EMBO reports

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RIP1 Negatively Regulates Basal Autophagic Flux through TFEB to Control Sensitivity to Apoptosis

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

Editor: Nonia Pariente

1st Editorial Decision 29 September 2014

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although the referees find the study potentially interesting, they also consider it preliminary and insufficiently conclusive at this stage. All of them note that further experiments are needed to provide convincing support for your claims, and also request a number of technical improvements of the data.

As the reports are below, I will not detail them here. However, it is clear that substantial additional work is necessary to causally show that RIPK1 regulates the sensitivity to external apoptosis stimuli through repression of basal autophagy by regulating TFEB. In this light, it would be crucial to strengthen the results shown in figure 4, as suggested by the referees. Please note that we would not consider a study that tones down the claims made, as mentioned by referee #2, but one that experimentally provides a causal link between the current observations and therefore conclusively supports the current claims of the study. The issues raised by the referees refer to critical experiments and should be addressed, with the exception of referee 3's third point. It would also be important to be more consistent in the use of cell lines and demonstrate reproducibility by repeating some critical experiments in multiple cell lines, including the RIPK1 knockout cells.

Please note that it is our policy to undergo one round of revision only and thus, acceptance of your

study will depend on the outcome of the next, final round of peer-review. I appreciate that experimentally addressing all the referee concerns would involve extensive additional work, and I would be open to extending our usual revision time of three months, should you feel time is the only constraint to a successful revision.

Please contact me if I can be of any assistance during the revision process. If you rather chose to seek rapid publication elsewhere, I would also welcome a message to that effect.

Referee reports

Referee #1:

In this work Yonekawa et al. screened for genes that affect growth/viability of cancer cells when cultured in different experimental conditions. They identified RIP1K as a protein that inhibits basal autophagy. They propose that regulation of basal autophagy by RIP1K is achieved by regulating the transcription factor TFEB. This is a very tiny story but given the central role of RIP proteins in the regulation of cell fate and the emerging role of autophagy in cancer, this work is important for the field. The paper is well written and the experiments shown are of fair quality. However in its current status some of the conclusions are supported by a limited number of experiments and need to be further validated.
Major Points:
- The demonstration that RIP1 deficiency induces autophagy via TFEB must be validated in the context of TFEB depletion.
- To demonstrate the role of ERK in mediating TFEB activation and autophagy induction in absence of RIP1, the reactivation of ERK should be performed in RIP1 KO/KD cells and TFEB localization and autophagy levels should be evaluated. This could be achieved by overexpression of wt or constitutively active MEK/ERK proteins.

- The claim that RIP1's ability to control basal autophagy affects the stimulus-selective response to FAS is not supported by the data (figure 4). To support this claim it is essential to show that inhibition of autophagy and TFEB in RIP1 depleted cells rescued the enhanced sensitivity to FAS treatment.

- The regulation of ERK by RIP1 was only marginally discussed and indeed no experiments were performed to show that re-expression of a kinase death domain RIP1 is able to restore ERK activity similarly to the WT RIP protein.

Minor points:

- The bands in Fig S3G are inverted.

- The statement: "Furthermore, expression of a kinase-dead RIP1 mutant (K45A) was capable of reducing autophagy in RIP1-/- MEFs to a similar extent as the wild-type
protein (Figure S3C)" is not correct since the figure showed an intermediate situation.

- The statement: "Moreover, starvation did not additionally increase autophagy in RIP1-knockdown HeLa cells relative to control cells (Figure 2D, S2B-C) suggesting that RIP1 primarily affects basal but not induced autophagy" is not clear to me, since in my opinion this observation suggests that is the starvation induced autophagy affected by RIP silencing.

-The regulation of MAPK by RIP1 should be discussed in greater detail in the introduction and more references should be inserted.

Referee #2:

In this paper the authors convincingly demonstrate that RIPK1 regulates basal autophagy, and

provide evidence that this is via regulation of TFEB. Overall, the work is well done and many of the results are convincing, although perhaps over-interpreted in places. My concerns, which I think can be addressed, follow.

1. The title of the paper suggests that RIPK1 regulates autophagic flux via TFEB, which in turn controls sensitivity to apoptosis. While they show that RIPK1 does each of these things, the connections between these are not at all shown. What they do show is that RIPK1 controls autophagic flux, affects TFEB, and affects sensitivity to apoptosis, but in no case do they show that these are linked, except by speculation. At the very least, the title should be modified, depending on the results of subsequent experiments, to soften this conclusion.

2. Figure 2C reports values of "high autophagy" which are not at all indicated by the examples of the FACS profiles reported in the supplemental data. It might therefore be useful if the actual FACS used to obtain these values (or an example, since it appears that these were done multiple times) be provided in the supplemental data. What is shown is always closer to 40-50%, rather than the 70-80% they report in the main text. This should be reconciled.

3. In Figure 3G it is clear that the blot for p-ERK is not a reprobe of the blot for ERK, based on the appearance of the ERK blot vs that of p-ERK, yet only one loading control is shown (implying that they are). The authors should re-evaluate this figure and rectify this concern. In general, it is always preferable to strip and reprobe the same blot for phosphorylation. [If this was, indeed, the same blot, the authors may want to show the entire blots in supplemental, as some readers will be concerned by the apparent discrepancy].

4. A more major concern relates to #1 (also see #5). While the ability of RIPK1 to affect basal autophagic flux, and to affect function of TFEB are fairly convincing, it is not clear that simply increasing TFEB activity to the extent shown is sufficient to increase autophagic flux. At the very least, the authors should assess ERK activity with or without RIPK1 (or the RIPK1 mutants) and the effects of inhibiting ERK. Ideally, they should also assess if silencing (even partially) TFEB impacts on the increase in autophagic flux seen upon knockdown of RIPK1.

5. Next, the authors wish to show that the changes in autophagic flux induced by silencing RIPK1, presumably mediated by TFEB, affect sensitivity to apoptosis induced by CD95 vs TRAILR ligation. This connection is not shown. The simple prediction is that inhibition of ERK should produce the same pattern of sensitivity as caused by RIPK1 silencing (It may, of course, not be this simple, as ERK has a number of effects). Therefore, it may be important to assess if silencing (or partial silencing) of TFEB reverses these changes in sensitivity caused by silencing of RIPK1, which would help to support the conclusion they wish to make. Further, it is well described that silencing of RIPK1 sensitizes to TNF-induced apoptosis (with low does CHX). It might be useful to assess this as well, but not essential.

6. The RHIM domain of RIPK1 is important for its interactions with RIPK3 and perhaps other proteins, and mutations in the RHIM are known to affect these interactions without affecting other functions of RIPK1. It would be useful to know how these affect the suppression of basal autophagy in RIPK1-/- MEF. It would also be very helpful to know if the effects of RIPK1 are independent of RIPK3, (they conclude this based on the use of kinase inactive RIPK1, and the effects of necrostatins, but it is now clear that RIPK1 can inhibit RIPK3 function when RIPK1 is kinase inactive. Given that most of the experiments involve exposure to interferon-inducing conditions-e.g. siRNA, shRNA, transient transfections-a more formal demonstration that RIPK3 is not involved would be helpful).

7. As a minor point, the use of RIP1 (and RIP3) is discouraged, as the official names for these proteins are RIPK1 and RIPK3.

Referee #3:

Review 22nd September 2014

- You should be trying to help the work get published not necessarily in this journal but ultimately.
- Don't criticize an experiment unless you can tell the authors how they could do it better. "If you just want to throw darts," he would say, "go to the pub."
- Keep in mind that no one ever built a statue to a critic.
- Try to act as a peer in the process of peer review.

Science Signaling 2009 Michael Yaffe

Title: RIP1 Negatively Regulates Basal Autophagic Flux through TFEB to Control Sensitivity to Apoptosis

Manuscript #EMBOR-2014-39496V1

General Remarks

This study looks at the ability of RIPK1 to regulate autophagy. They identified RIPK1 as a gene whose knock-down was selected for in conditions that increase reliance on autophagy. The authors would like to show that this is because RIPK1 represses basal autophagy by regulating the transcription factor TFEB. Furthermore they claim that this autophagic function of RIPK1 can regulate sensitivity to extrinsic apoptosis stimuli.

The conclusions that can be drawn from the work are however extremely limited because the author use different cell lines to conclude different things. Thus Figure 1 uses 67NR cells for the screen, Figure 2 uses Hela cells with knock-down RIPK1 to demonstrate increased basal autophagy. Figure 3 uses Hela cells to show regulation of TFEB and Figure 4 uses MCF10A cells to show effects on cell death. Furthermore although they have an excellent tool of Ripk1-/- cells {plus minus} FLAG tagged RIPK1, which is far less prone to artefacts than shRNA they perform only one assay with this tool.

Overall the manuscript is well written but there are a number of imprecisions/mistakes.
Page 5 The authors cite Hadji et al as a paper about RIPK1 knock-down but Hadji et al is about FasL and the death induced is not even dependent upon RIPK1.
Page 6 the authors surely mean Figure S2D-G and Figure S2H instead of Figure S4D-G and Figure S4H, respectively.
Page 7 Necrostatin was PREVIOUSLY
Page 7 RIP1's ability to activate NF-κB is not involved IN

Specific Remarks
Figure 1 is not as informative as it could be. Please add the fold change (or equivalent raw data) instead of just the ranking. Also, please show the actual ATG12 knockdown efficiency and demonstrate it inhibits autophagy (such as LC3B blotting etc).
Figure 2A would like to see other markers of autophagy not just LC3-II, it's possible that LC3-II levels are affected but not other things. Authors need to show the extent of RIP1 knockdown.
Figure 2B Why switch to Chloroquine? Either do 2A with CQ or 2B with bafilomycin. How were cells counted, blind? Why >20 GFP-LC3? firstly it is completely arbitary and selected and other papers from these authors use >30. It would be much better to show the distribution. Secondly it says >30 in Figure legend.
Figure 2C What is high autophagy? It is not defined in the legend. It is described in methods and demonstrated in Supplementary Fig. 2, however the cut off for high autophagy seems very arbitary. Furthermore, the gating of cells according to Cherry/GFP fluorescence looks strange in Supplementary Fig. 2, there is a high "blip" of cells at 256 that are included within the "high" gate. What are these cells? They are clearly unusual, how can they be included in the analysis? Furthermore none of these distributions looks anything like the authors' representation in their recent Autophagy 10:7, 1327-1334; 2014 paper.
Supplementary Figure 2G As the author state in their earlier publications it is important to characterise cell lines with GFP/Cherry construct. In these MEFs where is the characterisation with a normal autophagic stimulus?

Figure 3A Changes in transcription are very small, I would have no confidence in them unless tested with multiple shRNAs or the reconstituted Ripk1-/- MEFs discussed earlier.
Figure 3C TFEB expression seems to down rather than specifically pTFEB being affected. Given

such slight effects, it would be necessary to confirm with the other siRNAs or the Ripk1-/- MEFs. The authors claim that there is increased mobility in the total TFEB Western blot, however to me it doesn't look like an increased mobility of TFEB, more like an angled gel.
Figure 3E. Small small differences in a luciferase assay could be due to even minor changes in experimental setup. N is not defined in legend.
Figure S3G. Bizarre situation where RIPK1 knock-down with #3 shRNA results in higher levels of RIPK1 than in the parental line? Also see an increase in pTFEB, this almost certainly discredits the hypothesis that RIPK1 represses TFEB, because this shRNA increased autophagy flux same as other 2 in Supplementary Fig. 2. It is possible that the authors have mixed up their labelling, but that is a disappointing error to make. And even if it is mixed up then it seems to destroy the correlation between RIPK1 knock-down and pTFEB levels because the level of knock-down with #3 is less than #1 but the effect on pTFEB is greater.

Figure 4 RIPK1 plays such an important DIRECT role in regulating apoptosis and necroptosis it would be absolutely necessary to exclude the obvious interpretation that what is affected in these cells is rather to do with complex 2 formation. Because no data is presented on the regulation of autophagy by RIP1 knockdown in these cells one cannot make a link between autophagy and RIP1-induced cell death in MCF10A cells. Furthermore, even if this data was shown, it would be a correlation and not proof of cause. In A and B, N is not defined.
Supplementary Fig. 4 reduction in FAP1 levels with RIPK1 shRNA is unconvincing.

Mw markers should be present in all Figures - it is a courtesy to the reader and an aid to the reviewer to include Mw markers.

1st Revision - authors' response 30 January 2015

Point by Point response:

Referee #1:

In this work Yonekawa et al. screened for genes that affect growth/viability of cancer cells when cultured in different experimental conditions. They identified RIP1K as a protein that inhibits basal autophagy. They propose that regulation of basal autophagy by RIP1K is achieved by regulating the transcription factor TFEB. This is a very tiny story but given the central role of RIP proteins in the regulation of cell fate and the emerging role of autophagy in cancer, this work is important for the field. The paper is well written and the experiments shown are of fair quality. However in its current status some of the conclusions are supported by a limited number of experiments and need to be further validated.

We appreciate the recognition of the importance and quality of our work and the reviewer's constructive suggestions for manuscript improvement. To further support our conclusions, and in response to reviewers' suggestions, our revised manuscript includes new data, including 7 new panels in the main figures and 15 new panels in the expanded view figures.

Major Points:
- The demonstration that RIP1 deficiency induces autophagy via TFEB must be validated in the context of TFEB depletion.

We agree with this assessment. In the revised manuscript, we have included new data (Figure 3H&3I) showing that knockdown of TFEB with shRNA in HeLa and MCF10A cells prevents the ability of RIP1 shRNA to induce increases in basal autophagy.

- To demonstrate the role of ERK in mediating TFEB activation and autophagy induction in absence of RIP1, the reactivation of ERK should be performed in RIP1 KO/KD cells and TFEB localization and autophagy levels should be evaluated. This could be achieved by overexpression of wt or constitutively active MEK/ERK proteins.

As suggested, we have utilized a constitutively active MEK construct, which we have stably expressed in HeLa cells. We have included new data (Figure 3G) showing that MEK activity in these cell prevents the ability of RIP1 shRNA to induce increases in basal autophagy compared to control vector.

- The claim that RIP1's ability to control basal autophagy affects the stimulus-selective response to FAS is not supported by the data (figure 4). To support this claim it is essential to show that inhibition of autophagy and TFEB in RIP1 depleted cells rescued the enhanced sensitivity to FAS treatment.

We have included new data in Figure 4G. While TFEB knockdown reduces the ability of RIP1 depletion to protect cells from TRAIL (Figure 4E-F), TFEB knockdown, in contrast, protects cells from increased sensitization to Fas ligand caused by RIP1 depletion (Figure 4G).

- The regulation of ERK by RIP1 was only marginally discussed and indeed no experiments were performed to show that re-expression of a kinase death domain RIP1 is able to restore ERK activity similarly to the WT RIP protein.

The activation by the kinase-deficient RIP1 in comparison to WT RIP protein is now shown in expanded view figure E9E.

Minor points:

- The bands in Fig S3G are inverted.

We apologize for the mistake, which other reviewers also noticed. Apparently, while dragging the blots in our graphics software, the blots became inverted left to right and also upside down. The figure has been corrected in the revised manuscript.

- The statement: "Furthermore, expression of a kinase-dead RIP1 mutant (K45A) was capable of reducing autophagy in RIP1-/- MEFs to a similar extent as the wild-type protein (Figure S3C)" is not correct since the figure showed an intermediate situation.

We have softened this statement to say that the kinase-dead RIP1 mutant (K45A) was capable of reducing autophagy in RIP1-/- MEFs without reference to the wild-type protein.

- The statement: "Moreover, starvation did not additionally increase autophagy in RIP1-knockdown HeLa cells relative to control cells (Figure 2D, S2B-C) suggesting that RIP1 primarily affects basal but not induced autophagy" is not clear to me, since in my opinion this observation suggests that is the starvation induced autophagy affected by RIP silencing.

We have changed this statement in the text.

-The regulation of MAPK by RIP1 should be discussed in greater detail in the introduction and more references should be inserted.

We wish that we could insert a greater amount of discussion on this point and in general, but we are extremely limited by the space requirements of the report format (25,000 characters for all text, including references, legends, and spaces), so we have inserted a reference to a review article that discusses RIP1 activation of ERK. Readers interested in more of the specifics of the activation of ERK may access this reference. While the specifics of regulation of ERK by RIP1 in some specific contexts (e.g. TNF signaling) are known, and RIP1 is known to activate ERK in both these pathways and after overexpression, we are not aware of any publications that have addressed the mechanism of ERK activation by RIP1 in the basal, unstimulated, state.

Referee #2:

In this paper the authors convincingly demonstrate that RIPK1 regulates basal autophagy, and provide evidence that this is via regulation of TFEB. Overall, the work is well done and many of the

results are convincing, although perhaps over-interpreted in places. My concerns, which I think can be addressed, follow.

We appreciate the recognition of the quality and convincingness of our work. We also appreciate the reviewer's constructive suggestions. To further support our conclusions, and in response to reviewers' suggestions, our revised manuscript includes new data, including 7 new panels in the main figures and 15 new panels in the expanded view figures.

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We agree with this assessment of the lack of data on the connections, which other reviewers also pointed out. We have included new data in our revised manuscript showing that knockdown of the essential autophagy protein ATG7 inhibits the ability of RIP1 knockdown to protect from TRAIL-induced cell death (Figure 4D), indicating that RIP1knockdown-induced protection from TRAIL of is at least in part due to RIP1 effects on autophagy. Induction of MEK (and subsequently ERK) activity or the knockdown of TFEB with shRNA in HeLa and MCF10A cells prevents the ability of RIP1 shRNA to induce increases in basal autophagy (Figure 3H&3I). Other new data also shows that TFEB knockdown reduces the ability of RIP1 depletion to protect cells from TRAIL (Figure 4E-F). In contrast, TFEB knockdown protects cells from increased sensitization to Fas ligand caused by RIP1 depletion (Figure 4G).

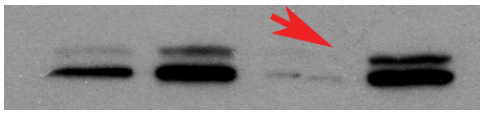
2. Figure 2C reports values of "high autophagy" which are not at all indicated by the examples of the FACS profiles reported in the supplemental data. It might therefore be useful if the actual FACS used to obtain these values (or an example, since it appears that these were done multiple times) be provided in the supplemental data. What is shown is always closer to 40-50%, rather than the 70-80% they report in the main text. This should be reconciled.

Actually, the supplemental data panel that is referred to (now Expanded View Figure E7A) IS one of the actual samples that were used to obtain the values reported in Figure 2C. We would like to suggest that, in our experience, the visual estimation of percentages by looking at these histograms when they are heavily shifted (very high autophagy) almost always underestimates (to the eye) the actual quantitation of the cells within the gate when done by the quantitative flow cytometry software. This is due to a substantial amount of horizontal spreading along the X-axis, which is less visually striking when compared to tall narrow peaks (such as are obtained when inhibiting flux with bafilomycin). Also we would like to point out that in Figure 2C and in the Expanded View Figure E7A, only one value actually is close to 70% in these two figures (that for RIP1 shRNA#1). The values for the cells with RIP1 shRNA#2 and #3 are closer to the 40-50% estimated by the reviewer, which range is also more consistent with the autophagy value ranges obtained thoughout most of the rest of the manuscript. The 70% of cells with puncta (in Figure 2B) probably is an overestimation of the amount of cells with high autophagy, since having LC3II formation is not the same as having actual autophagic flux.

3. In Figure 3G it is clear that the blot for p-ERK is not a reprobe of the blot for ERK, based on the appearance of the ERK blot vs that of p-ERK, yet only one loading control is shown (implying that they are). The authors should re-evaluate this figure and rectify this concern. In general, it is always preferable to strip and reprobe the same blot for phosphorylation. [If this was, indeed, the same blot, the authors may want to show the entire blots in supplemental, as some readers will be concerned by the apparent discrepancy].

We appreciate the reviewers close scrutiny and we agree that the appearance of the ERK vs. that of p-ERK in this blot would be concerning to me as a reviewer or as a reader. However, we have closely examined the original blots and found that we did not make a mistake. In fact, the autorads shown are from reprobing the same blot: first with p-ERK and then with ERK antibody. The reason for the dip that is seen in the ERK blot is not completely clear to us; as illustrated, none of the other antibodies showed a pronounced dip in this lane, although at higher contrast, one can see some

evidence of the dip in the p-ERK blot (shown by the red arrow below). The data has been moved out of the main figures and into the supplemental, since we needed more room in the main figures and the main point of the figure is that the MEK-ERK pathway is still intact in RIP1-knockdown cells when activated by other signaling pathways; we think this blot is sufficient to make this point.



4. A more major concern relates to #1 (also see #5). While the ability of RIPK1 to affect basal autophagic flux, and to affect function of TFEB are fairly convincing, it is not clear that simply increasing TFEB activity to the extent shown is sufficient to increase autophagic flux. At the very least, the authors should assess ERK activity with or without RIPK1 (or the RIPK1 mutants) and the effects of inhibiting ERK. Ideally, they should also assess if silencing (even partially) TFEB impacts on the increase in autophagic flux seen upon knockdown of RIPK1.

The effects of ERK inhibition on autophagy is shown in Expanded view Figure E9D. As mentioned previously in response to #1, the induction of MEK (and subsequently ERK) activity or the knockdown of TFEB with shRNA in HeLa and MCF10A cells prevents most of the ability of RIP1 shRNA to induce increases in basal autophagy (Figure 3H&3I), suggesting that it is TFEB, at least in part, that is responsible for changes in autophagy.

5. Next, the authors wish to show that the changes in autophagic flux induced by silencing RIPK1, presumably mediated by TFEB, affect sensitivity to apoptosis induced by CD95 vs TRAILR ligation. This connection is not shown. The simple prediction is that inhibition of ERK should produce the same pattern of sensitivity as caused by RIPK1 silencing (It may, of course, not be this simple, as ERK has a number of effects). Therefore, it may be important to assess if silencing (or partial silencing) of TFEB reverses these changes in sensitivity caused by silencing of RIPK1, which would help to support the conclusion they wish to make. Further, it is well described that silencing of RIPK1 sensitizes to TNF-induced apoptosis (with low does CHX). It might be useful to assess this as well, but not essential.

New data suggests that inhibition of ERK (actually MEK) does indeed produce the same pattern of sensitivity as RIP1 silencing in MCF10A cells, at least with respect to TRAIL sensitivity (Expanded View Figure E11A). TFEB is responsible, at least in part, for this inhibition, since TFEB knockdown inhibits about half of the protection offered by MEK inhibition. As mentioned previously in reponse to #1, our new data shows that TFEB knockdown reduces the ability of RIP1 depletion to protect cells from TRAIL (Figure 4E-F). In contrast, TFEB knockdown protects cells from increased sensitization to Fas ligand caused by RIP1 depletion (Figure 4G).

6. The RHIM domain of RIPK1 is important for its interactions with RIPK3 and perhaps other proteins, and mutations in the RHIM are known to affect these interactions without affecting other functions of RIPK1. It would be useful to know how these affect the suppression of basal autophagy in RIPK1-/- MEF. It would also be very helpful to know if the effects of RIPK1 are independent of RIPK3, (they conclude this based on the use of kinase inactive RIPK1, and the effects of necrostatins, but it is now clear that RIPK1 can inhibit RIPK3 function when RIPK1 is kinase inactive. Given that most of the experiments involve exposure to interferon-inducing conditions-e.g. siRNA, shRNA, transient transfections-a more formal demonstration that RIPK3 is not involved would be helpful).

We would like to have done an experiment with a RHIM-deficient RIP1 mutant. Unfortunately, we did not have the construct on hand and ran out of time to get it. Our use of kinase inactive RIPK1 and necrostatin was mostly to exclude the involvement of the kinase activity. We do not believe that RIP3 is involved, not only because of data with these agents, but also because RIP3 is not expressed at detectable levels in HeLa or MCF10A cell lines (see below).

(data not shown: article is currently in press in Cell Research "Methylation-Dependent Loss of RIP3 Expression in Cancer Represses Programmed Necrosis in Response to Chemotherapeutics")

7. As a minor point, the use of RIP1 (and RIP3) is discouraged, as the official names for these proteins are RIPK1 and RIPK3.

We have maintained the use of "RIP1" and "RIP3" solely for historical reasons (i.e. we studied these proteins before the nomenclature became standard). We may switch our nomenclature in future publications.

Referee #3:

Review 22nd September 2014

- You should be trying to help the work get published not necessarily in this journal but ultimately.
- Don't criticize an experiment unless you can tell the authors how they could do it better. "If you just want to throw darts," he would say, "go to the pub."
- Keep in mind that no one ever built a statue to a critic.
- Try to act as a peer in the process of peer review.

Science Signaling 2009 Michael Yaffe

Title: RIP1 Negatively Regulates Basal Autophagic Flux through TFEB to Control Sensitivity to Apoptosis

Manuscript #EMBOR-2014-39496V1

General Remarks

This study looks at the ability of RIPK1 to regulate autophagy. They identified RIPK1 as a gene whose knock-down was selected for in conditions that increase reliance on autophagy. The authors would like to show that this is because RIPK1 represses basal autophagy by regulating the transcription factor TFEB. Furthermore they claim that this autophagic function of RIPK1 can regulate sensitivity to extrinsic apoptosis stimuli.

We thank the reviewer for constructive suggestions for manuscript improvement. To further support our conclusions, and in response to reviewers' suggestions, our revised manuscript includes new data, including 7 new panels in the main figures and 15 new panels in the expanded view figures.

The conclusions that can be drawn from the work are however extremely limited because the author use different cell lines to conclude different things. Thus Figure 1 uses 67NR cells for the screen, Figure 2 uses Hela cells with knock-down RIPK1 to demonstrate increased basal autophagy. Figure 3 uses Hela cells to show regulation of TFEB and Figure 4 uses MCF10A cells to show effects on cell death.

We could have done better in the initial manuscript describing the reasons for cell selection. Initially, we used different cell lines for different reasons-- the 67NR cells with doxycycline-inducible expression of ATG12 shRNA are a nice model for such a shRNA screen and avoid the selective pressure against autophagy regulation in stable cells by making it inducible. However, these are mouse cells and have expression of RFP as a marker for ATG12 shRNA expression. They are therefore incompatible with many of our assays and reagents. However, once the screen has discovered a potential molecule, it is really not necessary to use these cells, since the findings are then confirmed in HeLa cells. HeLa cells worked as a nice model to demonstrate the effects of RIPK1 on autophagy. However, these cells are not sensitive to Fas, so we switched to MCF10 cells to measure effects on cell death so that we could use two different stimuli where autophagy activation have been reported to have opposite effects on cell death.

We now show data that indicate that RIP1 knockdown affects autophagy in MCF10A cells (Expanded View Figure E7 I-J) and that RIP1 knockdown in HeLa cells (in which we showed previously that RIP1 knockdown induces autophagy) protects from TRAIL-induced apoptosis (Figure 4E). We have included new data in our revised manuscript showing that knockdown of the essential autophagy protein ATG7 inhibits the ability of RIP1 knockdown to protect from TRAIL-induced cell death (Figure 4D), indicating that RIP1knockdown-induced protection from TRAIL of is at least in part due to RIP1 effects on autophagy. The knockdown of TFEB with shRNA in HeLa and MCF10A cells prevents the ability of RIP1 shRNA to induce increases in basal autophagy (Figure 3H&3I). Other new data also shows that TFEB knockdown reduces the ability of RIP1 depletion to protect cells from TRAIL (Figure 4E-F). In contrast, TFEB knockdown protects cells from increased sensitization to Fas ligand caused by RIP1 depletion (Figure 4G).

Furthermore although they have an excellent tool of Ripk1-/- cells {plus minus} FLAG tagged RIPK1, which is far less prone to artefacts than shRNA they perform only one assay with this tool.

We thought that the Rip1-/- cells would be a great tool as well. Unfortunately our Rip1-/- cells have proven to be somewhat problematic for use in the autophagy assays and responses. Although we have been able to show that restoration of RIP1 in these cells does indeed repress autophagy to some extent in these cells, consistent with our model, we have also determined that these cells are slightly deficient in autophagy responses in general. For instance they respond somewhat less than wild type cells in response to EBSS starvation. The basal level of autophagy in these cells is below what is predicted from transient knockdown experiment in WT MEFs Also differences with RIP1 restoration, although meaningful, were mild compared with what is predicted. It is possible that shRNA mediated effects are prone to artifacts, however, we believe that since we have shown that acute loss of RIP1 reduces cell proliferation and/or viability in some cell lines, that these cells may have been selected for wholesale reduction of autophagy or resistance to high autophagy during the process of spontaneous immortalization, and may not represent the full magnitude of response that acute knockdown enables.

This is somewhat reminiscent of a previous publication from our lab (Staskiewicz et al, *Autophagy*, 9(10): 1449-1450.), where we show that there seems to be selective pressure against aberrant levels of autophagy in some cell lines and where we conclude that acute knockdown of proteins in cells may be more relevant to observations with regard to regulating autophagic flux, since these cells have not adapted to different levels of autophagy through different adaptive pathways. We therefore have chosen to use acute knockdown as our primary tool in this publication.

Overall the manuscript is well written but there are a number of imprecisions/mistakes.
Page 5 The authors cite Hadji et al as a paper about RIPK1 knock-down but Hadji et al is about FasL and the death induced is not even dependent upon RIPK1.

The reviewer is correct that this paper is primarily about Fas Ligand. However, the citation of this paper is not an error. On page 211 of this publications under the subheading "**Arrayed shRNA Lethality Screens Identify CD95L and CD95 as Genes Critical for the Survival of Cancer Cells**" the reviewer will find that the authors of this paper collaborated with the HTS Core Facility at Memorial Sloan- Kettering Cancer Center (MSKCC) to examine survival gene candidates in its lethality screens (to investigate whether CD95 and CD95L were ever identified by such). Among the genes identified in this search of critical survival genes was RIP1 (see **Table S2** in the Hadji et al manuscript**: "Examples of Critical Survival Genes Identified in 12 Genome-Wide shRNA Lethality Screens, Related to Figure 1)".** Like Fas Ligand, RIP1 was identified in 9 of 12 cell lines Genome-Wide shRNA Lethality Screens as being important for full viability (at least the shRNA is highly selected against). This data is consistent with our present data that efficient RIP1 knockdown can reduce viability, or is is highly selected against, in some cell lines.

Page 6 the authors surely mean Figure S2D-G and Figure S2H instead of Figure S4D-G and Figure S4H, respectively.
Page 7 Necrostatin was PREVIOUSLY
Page 7 RIP1's ability to activate NF-κB is not involved IN

These mistakes have been corrected in our revised manuscript.

Specific Remarks
Figure 1 is not as informative as it could be. Please add the fold change (or equivalent raw data) instead of just the ranking. Also, please show the actual ATG12 knockdown efficiency and demonstrate it inhibits autophagy (such as LC3B blotting etc).

Raw data from read in the screen is now shown in Expanded View Figures E3-E5.
Data for ATG12 knockdown and LC3 western blots from the 67NR cells are now shown in Expanded View Figure E1.

Figure 2B Why switch to Chloroquine? Either do 2A with CQ or 2B with bafilomycin. How were cells counted, blind? Why >20 GFP-LC3? firstly it is completely arbitary and selected and other papers from these authors use >30. It would be much better to show the distribution. Secondly it says >30 in Figure legend.

We apologize for the switch between Bafilomycin and Chloroquine. We have had some staffing changes (and are currently understaffed) and so these experiments were done by different researchers with different preferences for reagents. However, since both bafilomycin and chloroquine inhibit autophagic flux late in the autophagic process before the degradation of LC3II, both can be used to block autophagic flux in a fairly similar manner, which is what is required in order to measure it in these assays.

After taking pictures of cells with puncta, the puncta are typically quantitated from the pictures without especial regard to which sample they came from. The data was then paired with the appropriate treatment conditions. So this is a partially blind quantitation, but not completely blind.

The legend for figure 2B is correct, the y axis label should read ">30 GFP-LC3". This has been corrected in the revised figures.

In our more recent experiment, the distribution of puncta in MCF10A cells was quantitated and is now shown in Extended View Figure E7I.

Figure 2C What is high autophagy? It is not defined in the legend. It is described in methods and demonstrated in Supplementary Fig. 2, however the cut off for high autophagy seems very arbitary.

The procedure for quantitation of high autophagy is now given in more detail in the supplemental methods section. We agree that the cutoff for high autophagy is somewhat arbitrary, however, the setting of high-autophagy gates in the flow cytometry software is always done with reference to Bafilomycin A_1-treated cells. As now detailed in the methods:

> "Cells with high autophagy were defined as those gated as having a high mCherry/GFP fluorescence ratio as illustrated in figures E7E and E8C. Bafilomycin A_1 –treated control cells were used to set this gate. Since bafilomycin A_1 inhibits autophagic flux, the bottom of the gate for each set of flow cytometry experiments was set at the rightward base of the bafilomycin A_1 treated cells, with the assumption that the cells under the bafilomycin A_1 curve represented cells with little or no autophagic flux, and that cells to the right of this curve would have higher autophagic flux. The quantitation gate was set such that no more than 2-5% of bafilomycin A_1 -treated cells from every given condition would be included in the gate. However, an exception to this rule was made in the case of MEF cells, due to a long rightward tail in the bafilomycin A_1 curve. For MEFs, therefore, up to 15% of bafilomycin A_1 -treated cells were allowed within the gate."

Furthermore, the gating of cells according to Cherry/GFP fluorescence looks strange in Supplementary Fig. 2, there is a high "blip" of cells at 256 that are included within the "high" gate. What are these cells? They are clearly unusual, how can they be included in the analysis? Furthermore none of these distributions looks anything like the authors' representation in their recent Autophagy 10:7, 1327-1334; 2014 paper.

The "blip" of cells at 256 are cells that have a higher mCherry to GFP fluorescence ratio than can be represented on the current scale, so they are stacked at the border of the histogram. (i.e. They are

cells with "super" high levels of autophagy, are not present in the control or Bafilomycin populations.) They are not particularly unusual other than their high autophagy. They therefore must be included in the analysis as "high autophagy" cells. As to our distributions looking different than the recent methods paper shows, we would have to disagree. These distributions in control and EBSS treated cells look similar when one looks at the proper scaling. The main difference between the representation in the methods paper and the current data is that the methods paper utilized relative scaling of the Y-axis to make it easier for the reader to see the rightward shift. The EBSS shift typically significantly reduces the amount of cell counts on the Y-axis when using the same scale as the control cells, since fluorescent events are spread out along the X-axis. In my opinion, this was an error on the part of the first author of the review paper in preparing the figure. For quantitation purposes using a gate, it would make no difference since a transformation along the Y-axis does not does not change the X-axis distribution, which is what is being quantitated (i.e. the mCherry/GFP ratio). What does change is the visual comparison of overlapping histograms. For the current paper, we have maintained the same Y axis scale, which we feel is the more accurate visual representation, even though it is sometimes hard to see a more evenly distributed mCherry/GFP shift (for example, see E7J where the peak for the RIP1 shRNA cells is much reduced, but the signal is spread in a wide distribution along the length of the X axis).

Supplementary Figure 2G As the author state in their earlier publications it is important to characterise cell lines with GFP/Cherry construct. In these MEFs where is the characterisation with a normal autophagic stimulus?

Supplementary Figure 2G also shows quantitation of the Tandem GFP/Cherry LC3 construct in MEF cell lines treated with EBSS. EBSS (or starvation-induced autophagy) is widely accepted by many in the field as the gold standard for autophagic stimuli.

Figure 3A Changes in transcription are very small, I would have no confidence in them unless tested with multiple shRNAs or the reconstituted Ripk1-/- MEFs discussed earlier.

It is true some of the changes in transcription are small, however they are in the same ranges as those reported by Settembre et al., (*Science* **332**(6036)**:** 1429-1433) in response to starvation or as regulated by TFEB. Moreover, the results were reproducible using multiple QPCR primer sets from multiple companies. The theory is that a large number of small changes in ATG genes induced by a transcription factor makes a large difference in autophagic flux. The recommendation of the editor was that we not address this particular concern.

Figure 3C TFEB expression seems to down rather than specifically pTFEB being affected. Given such slight effects, it would be necessary to confirm with the other siRNAs or the Ripk1-/- MEFs. The authors claim that there is increased mobility in the total TFEB Western blot, however to me it doesn't look like an increased mobility of TFEB, more like an angled gel.

Reduction in phospho-TFEB levels in response to a different RIP1 shRNA is confirmed by multiple experiments, and one other example is shown in Expanded View Figure E9A, which is perhaps more convincing. It is true that the gel is somewhat angled here (Figure 3C). Since the mobility aspect is not as convincing as we have seen in other blots, we have removed the text referring to increased mobility in the total TFEB for this blot.

Figure 3E. Small small differences in a luciferase assay could be due to even minor changes in experimental setup. N is not defined in legend.

Luciferase data was reproducible across multiple experiments and statistically significant. The luciferase differences are in the same ranges as those reported by Settembre et al., (*Science* **332**(6036)**:** 1429-1433) in response to starvation or as regulated by TFEB. N is now defined in the legend.

Figure S3G. Bizarre situation where RIPK1 knock-down with #3 shRNA results in higher levels of RIPK1 than in the parental line? Also see an increase in pTFEB, this almost certainly discredits the hypothesis that RIPK1 represses TFEB, because this shRNA increased autophagy flux same as other 2 in Supplementary Fig. 2. It is possible that the authors have mixed up their labelling, but that is a disappointing error to make. And even if it is mixed up then it seems to destroy the correlation

between RIPK1 knock-down and pTFEB levels because the level of knock-down with #3 is less than #1 but the effect on pTFEB is greater.

We apologize for the mistake, which other reviewers also noticed. Apparently, while dragging the blots in our graphics software, the blots became inverted left to right and also upside down. The figure has been corrected in the revised manuscript. As for a comparison between shRNA #3 and #1, these are from different gels, blots, and exposures, so we would recommend against such comparisons.

Figure 4 RIPK1 plays such an important DIRECT role in regulating apoptosis and necroptosis it would be absolutely necessary to exclude the obvious interpretation that what is affected in these cells is rather to do with complex 2 formation. Because no data is presented on the regulation of autophagy by RIP1 knockdown in these cells one cannot make a link between autophagy and RIP1-induced cell death in MCF10A cells. Furthermore, even if this data was shown, it would be a correlation and not proof of cause. In A and B, N is not defined.

No doubt RIP1 does play a direct role in apoptosis and complex II formation (we don't believe that RIP3 is expressed in these cells, so it is likely not an effect on necroptosis in these cells, see response to reviewer 2 , point #6). However, because RIP1 and TFEB knockdown (and autophagy inhibition) give different effects on TRAIL vs. FasL (i.e. protection vs. sensitization) we view it as highly unlikely that these differences are mediated solely by effects on complex II formation. We now show data that indicate that RIP1 knockdown affects autophagy in MCF10A cells (Expanded View Figure E7 I-J) and that RIP1 knockdown in HeLa cells (in which we showed previously that RIP1 knockdown induces autophagy) protects from TRAIL-induced apoptosis (Figure 4E). Moreover, we have included new data in our revised manuscript showing that delivery of ATG7 shRNA in MCF10A cells inhibits the ability of RIP1 knockdown to protect from TRAIL-induced cell death (Figure 4D). Importantly, knockdown of ATG7 in the low autophagy non-silencing control cells does not have much effect on cell death under these particular conditions. Other new data also shows that TFEB knockdown reduces the ability of RIP1 depletion to protect cells from TRAIL (Figure 4E-F). In contrast, TFEB knockdown protects cells from increased sensitization to Fas ligand caused by RIP1 depletion (Figure 4G). These data argue that the effects of RIP1 knockdown in these cells in response to TRAIL and FasL are at least in part due to RIP1 effects on autophagy, though it may be having multiple effects. N is now defined in the figure legends.

2nd Editorial Decision 02 March 2015

Thank you for your patience while we have reviewed your revised manuscript. We have now received the reports of referees 1 and 2. As you will see below, they both support publication of your study. Thus, I am making a decision now to avoid unnecessary loss of time.

I am writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once the following issues/corrections have been addressed:

- The legend to figure 3G, 3H and 3I states that these experiments were performed twice. Please note that neither errors nor significance can be calculated from two independent experiments only and you will need to use a higher number for their calculation.

- The P value seems to be missing from the legend to figures 2C and 2D.

- You have a large number of expanded view figures. As we can accommodate up to 5 main figures in the manuscript, and some of the expanded view figures show important results, please include one more figure in the main manuscript. I would suggest figure E8, E9 or, ideally, a combination of selected panels from both.

- The titles of the main figures are remarkably similar to each other. Indeed, that of figures 3 and 4

are exactly the same, which seems to be a mistake for figure 4. Some readers look mainly at the figures and their legends, so please make sure the titles are distinctive, self-explanatory and reflect the content of the figure.

- The Materials & Methods section is excessively succinct. Please note that the descriptions necessary for understanding the experiments performed must remain in the main text, although additional detailed information may be included as Expanded View. In particular, details of the shRNA screen ust be in the main text, but also ideally other important information. Please note that we could accomodate up to 30,000 characters to include this information.

After all remaining issues have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #1:

no other comments for the authors.

Referee #2:

The authors have thoroughly addressed my concerns.

2nd Revision - authors' response 24 March 2015

Thank you for your "accept in principle" decision for our original research report, entitled **"RIP1 Negatively Regulates Basal Autophagic Flux through TFEB to Control Sensitivity to Apoptosis"** for publication in *EMBO Reports* (*EMBOR-2014-39496V2)*. As requested by the senior editor, we have corrected the manuscript with regard to several issues:

- The experiments for figure 3G, 3H and 3I have been repeated to give an n=3.
- We have inserted the P value in the legend for figures 2C and 2D.
- We have included one more figure in the main manuscript and removed one figure from the expanded view figures.
- We have corrected titles of the main figures.
- We have transferred much of the Expanded View Materials & Methods to the main text

We hope that the revisions to the manuscript resolve any potential concerns and now be acceptable for publication in *EMBO Reports*.

3rd Editorial Decision 25 March 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal. You may be happy to know that I have commissioned a highlight to accompany your article, which will be written by Doug Green.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication.

