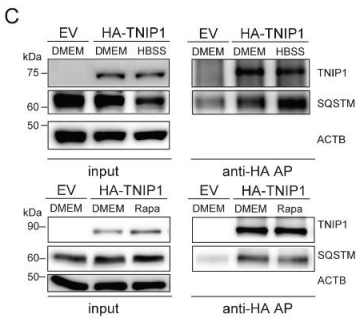
In Fig 2C, the authors should show fluorescent images with both BafA1-treated and untreated cells

As suggested by the reviewer, images showing mCherry-EYFP-TNIP1 upon BafA1-treatment has been added to Figure 2C.



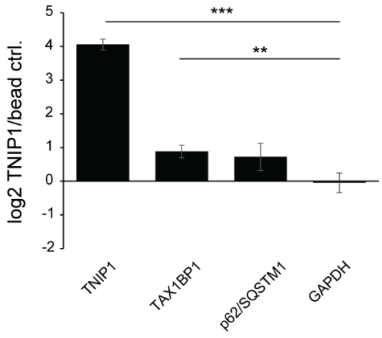
In Fig 3C upper panels, the authors should load the samples on the same gel and membrane.

Already in the original version, these samples were indeed run on the same gel/membrane. However, this gel contained additional, non-related samples which we cut-out. We repeated the experiments as suggested to avoid any confusion and added the data as **new Figure 3C**:



In Fig 3C, the authors should examine if endogenous TNIP1 interacts with endogenous p62 independently of autophagy induction.

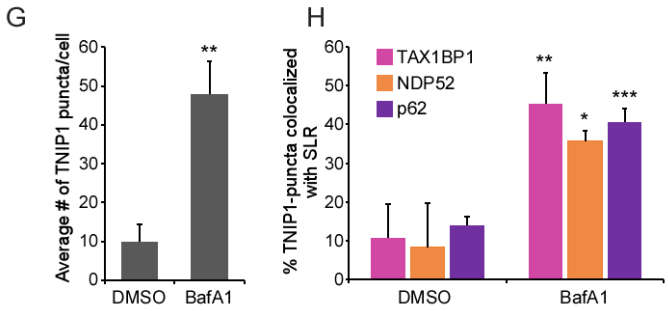
Indeed, we also identified p62 as well as TAX1BP1 as binding to endogenous TNIP1 under basal conditions and added this data as **new supplemental Figure S3A:**



SILAC-based, IP-MS analyses of anti-TNIP1 immunoprecipitations identified TAX1BP1 and p62/SQSTM1 as enriched compared to negative control IPs using beads only. GAPDH is shown as negative control. Shown are average values of three biological replicates. Error bars: std. dev., **: p<0.01, ***: p<0.001, T test.

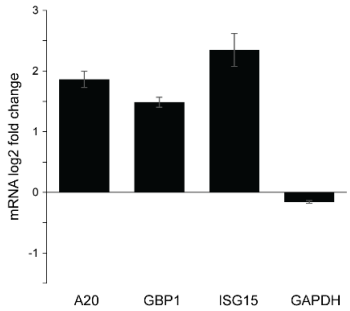
In Fig 3D, statistical analysis should be done.

As suggested, we quantified both the amount of TNIP1 puncta in untreated and BafA1-treated cells, as well as the percentage of these puncta which colocalized with the respective SLRs. The data has been added as **new figure 3G-H.**



In Fig 5G-H, the authors should examine if transcription of the indicated genes is altered by TNIP1 or not.

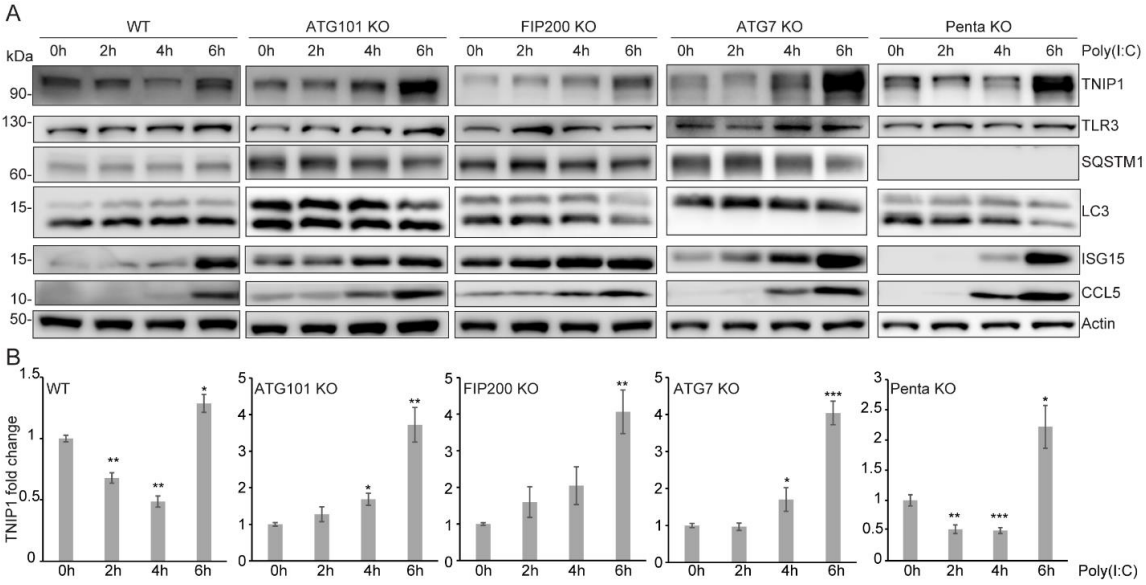
Indeed, as expected the transcription of these genes is modulated by TNIP1 KO. Thus, the observed changes in protein abundance do at least in part reflect changes on transcriptional level. We added transcription data as **new Figure 5H**:



TNIP1 represses translation of pro-inflammatory gene products. Whereas knockout of TNIP1 led to an increased abundance of indicated inflammatory proteins (G), which is likely due to transcriptional changes (H), re-expression of $TNIP1^{WT}$ or $TNIP1^{mLIR1+2}$ blunted this phenotype (I).

In Fig 6A, the authors should show the level of TLR3 in three types of cells.

As suggested we analyzed TLR3 abundance in all cell lines and added respective data as n**ew Figure 6A**. Poly(I:C) treatment appears to slightly increase TLR3 abundance in all cell lines, indicating that this is an autophagy independent phenomenon.



In Fig 6A-B, why did TNIP1 levels NOT increase in untreated ATG7 KO cells compared to untreated WT cells? TNIP1 levels increased in untreated Penta KO cells compared to untreated WT cells. The authors mentioned in the result section regarding Fig 2 that "TNIP1 is degraded by autophagy under basal conditions". Do they mean that ATG7-independent autophagy induces degradation of TNIP1 under basal conditions?

This is an interesting point. However, we find it difficult to directly compare TNIP1 levels in different cell lines as these may be masked by clonal effects. To highlight that *TNIP1* is differentially expressed in different clones, we performed qPCR analyses and added data as a **Figure 1 for review only**:

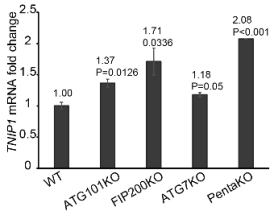


Figure 1 for review only: In different KO cell lines TNIP1 expression differs, likely due to clonal effects. Bar diagram shows quantification of three biological replicates, error bars: SEM. *: p<0.05, **: p<0.01, ***: p<0.001, T test.

To address the degradation pathways of TNIP1, we rather blocked autophagy-dependent, proteasomal or lysosomal degradation in different cell lines and analyze relative changes per cell line. We added new data as **new Figure 3D-E** (see comment above for reviewer 2). We have also tested mCherry-EYFP-TNIP1 in a selection of KOs (see comment for reviewer 2), where we saw a reduction, but not complete loss, of red-only TNIP1 dots in ATG7 and ATG16L1 KOs. This suggests that there is some degree ATG7-independent autophagic degradation of TNIP1, which could also explain why the basal levels of TNIP1 is not increased in the ATG7 KO cells. We added new text to make this more clear:

New text:

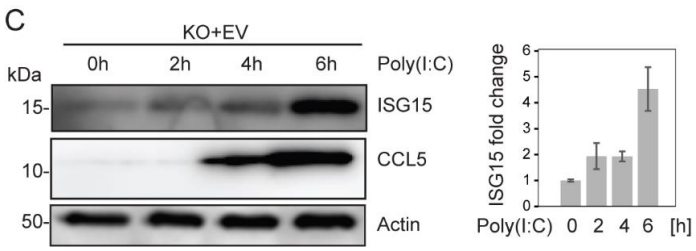
"To further investigate the basal turnover we analyzed the autophagic flux using mCherry-EYFP-TNIP1 in U2OS cells KO for FIP200, ATG9, ATG7 and ATG16L1. As quantified in Fig. 3I, FIP200 and ATG9 KO cells showed little to no red-only dots. This is consistent with the importance of these proteins in both ATG7-dependent and -independent autophagy (Goodwin et al., 2017). ATG7 and ATG16L1 KOs showed reduced formation of red-only dots, but not a complete loss, indicating that there may also be ATG7-independent degradation of TNIP1 under basal conditions. Altogether, these results suggest that autophagic degradation of TNIP1 under basal conditions is aided by SLRs, and that this turnover may be both ATG7-dependent and -independent."

In Fig 6D, what types of KO cells did the authors use?

Please excuse the lack of detail. These were KO1 cells. We added the info to the figure legend.

In Fig 6D, F, experiments using EV-transduced wild-type cells should also be performed under the same condition.

As suggested, we also used EV cells, which also responded to poly(I:C) treatment indicating that the functions of poly(I:C) are pleiotropic and not only depend on TNIP1 protein levels. We added respective data as **new suppl. Figure 4C**:



In Fig 6E, each sample should be compared to sample 5 (KO plus TNIP1-WT 0h). Why were the levels of TNIP1-LIR lower than that of TNIP1-WT at 0h? Why did TNIP1-LIR fail to suppress ISG15 expression at 0h? In Fig 5H, TNIP1-LIR suppressed ISG15 expression under basal conditions.

As stated above: all the generated transgenic cell lines might exhibit clonal effects, which makes a direct comparison of protein abundances between cell lines difficult/misleading. In the mentioned figure, we ectopically express the different TNIP1 variants in TNIP1 KO cell lines and we cannot rule out expression differences due to differences in transfection efficiencies (although we tried to select cell lines exhibiting protein abundances similar to endogenous levels). Therefore, we have to compare relative protein abundance differences between cell lines, i.e. relative response to a given stimulus.

In Fig 7F, what types of KO cells did the authors use?

Please excuse the lack of detail. These were KO1 cells. We added the info to the figure legend.

In Fig 7G, the authors should provide genetic evidence that TBK1 induces phosphorylation and subsequent autophagic degradation of endogenous TNIP1 in polyIC stimulated cells.

We thank the reviewer for suggesting these additional experiments to further support the proposed TBK1-dependent mechanism of TNIP1 degradation. To study the role of TBK1, we analyzed abundance differences of endogenous TNIP1 after poly(I:C) treatment of TBK1 KO compared to WT cells. As predicted by our model, we did observed poly(I:C)-dependent degradation of TNIP1 in wildtype HeLa (WT) cells, but not in two independent TBK1 KO lines. We added the new data as **new Figure 7H**:

H

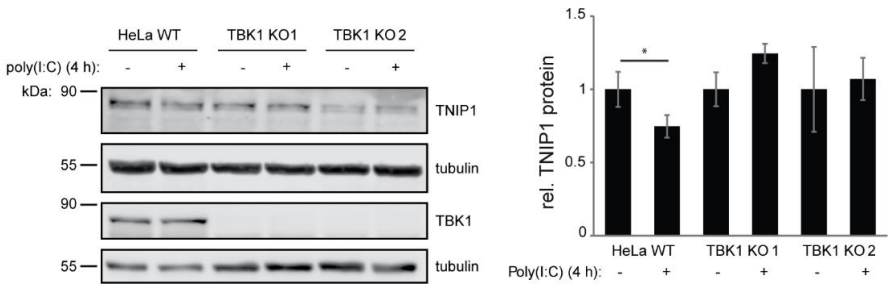


Figure 7H: Poly(I:C)-treatment of TBK KO cells does not induce TNIP1 degradation. One representative blot of three biological replicates is shown. Error bars, SEM ; * : $p<0.05$, t test.

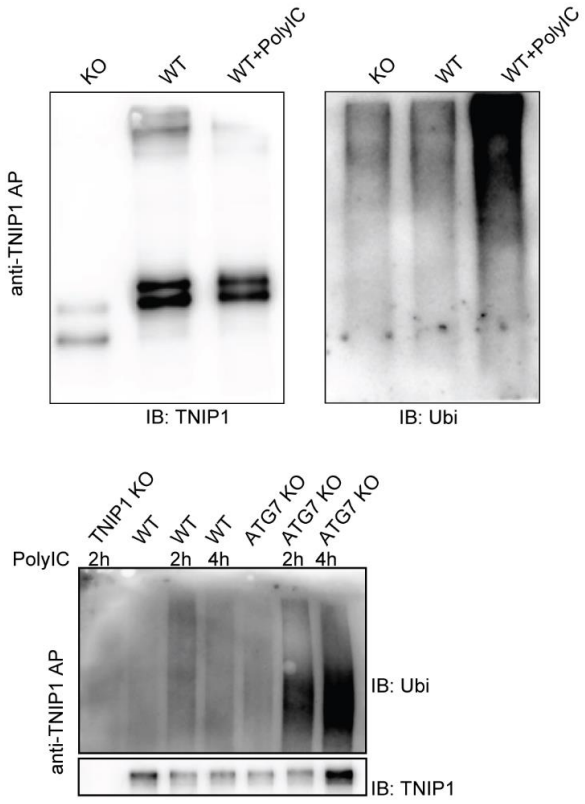
In Fig 7 and Fig S1, the authors should examine if phosphorylation of TNIP1 alters its ubiquitination in polyIC-stimulated cells.

This is indeed a very interesting question and we do observe increased ubiquitination levels in anti-TNIP1 affinity purifications after poly(I:C) stimulation (**see Figure 2 for review only**). This might indicate that TBK1 phosphorylation is a priming event for increased ubiquitination of TNIP1.

However, to clearly prove the underlying molecular mechanism, we would have to perform several additional time-consuming experiments and we feel that this is beyond the scope of our current

manuscript. Bottom-up proteomics is not an ideal method to study the crosstalk of PTMs. To address this, we would have to use an antibody-based approach expressing different site-mutants. We do not have a site-specific antibody detecting the TBK1 sites on TNIP1. In addition, we do not cover/know all ubiquitination sites of TNIP1 (see responses to reviewer 2 and supplemental figure 1F). Thus, a comprehensive analysis would take us minimally 6 months.

Figure 2 for review only:



In response to this comment, we included a respective statement in the discussion:

"Whether phosphorylation also influences the ubiquitination status of TNIP1 is currently no known and a potential crosstalk between the two PTMs will have to be addressed in future studies."

In Fig S3, the authors should examine if autophagic degradation of TNIP1 supports the production of inflammatory mediators, such as cytokines, interferons, and chemokines. A20, ISG15, and GBP1-5 are regulators of immune signaling pathways, but not direct mediators of inflammation.

Indeed, loss of TNIP1 leads to an upregulation of several chemokines. Among the top-100 most upregulated genes as detected by RNAseq, we identified four chemokines : CCL5, CXCL10, CXCL11,

and CCL20. Respective data are presented in supplemental Table S3B and we added a respective statement to the main manuscript.

“On mRNA level, we identified several chemokines which were more abundant in TNIP1 KO cells, CCL5 being the most differentially regulated gene (supplemental Table S3B).”

Due to the small size of chemokines and the limited number of respective tryptic peptides, we did not identify chemokines in our bottom-up expression proteomics approaches.

July 30, 2022

RE: JCB Manuscript #202108144R-A

Prof. Jörn Dengjel
University of Fribourg
Chemin du Musee 10
Fribourg, FR 1700
Switzerland

Dear Prof. Dengjel,

Thank you for submitting your revised manuscript "TBK1 phosphorylation activates LIR-dependent degradation of the inflammation repressor TNIP1." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add a scale bar to Figure 6D as well as MW markers to autoradiographs in 4D & 7E and Ponceau stains in 5A. MW markers should also be added to all ATG101 & Penta KO blots in Figure 3D since these originated from different gels than the WT and FIP200 KO blots.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. JCB strongly encourages the usage of violin plots instead of bar graphs. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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Reviewer #1 (Comments to the Authors (Required)):

The authors have addressed all my comments well and I have no further criticism.

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I have read over the revised manuscript and response to my previous comments. My major concerns have been satisfied with the newly provided analysis of SLR snd ATG loss of function cells.