THE EMBO JOURNAL

Manuscript EMBO-2015-93428

TFEB and TFE3 Are Novel Components of the Integrated Stress Response

Jose Martina, Heba Diab, Owen Brady, and Rosa Puertollano

Corresponding author: Rosa Puertollano, NIH

Review timeline:

Submission date:	07 July 2015
Editorial Decision:	12 August 2015
Resubmisison:	03 November 2015
Editorial Decision:	23 November 2015
Revision received:	07 December 2015
Accepted:	10 December 2015

Editor: Anne Nielsen

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 12 August 2015

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 the referees express interest in the work and topic in principle, they do not offer strong support for publication in The EMBO Journal.

I will not repeat all their individual points of criticism here, but it becomes clear that the referees find the depth of analysis to be too limited and that the study is thus too premature for them to support its publication here. We realize that referee #1 is slightly more positive about the overall merits of the study but we also had to notice that both referees together raise a number of serious concerns about the specificity, conclusiveness and functional outcome of the effects reported. Clearly, an extensive amount of further experimentation would be required to address the issues raised by both referees and to bring the study to the level of insight and significance required for publication in The EMBO Journal. Furthermore, the outcome of such experiments cannot be predicted at this point and would thus lie outside the scope and the timeframe of a revision. I therefore see little choice but to come to the conclusion that we cannot offer to publish the manuscript at this point.

Given these negative opinions from the referees, I am afraid we are unable to offer further steps towards publication in The EMBO Journal. If you were to undertake the efforts to extensively address all the concerns raised by the referees we could be willing to look at a new version of the manuscript at a later stage but this would have to be treated as a new independent submission.

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.

REFEREE REPORTS

Referee #1:

TFEB and TFE3 transcription factors have been known to be involved in adaptation to starvation or mTORC1 inactivation. In the current studies they found that these transcription factors are also involved in adaptation to cellular response to ER stress. Using tunicamycin, they found that TFE3 translocates to the nucleus and that this translocation involves PERK and calcineurin. Among the targets of TFE3/TFEB is ATF4 and other UPR genes. By CHIP-Seq analysis, they identified a number of other target genes in response to starvation. Finally, they showed that without TFEB/TFE3, cells have increased viability under ER stress conditions.

The studies link TFEB/TFE3 to control of UPR and in particular expression of the downstream UPR gene ATF4. Thus, the findings have novelty and importance. The involvement of PERK and calcineurin along with the conclusion that the process is independent of mTORC1 were not quite clearly addressed, however. There are also some other issues that would need to be further clarified/examined.

1. What is the effect of tunicamycin on mTORC1 signaling at the time points that have been used (16-24 hrs)?

2. It is possible that TFE3 does not translocate to the nucleus upon tunicamycin treatment in the PERK KO cells due to uncoupling of signals from ER stress to translation. Thus, mTORC1 (translation) stays active, hence TFEB remains cytosolic. In other words, the effect of PERK on TFE3 is more indirect. The authors should examine more carefully what happens to translation and mTORC1 signaling in the PERK KO upon tunicamycin treatment.

3. The effect of FK506 and calcineurin knockdown on TFE3 is quite minimal and therefore not convincing. What is the effect of FK506 on TFE3 phosphorylation? Would mutation of S321 have a more dramatic effect? Is the TFE3 and 14-3-3 binding disrupted by tunicamycin?

4. In Figure 4, why does the constitutively active TFEBS211A not have an effect on expression of ATF and other genes under basal conditions? The effect of this mutant on ATF4 RNA is also much less compared to the other targets (CHOP, GADD34, MCOLN1). Can the authors comment on this?

5. In Fig 6, it is not clear why this analysis was done on 2 hr starved cells. The rest of the studies have examined the effect of tunicamycin at 16 hrs. Thus, it seems that although there are a number of overlapping targets, this analysis does not quite support the main purpose of the study.

Referee #2:

TFE3 and TFEB are key transcription factors that regulate the lysosomal stress response. Under normal growth conditions, mTORC1 phosphorylates TFE3 and TFEB, leading to their cytoplasmic retention through binding to 14-3-3 proteins. Upon starvation, mTORC1 is inactivated, and TFE3 and TFEB are dephosphorylated by calcineurin, resulting in nuclear translocation of these transcription factors and transcriptional upregulation of genes involved in lysosomal biogenesis and autophagy through an enhancer element called CLEAR.

In this manuscript, the authors investigated the possibility that TFE3 and TFEB are involved in the unfolded protein response (UPR). They found that TFE3 and TFEB were dephosphorylated at least in part by calcineurin in a PERK-dependent manner in response to ER stress, translocated into the nucleus and activated transcription of ATF4. Transcriptional induction of ATF4 by TFE3 and TFEB resulted in transcriptional activation of lysosomal genes as well as the UPR target genes of the PERK pathway, leading to ER stress-induced apoptosis. The authors found the CLEAR element in the proximal region of the ATF4 promoter, to which TFE3 and TFEB directly bind to upon ER stress or starvation. Interestingly, TFE3/TFEB Double KO MEF cells were less susceptible to ER stress-induced cell death. Based on these observations, the authors proposed that TFE3 and TFEB

are novel components of the integrated stress response, which regulate an integrated cooperation between the UPR and the lysosomal stress response.

This is a potentially interesting paper, however, many questions or even controversies remain. This reviewer cannot recommend its publication in EMBO J. The data of the ChIP-seq experiment can be omitted because it was conducted in starved cells but not in tunicamycin-treated cells. The answers to my following questions are much more important. The data shown in Fig. 6FGH would confuse readers because the data suggest that activation of TFE3/TFEB by tunicamycin would be cytoproective but this is not the case (Fig. 7C).

1) Why wasn't ATF4 induced in starved cells (Fig. 1B), over 90% of which showed nuclear TFE3 (Fig. 1D), if TFE3/TFEB activate transcription of ATF4 through the clear motif (Fig. 5E) as the authors proposed?
2) Why did nuclear translocation of TFE3/TFEB take so long time (>16 h) in tunicamycin-treated cells (Fig. 1E)? The authors must at least show the time course of phosphorylation status of eIF2a and TFE3/TFEB as well as the time course of activation status of calcineurin (for example, measurement of nuclear translocation of NFAT as in Fig. 3F).
3) It is reasonable that overexpression of wild-type TFE3 induced ATF4, CHOP and GADD34 in cells starved or treated with tunicamycin (Fig. 4A and 4B) as TFE3 needed to be dephosphorylated. Then, why didn't overexpression of TFE3-S211A induce ATF4, CHOP and GADD34 in control cells (Fig. 4A and 4B)? As transcription of MCOLN1 was clearly induced by overexpression of TFE3-S211A in control cells (Fig. 4C), there is a clear difference in transcriptional induction mechanism between MCOLN1 and UPR targets, which must be revealed.
4) The authors showed that TFE3/TFEB Double KO MEF cells are less susceptible to ER stress-induced apoptosis (Fig. 7CD). This is surprising because previous papers reported that activation of TFEB is cytoprotective against stress. For instance, TFEB overexpression enhanced clearance of polyQ-expanded huntingtin (Sardiello et al., Science 2009), or mutant anti-trypsin, resulting in correction of hepatic disease (Pastor et al., EMBO Mol. Med. 2013). Moreover, TFEB is a promising therapeutic target for Pompe disease (Spampanato et al., EMBO Mol. Med. 2013). As the authors also described that TFE3 promotes cell adaptation to nutrient starvation (p. 5, line 15), the susceptibility of TFE3/TFEB Double KO MEF cells to nutrient deprivation as well as to treatment with the mTOR inhibitor Torin-1 must be examined.
5) Why was thapsigargin used only for the experiments shown in Fig. 3F?

Resubmission 03 November 2015

Thank you very much for your interest in our work and for providing us with the opportunity to submit a revised version of our manuscript entitled “TFEB and TFE3 Are Novel Components of the Integrated Stress Response”. We also would like to thank the reviewers for their very helpful comments. In this new version we have addressed all the concerns raised by the reviewers. This has resulted in 22 new datasets (Fig 3A, 3B, and 3D, Fig 5A, Fig 7A-E, Supplementary Fig S1C-H and S1J, Supplementary Fig S2A and B, Supplementary Fig S3C, S3D, and S3F) and 6 new Supplementary Tables. We also provide 5 new Supporting Figures attached to this rebuttal letter (Fig A-E). We have now repeated our ChIP-Seq analysis in MEFs both upon ER stress and starvation conditions. We found that 80% of the genes regulated by TFE3 following starvation are also modulated by this transcription factor in cells undergoing ER stress. Therefore we identified a common transcriptional network regulated by TFE3 under different stress conditions. In addition to lysosomal and autophagic genes, this network includes numerous genes implicated in cellular response to stress, ER homeostasis, and regulation of apoptosis. These new data provide relevant information to better understand the global role of TFE3 in the cellular response to stress. We have also extended our observations on the role of TFEB and TFE3 in cell fate by showing that starvation-induced ATF4 up-regulation and apoptosis is reduced in TFEB/TFE3-KO cells, and present additional mechanistic evidence supporting the mTORC1-independet activation of TFE3 upon ER stress. Finally, we provide point-by-point responses to the reviewer’s comments (see below).

Reviewer#1

TFEB and TFE3 transcription factors have been known to be involved in adaptation to starvation or mTORC1 inactivation. In the current studies they found that these transcription factors are also involved in adaptation to cellular response to ER stress. Using tunicamycin, they found that TFE3 translocates to the nucleus and that this translocation involves PERK and calcineurin. Among the targets of TFE3/TFEB is ATF4 and other UPR genes. By CHIP-Seq analysis, they identified a number of other target genes in response to starvation. Finally, they showed that without TFEB/TFE3, cells have increased viability under ER stress conditions. The studies link TFEB/TFE3 to control of UPR and in particular expression of the downstream UPR gene ATF4. Thus, the findings have novelty and importance.

RESPONSE: We thank the reviewer for the support

1. What is the effect of tunicamycin on mTORC1 signaling at the time points that have been used (16-24 hrs)?

RESPONSE: Please note that in the original version of our manuscript we had already shown that mTORC1 activity was not altered in ARPE-19 cells after 16 hours of tunicamycin treatment (Fig 3E in the revised version of the manuscript). To further corroborate these data, we have now performed a more detailed analysis of mTORC1 activity. ARPE-19 cells were treated with 5µg/ml tunicamycin for 6, 8, 16, and 24 hours. Quantification of three independent experiments did not reveal significant differences in the phosphorylation status of AKT or the mTORC1 substrates p70S6K and 4EBP following tunicamycin treatment (Supplementary Fig S1C-E). In addition, mTORC1 stayed associated with lysosomes at all times in tunicamycin-treated cells, further suggesting that mTOR remains active under ER stress conditions (Supplementary Fig S1F). Although we observed a slight increase in the amount of cytosolic mTOR at 24 hours tunicamycin treatment, Person's Coefficient analysis did not reveal significant differences in co-localization between mTOR and LAMP1 at any of the analyzed time-points (Supplementary Fig S1G). Therefore we confirm that in ARPE-19 cells, ER stress-induced TFE3 nuclear translocation does not require mTORC1 inactivation.

2. It is possible that TFE3 does not translocate to the nucleus upon tunicamycin treatment in the PERK KO cells due to uncoupling of signals from ER stress to translation. Thus, mTORC1 (translation) stays active, hence TFEB remains cytosolic. In other words, the effect of PERK on TFE3 is more indirect. The authors should examine more carefully what happens to translation and mTORC1 signaling in the PERK KO upon tunicamycin treatment.

RESPONSE: As mentioned by the reviewer, it has been described that cells exhibit a profound reduction in global translation rates following ER stress and this reduction is significantly attenuated in PERK and GCN2-KO MEFs (Harding et al., 1999; Deng et al., 2002). Following the reviewer's suggestion, we monitored mTORC1 signaling in wild-type and PERK-KO MEFs upon ER stress. In contrast with ARPE-19 cells, we found that prolonged tunicamycin treatment (>16 hours) caused a small reduction in mTORC1 activity, as assessed by measuring the phosphorylation status p70S6K and 4EBP (Supplementary Fig S1H). However, this reduction is not sufficient to induce TFE3 nuclear translocation, since the reduction in mTORC1 activity is more pronounced in PERK-KO cells and yet TFE3 remains retained in the cytosol. Therefore, our data argue against the possibility that increased mTORC1 activity in PERK-KO cells might be the reason for the lack of TFE3 nuclear translocation under ER stress conditions and support a direct role of PERK in TFE3 activation.

3. The effect of FK506 and calcineurin knockdown on TFE3 is quite minimal and therefore not convincing. What is the effect of FK506 on TFE3 phosphorylation? Would mutation of S321 have a more dramatic effect? Is the TFE3 and 14-3-3 binding disrupted by tunicamycin?

RESPONSE: Please note that the effect of FK506 and calcineurin knockdown that we observe on TFE3 activation is comparable to values reported by the Ballabio group for TFEB (Medina et al., 2015). They found that FK506 treatment or PPP3CB depletion caused a 20-30% reduction on starvation-induced TFEB nuclear translocation. These reduction ranges are very similar to the ones we report in Fig 3F and 3G. One aspect to consider is that FK506 may be inducing only a partial inhibition of calcineurin or that our calcineurin depletion may not be 100% efficient. Alternatively, additional factors might be involved in regulating TFE3 activation. These different possibilities are now further discussed in the text.

We have previously shown that mutation of S321 prevents binding of TFE3 to 14-3-3 and causes accumulation of TFE3 in the nucleus (Martina et al., 2014 and Fig 3A). Following the reviewer's suggestion, we now show that the TFE3 fraction that translocates to the nucleus in tunicamycin treated cells is not phosphorylated in S321 and does not bind to 14-3-3 (Fig 3C and 3D). Conversely, the TFE3 that remains in the cytosol is phosphorylated in S321 and remains bound to 14-3-3 (Fig 3C and 3D). We also provide additional evidence of the specificity of our phospho-TFE3 antibody. As expected, our phospho-TFE3 antibody efficiently recognized TFE3-wild type but not the TFE3-S321A mutant (Fig 3B).

4. In Figure 4, why does the constitutively active TFEBS211A not have an effect on expression of ATF and other genes under basal conditions?

RESPONSE: We apologize if we did not make this point sufficiently clear. It is well established that in basal condition ATF4 is almost undetectable due to the short half-life of the protein and low translation efficiency of the ATF4 mRNA. Activation of PERK (under ER stress) or GCN2 (under starvation) results in phosphorylation and inhibition of the general translation factor eIF2-alpha, thus causing a reduction in the translation of wide variety of mRNAs. However, a few mRNAs, such as ATF4, show increased translation upon eIF2-alpha phosphorylation, thus resulting in increased ATF4 protein levels. Since over-expression of TFEB-S211A or TFE3 do not cause stress or increased eIF2-alpha phosphorylation per se, we do not expect to see increased ATF4 protein levels, or its transcriptional targets CHOP and GADD34, in TFEB-S211A and TFE3-expressing cells under basal conditions, and this is exactly what we show in Fig 5A. Therefore, even though TFEB and TFE3 induce increased levels of ATF4 mRNA (see below) this mRNA is not efficiently translated under non-stress conditions. This point is now further explained in the text. In addition, levels of phospho-eIF2alpha are now shown in Fig 5A.

The effect of this mutant on ATF4 RNA is also much less compared to the other targets (CHOP, GADD34, MCOLN1). Can the authors comment on this?

RESPONSE: We thank the reviewer for the opportunity to clarify this point. There are several considerations to take into account: 1) ATF4 mRNA is particularly unstable. It has been previously shown that ATF4 mRNA is short-lived (Dey et al., 2010). This could explain that, even if we over-express TFEB-S211A for 36-40 hours, when we do our qRT-PCR experiments we are only quantifying the mRNA that has accumulated in the last couple of hours. To confirm this possibility, we measured ATF4 mRNA half-life in wild-type MEFs using actinomycin D (Dey et al., 2010). In agreement with previous reports, we confirmed that ATF4 mRNA is very unstable with a half-life of less than 1.5 hours. In contrast, MCOLN1 mRNA is much more stable, with a half-life > 6 hours (Fig A in this rebuttal letter). Importantly, TFEB-S211A over-expression did not change ATF4 mRNA half-life, indicating the increased ATF4 mRNA levels observed in TFEB-S211A-expressing cells are not due to increased ATF4 mRNA stability but rather to increased transcription (Fig B in this rebuttal letter).

2) MCOLN1 is one of the best TFEB/TFE3 targets. In ARPE-19 we observe a very robust increase in MCOLN1 mRNA levels upon TFEB or TFE3 over-expression. This may in part be due to the fact that the basal MCOLN1 mRNA and protein levels are very low in ARPE-19 cells (Martina et al., 2014). However, for most lysosomal and autophagy genes we, and others have reported increases between 1.5 and 5 fold following TFEB or TFE3 over-expression (Sardiello et al., 2009; Martina et al., 2012; Martina et al., 2014). To further compare expression of ATF4 and lysosomal genes, we over-expressed TFEB-S211A in MEFs and measured ATF4, MCOLN1, and LAMP1 mRNA levels by qRT-PCR. As seen in a new panel in Supplementary Fig S1J, we observed a 1.5 fold increase in the ATF4 mRNA levels upon TFEB over-expression, while the levels of MCOLN1 and LAMP1 mRNA increased 2.5 fold. Those increases are very reproducible, significant, and comparable in magnitude to previous publications on the effects of TFEB and TFE3 activation on lysosomal and autophagic gene expression (Settembre et al., 2011; Settembre et al., 2013; Martina et al., 2014).

In summary, the 1.5 fold increased in the levels of ATF4 mRNA that we observe in basal conditions upon TFEB-S211A over-expression is in the range of what it has been observed for other TFEB targets. We think that the short half-life of ATF4 mRNA in basal conditions contributed to this modest (although very reproducible and significant) up-regulation. It is important to keep in mind that endogenous TFEB and TFE3 remain in the cytosol under basal conditions and probably only contribute to ATF4 expression following ER or starvation stress. The increased TFE3 binding to the

CLEAR element present in the ATF4 promoter reported in our ChIP-Seq and ChIP-qPCR experiments under stress conditions, the increased ATF4 mRNA and protein levels induced by TFEB and TFE3 over-expression, and the reduced ATF4 induction in TFEB/TFE3 knockout cells collectively support the role of TFEB and TFE3 in ATF4 regulated expression.

5. In Fig 6, it is not clear why this analysis was done on 2 hr starved cells. The rest of the studies have examined the effect of tunicamycin at 16 hrs. Thus, it seems that although there are a number of overlapping targets, this analysis does not quite support the main purpose of the study.

RESPONSE: While the role of TFEB and TFE3 in ER stress is one of the main points of our study, we think that the TFE3-dependent regulation of ATF4 expression under nutrient deprivation as well as the identification of common regulatory links between different stress pathways are equally relevant. In addition, our study is the first to use ChIP-seq to identify TFE3 targets in starved cells and we consider these data to be an important part of our work. However, we agree with the referee in that it would also be interesting to identify TFE3 targets in cells subjected to ER stress.

For this reason we have now repeated our ChIP-seq analysis in MEFs cells both under starvation and ER stress conditions. MEFs were starved for 2 hours (note that MEFs are very sensitive to starvation and start dying by apoptosis after prolonged [>6 hours] starvation. In addition, TFE3 is fully activated and largely accumulated in the nucleus by 2 hours starvation) or treated with tunicamycin for 16 hours (at this time we see a significant accumulation of TFE3 in the nucleus while cells are still reasonable healthy). TFE3 pulled-down 1,391 genes in tunicamycin-treated cells and 1,169 genes in starved cells. None of those genes were observed when we used an irrelevant immunoglobulin or when we performed the experiments in TFE3-KO cells. Notably, 901 TFE3 targets overlapped between the two stress conditions. This means that close to 80% of the genes regulated by TFE3 in starvation conditions are also modulated by TFE3 under ER stress. These results are now shown in Fig 7A-E, Supplementary Fig S2A and B, and Supplementary Tables S1-5. Some of the main conclusions drawn from our ChIP-seq analysis are the following: 1) we confirmed increased TFE3 binding to the ATF4 promoter under ER stress and starvation conditions, 2) we confirmed TFE3-mediated regulation of numerous lysosomal and autophagic genes under ER stress conditions, 3) we identify a common transcriptional network regulated by TFE3 under different stress conditions, and 4) we found that, in addition to lysosomal and autophagic genes, this network includes numerous genes implicated in cellular response to stress, ER homeostasis, and regulation of apoptosis. Therefore, our ChIP-seq data provide relevant information to better understand the global role of TFE3 in the cellular response to stress.

Referee #2:

TFE3 and TFEB are key transcription factors that regulate the lysosomal stress response. Under normal growth conditions, mTORC1 phosphorylates TFE3 and TFEB, leading to their cytoplasmic retention through binding to 14-3-3 proteins. Upon starvation, mTORC1 is inactivated, and TFE3 and TFEB are dephosphorylated by calcineurin, resulting in nuclear translocation of these transcription factors and transcriptional upregulation of genes involved in lysosomal biogenesis and autophagy through an enhancer element called CLEAR.

In this manuscript, the authors investigated the possibility that TFE3 and TFEB are involved in the unfolded protein response (UPR). They found that TFE3 and TFEB were dephosphorylated at least in part by calcineurin in a PERK-dependent manner in response to ER stress, translocated into the nucleus and activated transcription of ATF4. Transcriptional induction of ATF4 by TFE3 and TFEB resulted in transcriptional activation of lysosomal genes as well as the UPR target genes of the PERK pathway, leading to ER stress-induced apoptosis. The authors found the CLEAR element in the proximal region of the ATF4 promoter, to which TFE3 and TFEB directly bind to upon ER stress or starvation. Interestingly, TFE3/TFEB Double KO MEF cells were less susceptible to ER stress-induced cell death. Based on these observations, the authors proposed that TFE3 and TFEB are novel components of the integrated stress response, which regulate an integrated cooperation between the UPR and the lysosomal stress response.

This is a potentially interesting paper, however, many questions or even controversies remain. This reviewer cannot recommend its publication in EMBO J.

he data of the ChIP-seq experiment can be omitted because it was conducted in starved cells but not in tunicamycin-treated cells.

RESPONSE: While the role of TFEB and TFE3 in ER stress is one of the main points of our study, we think that the TFE3-dependent regulation of ATF4 expression under nutrient deprivation as well as the identification of common regulatory links between different stress pathways are equally relevant. In addition, our study is the first to use ChIP-seq to identify TFE3 targets in starved cells and we consider these data to be an important part of our work. However, we agree with the referee in that it would also be interesting to identify TFE3 targets in cells subjected to ER stress.

For this reason we have now repeated our ChIP-seq analysis in MEFs cells both under starvation and ER stress conditions. MEFs were starved for 2 hours (note that MEFs are very sensitive to starvation and start dying by apoptosis after prolonged [>6 hours] starvation. In addition, TFE3 is fully activated and largely accumulated in the nucleus by 2 hours starvation) or treated with tunicamycin for 16 hours (at this time we see a significant accumulation of TFE3 in the nucleus while cells are still reasonable healthy). TFE3 pulled-down 1,391 genes in tunicamycin-treated cells and 1,169 genes in starved cells. None of those genes were observed when we used an irrelevant immunoglobulin or when we performed the experiments in TFE3-KO cells. Notably, 901 TFE3 targets overlapped between the two stress conditions. This means that close to 80% of the genes regulated by TFE3 in starvation conditions are also modulated by TFE3 under ER stress. These results are now shown in in Fig 7A-E, Supplementary Fig S2A and B, and Supplementary Tables S1-5. Some of the main conclusions drawn from our ChIP-seq analysis are the following: 1) we confirmed increased TFE3 binding to the ATF4 promoter under ER stress and starvation conditions, 2) we confirmed TFE3-mediated regulation of numerous lysosomal and autophagic genes under ER stress conditions, 3) we identify a common transcriptional network regulated by TFE3 under different stress conditions, and 4) we found that, in addition to lysosomal and autophagic genes, this network includes numerous genes implicated in cellular response to stress, ER homeostasis, and regulation of apoptosis. Therefore, our ChIP-seq data provide relevant information to better understand the global role of TFE3 in the cellular response to stress.

The answers to my following questions are much more important.

The data shown in Fig. 6FGH would confuse readers because the data suggest that activation of TFE3/TFEB by tunicamycin would be cytoproective but this is not the case (Fig. 7C).

RESPONSE: We propose that TFEB and TFE3 may have a dual role in promoting survival or cell death depending on the duration and strength of the stress, the cell type, and other environmental factors. Similar dual roles have been reported for other transcription factors. In addition to ATF4 that we discuss in this manuscript, it is well established that p53 stimulates expression of pro-survival genes in response to weak or moderate stress insults. The promoters of these genes are very sensitive to even low p53 activation and usually activate very quickly after the stress is applied. On the contrary, pro-apoptotic genes are activated in response to intense stress and with a significant delay as compared to pro-survival genes (Sablina et al., 2005; Vousden and Prives, 2009). Likewise, we propose that the TFEB and TFE3-mediated increase in autophagy, lysosomal biogenesis, and expression of regulators of ER homeostasis, facilitate cell survival, whereas the sustained ATF4 up-regulation and expression of other apoptotic genes lead to cell death. In addition, we think that the data shown in Fig 7F-H are relevant, since they show that TFEB induces up-regulation of several important regulators of the cellular stress response, thus confirming our ChIP-seq data.

1. Why wasn't ATF4 induced in starved cells (Fig. 1B), over 90% of which showed nuclear TFE3 (Fig. 1D), if TFE3/TFEB activate transcription of ATF4 through the clear motif (Fig. 5E) as the authors proposed?

RESPONSE: The reviewer is correct in that the increase in ATF4 protein levels following starvation is not very efficient in ARPE-19 cells. We have now performed a more complete time course assessing ATF4 protein levels at 4, 8, and 16 hours after starvation and obtained similar results (Fig C in this rebuttal letter). Please note that, as we reported previously, TFEB-S211A over-expression significantly increased ATF4 levels at all times. We also analyzed ATF4 induction in MEFs. In this cell type we observed a robust ATF4 protein up-regulation after 2-4 hours starvation (Supplementary Fig S3C and Fig D attached to this cover letter). Similar to our observations in

ARPE-19, we found that TFEB-S211A over-expression further enhanced ATF4 up-regulation (Fig D).

2. Why did nuclear translocation of TFE3/TFEB take so long time (>16 h) in tunicamycin-treated cells (Fig. 1E)? The authors must at least show the time course of phosphorylation status of eIF2a and TFE3/TFEB as well as the time course of activation status of calcineurin (for example, measurement of nuclear translocation of NFAT as in Fig. 3F).

RESPONSE: As seen in Fig 8A, there is a shift in the TFEB and TFE3 electrophoretic mobility after 16 and 24 hours tunicamycin treatment, that is the time at which we observe TFEB and TFE3 nuclear translocation. We and others have previously described that this shift is indicative of TFEB and TFE3 de-phosphorylation. Also, as requested by the reviewer, levels of phospho-eIF2alpha are now shown in Fig 5A.

The rapid mTORC1 inactivation that follows nutrient deprivation explains the quick TFE3 de-phosphorylation and nuclear translocation observed under these conditions. Since mTORC1 remains active in tunicamycin-treated cells, TFE3 de-phosphorylation likely requires sustained activation of calcineurin and other phosphatases. In agreement with this idea, it has been shown that prolonged ER stress triggers calcium release from the ER, thus resulting in a low but persistent increase in cytosolic calcium (Deniaud et al., 2008; Høyer-Hansen Jäättelä M, 2007). In addition, PERK has been proposed to be an essential component of mitochondrial associate ER membranes (MAMs) that are critical for calcium transference from ER to mitochondria under sustained ER stress (Verfaillie et al., 2012). We favor the hypothesis that direct calcineurin activation by PERK at late times of ER stress contributes to TFE3 activation, thus explaining the lack of TFE3 nuclear translocation in PERK-KO cells.

3. It is reasonable that overexpression of wild-type TFE3 induced ATF4, CHOP and GADD34 in cells starved or treated with tunicamycin (Fig. 4A and 4B) as TFE3 needed to be dephosphorylated. Then, why didn't overexpression of TFE3-S211A induce ATF4, CHOP and GADD34 in control cells (Fig. 4A and 4B)?

RESPONSE: We apologize if we did not make this point sufficiently clear. It is well established that in basal condition ATF4 is almost undetectable due to the short half-life of the protein and low translation efficiency of the ATF4 mRNA. Activation of PERK (under ER stress) or GCN2 (under starvation) results in phosphorylation and inhibition of the general translation factor eIF2-alpha, thus causing a reduction in the translation of wide variety of mRNAs. However, a few mRNAs, such as ATF4, show increased translation upon eIF2-alpha phosphorylation, thus resulting in increased ATF4 protein levels. Since over-expression of TFEB-S211A or TFE3 do not cause stress or increased eIF2-alpha phosphorylation per se, we do not expect to see increased ATF4 protein levels, or its transcriptional targets CHOP and GADD34, in TFEB-S211A and TFE3-expressing cells under basal conditions, and this is exactly what we show in Fig 5A. Therefore, even though TFEB and TFE3 induce increased levels of ATF4 mRNA (see below) this mRNA is not efficiently translated under non-stress conditions. This point is now further explained in the text. In addition, levels of phospho-eIF2alpha are now shown in Fig 5A.

As transcription of MCOLN1 was clearly induced by overexpression of TFE3-S211A in control cells (Fig. 4C), there is a clear difference in transcriptional induction mechanism between MCOLN1 and UPR targets, which must be revealed.

RESPONSE: We thank the reviewer for the opportunity to clarify this point. There are several considerations to take into account:

1) ATF4 mRNA is particularly unstable. It has been previously shown that ATF4 mRNA is short-lived (Dey et al., 2010). This could explain that, even if we over-express TFEB-S211A for 36-40 hours, when we do our qRT-PCR experiments we are only quantifying the mRNA that has accumulated in the last couple of hours. To confirm this possibility, we measured ATF4 mRNA half-life in wild-type MEFs using actomycin D, following the protocol previously described by Dev et al. In agreement with previous reports, we confirmed that ATF4 mRNA is very unstable with a half-life of less than 1.5 hours. In contrast, MCOLN1 mRNA is much more stable, with a half-life > 6 hours (Fig B in this rebuttal letter). Importantly, TFEB-S211A over-expression did not change ATF4 mRNA half-life, indicating the increased ATF4 mRNA levels observed in TFEB-S211A-expressing

cells are not due to increases ATF4 mRNA stability but rather to increased transcription (Fig C in this rebuttal letter).

2) MCOLN1 is one of the best TFEB targets. In ARPE-19 we observed very robust increase in MCOLN1 mRNA levels upon TFEB or TFE3 over-expression. This may in part be due to the fact that the basal MCOLN1 mRNA and protein levels are very low in ARPE-19 cells (Martina et al., 2014). However, for most lysosomal and autophagy genes we and others have reported increases between 1.5 and 5 fold following TFEB or TFE3 over-expression (Sardiello et al., 2009; Martina et al., 2012; Martina et al., 2014). To further compare expression of ATF4 and lysosomal genes, we over-expressed TFEB-S211A in MEFs and measured ATF4, MCOLN1, and LAMP1 mRNA levels by qRT-PCR. As seen in a new panel in Supplementary Fig S1J, we observed a 1.5 fold increase in the ATF4 mRNA levels upon TFEB over-expression, while the levels of MCOLN1 and LAMP1 mRNA increased 2.5 fold. Those increases are very reproducible, significant, and comparable in magnitude to previous publications on the effects of TFEB and TFE3 activation on lysosomal and autophagic gene expression (Sardiello et al., 2011; Settembre et al., 20313; Martina et al., 2014).

In summary, the 1.5 fold increased in the levels of ATF4 mRNA that we observe in basal conditions upon TFEB-S211A over-expression is in the range of what it has been observed for other TFEB targets. We think that the short half-life of ATF4 mRNA in basal conditions contributed to this modest (although very reproducible and significant) up-regulation. It is important to keep in mind that endogenous TFEB and TFE3 remain in the cytosol under basal conditions and probably only contribute to ATF4 expression following ER or starvation stress. The increased TFE3 binding to the CLEAR element present in the ATF4 promoter reported in our ChIP-seq and ChIP-qPCR experiments under stress conditions, the increased ATF4 mRNA and protein levels induced by TFEB and TFE3 over-expression, and the reduced ATF4 induction in TFEB/TFE3 knockout cells collectively supports the role of TFEB and TFE3 in ATF4 regulated expression.

4. The authors showed that TFE3/TFEB Double KO MEF cells are less susceptible to ER stress-induced apoptosis (Fig. 7CD). This is surprising because previous papers reported that activation of TFEB is cytoprotective against stress. For instance, TFEB overexpression enhanced clearance of polyQ-expanded huntingtin (Sardiello et al., Science 2009), or mutant anti-trypsin, resulting in correction of hepatic disease (Pastor et al., EMBO Mol. Med. 2013). Moreover, TFEB is a promising therapeutic target for Pompe disease (Spampanato et al., EMBO Mol. Med. 2013). As the authors also described that TFE3 promotes cell adaptation to nutrient starvation (p. 5, line 15), the susceptibility of TFE3/TFEB Double KO MEF cells to nutrient deprivation as well as to treatment with the mTOR inhibitor Torin-1 must be examined.

RESPONSE: As requested by the reviewer, we have now addressed starvation stress response in TFEB/TFE3 KO MEFs. Importantly, we found that ATF4 induction following starvation was dramatically reduced in TFEB/TFE3 KO MEFs (Supplementary Fig S3C). Consistent with these results, we observed that TFEB/TFE3 KO cells are significantly more resistant to starvation-induced apoptosis (Supplementary Fig S3D). Moreover, we now show that prolonged expression (40 hours) *of either TFEB-S211A or TFE3-S321A in MEFs is sufficient to cause a significant reduction in cell viability (Supplementary Fig S3F). All together, our data confirm that TFEB and TFE3 contribute to cell death under conditions of prolonged ER or starvation stress.*

We are well aware of the potential use of TFEB as a therapeutic target for the treatment of neurological and lysosomal diseases. In fact our laboratory has contributed to some of the studies mentioned by the reviewer. However, we do not think our results are in conflict with these studies. As we mention in the discussion, we propose that TFEB and TFE3 may have a dual role, helping cells to survive when the stress is not too severe but facilitating apoptosis if cells can not survive. It seems clear that the autophagy induction, increased lysosomal activity, and lysosomal biogenesis induced by TFEB and TFE3 are beneficial to achieve cellular clearance in those disorders caused by cytosolic protein aggregation or defects in the autophagic/lysosomal pathway. However, under our experimental conditions we are inducing a very severe and prolonged stress (>16 hours of nutrient deprivation or accumulation of missfolded protein in the ER) and under these conditions TFEB and TFE3 contribute to apoptosis. A dual role for other transcription factors in cell survival has been reported, including ATF4, p53, and several members of the FOXO family (Budanov 2014; Wang et al., 2014). In those cases it is clear that whether or not cellular stress triggers cell death or cell survival programs is determined by a set of different factors, among them the initial stress stimulus, cell type, and environmental factors. We proposed that the same is applicable to TFEB

and TFE3. Please note that we have now expanded the Discussion section to incorporate some of these considerations.

5. Why was thapsigargin used only for the experiments shown in Fig. 3F?

RESPONSE: The Ballabio group has recently shown that thapsigargin causes TFEB activation in HeLa cells and this process is dependent on calcineurin (Medina et al., 2015). In our hands, prolonged thapsigargin treatment of MEFs or ARPE-19 cells adversely impacted cell viability. In addition, long-term thapsigargin treatment inhibits mTOR activity through calcium-dependent induction of AMPK (Hoyer-Hansen et al., 2007), thus complicating the interpretation of the results.

To further support our claim that ER stress causes TFEB and TFE3 activation in a more physiological setting, we have now analyzed endogenous TFE3 localization in TSC2-KO MEFs. It has been reported that TSC2 depletion results in chronic ER stress (Ozcan et al., 2008). As expected, we observed a significant increased in nuclear TFE3 in TSC2-KO cells (Fig E in this rebuttal letter).

2nd Editorial Decision 23 November 2015

Thank you for submitting a new version of your manuscript to The EMBO Journal. Your study has now been seen by the two original referees (comments included below) and as you will see they both find that all major criticisms have been addressed.

However before we can proceed to officially accept your manuscript for publication here, I would ask you to address the following minor points in a final revision.

-> Please rephrase/tone down the statements on page 12 and 13 pointed out by ref #1.

-> Please provide us with a filled-out author checklist as described in our guide to authors (http://emboj.embopress.org/authorguide)

-> Please make sure that the number of replicas underlying statistical significance calculations is included in the relevant figure legends and that all statistics are adequately powered (n{greater than or equal to}3)

-> Please also include a database accession code for the ChIP-seq data generated in the study. If the final code is not yet available you can insert a placeholder text in the materials and methods section and add the final number at proof stage.

-> We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format. The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data".

-> As you may know papers published in The EMBO Journal contain a 'Synopsis' to further enhance their discoverability. The synopsis consists of a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to include your suggestions for bullet points. In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

Please feel free to contact me with any questions. I look forward to receiving your final revision.

REFEREE REPORTS

Referee #1:

In this revision, the authors have addressed satisfactorily this reviewer's questions and comments. More importantly, they have now performed genome-wide ChIP-seq analysis to compare TFE3-dependent gene expression of tunicamycin-treated vs starved cells and identified genes involved in ER stress response. Thus, the studies provide new insights on the regulation of TFEB/TFE3 by ER stress and the corresponding target genes.

minor comment:
top of page 12: The concluding statement "These results indicate that TFE3 and TFEB promote autophagy and lysosomal biogenesis in response to ER stress." is an overstatement. The data showing reduction of autophagy-related genes by TFEB/TFE3 knockdown only suggest TFEB/TFE3 involvement but more careful analysis on autophagy during ER stress would need to be conducted to support such statement.

top paragraph page 13: "All together, our data suggest that TFEB increases transcriptional up-regulation of ATF (under stress conditions)"

Referee #2:

The authors fully revised the manuscript, which the reviewer thinks is suitable for publication in EMBO Journal.
