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Rab35 GTPase recruits NPD52 to autophagy targets

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Transaction Report:

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

1st Editorial Decision 20 February 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, referee #1 and #3 appreciate your data. However, they also think that more insight is required to warrant further consideration here.
Importantly, a xenophagy/mitophagy specific effect versus a general role in autophagy needs to be better analyzed (referee #1, point 1-2, minor point 7), and more insight into Rab35/NDP52 recruitment to GAS/mitochondria is needed (referee #1, point 3; referee #3, points 4, 6, 7). More information and controls are furthermore required (referee #1, minor points 1-6; referee #2, points 2-5, 7; referee #3, points 1-3, 5).

Should you be able to address the referees' criticisms in full, we could consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort. So please consider your options carefully. Should you not to be able to extend your analyses as outlined above, it might be in your best interest to seek publication elsewhere.

Thank you for the opportunity to consider your work for publication. Please get in touch with me should you have any questions regarding the revision. I look forward to your revision.

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REFEREE REPORTS

Referee #1:

The manuscript of Minowa-Nozawa et al., focuses on further characterizing the role of Rab GTPases and TBC/RabGAPs in autophagy. In particular, through examining the effect of TBC1D10A on xenophagy, the authors identified the small GTPase, Rab35 as an important regulator of autophagy. Via using multiple techniques, the authors nicely demonstrate that active Rab35 is involved in the recruitment of the autophagy receptor NDP52 to damaged bacteria-containing vacuoles, following infection with Group A Streptococcus (GAS). Interestingly, data presented in this manuscript suggests that Rab35-mediated NDP52 recruitment is also relevant for recognizing damaged mitochondria in mitophagy as well as regulating the autophagy flux under basal and starvation-induced autophagy.

Work presented in this manuscript provides interesting mechanistic insight into target recognition following stimulation of selective autophagy and identifies Rab35 and TBC1D10A as important autophagy regulators. The authors need to address a few points to fully support the proposed model and increase the impact of their work.

Major Comments:

(1) The authors investigate the effect of 30 TBC/RabGAPs on the formation of GAS-containing LC3-positive vacuoles. This serves as the basis for the remainder of the study. While the manuscript mainly focuses on xenophagy, the authors propose that Rab35-mediated NDP52 recruitment to LC3-positive membranes is important for other modes of autophagy. In order to further support this claim, the authors should also investigate the effect of the tested TBC/RabGAPs, or at least TBC1D10A, on mitophagy, basal autophagy and starvation-induced autophagy. This will give a better indication of whether TBC/RabGAPs that affect xenophagy and their associated Rab GTPases are truly universal regulators of autophagy or whether their effects are limited and specific to xenophagy.

(2) In Fig EV1A, expression of TBC1D10A results in the increased formation of LC3-positive vesicles that do not surround GAS. The authors hint at a possibility of TBC1D10A-mediated regulation of autophagy, thus it would be important to determine whether these vesicles are specific to GAS infection or if they also occur under basal conditions.

(3) The authors provide compelling evidence indicating the requirement of NDP52 in GAS-specific xenophagy. They nicely demonstrate that TBC1D10A GAP activity suppresses NDP52 recruitment to GAS. Consistently, Rab35 KO also attenuates NDP52 recruitment. Accordingly, they propose a model in which Rab35-mediated recruitment serves as a target recognition tool for the autophagy machinery. The authors also demonstrate that the Ub-binding NDP52 mutant (D439K) displays reduced recruitment to GAS. However, the authors do not comment on whether Ub-binding represents a Rab35-independent recruitment mechanism. Since the proposed model suggests that Rab35 recruits NDP52 via direct binding in a TBK1-dependent manner, it would be important to explore the effect of this mutation on Rab35-NDP52 binding. To investigate this further the authors should also examine the effect of this mutant on the formation of GAS-containing LC3-positive vacuoles.

Minor Comments:

(1) The TBC/RabGAPs screen described in the manuscript serves as an important basis for the reminder of the data. While the quantification provided in Fig 1A provides a comprehensive summary of the effect of expressing different TBC/RabGAPs on xenophagy, it would be useful to also include representative images from the screen in an extended figure to help consolidate the screen results.

(2) The authors utilize biochemical analysis of LC3-II levels to determine the effect of TBC1D10A on autophagy. In DMSO treated cells, expression of TBC1D10A inhibits LC3-II accumulation over

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time following GAS infection. The authors suggest that this is likely due to reduced autophagosome formation specifically in response to GAS infection. However, expression of TBC1D10A in bafilomycin A1 treated cells is not associated with any LC3-II accumulation defects. This contradicts the authors' proposed hypothesis, as reduced autophagosome formation following infection should still result in reduced overall levels of LC3-II even if the autophagy flux is blocked. As indicated in the figure legends, cells were infected in the presence of DMSO or bafilomycin A1 and incubated for the indicated number of hours. It is, therefore, possible that bafilomycin A1 treatment might have already influenced the autophagy flux, irrespective of GAS infection, with the treatment itself causing the observed LC3-II accumulation. If that is the case, bafilomycin A1 treatment, using this experimental set up, provides no real scientific information. This should be reflected in the authors' interpretation of the results. The authors should investigate this by comparing LC3-II lipidation levels upon FLAG, FLAG-TBC1D10A and FLAG-TBC1D10A R160K in the presence of DMSO or bafilomycin A1 under basal conditions as well as upon GAS infection for 0, 2 and 4hrs. This should also be considered for Fig 2G where the authors investigate the effect of NDP52 and Rab35 KO on LC3-II levels in the presence of DMSO or E64d Pepstatin A.

(3) The Rab27A KD presented in Fig EV3A is not optimal. The authors should, therefore, be careful in interpreting the results following the Rab27A KD. It is possible that the lack of an effect on the number of GAS-containing LC3-positive vacuoles and NDP52 recruitment to GAS is due to the presence of sufficient amounts of Rab27A in cells, despite the KD. This should be reflected in the authors' interpretation of the results. To conclusively determine whether NDP52 recruitment is specific to Rab35, the authors should optimize their KD or generate Rab27A KO cells then reevaluate the contribution of Rab27A to the TBC1D10A phenotype.

(4) The authors demonstrate that Rab35 KO reduces the number of GAS-containing LC3-positive vacuoles. However, expressing EmGFP-tagged Rab35 WT, Rab35 Q67A or Rab35 S22N all fail to rescue the KO phenotype. The authors propose that this is potentially due to the requirement of an intricate balance of GDP and GTP-bound levels of Rab35. While this might be true for both the constitutively active mutant (Q67A) and the dominant-negative mutant (S22N), it does not explain the results observed upon expression of Rab35 WT. In the discussion, the authors acknowledge this, however, to try and explain these results, the authors should examine the functionality of their constructs. For example, it is possible that the expressed EmGFP-tagged Rab35 WT might have GDP-GTP cycling defects or altered subcellular localization.

(5) In the manuscript, the authors propose that direct binding of Rab35 to NDP52 facilitates its recruitment to LC3-positive vesicles in xenophagy, mitophagy, basal autophagy and starvation-induced autophagy. They also convincingly demonstrate that this interaction is mediated via the zinc finger domain (Zn). Indeed, in both GAS xenophagy and mitophagy the NDP52 Zn mutant displayed impaired recruitment, supporting the proposed hypothesis. In contrast, recruitment of this mutant was not affected under basal autophagy or starvation-induced autophagy. Yet, the authors demonstrate a Rab35-dependent NDP52 recruitment in basal autophagy and starvation-induced autophagy. The authors should, therefore, provide a potential explanation for the observed result. Since they demonstrate that TBK1 activation might be involved in NDP52 recruitment through modulating Rab35-NDP52 binding, they should investigate whether TBK1 activation facilitates the interaction of the NDP52 Zn mutant with Rab35, particularly since some residual binding was still observed upon deletion of the Zn domain in Fig 3E. The ability of this mutant to form dimers with endogenous NDP52 should also be investigated.

(6) The autophagy-mediated p62 degradation in WT cells presented in Fig 4C is not very obvious. The authors should provide a WB that is more reflective of the provided quantification.

(7) The authors suggest that Rab35-mediated NDP52 recruitment represents a universal mechanism for autophagy regulation. They also propose that this process is mediated via direct binding of Rab35 and NDP52 in a TBK1 activation-dependent manner. The requirement of TBK1 is nicely demonstrated in both GAS xenophagy and mitophagy. Thus, to further demonstrate that this is a universal autophagy regulatory mechanism, the importance of TBK1 for Rab35-mediated NDP52 recruitment should also be demonstrated for basal autophagy and starvation-induced autophagy.

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Referee #2:

In this study the authors report that Rab35 plays a role in recruitment of NDP52 to various targets. There seem to be effects not only on the receptor NDP52 but also on maturation. The proposed targets for Rab35 encompass streptococci, mitochondria and starvation-induced autophagosomes. Rab35 is a small GTPase shown to functionally interact with ARF6 and to play a role in cell migration as well as more recently in activation of Akt in cancer cells. Rab35 has already been shown to affect receptor trafficking to lysosomes. Now, these authors present data that Rab35 controls autophagy primarily through NDP52. The most significant and convincing finding is that Rab35 and NDP52 directly interact. Other aspects seem a bit underwhelming.

1. NDP52 is present in human cells but murine cells are devoid of NDP52 (the mouse is a natural mutant lacking NDP52). If NDP52 were to be so critical for Rab35 action and autophagy, what about autophagy in other species and what about Rab35 function in general?
2. The authors use Cherry-LC3 as a marker of autophagosomes in their screen with Rab GAPs. Cherry-LC3 will end in lysosomes and thus the screen is more correctly defined as lysosomal screen and not autophagosomal. If their screen was directed at autophagosomes, how come no TBC1D5, TBC1D14, TBC1D15, TBC1D17 and TBC1D25 (identified in previous autophagy screens with TBC1D Rab GAPs; see Lamb et al.), the previously reported GAPs did not show up? Is it fair/appropriate to refer to Cherry-LC3 as "autophagosomal formation"?
3. The CCCP experiments need to be repeated with AO due to the now well known issues with CCCP.
4. The stated relationships between mitophagy and Rab35 in Fig. 5 are not convincing.
5. Starvation autophagy and NDP52-Rab35. This is even less convincing. It is unclear to this reviewer what the authors use as a premise, what they are presenting and concluding. Are they saying that NDP52 drives starvation induced autophagy (nonselective) through selective autophagy? Totally confusing.
6. Given that mouse has no NDP52 the point about the role of Rab35-NDP52 in starvation induced autophagy appears at best dubious.
7. The mechanism of how TBK1 increases NDP52 interactions with Rab35 seems unclear.
8. The reference for p62 phosphorylation by TBK1 is grossly misquoted on line 179 and the actual study that demonstrated this is not even cited. However, other parts of the study are well referenced and the authors are to be commended for good and in depth literature analysis, which they utilized to the fullest to repeat the known relationships in their study.

Referee #3:

Selective autophagy including xenophagy and mitophagy is well known and essential intracellular degradation system. Previous reports have shown that several autophagy receptors such as p62, OPTN, and NDP52 provide a link between ubiquitin on the cargo substrates and LC3 on autophagic membranes. Minowa-Nozawa et al. in this study identified that Rab35 directs NDP52 to the cargo substrates during GAS infection (xenophagy) and mitophagy. This is an interesting study. They provided novel and solid data supporting their conclusion. They examined not only GAS-infected autophagy, but also Parkin-induced mitophagy. Although I recommend their study for publication in the EMBO J, the authors have to answer my comments below before acceptance.

Major comments:
1) To which organelle does endogenous Rab35 localize under a basal condition?

2) Fig EV 3A
Rab27 was not efficiently knocked down by miR-RNA. The authors should use a different construct or alternative approach.

3) Fig EV 3D
Testing whether Rab35 is involved in endosomal and/or lysosomal functions is very important. The cathepsin B assay is not sufficient to confirm normal lysosomal functions in Rab35 KO cells. The authors should at least test and compare 1) lysosomal pH and 2) endosome/lysosome morphologies.

4) Based on the results the authors provided in this study, Rab35 functions upstream of NDP52 for

NDP52 recruitment to GAS. Consistent with this, the immunoelectron microscopy (Fig EV 9B) beautifully indicates that Rab35 was recruited onto bacteria-containing endosomal membranes. On the other hand, how Rab35 is recruited to the damaged mitochondria during mitophagy still remains unknown. Does Rab35 directly attach to damaged mitochondrial membranes during mitophagy? Immunoelectron microscopy upon mitophagy should answer this important question. Furthermore, what recruits Rab35 to the place (endosomes during GAS infection and damaged mitochondria during mitophagy) where autophagosome formation will occur?

5) Fig EV 6A
In Fig EV 4C, EmGFP-Rab35 S22N was nicely merged with mCherry-NDP52 in cells, but the BiFC assay in Fig EV 6A showed no interaction between Rab35 S22N and NDP52. How do the authors explain this conflict?

6) Fig 5F
Based on the fractionation assay, just the expression level of endogenous Rab35 seemed to increase after CCCP treatment while authors suggest 'recruitment' of Rab35 to the damaged mtiochondria. Does accumulation of Rab35 in the mitochondrial fraction result from the recruitment, inhibition of the degradation, or increase of the protein expression?

7) Is Rab35 able to be recruited to the damaged mitochondria in NDP52 KO cells during mitophagy?

Minor comments:

1) Magnified images should be provided even if the signals (such as Fig EV1 A GFP-TBC1D10A, Fig1 H GFP-TBC1D10A, Fig 2 C mCherry-NDP52, and so on) were not found on or near infected bacteria.

2) Fig 1C
The amounts of LC3-II in Bafilomycin A1 0h were higher than those in DMSO 0h. The authors should mention in the legend at which time point Bafilomycin A1 was added, before the infection or at the time of the infection? The same comment is valid for Fig 2 G.

3) Fig EV 3C
Magnified images are required. According to the images, Rab35 signal appeared in some cells only after GAS infection. To which organelles does endogenous Rab35 localize? Confocal Micrographs of Rab35 staining without GAS infection should be shown.

4) Fig 3E
The amounts of GFP-Rab35 pulled down with the different GST-NDP52 constructs should be quantified.

5) Fig 3F
Do in vitro interactions between Rab35 and NDP52 differ for different GTP-bound states? Test the same binding assay using MBP-Rab35 GTP- and GDP-bound mutants.

6) Fig EV 7B
Overexpressed Rab35 inhibited NDP52 recruitment during GAS infection (Fig EV 4C). Is the same inhibition observed for mitophagy?

7) Fig 6A-D
On the microscopic images, GFP-LC3 puncta increased upon starvation treatment, but on WB, LC3-II decreased. Why?

8) Fig 4A and B
In WT cells, most of all mCherry-EmGFP-NDP52 dots were mCherry-positive but EmGFP-negative. Does it mean that most of the exogenous NDP52 was included inside lysosomes? Same comments for Fig 6E.

9) Fig7 A

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Does TBK1 stably form a complex with NDP52 and Rab35?

Typo errors and etc.
1) Line 148: Rad35 should be Rab35.

2) The legend of Fig 1G is for Fig 1H and vice versa.

3) Fig 5C: The merged images lack the green channel (EmGFP-Parkin).

4) Fig EV 7B: EmGFP-Rab35 labeled above the image should be mCherry-Rab35.

1st Revision - authors' response 18 May 2017

Dear Reviewers, We sincerely appreciate the helpful comments regarding the original version of our manuscript. We have considered all of the referees' comments and have attempted to respond to the questions with additional experimental data in the revised version of our manuscript. Our detailed point-to-point responses to each comment are provided below. The modified sections in the revised manuscript are highlighted in red. We have indicated the figure number where modifications relevant to each of the comment made by the referees have been made. We hope that these explanations and revisions are satisfactory.

Referee #1:

Comment #1-1) *The manuscript of Minowa-Nozawa et al., focuses on further characterizing the role of Rab GTPases and TBC/RabGAPs in autophagy. In particular, through examining the effect of TBC1D10A on xenophagy, the authors identified the small GTPase, Rab35 as an important regulator of autophagy. Via using multiple techniques, the authors nicely demonstrate that active Rab35 is involved in the recruitment of the autophagy receptor NDP52 to damaged bacteria-containing vacuoles, following infection with Group A Streptococcus (GAS). Interestingly, data presented in this manuscript suggests that Rab35-mediated NDP52 recruitment is also relevant for recognizing damaged mitochondria in mitophagy as well as regulating the autophagy flux under basal and starvation-induced autophagy.*
Work presented in this manuscript provides interesting mechanistic insight into target recognition following stimulation of selective autophagy and identifies Rab35 and TBC1D10A as important autophagy regulators. The authors need to address a few points to fully support the proposed model and increase the impact of their work.

Response to comment #1-1) We appreciate your positive and polite comment and helpful suggestions to our study. To further confirm our results, we performed additional experiments described below.

Comment #1-2) *The authors investigate the effect of 30 TBC/RabGAPs on the formation of GAS-containing LC3-positive vacuoles. This serves as the basis for the remainder of the study. While the manuscript mainly focuses on xenophagy, the authors propose that Rab35-mediated NDP52 recruitment to LC3-positive membranes is important for other modes of autophagy. In order to further support this claim, the authors should also investigate the effect of the tested TBC/RabGAPs, or at least TBC1D10A, on mitophagy, basal autophagy and starvation-induced autophagy. This will give a better indication of whether TBC/RabGAPs that affect xenophagy and their associated Rab GTPases are truly universal regulators of autophagy or whether their effects are limited and specific to xenophagy.*

Response to comment #1-2) As suggested by the referee, we have examined whether TBC1D10A is involved in NDP52 recruitment in mitophagy, basal and starvation-autophagy. Overexpression of TBC1D10A significantly suppressed NDP52 recruitment to depolarized mitochondria after mitochondrial depolarization by antimycin A and oligomycin (AO) treatment, whereas the R160K mutant did not, suggesting that GAP activity of TBC1D10A negatively regulates NDP52 recruitment to damaged mitochondria during mitophagy (Fig EV9E and F). Furthermore, overexpression of TBC1D10A significantly reduced endogenous NDP52 recruitment to basal and starvation-induced autophagosomes, whereas that of R160K mutant did not, suggesting that GAP

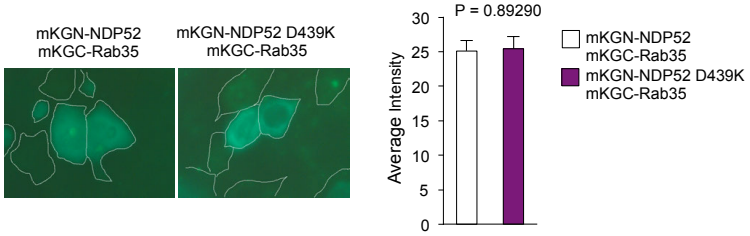
activity of TBC1D10A negatively regulates NDP52 recruitment in basal and starvation-induced autophagy (Fig EV11A and B). We therefore concluded that TBC1D10A is also universal regulator of autophagy, as is the case with Rab35.

Comment #1-3) *In Fig EV1A, expression of TBC1D10A results in the increased formation of LC3-positive vesicles that do not surround GAS. The authors hint at a possibility of TBC1D10A-mediated regulation of autophagy, thus it would be important to determine whether these vesicles are specific to GAS infection or if they also occur under basal conditions.*

Response to comment #1-3) We think that our selection of confocal pictures led to a misunderstanding. HeLa cells that have a number of LC3-positive vesicles in the cytoplasm sometimes exist even in the control condition (EmGFP control cells), probably because transfection has some kind of effect on these cells. Although we observed and took pictures of LC3-positive vesicles that do not surround GAS, during at least GAS infection, we could not find a significant difference between control cells and TBC1D10A-overexpressing cells. Therefore, we have replaced these images so as not to cause misunderstanding in the revised manuscript (Fig EV2A).
On the other hand, under the basal condition, overexpression of TBC1D10A resulted in increased formation of GFP-LC3-positive autophagosomes (Fig EV11A), and NDP52 recruitment to basal autophagosomes was suppressed by TBC1D10A compared to the control (Fig EV11A and B), suggesting that TBC1D10A is involved in basal autophagy via NDP52.

Comment #1-4) *The authors provide compelling evidence indicating the requirement of NDP52 in GAS-specific xenophagy. They nicely demonstrate that TBC1D10A GAP activity suppresses NDP52 recruitment to GAS. Consistently, Rab35 KO also attenuates NDP52 recruitment. Accordingly, they propose a model in which Rab35-mediated recruitment serves as a target recognition tool for the autophagy machinery. The authors also demonstrate that the Ub-binding NDP52 mutant (D439K) displays reduced recruitment to GAS. However, the authors do not comment on whether Ub-binding represents a Rab35-independent recruitment mechanism. Since the proposed model suggests that Rab35 recruits NDP52 via direct binding in a TBK1-dependent manner, it would be important to explore the effect of this mutation on Rab35-NDP52 binding. To investigate this further the authors should also examine the effect of this mutant on the formation of GAS-containing LC3-positive vacuoles.*

Response to comment #1-4) As suggested by the referee, to determine whether interaction of NDP52 with ubiquitin is required for interaction of NDP52 with Rab35, we investigated the interaction between the NDP52 D439K mutant and Rab35. Immunoprecipitation showed that the EmGFP-NDP52 D439K mutant interacted with FLAG-Rab35 similarly to the control (Fig EV7A). Similarly, the fluorescence protein fragment complementary assay also demonstrated that the interaction of NDP52 D439K with Rab35 was not inhibited (please see reviewer figure below). These results suggest that NDP52-ubiquitin binding is not essential for NDP52-Rab35 binding. Therefore, because the NDP52 D439K mutant not a focus of this study on Rab35, we did not examine the effect of the NDP52 D439K mutant on the formation of GAS-containing LC3-positive vacuoles. On the other hand, we also investigated whether Rab35 is involved in the NDP52-ubiquitin interaction by using PLA. Compared to the wild-type cells, fewer PLA signals in the cytoplasm were observed in Rab35 knockout cells (Fig 2G and H), suggesting that Rab35 promotes the interaction between NDP52 and ubiquitin.



Comment #1-5) *The TBC/RabGAPs screen described in the manuscript serves as an important basis for the reminder of the data. While the quantification provided in Fig 1A provides a comprehensive summary of the effect of expressing different TBC/RabGAPs on xenophagy, it would be useful to also include representative images from the screen in an extended figure to help consolidate the screen results.*

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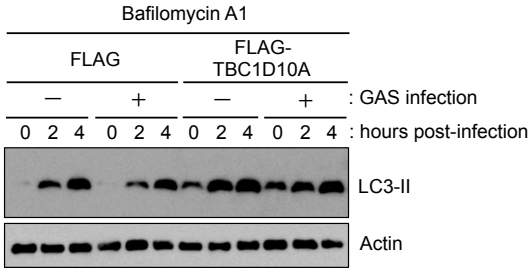
Response to comment #1-5) As suggested, we have added these representative images from the screen for 30 TBC/RabGAPs (Fig EV1).

Comment #1-5) *The authors utilize biochemical analysis of LC3-II levels to determine the effect of TBC1D10A on autophagy. In DMSO treated cells, expression of TBC1D10A inhibits LC3-II accumulation over time following GAS infection. The authors suggest that this is likely due to reduced autophagosome formation specifically in response to GAS infection. However, expression of TBC1D10A in bafilomycin A1 treated cells is not associated with any LC3-II accumulation defects. This contradicts the authors' proposed hypothesis, as reduced autophagosome formation following infection should still result in reduced overall levels of LC3-II even if the autophagy flux is blocked. As indicated in the figure legends, cells were infected in the presence of DMSO or bafilomycin A1 and incubated for the indicated number of hours. It is, therefore, possible that bafilomycin A1 treatment might have already influenced the autophagy flux, irrespective of GAS infection, with the treatment itself causing the observed LC3-II accumulation. If that is the case, bafilomycin A1 treatment, using this experimental set up, provides no real scientific information. This should be reflected in the authors' interpretation of the results. The authors should investigate this by comparing LC3-II lipidation levels upon FLAG, FLAG-TBC1D10A and FLAG-TBC1D10A R160K in the presence of DMSO or bafilomycin A1 under basal conditions as well as upon GAS infection for 0, 2 and 4hrs. This should also be considered for Fig 2G where the authors investigate the effect of NDP52 and Rab35 KO on LC3-II levels in the presence of DMSO or E64d Pepstatin A.*

Response to comment #1-5) To determine whether bafilomycin A1 treatment causes LC3-II accumulation, irrespective of GAS infection, we first examined LC3-II accumulation in non-infected and GAS infected cells under bafilomycin A1 treatment. GAS infection under bafilomycin A1 treatment increased LC3-II levels with time. However, almost the same LC3-II levels were observed in non-infected cells under bafilomycin A1 treatment (please see reviewer figure below). Similar relationships were also observed with E64d and Pepstatin A treatment. As anticipated by the referee, during GAS infection, bafilomycin A1 treatment and E64d and Pepstatin A treatment cannot provide real scientific information. As a result, we have deleted these results and shown only the results obtained under non-treatment (Fig 1C and D, 2I and J).



Comment #1-6) *The Rab27A KD presented in Fig EV3A is not optimal. The authors should, therefore, be careful in interpreting the results following the Rab27A KD. It is possible that the lack of an effect on the number of GAS-containing LC3-positive vacuoles and NDP52 recruitment to GAS is due to the presence of sufficient amounts of Rab27A in cells, despite the KD. This should be reflected in the authors' interpretation of the results. To conclusively determine whether NDP52 recruitment is specific to Rab35, the authors should optimize their KD or generate Rab27A KO cells then reevaluate the contribution of Rab27A to the TBC1D10A phenotype.*

Response to comment #1-6) As suggested, we generated Rab27A knockout cell lines (Fig EV4A), and examined NDP52 recruitment and autophagosome formation in the knockout cells. Rab27A knockout did not affect NDP52 recruitment and autophagosome formation in response to invading bacteria, whereas these processes were significantly suppressed by Rab35 knockout (Fig 2A-D). Thus, we concluded that the inhibitory effects of NDP52 recruitment on GAS and autophagosome formation by TBC1D10A are specifically mediated by Rab35.

Comment #1-7) *The authors demonstrate that Rab35 KO reduces the number of GAS-containing LC3-positive vacuoles. However, expressing EmGFP-tagged Rab35 WT, Rab35 Q67A or Rab35 S22N all fail to rescue the KO phenotype. The authors propose that this is potentially due to the*

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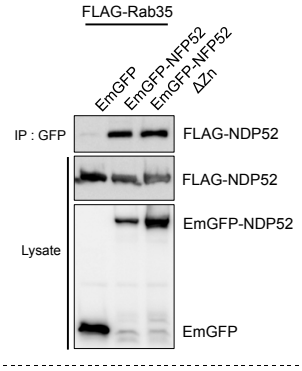
requirement of an intricate balance of GDP and GTP-bound levels of Rab35. While this might be true for both the constitutively active mutant (Q67A) and the dominant-negative mutant (S22N), it does not explain the results observed upon expression of Rab35 WT. In the discussion, the authors acknowledge this, however, to try and explain these results, the authors should examine the functionality of their constructs. For example, it is possible that the expressed EmGFP-tagged Rab35 WT might have GDP-GTP cycling defects or altered subcellular localization.

Response to comment #1-7) To examine whether proper expression of Rab35 can rescue the knockout phenotype, we constructed cell lines stably expressing Rab35 in the knockout background, which were made to express a similar level of Rab35 compared with endogenous Rab35 by using lentiviral vectors, and tested whether these cell lines showed rescue of the inhibition of NDP52 recruitment and autophagosome formation. Stable expression of EmGFP-tagged Rab35 and Rab35 Q67A fully rescued the inhibition, whereas that of Rab35 S22N did not (Fig EV5E-G). These results suggest that GTP-bound Rab35 regulates NDP52 recruitment to GAS.

Comment #1-8) *In the manuscript, the authors propose that direct binding of Rab35 to NDP52 facilitates its recruitment to LC3-positive vesicles in xenophagy, mitophagy, basal autophagy and starvation-induced autophagy. They also convincingly demonstrate that this interaction is mediated via the zinc finger domain (Zn). Indeed, in both GAS xenophagy and mitophagy the NDP52 Zn mutant displayed impaired recruitment, supporting the proposed hypothesis. In contrast, recruitment of this mutant was not affected under basal autophagy or starvation-induced autophagy. Yet, the authors demonstrate a Rab35-dependent NDP52 recruitment in basal autophagy and starvation-induced autophagy. The authors should, therefore, provide a potential explanation for the observed result. Since they demonstrate that TBK1 activation might be involved in NDP52 recruitment through modulating Rab35-NDP52 binding, they should investigate whether TBK1 activation facilitates the interaction of the NDP52 Zn mutant with Rab35, particularly since some residual binding was still observed upon deletion of the Zn domain in Fig 3E. The ability of this mutant to form dimers with endogenous NDP52 should also be investigated.*

Response to comment #1-8) As suggested by the referee, to investigate whether a region of NDP52 other than Zn domain is involved in the interaction with Rab35 via TBK1 activity, we performed a fluorescence protein fragment complementary assay in presence or absence of BX795. The interaction of NDP52 with Rab35 was inhibited by BX795, whereas the interaction of NDP52ΔZn with Rab35 was also reduced (Fig EV12F and G). On the other hand, we investigated whether the NDP52ΔZn mutant is able to form dimers. As endogenous NDP52 was difficult to detect in immunoprecipitated samples with overexpression of NDP52, we used cells co-expressing EmGFP-NDP52ΔZn and FLAG-NDP52. Immunoprecipitation showed that the NDP52ΔZn mutant did not lose the ability to form dimers (please see reviewer figure below). These results suggest that a region of NDP52 other than the Zn domain might promote the interaction with Rab35 via TBK1 activity.



Comment #1-9) *The autophagy-mediated p62 degradation in WT cells presented in Fig 4C is not very obvious. The authors should provide a WB that is more reflective of the provided quantification.*

Response to comment #1-9) As suggested, we have replaced the western blot image with a representative one (Fig 4C).

Comment #1-10) *The authors suggest that Rab35-mediated NDP52 recruitment represents a universal mechanism for autophagy regulation. They also propose that this process is mediated via direct binding of Rab35 and NDP52 in a TBK1 activation-dependent manner. The requirement of TBK1 is nicely demonstrated in both GAS xenophagy and mitophagy. Thus, to further demonstrate that this is a universal autophagy regulatory mechanism, the importance of TBK1 for Rab35-mediated NDP52 recruitment should also be demonstrated for basal autophagy and starvation-induced autophagy.*

Response to comment #1-10) As suggested by the referee, we investigated the involvement of TBK1 in basal autophagy and starvation-induced autophagy. TBK1 knockout suppressed endogenous NDP52 recruitment to basal and starvation-induced autophagosomes (Fig. EV13G, H), indicating that TBK1 is involved in Rab35-mediated NDP52 recruitment in basal autophagy and starvation-induced autophagy.

Referee #2:

Comment #2-1) *In this study the authors report that Rab35 plays a role in recruitment of NDP52 to various targets. There seem to be effects not only on the receptor NDP52 but also on maturation. The proposed targets for Rab35 encompass streptococci, mitochondria and starvation-induced autophagosomes. Rab35 is a small GTPase shown to functionally interact with ARF6 and to play a role in cell migration as well as more recently in activation of Akt in cancer cells. Rab35 has already been shown to affect receptor trafficking to lysosomes. Now, these authors present data that Rab35 controls autophagy primarily through NDP52. The most significant and convincing finding is that Rab35 and NDP52 directly interact. Other aspects seem a bit underwhelming.*

Response to comment #2-1) First, we thank the referee for careful reading our manuscript and for giving useful comments. We have performed several experiments as described below.

Comment #2-2) *NDP52 is present in human cells but murine cells are devoid of NDP52 (the mouse is a natural mutant lacking NDP52). If NDP52 were to be so critical for Rab35 action and autophagy, what about autophagy in other species and what about Rab35 function in general?*

Response to comment #2-2) The referee is correct that NDP52 is not expressed in murine cells. In fact, expression databases suggest that it is not expressed in any tissue except ES cells (http://biogps.org/#goto=genereport&id=76815). On the other hand, it has been reported that NDP52 expression is regulated by Nrf2 in the mouse brain and that NDP52 is involved in the clearance of phosphorylated tau (Chulman et al. 2014, Nat Commun). In light of these points, we elucidated the regulatory mechanism of human NDP52 by using human cell lines and a human pathogen in this study. The importance of human NDP52 is well known in xenophagy and mitophagy and basal autophagy (Thurston et al., Nat Immunol. 2009; Lazarou et al., Nature. 2015; Tumbarello et al., Nat Cell Biol., 2012), as shown in the manuscript. Rab35 might also play a role in the clearance of phosphorylated tau through recruiting NDP52 in other species. However, if we want to know accurately how NDP52-Rab35 functions in other species, we need to investigate these functions and regulatory mechanism in every species in which these proteins are expressed because NDP52 homologues have various structures in every species (Tumbarello et al. 2015, PLoS Pathog). We are also interested in how NDP52-Rab35 functions in other species. However, our data cannot reveal these points, and this suggestion is outside the scope of the study this time.

Comment #2-3) *The authors use Cherry-LC3 as a marker of autophagosomes in their screen with Rab GAPs. Cherry-LC3 will end in lysosomes and thus the screen is more correctly defined as lysosomal screen and not autophagosomal. If their screen was directed at autophagosomes, how come no TBC1D5, TBC1D14, TBC1D15, TBC1D17 and TBC1D25 (identified in previous autophagy screens with TBC1D Rab GAPs; see Lamb et al.), the previously reported GAPs did not show up? Is it fair/appropriate to refer to Cherry-LC3 as "autophagosomal formation"?*

Response to comment #2-3) As the referee mentioned, mCherry-LC3 localizes in both autophagosomes and autolysosomes. However, during GAS infection, almost all mCherry-EmGFP-LC3 surrounding GAS appears yellow in LAMP1 (marker of lysosome)-positive autolysosomes (please see reviewer figure below), indicating that both mCherry-LC3 and EmGFP-LC3 localize to

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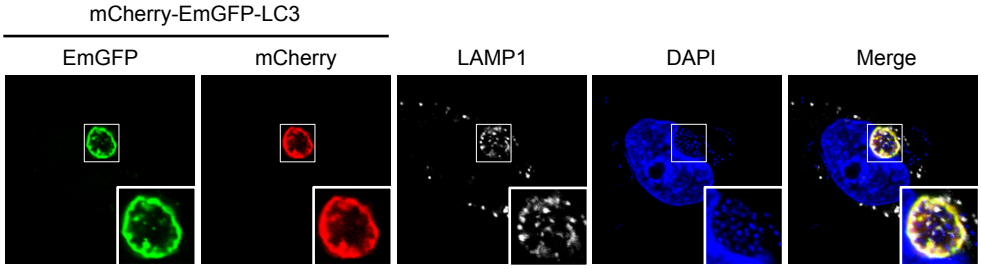
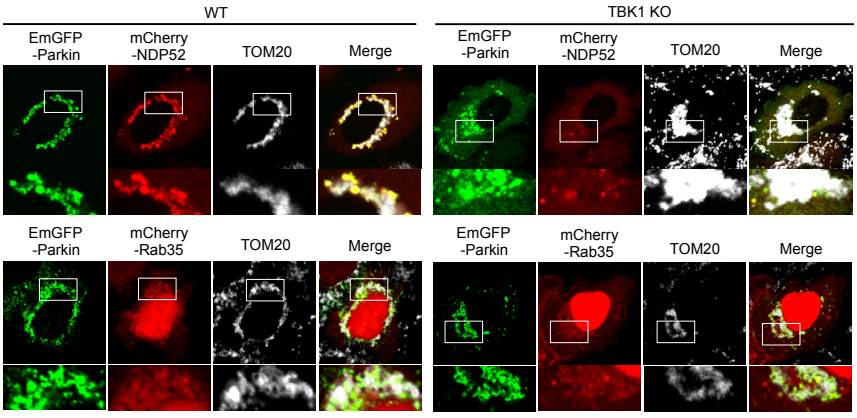
autophagosomes and autolysosomes during GAS infection. For this reason, we consider that we have observed both autophagosomes and autolysosomes in this screening experiments. However, we intended to select RabGAPs that decreased LC3-positive bacteria (i.e., negative regulator of autophagosome formation), and this decrease should be observed regardless of whether we use GFP-LC3 or mCherry-LC3. Thus, we do not consider that using mCherry-LC3 as a marker is inappropriate in these experiments.



On the other hand, in this study, we first aimed to identify a novel Rab GTPase that regulates selective autophagy during GAS infection. As a result of the experiments in this study, we demonstrated that Rab35 and TBC1D10A are master regulators in multiple forms of autophagy. Rab7 is reported a common regulator in GAS infection- and starvation-induced autophagy (Yamaguchi et al. 2009, PLoS Pathog; Maximiliano et al. 2004, J Cell Sci). However, we have previously reported that Rab9A, Rab23, Rab17, and Rab30 are autophagy regulators in GAS infection-induced autophagy, but these Rabs are dispensable for starvation-induced autophagy (Nozawa et al. 2012, Cellular Microbiology; Haobam et al. 2014, Cellular Microbiology; Oda et al. 2016, PLoS One). Additionally, Rab11 is a regulator in starvation-induced autophagy, but not in GAS-induced autophagy (Longatti et al. 2012, J Cell Biol; Haobam et al. 2014, Cellular Microbiology). In a previous autophagy screen for TBC/RabGAPs that the referee might be referring to (Longatti et al. 2012, J Cell Biol) GFP–LC3-II accumulation was evaluated with addition of a lysosomal protease inhibitor; therefore, their results reflect autophagosome formation. Therefore, their results might be largely different from our results in GAS infection-induced autophagy.

Comment #2-4) *The CCCP experiments need to be repeated with AO due to the now well known issues with CCCP.*

Response to comment #2-4) We agree with that we need to investigate whether mitochondrial depolarization by antimycin A and oligomycin (AO) treatment also leads to similar results. NDP52 recruitment to depolarized mitochondria was also inhibited by AO treatment in Rab35 knockout cells, as with CCCP treatment (Fig EV9B and C). In addition, overexpression of TBC1D10A significantly suppressed NDP52 recruitment to depolarized mitochondria after AO treatment, whereas expression of the R160K mutant did not (Fig EV9E and F). Furthermore, TBK1 knockout significantly suppressed NDP52 recruitment to depolarized mitochondria after AO treatment, as with CCCP treatment (please see reviewer figure below). These results suggest that similar results are obtained from both methods, and that Rab35, TBC1D10A, and TBK1 are involved in NDP52 recruitment to damaged mitochondria in mitophagy.



Comment #2-5) *The stated relationships between mitophagy and Rab35 in Fig. 5 are not convincing.*

Response to comment #2-5) We demonstrated that Rab35 is involved in NDP52 recruitment to damaged mitochondria during mitophagy in this study. On the other hand, NDP52 was reported to recruit autophagy factors ULK1, DFCP1, and WIPI1 to focal spots proximal to mitochondria (Lazarou et al. 2015, Nature). Rab35 may also recruit these factors to mitochondria via NDP52. However, according to this report, as the NDP52 single knockout was unable to verify this phenotype, there is a high probability that the same problem would occur with the Rab35 single knockout. At present, it is difficult for us to generate knockout cell lines with editing of several genes, and to further analyze them in detail. However, we consider that it is clear that Rab35 is involved in mitophagy by recruiting NDP52.

Comment #2-6) *Starvation autophagy and NDP52-Rab35. This is even less convincing. It is unclear to this reviewer what the authors use as a premise, what they are presenting and concluding. Are they saying that NDP52 drives starvation induced autophagy (nonselective) through selective autophagy? Totally confusing.*

Response to comment #2-6) Thank you for pointing out the ambiguity of our statement. We have corrected these issues in the manuscript. We had two premises in this study. One is that TBK1, which interacts and phosphorylates with NDP52, is necessary for autophagosome maturation during starvation-induced autophagy, as well as *M. tuberculosis* var. bovis BCG-induced autophagy starvation-induced autophagy (Manohar et al. 2012, Immunity Fig 2D-G). The other is that autophagy receptors including NDP52 contribute to the fusion of the autophagosome with TOM1-expressing endosomes by directly binding the motor protein myosin VI (Tumbarello et al., 2012). NDP52 has multiple roles in autophagy; NDP52 initially acts as a receptor for cargo recognition and autophagosome biogenesis and then in later stages, it is involved in autophagosome maturation and fusion with the lysosome. NDP52 was reported to localize to the basal autophagosome. However, it is unclear whether NDP52 localizes to starvation-induced autophagosomes and how subcellular localization of NDP52 is regulated. Therefore, we first examined the subcellular localization of NDP52 and starvation-induced autophagosomes. NDP52 was detected in basal and starvation-induced autophagosomes (Fig EV10A), and recruitment of NDP52 to basal and starvation-induced autophagosomes was regulated by Rab35, TBC1D10A, and TBK1 (Fig 6G and H, EV11A and B, EV13G and H). In addition, TBK1 was involved in autophagosome maturation in HeLa cells (Fig EV13E and F), consistent with a previous report. Similarly, Rab35 and NDP52 were involved in autophagosome maturation (Fig 6E and F). Thus, Rab35 and TBK1 regulate NDP52 recruitment to starvation-induced and basal autophagosomes, and TBC1D10A negatively regulates this process by exchanging GTP-Rab35 with GDP-Rab35. In starvation-induced and basal autophagy, NDP52 would play a role in connecting the autophagosome with myosin VI only when the autophagosome fuses with the lysosome, but it would not act as a receptor for cargo recognition.

Comment #2-7) *Given that mouse has no NDP52 the point about the role of Rab35-NDP52 in starvation induced autophagy appears at best dubious.*

Response to comment #2-7) As mentioned above, we investigated the role of human Rab35-NDP52 in this study. Just because a certain protein is not expressed in mice, it does not mean that it is not required in humans. The mouse is the model organism for human physiology and disease most used among mammals. Therefore, when scientists (including us) investigate autophagy receptors and selective autophagy using murine cells or mouse models, we must give due and careful consideration to the fact that NDP52 is not expressed in murine cells. However, because we did not use mice and did not mention murine NDP52 in this study, we consider that this comment is beside the point.

Comment #2-8) *The mechanism of how TBK1 increases NDP52 interactions with Rab35 seems unclear.*

Response to comment #2-8) To investigate the mechanism of NDP52-Rab35 interaction via TBK1 activation, we performed a fluorescence protein fragment complementary assay against NDP52ΔZn and Rab35 in the presence or absence of BX795. The interaction of NDP52 with Rab35 was

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inhibited by BX795, whereas the interaction of NDP52ΔZn with Rab35 was also reduced (Fig EV12F and G), indicating that a region of NDP52 other than the Zn domain facilitates the interaction with Rab35 via TBK1 activity. Given that the coiled-coil domain is believed to be involved in NDP52 dimerization (Korioth et al. J Cell Biol. 1995), it may increase the avidity of the interaction via TBK1 activity. Recently, TBK1 was reported to phosphorylate the coiled-coil and SKICH domains in NDP52 (Benjamin et al. 2016, PNAS) The phosphorylation of the coiled-coil domain in NDP52 via TBK1 may play an important role in the interaction of NDP52 with Rab35.

Comment #2-9) *The reference for p62 phosphorylation by TBK1 is grossly misquoted on line 179 and the actual study that demonstrated this is not even cited. However, other parts of the study are well referenced and the authors are to be commended for good and in depth literature analysis, which they utilized to the fullest to repeat the known relationships in their study.*

Response to comment #2-9) We have corrected the references pointed out in the manuscript. We appreciate your positive comment.

Referee #3:

Comment #3-1) *Selective autophagy including xenophagy and mitophagy is well known and essential intracellular degradation system. Previous reports have shown that several autophagy receptors such as p62, OPTN, and NDP52 provide a link between ubiquitin on the cargo substrates and LC3 on autophagic membranes. Minowa-Nozawa et al. in this study identified that Rab35 directs NDP52 to the cargo substrates during GAS infection (xenophagy) and mitophagy. This is an interesting study. They provided novel and solid data supporting their conclusion. They examined not only GAS-infected autophagy, but also Parkin-induced mitophagy. Although I recommend their study for publication in the EMBO J, the authors have to answer my comments below before acceptance.*

Response to comment #3-1) First, we wish to thank the referee for the positive comments and helpful suggestions for our study. To further confirm our results, we performed additional experiments as described below.

Major comments:

Comment #3-2) *To which organelle does endogenous Rab35 localize under a basal condition?*
Response to comment #3-2) As suggested, we examined where endogenous Rab35 localizes under the basal condition. Endogenous Rab35 clearly colocalized with HSP60, a marker of mitochondria, whereas clear colocalization of endogenous Rab35 with Rab7A, a marker of late endosomes; EEA1, a marker of early endosomes; and Rab11A and TfR, markers of recycling endosomes, could not be detected. In addition, we found that endogenous Rab35 localized directly under mCherry-ATP1A1, a marker of the plasma membrane. We conclude that endogenous Rab35 was localized in mitochondria and directly under the plasma membrane (Fig EV4H).

Comment #3-3) *Rab27 was not efficiently knocked down by miR-RNA. The authors should use a different construct or alternative approach.*

Response to comment #3-3) As suggested, we generated Rab27A knockout cell lines (Fig EV4A), and examined NDP52 recruitment and autophagosome formation in these knockout cells. Rab27A knockout did not affect NDP52 recruitment and autophagosome formation in response to invading bacteria, whereas Rab35 knockout significantly suppressed these processes (Fig 2A-D). Thus, we concluded that the inhibitory effects of NDP52 recruitment to GAS and autophagosome formation by TBC1D10A are specifically mediated by Rab35.

Comment #3-4) *Testing whether Rab35 is involved in endosomal and/or lysosomal functions is very important. The cathepsin B assay is not sufficient to confirm normal lysosomal functions in Rab35 KO cells. The authors should at least test and compare 1) lysosomal pH and 2) endosome/lysosome morphologies.*

Response to comment #3-4) As suggested by the referee, we examined endosome/lysosome

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morphology and lysosomal pH in Rab35 knockout cells. Rab35 knockout did not alter endosome/lysosome morphology and lysosomal pH (Fig EV4J).

Comment #3-5) *Based on the results the authors provided in this study, Rab35 functions upstream of NDP52 for NDP52 recruitment to GAS. Consistent with this, the immunoelectron microscopy (Fig EV 9B) beautifully indicates that Rab35 was recruited onto bacteria-containing endosomal membranes. On the other hand, how Rab35 is recruited to the damaged mitochondria during mitophagy still remains unknown. Does Rab35 directly attach to damaged mitochondrial membranes during mitophagy? Immunoelectron microscopy upon mitophagy should answer this important question. Furthermore, what recruits Rab35 to the place (endosomes during GAS infection and damaged mitochondria during mitophagy) where autophagosome formation will occur?*

Response to comment #3-5) As suggested by the referee, we investigated where Rab35 localizes on induction of mitophagy. Immunoelectron microscopy analysis revealed that Rab35 was localized on the mitochondrial membrane under mitochondrial depolarization with CCCP (Fig 5C). In response #3-2, we also showed that endogenous Rab35 was clearly colocalized with HSP60, a marker of mitochondria, under the basal condition. Therefore, although Rab35 actually accumulates on the mitochondrial membrane during mitophagy, a significant amount of Rab35 might be present on the mitochondrial membrane even in basal the condition. Regarding the recruitment mechanism of Rab35, activation of Rab GTPase (exchanging from the GDP-bound form to the GTP-bound form) is known to be regulated by GEF (guanine nucleotide exchange factors), and RabGDI (Rab-specific GDP dissociation inhibitor) is known to recruit inactive Rab from the acceptor to donor membranes. Because DENND1A, DENND1B, DENND1C, and folliculin have been reported as GEFs for Rab35, these proteins and RabGDI1 or 2 may regulate Rab35 recruitment to target membrane. These issues would be important to clarify for further analysis of the molecular mechanism of Rab-mediated autophagy.

Comment #3-6) *In Fig EV 4C, EmGFP-Rab35 S22N was nicely merged with mCherry-NDP52 in cells, but the BiFC assay in Fig EV 6A showed no interaction between Rab35 S22N and NDP52. How do the authors explain this conflict?*

Response to comment #3-6) To reveal why Rab35 S22N puncta in the cytoplasm exist, we investigated the subcellular localization of Rab35 S22N puncta. As a result, we found that DENDD1A, one of Rab35 GEF, and RabGDI1, and RabGDI2, which recruit inactive Rab from the acceptor to donor membranes, clearly colocalized with Rab35 S22N puncta (please see reviewer figure below). Therefore, inactive Rab35 may accumulate during interaction with these proteins or in the process of recruitment to target membranes. Importantly, we constructed HeLa cells stably expressing EmGFP-Rab35 constructs in the knockout background, and found that Rab35 S22N-positive puncta were significantly decreased in this cell line, implying that overexpression of Rab35 S22N mutants caused the formation of artificial structures (Fig EV5E). Because colocalization of Rab35 S22N with NDP52 dose not mean that Rab35 S22N interacts with NDP52, we further confirmed GTP form-dependent binding of Rab35 with NDP52 in vitro (Fig 3H), and we concluded that active Rab35 specifically binds to NDP52.

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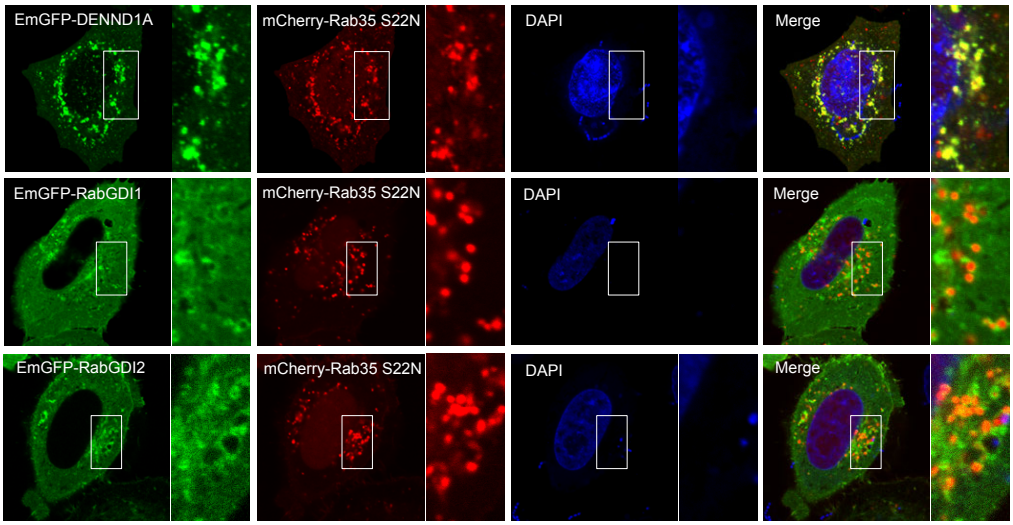

Comment #3-7) *Based on the fractionation assay, just the expression level of endogenous Rab35 seemed to increase after CCCP treatment while authors suggest 'recruitment' of Rab35 to the damaged mtiochondria. Does accumulation of Rab35 in the mitochondrial fraction result from the recruitment, inhibition of the degradation, or increase of the protein expression?*

Response to comment #3-7) As suggested by the referee, to confirm the underlying causes of accumulation of Rab35 in the mitochondrial fraction, we examined the total expression level of endogenous Rab35 after CCCP treatment. The total expression level of NDP52 in wild-type cells decreased with time after CCCP treatment, whereas that of Rab35 was not changed. Consequently, we concluded that accumulation of Rab35 in the mitochondrial fraction results from recruitment.

Comment #3-8) *Is Rab35 able to be recruited to the damaged mitochondria in NDP52 KO cells during mitophagy?*

Response to comment #3-8) As suggested by the referee, we examined Rab35 recruitment to damaged mitochondria in NDP52 knockout cells during mitophagy. Knockout of NDP52 did not affect Rab35 recruitment to depolarized mitochondria, suggesting that NDP52 is not involved in Rab35 recruitment to depolarized mitochondria

Minor comments:

Comment #3-9) *Magnified images should be provided even if the signals (such as Fig EV1 A GFP-TBC1D10A, Fig1 H GFP-TBC1D10A, Fig 2 C mCherry-NDP52, and so on) were not found on or near infected bacteria.*

Response to comment #3-9) As suggested, we have added magnified images in every figure showing bacteria.

Comment #3-10) *The amounts of LC3-II in Bafilomycin A1 0h were higher than those in DMSO 0h. The authors should mention in the legend at which time point Bafilomycin A1 was added, before the infection or at the time of the infection? The same comment is valid for Fig 2 G.*

Response to comment #3-10) As suggested by another referee, we examined LC3-II accumulation in non-infected and GAS-infected cells under bafilomycin A1 treatment, as well as under E64d and Pepstatin A treatment. As a result, we found that during GAS infection, bafilomycin A1 treatment and E64d and Pepstatin A treatment cannot provide real scientific information. Thus, we have deleted these results and showed results only under non-treatment (Fig 1C and D, 2I and J), as the effects of these treatments are not directly related to this study.

Comment #3-11) *Magnified images are required. According to the images, Rab35 signal appeared in some cells only after GAS infection. To which organelles does endogenous Rab35 localize? Confocal Micrographs of Rab35 staining without GAS infection should be shown.*

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Response to comment #3-11) As suggested, we have added confocal micrographs of Rab35 staining without GAS infection in Fig EV4H. As described above, we conclude that endogenous Rab35 localized in mitochondria and directly under the plasma membrane.

Comment #3-12) *The amounts of GFP-Rab35 pulled down with the different GST-NDP52 constructs should be quantified.*

Response to comment #3-12) To determine the extent of interaction between Rab35 and NDP52 deletion mutants, we performed fluorescence protein fragment complementary assay. We found that ΔSKICH, ΔLIR, ΔCC, Δ350, and ΔZn generated similar fluorescence intensities as wild-type NDP52, whereas the fluorescence was drastically decreased when ΔCC and ΔZn were expressed (Fig EV6B and C), suggesting that the NDP52-Rab35 interaction is involved in the coiled-coil and zinc finger domains in NDP52.

Comment #3-13) *Do in vitro interactions between Rab35 and NDP52 differ for different GTP-bound states? Test the same binding assay using MBP-Rab35 GTP- and GDP-bound mutants.*

Response to comment #3-13) To investigate whether GTP bound-formed Rab35 specifically binds NDP52 in vitro, we performed an in vitro binding assay. We found that recombinant MBP-NDP52 specifically bound to GTP-loaded GST-Rab35, but not to GDP-loaded GST-Rab35 (Figs 3H), suggesting that GTP-bound Rab35 directly interacts with NDP52.

Comment #3-14) *Overexpressed Rab35 inhibited NDP52 recruitment during GAS infection (Fig EV 4C). Is the same inhibition observed for mitophagy?*

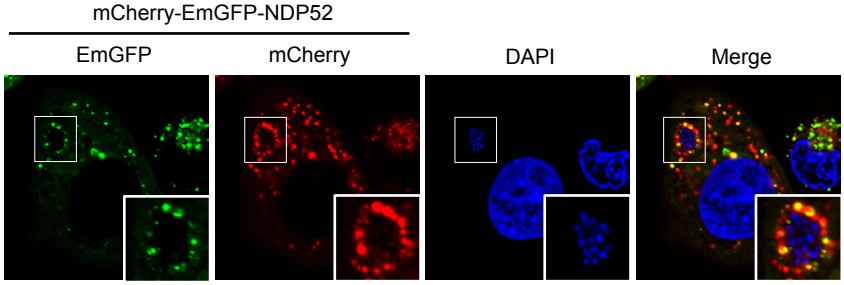
Response to comment #3-14) We did not investigate whether overexpression of Rab35 inhibits NDP52 recruitment during mitophagy in detail, as we consider that this is of minor importance. However, similar inhibition was observed in some experiments.

Comment #3-15) *On the microscopic images, GFP-LC3 puncta increased upon starvation treatment, but on WB, LC3-II decreased. Why?*

Response to comment #3-15) At present, we cannot explain this phenomenon pointed out by the referee. However, a similar phenomenon was reported in several previous studies. For example, see Jiang et al, 2014. Mol Biol Cell and Carroll et al, 2013. Dev Cell. Endogenous LC3 may have already been degraded by the lysosome under the starvation condition applied for 2 h.

Comment #3-16) *In WT cells, most of all mCherry-EmGFP-NDP52 dots were mCherry-positive but EmGFP-negative. Does it mean that most of the exogenous NDP52 was included inside lysosomes? Same comments for Fig 6E.*

Response to comment #3-16) In Fig 6E, we show a representative image to allow the readers to easily understand the results. However, not all cells showed mCherry-EmGFP-NDP52 dots that were mCherry-positive but EmGFP-negative. We were able to observe many cells in which only a part of mCherry-EmGFP-NDP52 dots were mCherry-positive but EmGFP-negative (please see reviewer figure below). Thus, we cannot state that most of the exogenous NDP52 was included inside lysosomes.



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Comment #3-17) *Does TBK1 stably form a complex with NDP52 and Rab35?*

Response to comment #3-17) TBK1 has been already reported to form a complex with NDP52 (Thurston et al, 2009. Nat Immunol). In this study, we demonstrated that NDP52 forms a complex with Rab35. Therefore, TBK1 may stably form a complex with NDP52 and Rab35.

Typos, errors, etc:

Comment #3-18) *Line 148: Rad35 should be Rab35.*

Response to comment #3-18) As suggested, we corrected the misspelled “Rad35” to “Rab35”.

Comment #3-19) *The legend of Fig 1G is for Fig 1H and vice versa.*

Response to comment #3-19) As suggested, we corrected the referring number.

Comment #3-20) *Fig 5C: The merged images lack the green channel (EmGFP-Parkin).*

Response to comment #3-20) To facilitate visualization of NDP52-TOM20 colocalization, we only showed the merged images of NDP52 and TOM20. We have changed the label from “Merge” to “mCherry-NDP52/TOM20” in Fig 5D in the revised manuscript.

Comment #3-21) *Fig EV 7B: EmGFP-Rab35 labeled above the image should be mCherry-Rab35.*

Response to comment #3-21) As suggested, we corrected our mistake.

2nd Editorial Decision 09 June 2017

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

As you will see, referee #1 now supports publication, while referee #2 remains critical and referee #3 thinks that a few clarifications are still needed. I would thus like to ask you to address referee #2's concern by clearly discussing in your manuscript differences between mice and human in regard to NDP52 and to address the remaining concerns of referee #3.

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

REFEREE REPORTS

Referee #1:

The authors have improved the manuscript by successfully responding to all major questions. No further comments.

Referee #2:

1. General summary and opinion about the principle significance of the study, its questions and findings: The authors have attempted to address several points, and many of them satisfactorily. However, there is one major, key point brought up twice in the previous round of review that the reviewers unfortunately chose to ignore or deflect. They appear to push aside the fact that the mouse, the key model mammalian organism, does not have NDP52 and yet they base their specific and universal conclusions on NDP52 suggesting this is the most important regulator of autophagy. This cannot be so, and this issue cannot be easily skirted. Unfortunately, the athors' responses and stance is disappointing, and they chose to dismiss and conveninetly push aside this key conceptual

criticism.

2. Specific major concerns essential to be addressed to support the conclusions.
The authors chose NDP52 as the key player, and it is incumbent upon these authors to appropriately select their model systems and outputs.

I have no minor concerns.

Referee #3:

In the revised experiments, the authors have answered most of the questions I raised. However, I have several questions and comments for the new results and figs, which should be answered before acceptance.

Although Fig EV4H showed mitochondrial localization of endogenous Rab35, other figs such as Fig3A, Fig5B, Fig EV4C, Fig EV12A for endogenous Rab35, and Fig 7B, Fig 7G, Fig EV5A, Fig EV5C, Fig EV5E, Fig EV8B and Fig EV9A for overexpressed Rab35 do not support it. As I think Rab35 localization is important because Rab35 can recruit NDP52 to the cargo where Rab35 localizes, the authors should clearly explain the different Rab35 localization in different Figs.
The authors mentioned in the main text that knockout of NDP52 did not affect Rab35 recruitment to depolarized mitochondria..., but in Fig EV9A, it's hard to see Rab35 recruitment in either WT or NDP52 KO cells.

Fig EV5E
The authors should confirm the similar level of EmGFP-Rab35 to the endogenous one by WB.

Fig EV9E
How did authors guarantee that the cells in fig EV9E express FLAG-TBC1D10A?

2nd Revision - authors' response 04 July 2017

Dear Reviewers, We sincerely appreciate the helpful comments and suggestions regarding our manuscript. We have considered all of the referees' comments and have accordingly provided additional experimental data in this revised version of our manuscript. Our detailed point-to-point responses to each comment are provided below. The modified sections in the revised manuscript are highlighted in red. We have indicated the figure number where modifications relevant to each of the comments made by the referees have been made. We hope that these explanations and revisions are satisfactory.

Referee #1:

The authors have improved the manuscript by successfully responding to all major questions. No further comments.

Response to comment #1-1) We appreciate that the referee supports the publication of our manuscript.

Referee #2:

1. General summary and opinion about the principle significance of the study, its questions and findings: The authors have attempted to address several points, and many of them satisfactorily. However, there is one major, key point brought up twice in the previous round of review that the reviewers unfortunately chose to ignore or deflect. They appear to push aside the fact that the mouse, the key model mammalian organism, does not have NDP52 and yet they base their specific and universal conclusions on NDP52 suggesting this is the most important regulator of autophagy. This cannot be so, and this issue cannot be easily skirted. Unfortunately, the athors' responses and

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stance is disappointing, and they chose to dismiss and conveninetly push aside this key conceptual criticism. Specific major concerns essential to be addressed to support the conclusions.
The authors chose NDP52 as the key player, and it is incumbent upon these authors to appropriately select their model systems and outputs.

Response to comment #2-1) We thank the referee for the time and effort spent in reviewing our manuscript. We apologize if our previous responses could not convey our intentions and caused misunderstanding. The referee indicated that "murine cells are devoid of NDP52 (the mouse is a natural mutant lacking NDP52)," and this point was the underlying premise for the referee's critique. However, we respectfully believe that the referee's position is not scientifically supported.

The main reasons for this are as follows:

i) Johnson and colleagues have shown that NDP52 is present in mouse brains and colocalizes with both phosphorylated tau and intracellular amyloid-beta, as well as with ATG proteins (Jo et al., Nat Commun. 2014; Kim et al., Biochem Biophys Res Commun 2014).
ii) Expression of NDP52 was shown in mouse macrophages in another recent report (Zhong et al., Cell 2016).
iii) Recent evidence indicates the existence of mouse NDP52/Calcoco2 (https://www.ncbi.nlm.nih.gov/nucleotide/402169572?report=genbank&log$=nuclalign&blast_rank=2&RID=M7UCXPPZ014), and the mouse NDP52/Calococo2 gene was included in a phylogenetic tree (Please refer Fig. 1B, Tumbarello et al, PLoS Pathog. 2015).
In contrast, we could not find (and the reviewer has not cited) any report showing that NDP52 is not expressed in mice. However, we agree with the referee that the mouse is one of the most important model organisms. In light of these points, because mouse NDP52 is a truncated form lacking the C-terminal zinc finger domain, we have added a discussion about the structural and regulatory differences of NDP52 between mice and humans (please see line 444-449 in our manuscript). Additionally, it remains unclear whether mouse NDP52, which lacks ubiquitin and Rab35-binding domains, functions as autophagy receptor to bind ubiquitinated targets and LC3 and does not actually interact with these proteins. These important issues have to be addressed in future studies.

Referee #3:

In the revised experiments, the authors have answered most of the questions I raised. However, I have several questions and comments for the new results and figs, which should be answered before acceptance.

Response to comment #3-1) We thank the referee for the fruitful comments and suggestions. Our responses to the referee's comments are as follows.

Although Fig EV4H showed mitochondrial localization of endogenous Rab35, other figs such as Fig3A, Fig5B, Fig EV4C, Fig EV12A for endogenous Rab35, and Fig 7B, Fig 7G, Fig EV5A, Fig EV5C, Fig EV5E, Fig EV8B and Fig EV9A for overexpressed Rab35 do not support it. As I think Rab35 localization is important because Rab35 can recruit NDP52 to the cargo where Rab35 localizes, the authors should clearly explain the different Rab35 localization in different Figs.
The authors mentioned in the main text that knockout of NDP52 did not affect Rab35 recruitment to depolarized mitochondria..., but in Fig EV9A, it's hard to see Rab35 recruitment in either WT or NDP52 KO cells.

Response to comment #3-2) In contrast to Tom20 and HSP60, because endogenous Rab35 localizes not only in mitochondria but also in the cytoplasm, it is difficult to confirm the mitochondrial localization of endogenous Rab35 without simultaneously using a mitochondrial marker, as shown in Fig 5B and Fig EV1H (Fig 5B and Fig EV4H in previous manuscript). In contrast, during GAS infection, mitochondrial morphology is distinctly different from that under basal conditions, and GAS infection causes mitochondrial fragmentation (please see reviewer figure A below). We examined the subcellular localization of endogenous Rab35 and mitochondria during GAS infection. It was difficult to verify a clear colocalization of endogenous Rab35 with mitochondria (please see reviewer figure B below). Mitochondrial fragmentation or Rab35 recruitment to bacteria containing endosomes might change the subcellular localization of

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endogenous Rab35. However, it was not easy to confirm the colocalization of exogenous Rab35 with mitochondria in GAS infection or without infection (basal condition) when HeLa cells overexpressed Rab35. To our knowledge, there is no report that exogenous Rab35 colocalizes with mitochondria. However, under basal conditions, we were able to observe the colocalization of exogenous Rab35 with mitochondria in HeLa cells stably expressing EmGFP-Rab35 in the knockout background (please see reviewer figure C below). These results imply that excessive transient expression of Rab35 by transfection may have prevented us from detecting the natural localization of Rab35. With regard to Rab35 recruitment to depolarized mitochondria in WT and NDP52 KO cells, we consider that our selection of confocal pictures led to a misunderstanding, as the referee pointed out. We have replaced these images with other images in the revised manuscript (Fig EV3A).

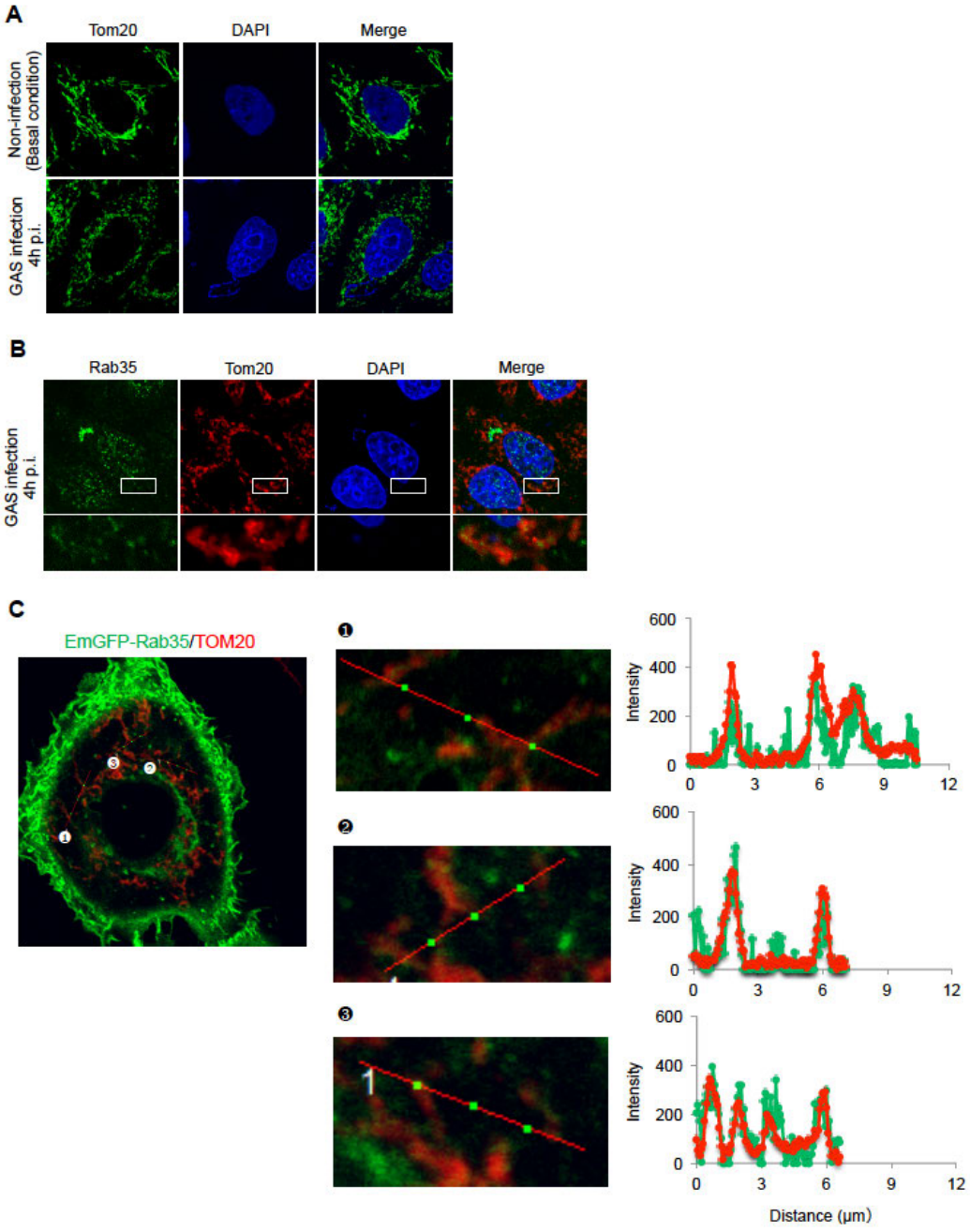

Fig EV5E: The authors should confirm the similar level of EmGFP-Rab35 to the endogenous one by WB.

Response to comment #3-3) As suggested by the referee, the requested blots have been added (Fig EV2E).

Fig EV9E: How did authors guarantee that the cells in fig EV9E express FLAG-TBC1D10A?

Response to comment #3-4) We ensured that the cells expressed FLAG-TBC1D10A or its mutant by labeling with an anti-FLAG antibody. We have added confocal images showing the labeling with the anti-FLAG antibody (Fig EV3E).

3rd Editorial Decision 21 July 2017

Thank you for submitting your revised manuscript to us. As you will see, I ran this final version again by referee #3, who is now supporting publication. I am thus pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. Congratulations!

REFEREE REPORT

Referee #3: The authors have answered all my questions. No further comments.

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Corresponding Author Name:	Ichiro Nakagawa
Journal Submitted to:	The EMBO Journal
Manuscript Number:	EMBOJ-2017-96463R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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

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1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We chose the sample size and the number of cell per measurement according to the previous studies in the same field. We used biological replicates-triplicates. This is a standard setup.
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5. For every figure, are statistical tests justified as appropriate?	Statistical analyses were chosen appropriately to the best of our knowledge.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistically significant differences between groups were determined using two-tailed Student t-test when tha data exhibited normal distribution.
Is there an estimate of variation within each group of data?	Standard error of the mean were calculated as indicated.
Is the variance similar between the groups that are being statistically compared?	See above.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog numbers or clone numbers have been provided in the materials and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HeLa and HEK293T cell lines originated from ATCC. Cells are tested regularly for their identity and are confirmed as mycoplasma free using a commercial kit.

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18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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