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The IKK-related kinase TBK1 activates mTORC1 directly in response to growth factors and innate immune agonists

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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 06 December 2016

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

As you will see, the referees appreciate the identification of TBK1 mediated mTORC1 activation. However, they also think that more insight is needed and that your conclusions would need confirmation in a more physiologically relevant setting (primary cells; infection assays; see also report from referee #1 and from the advisor). Given these opinions and the fact that the outcome of addressing these issues is rather unclear at this stage, I see no other choice but to return your manuscript to you with the message that we cannot offer to publish it in The EMBO Journal. Having said this, I would be prepared to take a fresh look at a revised version, should you be able to fully address the referees/advisors comments and to thus provide more physiological relevant insight into the link between TBK1 and mTORC1.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

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REFEREE REPORTS

Referee #1:

In this work Bodur et al have explored the role of TBK1 in activating of mTORC1 and the impact on signaling by growth factors and pattern recognition receptors. The paper follows a structure where Figure 1 and 2 shows that TBK1 can phosphorylate mTOR and activate mTORC1 under overexpression conditions. Figure 3-5 and 6-7 explore this in settings of growth factor and TLR stimulation, respectively. Finally, Figure 8 examines the effect of the observed phenomenon in TLR-induced IFNbeta expression. Although, the basic observation that TBK1 can activate mTORC1 is interesting, the work is very descriptive, and does not provide much mechanistic information. As such the work can appear underdeveloped, despite the large number of data.

SPECIFIC POINTS:
1. A recent study showed a role of the mTOR downstream kinase S6K in cGAS-STING signaling to IRF3 and IFN expression (Nat Immunol. 2016 May;17(5):514-22.). It is essential that the authors provide a mechanistic link between mTORC1/S6K and IFN expression.

2. There are no data from primary human cells. The work would gain if key data are confirmed in primary human cells.

3. At the methodological level, largely all data are based on western blotting. However, these data provide limited information on subcellular locations of action of the molecules involved. Therefore, the authors should use confocal microscopy to demonstrate the TBK1 colocalizes with mTOR and Raptor, and provide details on the kinetics.

4. Most conclusions on mTOR are based on use of small molecule inhibitors. Key conclusions should be supported by genetic data.

Referee #2:

Bodur et al have shown that the innate immunity kinase, TBK1 phosphorylates mTOR, as part of mTORC1, at Ser2159. This phosphorylation promotes the catalytic activity of mTOR and mTORC1/mTORC2 signaling. Interestingly, the TBK1-mediated mTORC1 activation appears to be stimulus-specific since EGFR and TLR/TLR4, but not IR stimulation of S6K can be abrogated in the absence of TBK1. They also analyzed how mTORC1 function is required for production of certain cytokines in response to TLR3/4 activation in macrophages using a cytokine array.

Overall the study provides new insights on how mTOR (mTORC1) can be directly phosphorylated at a regulatory site by a kinase that is involved in inflammatory responses. The combined use of pharmacological inhibitors, knockout cells and transfection experiments provides supportive data on regulation of S2159 phosphorylation and mTORC1 signaling by TBK1. There is little known on kinases that directly phosphorylate mTOR so this study addresses that gap in our knowledge. Some other questions/comments below should be addressed to strengthen the conclusion.

1. The effect of amlexanox on mTOR S2159 phosphorylation in MEFs (Figure 1F) is not quite so convincing. While the inhibitors seemed to have a pretty strong effect on mTORC1 signaling, its effect on S2159 phosphorylation itself is weak. It seems that both the in vitro phosphorylation data and the in vivo phosphorylation via agonist stimulation are quite robust so it's possible that the inhibitors may not be so specific or the LPS-triggered phosphorylation is not via TBK1. Amlexanox did not seem to decrease TBK phosphorylation while BX did, so the authors should clarify the effects of these inhibitors on TBK1-mediated mTORC1 phosphorylation.

2. The finding that S6K1 phosphorylation occurs upon EGF treatment despite the absence of Akt phosphorylation in Rictor-deficient MEFs does not necessarily indicate that this occurs via TBK1. Could this phosphorylation be inhibited by amlexanox? Is TBK1 activated by EGF in the absence of rictor? Knockdown of TBK1 in the rictor-/- MEFs or knockdown of rictor on the TBK-/- MEF

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should address the contribution of these two kinases on mTORC1 signaling under EGF stimulatory conditions.

3. Is the phosphorylation of S2159 also distinctly regulated by EGF and insulin?

4. The authors propose that mTORC1 phosphorylation by different kinases could act like a rheostat. They should compare the degree of S6K phosphorylation and mTOR S2481 in the presence of different stimuli (eg LPS, amino acid, EGF alone and in combination) to support such statement.

5. While Figure 8 supports that triggering TLR3/4 affects its downstream targets in an mTORC1-dependent manner, this does not quite address the importance of S2159, ie the effects could just be indirect.

Advisor:

The topic is exciting and relevant but overall the submitted manuscript is not providing sufficient biological or molecular data to support a critical role for TBK1 in activating TORC1 and 2. Most of presented data derive from in tissue cultured cells while more focused functional assays where TBK1-TORC pathways may be studied have not been evaluated. The authors discuss a lot about the potential links but no attempts are made to study them. Also the molecular details or explaining the link between TBK1 and the TOR pathway are missing.

1st Revision - authors' response 27 June 2017

Point-by-Point Responses to Referee Comments:

Referee #1:
In this work Bodur et al have explored the role of TBK1 in activating of mTORC1 and the impact on signaling by growth factors and pattern recognition receptors. The paper follows a structure where Figure 1 and 2 shows that TBK1 can phosphorylate mTOR and activate mTORC1 under overexpression conditions. Figure 3-5 and 6-7 explore this in settings of growth factor and TLR stimulation, respectively. Finally, Figure 8 examines the effect of the observed phenomenon in TLR-induced IFNbeta expression. Although, the basic observation that TBK1 can activate mTORC1 is interesting, the work is very descriptive, and does not provide much mechanistic information. As such the work can appear underdeveloped, despite the large number of data.

SPECIFIC POINTS:
1. A recent study showed a role of the mTOR downstream kinase S6K in cGAS-STING signaling to IRF3 and IFN expression (Nat Immunol. 2016 May;17(5):514-22.). It is essential that the authors provide a mechanistic link between mTORC1/S6K and IFN expression.
Yes, good point. We have investigated further how mTORC1 promotes production of IFNb. We include new data showing that rapamycin suppresses the cytosolic to nuclear translocation of IRF3, the transcription factor that induces IFNb gene expression, in both cultured RAW264.7 macrophages (Figure 7D) and primary mouse BMDMs (Figure 7E). We have also cited the paper mentioned above in the Discussion, Wang et al. 2016.

2. There are no data from primary human cells. The work would gain if key data are confirmed in primary human cells.
While we have not included new data using primary human cells, we have included several new experiments using primary mouse cells, specifically bone marrow-derived macrophages (BMDMs). We show that in primary mouse BMDMs, rapamycin suppresses IFNb production (Figure 7C) by blunting the translocation of IRF3 from the cytosol to nucleus (Figure 7E). We hope that analysis of primary mouse cells provides sufficient physiological relevance.

3. At the methodological level, largely all data are based on western blotting. However, these data provide limited information on subcellular locations of action of the molecules involved. Therefore,

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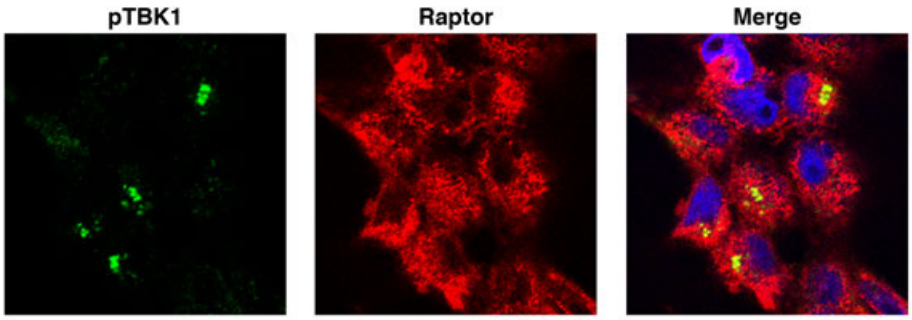
the authors should use confocal microscopy to demonstrate the TBK1 colocalizes with mTOR and Raptor, and provide details on the kinetics.
This point is important and has been on our radar for some time. We have spent time investigating the potential co-localization of endogenous TBK1 and raptor, a partner protein found exclusively in mTORC1 but not mTORC2, by immunofluorescence confocal microscopy of fixed cells. We validated total TBK1 and P-TBK S172 antibodies using TBK1+/+ vs TBK1-/- MEFs (data not shown). Total TBK1 appears to be distributed all over the cell, likely on diverse membranes. The distribution of P-TBK1, however, which marks active TBK1, shows a more restricted staining pattern. We therefore attempted to co-localize P-TBK1 with raptor under steady-state conditions and in response to EGF-receptor and TLR3 activation. First, the overall distribution of P-TBK1 did not obviously alter upon stimulation (data not shown). Second, raptor distributes all over the cell, likely associated with many membranes. When we merge P-TBK1 and raptor in cells under steady-state conditions, we indeed observe co-localization (see images below). Thus, subpopulations of active TBK1 and mTORC1 indeed localized near each other on a membrane compartment. We have not included these microscopic data in the manuscript, however, as the significance of such co-localization is unclear due to the widespread distribution of raptor. We plan to continue investigating the subcellular localization of active TBK1 relative to mTORC1 using alternate approaches.

pTBK1 and Raptor localization: HEK293 cells; steady-state conditions



4. Most conclusions on mTOR are based on use of small molecule inhibitors. Key conclusions should be supported by genetic data.
The original submission analyzed mTORC1 signaling by several genetic approaches as well as by pharmacologic inhibition of TBK1 with amlexanox: We showed reduced EGF-stimulated mTORC1 signaling in TBK1$^{-/-}$ MEFs (relative to TBK1$^{+/+}$ MEFs) (current Figures 2A; 2D; 2F) and rescue of mTORC1 signaling upon re-introduction of wild type TBK1 into TBK1$^{-/-}$ MEFs (current Figure 2B); we used siRNA to knockdown TBK1 in RAW264.7 macrophages (current Figure S5); and we chemically knocked-out mTORC1 with rapamycin and showed that mTORC1 signaling was reduced in HEK293 cells and RAW264.7 macrophages expressing a rapamycin-resistant (RR)-S2159A mTOR allele relative those expressing wild type RR-mTOR (current Figures 4A; 6B; 6C).

In this revised submission, we include new data showing that in addition to knockdown of TBK1 with siRNA (Figure S5), knockdown of TBK1 with shRNA (Figure 5B) also reduces mTORC1 signaling in RAW264.7 macrophages. Importantly, we include new data showing that primary macrophages isolated from mTOR S2159A knock-in mice show reduced mTORC1 signaling in response to TLR3 and TLR4 activation relative to wild type macrophages (Figures 8A and 8B).

Referee #2:
Bodur et al have shown that the innate immunity kinase, TBK1 phosphorylates mTOR, as part of mTORC1, at Ser2159. This phosphorylation promotes the catalytic activity of mTOR and mTORC1/mTORC2 signaling. Interestingly, the TBK1-mediated mTORC1 activation appears to be stimulus-specific since EGFR and TLR/TLR4, but not IR stimulation of S6K can be abrogated in the absence of TBK1. They also analyzed how mTORC1 function is required for production of certain cytokines in response to TLR3/4 activation in macrophages using a cytokine array.

Overall the study provides new insights on how mTOR (mTORC1) can be directly phosphorylated at a regulatory site by a kinase that is involved in inflammatory responses. The combined use of

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pharmacological inhibitors, knockout cells and transfection experiments provides supportive data on regulation of S2159 phosphorylation and mTORC1 signaling by TBK1. There is little known on kinases that directly phosphorylate mTOR so this study addresses that gap in our knowledge. Some other questions/comments below should be addressed to strengthen the conclusion.

1. The effect of amlexanox on mTOR S2159 phosphorylation in MEFs (Figure 1F) is not quite so convincing. While the inhibitors seemed to have a pretty strong effect on mTORC1 signaling, its effect on S2159 phosphorylation itself is weak. It seems that both the in vitro phosphorylation data and the in vivo phosphorylation via agonist stimulation are quite robust so it's possible that the inhibitors may not be so specific or the LPS-triggered phosphorylation is not via TBK1. Amlexanox did not seem to decrease TBK phosphorylation while BX did, so the authors should clarify the effects of these inhibitors on TBK1-mediated mTORC1 phosphorylation.
We agree that the effect of amlexanox on LPS stimulated mTOR S2159 phosphorylation in cultured RAW 264.7 macrophages was not as strong as the effects of the other TBK1 inhibitor, BX-795, as shown in the original submission. Amlexanox may not be as strong an inhibitor toward TBK1 as BX-795, as suggested by the referee. We have removed the amlexanox data from the revised manuscript. Figure 1H shows clearly that BX-795 suppresses both TLR3- and TLR4-induced mTOR S2159 phosphorylation.

2. The finding that S6K1 phosphorylation occurs upon EGF treatment despite the absence of Akt phosphorylation in Rictor-deficient MEFs does not necessarily indicate that this occurs via TBK1. Could this phosphorylation be inhibited by amlexanox? Is TBK1 activated by EGF in the absence of rictor? Knockdown of TBK1 in the rictor-/- MEFs or knockdown of rictor on the TBK-/- MEF should address the contribution of these two kinases on mTORC1 signaling under EGF stimulatory conditions.
Yes, agreed. We have included new data showing that shRNA-mediated knockdown of TBK1 reduces mTORC1 signaling in rictor$^{-/-}$ MEFs. Thus, in cells with an impaired mTORC2-Akt P-S473 axis, mTORC1 signaling indeed requires TBK1. These data can be seen in Figure 3B.

3. Is the phosphorylation of S2159 also distinctly regulated by EGF and insulin?
Great question. We have included new data showing that EGF but not insulin stimulation of MEFs increases mTOR S2159 phosphorylation (Figure 2H). Importantly, this increase occurs in TBK1 wild type but not null MEFs. These data suggest that TBK1 is required for EGF- but not insulin-stimulated mTORC1 signaling because the EGF-receptor pathway but not the insulin-receptor pathway activates TBK1, an idea considered in the Discussion.

4. The authors propose that mTORC1 phosphorylation by different kinases could act like a rheostat. They should compare the degree of S6K phosphorylation and mTOR S2481 in the presence of different stimuli (e.g. LPS, amino acid, EGF alone and in combination) to support such statement.
We attempted a few experiments to address this point, but differences in the magnitude of mTORC1 activation by different stimuli made it difficult to demonstrate synergistic activation by combinations of signals. Careful dose responses for each stimulus to find a sub-maximal dose at the right time point would be required. As we felt we had other experimental priorities to pursue, we cannot address this point experimentally at the moment. We have removed the sentence proposing a "rheostat-like mechanism" for mTORC1 regulation by diverse stimuli from the revised manuscript.

5. While Figure 8 supports that triggering TLR3/4 affects its downstream targets in an mTORC1-dependent manner, this does not quite address the importance of S2159, i.e. the effects could just be indirect.
Yes, absolutely agreed. To address this important point, we have generated genetically modified mice using CRISPR/Cas9 genome editing technology bearing a germline knock-in mTOR S2159A allele, referred to in the manuscript as mTOR$^{A/A}$ mice in Figure 8. By studying primary macrophages from these mice, we demonstrate that TLR3-induced IFNb production (Figure 8C) and IRF3 translocation (Figure 8D) require mTOR S2159 phosphorylation. Moreover, we demonstrate that mTOR S2159 phosphorylation is required for TLR3- and TLR4- induced mTORC1 signaling (Figures 8A and 8B).

Advisor:

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The topic is exciting and relevant but overall the submitted manuscript is not providing sufficient biological or molecular data to support a critical role for TBK1 in activating TORC1 and 2. Most of presented data derive from in tissue cultured cells while more focused functional assays where TBK1-TORC pathways may be studied have not been evaluated. The authors discuss a lot about the potential links but no attempts are made to study them. Also, the molecular details or explaining the link between TBK1 and the TOR pathway are missing.

The original submission demonstrated that TBK1 phosphorylates mTOR S2159 within mTORC1 (see current Figure 1). By chemically knocking out mTORC1 function with rapamycin and ectopically expressing rapamycin-resistant wild type vs. S2159 mTOR in two cell types (HEK293; RAW264.7), we demonstrated in the original submission that this phosphorylation event promotes mTORC1 signaling in response to EGF and innate immune signals (see current Figures 4A, 4E, 5B, 5C). In addition, we showed that ectopically expressed mTOR S2159A shows impaired autophosphorylation on S2481 in response to EGF relative to wild type mTOR, thus demonstrating that mTOR P-S2159 promotes mTOR catalytic activity (see Figure 4E).
Taken together, these data provide a molecular mechanism by which TBK1 activates mTORC1, demonstrating that TBK1 phosphorylates mTOR S2159 to promote mTORC1 catalytic signaling and downstream signaling.

We go further in this revised manuscript to show that primary macrophages derived from mTOR S2159A knock-in mice (generated by CRISPR-Cas9 genome editing) show reduced mTORC1 signaling relative to wild type macrophages in response to innate immune signals (see Figures 8A, 8B). Moreover, by studying the S2159A primary macrophages, we show that mTOR S2159 phosphorylation promotes IFNb production by inducing IRF3 nuclear translocation (see Figures 8C, 8D). We hope that these data provide sufficient molecular mechanism for how TBK1 activates mTORC1 and sufficient biologic relevance for the TBK1-mTORC1 axis in regulation of innate immune function.

2nd Editorial Decision 25 July 2017

Thank you for resubmitting your manuscript for consideration by the EMBO Journal. It has now been seen by the original referees again whose comments are enclosed. As you will see, all three referees express interest in your manuscript and are now broadly in favour of publication, pending satisfactory minor revision.

I would thus like to ask you to address the remaining concerns of referee #3 by providing a further revised manuscript.

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

REFEREE REPORTS

Referee #1:

I find that the authors have improved the work significantly during revision, and now provide key mechanistic data that promote novelty and connection to the existing literature.

Referee #2:

The authors have addressed my main comments satisfactorily. The studies provide great insights on how mTOR could be regulated directly by an immune-related kinase in response to specific stimuli. There are not very many studies that address a more direct regulation of mTOR, hence, this study is significant and reveal new mechanisms.

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Referee #3:

In the manuscript by Bodur et al the authors identified TBK1 as a new kinase controlling mTORC1 signaling in the EGFR and TLR3/4 pathway. Authors provide evidence that TBK1 phosphorylates mTORC1 directly at S2159, thereby controlling mTORC1 activity. Furthermore, they show that mTORC1 S2159 phosphorylation by TBK1 is critical for downstream signaling events. Most interestingly, the authors linked the TBK1-mediated mTORC1 S2159 phosphorylation to IRF3 activation, driving IFNß secretion. Thereby they identified a new TBK1-mTORC1 signaling axis in the type I interferon response.

Taken together, this is a very interesting study providing new insights into mTORC1 and INFß signaling. The authors managed to extend the first version of the manuscript significantly, by showing diminished IFR3 activation and IFNß secretion in primary BMDMs derived from mTOR S2159A knock-in mice. This work will therefore be interesting for a wide readership. Some comments below should be addressed to strengthen the manuscript:

• Fig1J: Due to high variability between the mice sample, as seen in e.g. the p-4EBP1 T37/46 staining, authors should analyze more mice and/or provide any statistics. This would support their finding that LPS activates mTOR signaling and most importantly induces mTOR S2159 phosphorylation in vivo.

• Fig2: Proof of TBK1 activation is missing upon EGF stimulation. A pTBK1 S172 staining should be included in any of these panels to test if TBK1 gets activated after growth factor treatment.

• Fig2C: Authors claims that EGFR signaling is not affected in TBK1 -/- MEFs. However, they show just a single phosphorylation site (pERK) as a read-out for proper signaling. The panel should be extended by additional stainings for e.g. pAKT S472 or pSTAT3 etc.

• Fig7D and 8D: The quality of the IF images should be improved, since the IF images do not clearly reflect the quantified results. Also, a higher resolution would be appreciated (zoom in, to show a single cell). For IF, the authors should maybe consider to use another cell type, which is more suitable for imaging. As an alternative approach, the authors could analyze IRF3 translocation/activation using biochemical approaches, like subcellular fractionation or IRF3 dimerization in a native PAGE.

2nd Revision - authors' response 10 October 2017

Referee #3:

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We have included a new graph as part of Figure 1J in the revised manuscript that shows a statistically significant fold-increase in mean P-mTOR-2159 over total mTOR +/- SD in response to

*LPS vs. control PBS injection in vivo in spleen tissue of wild type mice from three (3) independent experiments (n=3 mice total). *p=.004: LPS injected mice relative to untreated controls by paired t-test (two-tailed). We agree that the quality of the P-4EBP1 blots were poor and have therefore removed these images from the figure.*

• **Fig2:** Proof of TBK1 activation is missing upon EGF stimulation. A pTBK1 S172 staining should be included in any of these panels to test if TBK1 gets activated after growth factor treatment.
Reviewer #3 makes an excellent point. In fact, EGF-receptor signaling does not increase TBK1 phosphorylation on its activation loop site, S172. Different lab members have failed to detect increased P-TBK1-S172 upon stimulation of MEFs or HEK293 cells with EGF. It is important to note that in independent experiments using poly(I:C) and LPS to activate TLR3/4 signaling, we observe increased P-TBK1-S172 consistently. At first we found our inability to observe increased P-TBK1-S172 upon EGF stimulation perplexing, particularly because a paper published by Ou et al (2011) (Mol Cell 41(4): 458-70) demonstrated that cellular EGF stimulation increases the ability of immunoprecipitated TBK1 to phosphorylate GST-IRF3 in vitro, indicating that EGF increases TBK1 intrinsic catalytic activity. Interestingly, no P-TBK1-S172 western blots were included in this Ou et al. paper to show how EGF affects TBK1 S172 phosphorylation in intact cells. Indeed, we found the absence of these blots odd, but now we surmise that the authors may have been unable to detect increased P-TBK1-S172 upon EGF stimulation of intact cells, just like us. What do these observations mean? While we do not know at the moment, these data may suggest that EGF-receptor signaling does not increase TBK1 intrinsic catalytic activity, which can be monitored by phosphorylation on the TBK1 activation loop site (S172). In this case, basal TBK1 kinase activity would "prime" mTOR for EGF-stimulated activation of mTORC1. Alternately, the data may suggest that the mechanism by which innate immune agonists vs. EGF activate TBK1 differs; in this scenario, immunoblotting with P-TBK1-S172 antibodies does not represent a reliable read-out for TBK1 activation in response to EGF.

Due to the negative nature of these data, we chose to exclude P-TBK1-S172 blots from EGF experiments in the original submissions of this manuscript. Reviewer #3 clearly finds this issue important, however, as do we. We have therefore included two (2) new figures demonstrating the inability of EGF to increase P-TBK1-S172 in MEFs (EV2A) or HEK293/TLR3 cells (EV2B). Importantly, positive controls were included: The MEF experiment shows that LPS indeed increased P-TBK1-S172, as expected. The HEK293/TLR3 cell experiment shows that poly(I:C) indeed increased P-TBK1-S172, as expected. In both experiments, EGF and LPS (MEFs) or EGF and poly (I:C) (HEK293/TLR3 cells) increased mTORC1 signaling, as monitored by increased S6K1 T389 phosphorylation. As these experiments include critical positive controls, we now feel more comfortable to include these "negative" data in the manuscript. The text has been modified accordingly in the Results (see new text on pg. 9) and Discussion (see new text on pg. 16) to explain that cellular EGF stimulation does not increase P-TBK1-S172.

• **Fig2C**: Authors claims that EGFR signaling is not affected in TBK1 -/- MEFs. However, they show just a single phosphorylation site (pERK) as a read-out for proper signaling. The panel should be extended by additional stainings for e.g. pAKT S472 or pSTAT3 etc.
We have performed a new experiment to replace the former (Figure 2C) demonstrating that EGF-receptor signaling remains intact in TBK1$^{-/-}$ MEFs, as determined by immunoblotting with P-EGFR-Y1173, P-MAPK-T202/Y204, and P-STAT3-Y705 antibodies. The text was edited appropriately on pg. 8. We cannot use P-Akt-S473 as a readout, as data provided in this paper (see Figures 2A and 2D) indicate that EGF stimulated mTORC2 signaling is also impaired in TBK1$^{-/-}$ MEFs.

• **Fig7D and 8D:** The quality of the IF images should be improved, since the IF images do not clearly reflect the quantified results. Also, a higher resolution would be appreciated (zoom in, to show a single cell). For IF, the authors should maybe consider to use another cell type, which is more suitable for imaging. As an alternative approach, the authors could analyze IRF3 translocation/activation using biochemical approaches, like subcellular fractionation or IRF3 dimerization in a native PAGE.
In Figures 7D, 7E, and 8D we have replaced the original confocal images showing IRF3 localization with zoomed-in images to improve resolution. We hope that it is now more clear that the images reflect the quantified results. We agree that the suggested biochemical approach (fractionation) would provide additional evidence that the TBK1-mTORC1 axis promotes the cytoplasmic to nuclear translocation of IRF3; we plan to employ this approach in future work. In

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addition, we plan to investigate a role for the TBK1-mTORC1 axis in control of IRF3 dimerization using native PAGE in future work.

***Additional comment**: Please note that we have provided quantitation for the level of mTORC1 signaling in $mTOR^{+/+}$ vs $mTOR^{A/A}$ primary-BMDMs isolated from genome edited mice in response to poly (I:C) (Figure 8A) and LPS (Figure 8B). The graph quantitates three independent experiments each with n=1 (n=3 total). The level of P-S6K1-T389 normalized to total protein in $mTOR^{+/+}$ macrophages stimulated with poly (I:C) or LPS was set at 100%. Confidence intervals at 95% indicate a statistical difference between S6K1 T389 phosphorylation in $mTOR^{+/+}$ vs. $mTOR^{A/A}$ BMDMs in response to both innate immune ligands.*

3rd Editorial Decision 11 October 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. I appreciate the introduced changes, and I would like to publish your work, pending the incorporation of the following:

- the p-STAT-Y705 levels are already high without EGF treatment in TKB-/- cells; please mention this when discussing these data in the manuscript text
- the source data for figure 2F do not match the main figure data for the p4EBP1-T70 staining and for the IB Akt staining (one too many lanes displayed for the latter); please rectify

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

3rd Revision - authors' response 11 October 2017

Thank you very much for your interest in publishing our manuscript entitled, “The IKK-related kinase TBK1 activates mTORC1 directly in response to growth factors and innate immune agonists”.

We modified the manuscript in the following ways, as you requested:

We added the following text to pg. 7 of the Results section: “We noted that $TBK1^{-/-}$ MEFs exhibit increased basal levels of P-STAT3-Y705 relative to $TBK1^{+/+}$ MEFs. While we do not know the reason for this phenomenon at this time, we speculate that it may result from reduced TBK1- or mTORC1-mediated negative feedback in $TBK1^{-/-}$ MEFs, leading to elevated P-STAT3.”

4th Editorial Decision 12 October 2017

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.
Congratulations on this nice work!

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Diane C. Fingar
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2016-96164R

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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was not limiting in experiments employing established cell lines; each experiment was performed independently at least three times (and often more). For analysis of IFNb production in primary bone marrow derived macrophages (BMDMs), experiments were performed at least 3 times, and cells were plated and assayed as biologic triplicates, as indicated in the figure legends. For analysis of IRF3 translocatation in primary BMDMs by microscopy, a large number of cells per condition were quantified (at least 400 in Figure 7D; 380 in Figure 7E; 270 in Figure 8D; 95 in EV5E) , as indicated in the figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA; no animal studies
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No pre-established criteria for inclusion/ exclusion
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No
For animal studies, include a statement about randomization even if no randomization was used.	NA; no animal studies
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA; no animal studies
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Shapiro-Wilk test was used to assess normal distribution in the data sets.
Is there an estimate of variation within each group of data?	Yes. Standard deviation or standard error of the mean was calculated and shown on the graphs.
Is the variance similar between the groups that are being statistically compared?	Yes. F-test was used to ensure the variance between the compared groups is not significantly different.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jji.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies to the following proteins were used in this work: Myc-9E10 (#MMS-150P) and HA.11 (#MMS-101P) monoclonal antibodies for immunoprecipitation and immunoblotting were from Covance, now Biolegend. Flag-M2 monoclonal antibody was from Sigma (#F3165). AU1 monoclonal antibody was from Biolegend (#903101). The following commercial antibodies were from Cell Signaling Technology: mTOR (#2972); P-S6K1-T389 (rabbit monoclonal 108D2; #9234); P-4EBP1-T37/46 (#9459); P-4EBP1-T70 (#9455); P-4EBP1-S65 (#9451); non-P-4EBP1-T46 (#4923); 4EBP1 (#9452); GST (#2625); P-Akt-S473 (#4060); Akt (#9272); P-TBK1-S172 (#5483); TBK1 (#3504); P-IKKe-S172 (#8766); IKKe (#3416); P-IRF3-S396 (#4947); IRF3 (#4302). P-mTOR-P-S2481 was from Millipore (#09-343). Commercial polyclonal antibodies to raptor were from Millipore (#09-217). Several polyclonal antibodies to the following proteins were generated in-house using a Covance custom antibody service, as described {Acosta-Jaquez, 2009 #1933}: Raptor (amino acids 1-17 or 885-901; human); mTOR (amino acids 221-237; rat); rictor (amino acids 6-20; human); S6K1 (amino acids 485-502 of the 70 kDa isoform; rat). P-mTOR--S2159 antibodies (amino acids 2154-2163; rat) were generated in collaboration with Millipore (#ABS79), as described {Ekim, 2011 #415}.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	This work employed the following cultured cell lines: HEK293 (originally from Dr. John Blenis's lab at Harvard Medical School); HEK293/TLR3 (shared by Dr. Kate Fitzgerald; UMass Medical School-Worcester; TBK1+/+ vs. TBK1-/- MEFs (shared by Dr. Alan Saltiel; formerly at Univ. of Michigan Medical School and now at UCSD); and RAW264.7 macrophages (shared by Dr. Alan Saltiel; formerly at Univ. of Michigan Medical School and now at UCSD). These cell lines were last tested for mycoplasma in fall 2016. We typically test our cell lines once per year for mycoplasma when we have no concerns regarding potential mycoplasma contamination (i.e. no changes in proliferation rate, morphology, survival, transfection efficiency, or any other cell behavior). For many of the experiments that employed these established cell lines, the lines have not been STR profiled, which represents a relatively new NIH recommendation. The lab's current stocks of HEK293 cells (as well as U2OS, HepG2, and HeLa) were acquired recently from ATCC and thus have been STR profiled and validated.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mus musculus; mostly C57/BL6 background; primary bone marro derived macrophages (BMDMs) from male mice 8-16 weeks old were studied; germ-line mTOR was genetically engineered using CRISPR-Cas9 to substitute an Ala for Ser at amino acid 2159.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All work with mice has been approved by the University of Michigan's Institutional Animal Care & Use Committee (IACUC), protocol # PRO00004771.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes, we confirm compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA; no human subjects
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA; no large data sets were generated in this work.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No, this work does not fall under dual use research restrictions.