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A Lysosome-to-Nucleus Signaling Mechanism Senses and Regulates the Lysosome via mTOR and TFEB

Carmine Settembre, Roberto Zoncu, Diego L. Medina, Francesco Vetrini, Serkan Erdin, Serpil Uckac Erdin, Tuong Huynh, Mathieu Ferron, Gerard Karsenty, Michel C. Vellard, Valeria Facchinetti, David Sabatini and Andrea Ballabio

Corresponding author: Andrea Ballabio, TIGEM

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1st Editorial Decision 03 January 2012

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are shown below. As you will see, while both referees consider the study as interesting and potentially very important, they both think that a few additional experiments will be needed before they can support publication. Referee 1 thinks that more evidence that mTORC1 directly phosphorylates TFEB would be needed and that the phosphorylation site would need to be identified. Furthermore, he/she thinks that data on endogenous TFEB should be included. Both points are also raised by referee 2. Referee 2 further thinks that the discrepancies to Pena-Llopis et al. 2010 should be addressed more directly by analysing more comparable experimental systems... Taking together all issues raised, we will therefore be happy to consider a revised version of the paper that addresses the issues raised by the referees, in particular those mentioned above, in an adequate manner.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

This is an interesting study in which the authors provide evidence that mTORC1 controls the localisation and hence the TFEB transcription factor. The data suggest that mTORC1 phosphorylate TFEB at the lysosome promoting its non-nuclear localisation and thereby inhibiting expression of genes involved in lysosomal biogenesis. When mTORC1 is inhibited with pharmacological agents or by starvation TFEB is dephosphorylated and enters the nucleus where it can regulate gene expression. Consistent with this data key regulators of mTORC1 such as the RAG GTPases can be shown to control TFEB activity in a manner which parallels mTORC1 activity/lysosomal localisation.

There are some important molecular details that required to complete this study. These would need to be addressed prior to publication

1. The authors should also establish whether mTORC1 can directly phosphorylate TFEB in vitro

2. The key mTORC1 phosphorylation sites on TFEB need to be identified.

3. Mutation of the key mTORC1 phosphorylation site(s) should render TFEB constitutively active within the nucleus and independent of mTORC1, mTORC1 inhibition and RAG GTPases.

4. All the data concerning phosphorylation and localisation of TFEB seems to be undertaken with overexpression of a FLAG-TFEB construct. It would be essential to have at least one convincing figure showing that endogenous TFEB is regulated by mTORC1. Ie demonstrate that endogenous TFEB undergoes a bandshift collapse on immunoblot analysis after Torin treatment. Also Torin should be shown to promote nuclear localisation of endogenous TFEB.

Referee #2

The studies by Settembre, Zoncu et al link mTORC1 to TFEB regulation in response to changes in nutritional and lysosomal status. Previously, the Ballabio group has reported the role of TFEB in autophagic/lysosomal responses and that TFEB is regulated by MAPK and not mTOR based on the use of ERK inhibitor vs rapamycin. They have now compared inhibitors to MAPK with other mTOR inhibitors and have come to the conclusion that blocking mTOR with active site inhibitor rather than ERK inhibition achieved more potent effects on TFEB regulation. The involvement of mTORC1 was further supported by findings that the Rag GTPase, which mediates mTORC1 activation by amino acids at the lysosomal surface, can inhibit TFEB nuclear localization upon knockdown or expression of a dominant negative form. They propose a model whereby TFEB and mTORC1 interact at the lysosome surface where TFEB can get phosphorylated presumably by mTORC1 to prevent its nuclear localization under nutrient replete conditions.

Overall, the studies are interesting and extend previous knowledge on regulation of TFEB and support previous findings that mTORC1 is involved in regulating TFEB and lysosomal gene expression. The evidence on TFEB nuclear localization under mTOR-inhibitory conditions is strong but there is not enough data to support that colocalization of TFEB with mTORC1 allows its phosphorylation by mTORC1. Analysis of the role of mTORC1 in the regulation of lysosomal expression genes was also minimal. Finally, it is hard to ignore the discrepancies with previous studies supporting a more complicated role of mTORC1 in TFEB regulation. This could perhaps be addressed either by generating more specific antibodies to endogenous TFEB or test TFEB-GFP regulation in TSC-/- MEFs using Torin1.

1. A major shortcoming of these studies is the reliance on overexpressed TFEB for all the analysis performed. The authors point out that the Bethyl antibody used in a previous study by Pena-Llopis et al did not work in their hands and noted that Bethyl claimed that this TFEB antibody did not pass QC (was this exactly the same antibody used by the Pena-Llopis group?). Since the Pro-Tec antibody seemed to work, the authors should make an effort to test this antibody in the TSC MEFs used by the Pena-Llopis group.

2. Further analysis to support that TFEB is indeed phosphorylated under amino acid replete conditions should be performed (Fig. 2E). The use of phosphatases should at least be examined. Furthermore, this group has previously used Ser to Ala mutants of TFEB to analyze their effects on nuclear accumulation. These mutants or putative mTOR-targeted sites should be examined.

3. In Fig. 2G, there seems to be a small but nevertheless not negligible effect of knockout of SIN on nuclear accumulation accumulation of TFEB. To rule out that mTORC2 is not involved, siRNA should be performed on mTORC2 components. Fig 2H should also include immunoprecipitation with mTORC2 components as control.

4. Although the authors present data that mTORC1 and TFEB colocalize to the lysosomes upon Torin1 treatment, there is no evidence to support that phosphorylation of TFEB by mTORC1 occurs on the lysosomal surface.

5. Fig. 4 nicely shows the effect of Rags (CA vs DN) on TFEB nuclear localization under amino acid depleted/repleted conditions. Would Torin1 treatment of cells expressing RagCA increase nuclear acculumation of TFEB?

6. Regulation of expression of lysosomal genes by mTORC1 should be further examined in mTORC1 knockdown cells. Why are the control levels different in Fig. 5B and 5C?

1st Revision - authors' response 16 January 2012

REVIEWER 1

1. The authors should also establish whether mTORC1 can directly phosphorylate TFEB in vitro

To test whether mTORC1 directly phosphorylates TFEB, we performed radioactive kinase assays in vitro with highly purified proteins. The results unequivocally show that TFEB is phosphorylated by mTORC1. Phosphorylation of the canonical mTORC1 substrate S6K1 was used as a positive control. Importantly, TFEB phosphorylation by mTORC1 was completely abolished by treating samples with the mTOR inhibitor Torin1. Finally, mutation of S142 into Alanine in TFEB strongly decreased mTORC1-dependent phosphorylation, indicating that S142 is a direct mTORC1 phosphorylation site (see also response to point 2). These data have been included in the new Figure 3B and discussed in the text.

2) The key mTORC1 phosphorylation sites on TFEB need to be identified
3) Mutation of the key mTORC1 phosphorylation site(s) should render TFEB constitutively active within the nucleus and independent of mTORC1, mTORC1 inhibition and RAG GTPases.

We previously showed that TFEB nuclear translocation is regulated by the phosphorylation state of serine S142. Now we generated a phospho-specific antibody that recognizes TFEB only when phosphorylated in position 142. This antibody fails to recognize TFEB in cells starved or treated with the mTOR inhibitor Torin1, further indicating that S142 is phosphorylated by mTOR. In addition, we mutagenized twelve TFEB residues, which were predicted to be phosphorylated by mTORC1 by bioinformatic analysis, into alanines and tested the effects of each of these mutations on TFEB subcellular localization. This analysis revealed that serine S211, in addition to S142, controls TFEB subcellular localization. We included the description of the experiments in a new paragraph in the revised version of the manuscript and included the data in the new Figure 3.

4) All the data concerning phosphorylation and localization of TFEB seems to be undertaken with overexpression of a FLAG-TFEB construct. It would be essential to have at least one convincing figure showing that endogenous TFEB is regulated by mTORC1. I.e. demonstrate that endogenous TFEB undergoes a bandshift collapse on immunoblot analysis after Torin treatment. Also Torin should be shown to promote nuclear localisation of endogenous TFEB.

The detection of endogenous TFEB has been hampered by the low amounts of this protein in most cell types and by the difficulties that we and others have encountered in obtaining and generating suitable antibodies. After testing 9 different antibodies (some commercially available and others generated by us) we finally found one antibody (My biosource) that is able to recognize the endogenous TFEB by both immunoblotting and immunofluorescence analyses. Using TFEB-/- primary hepatocytes, which were obtained from an Albumin CRE conditional KO mouse in which TFEB was deleted in the liver, we verified that this antibody indeed recognizes endogenous TFEB (see Figure S1). We then used this antibody to stain HEK-293T cells cells as well as mouse embryonic fibroblasts under different conditions: untreated, amino acid-starved, chloroquine-treated and Torin1-treated. The results clearly show that the endogenous TFEB protein behaved in exactly the same way as FLAG- or GFP-tagged TFEB (i.e. largely cytoplasmic in control cells, completely nuclear in starved and drug-treated cells). Data on endogenous TFEB have been included in Figures 1C, 2E, S8 and S9 and a corresponding discussion has been included in the text.

REVIEWER 2

1) A major shortcoming of these studies is the reliance on overexpressed TFEB for all the analysis performed. The authors point out that the Bethyl antibody used in a previous study by Pena-Llopis et al did not work in their hands and noted that Bethyl claimed that this TFEB antibody did not pass

QC (was this exactly the same antibody used by the Pena-Llopis group?). Since the Pro-Tec antibody seemed to work, the authors should make an effort to test this antibody in the TSC MEFs used by the Pena-Llopis group.

As suggested by the reviewer, we analyzed the endogenous TFEB and found that it behaves in the same way as the exogenously expressed tagged versions. Data on endogenous TFEB have been included in Figures 1C, 2E, S8 and S9 and a corresponding discussion has been included in the text (see also response to point 4 of Reviewer 1). In addition, we tested TFEB regulation using exactly the same system used by Pena-Llopis et al., namely TSC-/- cells. We examined the localization of TFEB-GFP and of endogenous TFEB in wild type vs TSC-/- cells under different conditions. Under normal growth conditions, both wild type and TSC-/- MEFs displayed a predominantly cytoplasmic localization of TFEB. Moreover, amino acid starvation, chloroquine and Torin1 treatments induced nuclear translocation of TFEB-GFP and of endogenous TFEB in both wild type and TSC-/- cells, in complete concordance with our results in other cell types. Finally, Torin1-induced accumulation of TFEB on lysosomes was also clearly visible in both cell types. These data have been included in the new Figures S8 and S9. Concerning the issue of the Bethyl antibody, while we can confirm that Bethyl only had one TFEB antibody and that this antibody did not pass the quality control, we cannot be sure that the one that was used by Pena-Llopis et al. was indeed "the same batch" that we have used. Therefore, we decided to delete any data or reference to this antibody in our paper.

Collectively, these results confirm our earlier observations, and strongly support a model where mTORC1 *suppresses* nuclear translocation and *inhibits* TFEB activity, (contrary to what was proposed by Pena-Llopis et al.). Moreover, these data argue that the lysosomal/amino acid inputs to mTORC1 dominate over growth factor inputs in controlling TFEB localization. The discrepancies between our data and model with those of Pena Llopis et al. have been discussed more in details in the new version of the manuscript.

2) Further analysis to support that TFEB is indeed phosphorylated under amino acid replete conditions should be performed (Fig. 2E). The use of phosphatases should at least be examined. Furthermore, this group has previously used Ser to Ala mutants of TFEB to analyze their effects on nuclear accumulation. These mutants or putative mTOR-targeted sites should be examined.

We previously showed that TFEB nuclear translocation is regulated by the phosphorylation state of serine S142. Now we generated a phospho-specific antibody that recognizes TFEB only when phosphorylated in position 142. This antibody fails to recognize TFEB in cells starved for nutrients or treated with the mTOR inhibitor Torin1, further indicating that S142 is phosphorylated by mTOR. Furthermore, we found that another serine (S211), in addition to S142, controls TFEB subcellular localization (see also response to points 2 and 3 of Reviewer 1). We included the description of the experiments in a new paragraph in the revised version of the manuscript and we generated an additional figure containing these data (new Figure 3). The treatment of nutrient-

stimulated cells with phosphatases resulted in a pronounced TFEB band shift, suggesting that TFEB is indeed phosphorylated at several residues. However, these data have not been included in the paper as they do not contribute significant information at this stage.

3) In Fig. 2G, there seems to be a small but nevertheless not negligible effect of knockout of SIN on nuclear accumulation accumulation of TFEB. To rule out that mTORC2 is not involved, siRNA should be performed on mTORC2 components.

Figure 2G shows that Torin 1 causes a similar TFEB band shift and nuclear translocation in both wild type and Sin1-/- KO MEFs, which lack functional mTORC2. This suggests that in Sin1-/- cells TFEB can still be phosphorylated by mTOR and retained in cytoplasm, thus ruling out mTORC2. The figure shows two independent blots and thus a quantitative comparison cannot be performed between them; rather the relative increase in the nuclear fraction between untreated and Torin 1 treated should be considered. This relative increase was identical between the wild type and Sin1-/- MEFs. To avoid confusion, we substituted the blot of the nuclear fraction of TFEB in Sin1-/- cells with a blot that was exposed for a shorter period. In conclusion, these data, along with the lack of binding to mTORC2 components (new Figure 2H), strongly argue against a direct involvement of mTORC2 in controlling TFEB localization.
As genetic loss of Sin1 completely disrupts mTORC2 signaling, while SiRNA would only partially abolish it, we do not expect that performing siRNA on mTORC2 components would contribute to the interpretation of the results.

Fig 2H should also include immunoprecipitation with mTORC2 components as control.

We have now performed immunoblotting analysis of the mTORC2 components Rictor and mSin1 showing that TFEB does not bind to these proteins, further supporting a specific regulation of TFEB by mTORC1 and not by mTORC2. These data have been included in the new Figure 2H.

4. Although the authors present data that mTORC1 and TFEB colocalize to the lysosomes upon Torin1 treatment, there is no evidence to support that phosphorylation of TFEB by mTORC1 occurs on the lysosomal surface.

Our newly generated in vitro kinase assay data (see also response to point 1 of Reviewer 1) clearly demonstrate that mTORC1 directly phosphorylates TFEB (see Figure 3). These data, together with the notion that when mTORC1 is active it resides on the lysosomal surface, strongly suggest that TFEB phosphorylation by mTORC1 occurs at the lysosome.

5. Fig. 4 nicely shows the effect of Rags (CA vs DN) on TFEB nuclear localization under amino acid depleted/repleted conditions. Would Torin1 treatment of cells expressing RagCA increase nuclear acculumation of TFEB?

We have used Torin1 on cells co-expressing TFEB and RagCA, as suggested by this reviewer. Similar to control cells, Torin1 induced a massive translocation of TFEB to the nucleus in RagCA expressing cells. This result indicates that the Rags control TFEB localization mainly through mTORC1, and that inhibition of mTORC1 kinase activity is dominant over constitutive Rag activation. These data are included in the new Figure S6.

6. Regulation of expression of lysosomal genes by mTORC1 should be further examined in mTORC1 knockdown cells. Why are the control levels different in Fig. 5B and 5C?

We have performed several attempts to knockdown mTORC1 in hepatocytes. This has always resulted in either low efficiency or high toxicity. The use of Torin1 circumvents this problem since it inhibits completely mTOR signaling for a short period, thus limiting the toxicity. Figure 5B and 5C (now renamed Figure 6B and 6C) shows the increase of the expression levels of several lysosomal/autophagic genes following chloroquine and Torin1 treatment, respectively. The values shown in Figure 5B and 5C were obtained by comparing stimulated cells versus untreated cells carrying the same genotype. For example chloroquine treatment increases the expression level of SQSTM1 7 fold in treated wild type cells compared to untreated wild type cells. A similar treatment results in an only 4 fold increase in TFEB KO cells compared to untreated KO cells. To improve clarity we revised the figure by representing expression levels as % values of treated over untreated cells.

2nd Editorial Decision 26 January 2012

Thank you for sending us your revised manuscript. Both referees have now seen it again and support publication (please see below). The paper will therefore be publishable in The EMBO Journal.

Prior to formal acceptance, there are a number of editorial issues that need further attention:

* Please make sure that the titles and the order of chapters comply with EMBO Journal format (please refer to the authors instructions).

* Please add an author contribution section and a conflict of interest statement to the main body of the manuscript text after the acknowledgements section.

* Please upload one individual file per figure.

* Please add scale bars and explanations to figures: 2C, 3E, S4, S5.

* Please add the statistical details to the legends of figures 6B,C; S7

I am looking forward to the final version of your manuscript. Thank you for your kind cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The authors have adequately revised this study and I recommend that it be accepted

Referee #2

In this revision, the authors present additional data to support that mTORC1 can directly phosphorylate TFEB. Furthermore, they identify 2 sites that could control TFEB localization via mTORC1. They also attempted to address the discrepancy between their results vs that of Pena-Llopis et al by examining TFEB localization in TSC-deficient cells. Although they confirmed their model of negative regulation of TFEB by mTORC1, it remains unclear why their findings are different from Pena-Llopis et al.

1st Revision - authors' response 26 January 2012

Thank you very much for accepting our paper for publication in EMBO journal. We have made all the last changes that you recommended and re-submitted the final version.

