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TFEB CONTROLS VASCULAR DEVELOPMENT BY REGULATING THE PROLIFERATION OF ENDOTHELIAL CELLS

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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 23rd October 2017

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

As you will see, the referees acknowledge that your findings are of value. However, they also think that the definitive insight offered is not sufficient for publication here. The referees note that the link between the TFEB deficiency phenotype and altered VEGFR2 levels and signaling is not causally shown, and that the physiological significance of the VEGFR2 phenotype remains unclear. They further note some inconsistencies in the data provided.

Given these opinions, I am afraid we cannot offer to publish your manuscript in The EMBO Journal.

Thank you in any case for the opportunity to consider this manuscript. I am sorry that I couldn't bring better news, but I hope nevertheless that you will find our referees' comments helpful.

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REFEREE REPORTS

Referee #1:

Doronzo et al describe the features of Tfeb, a transcription factor expressed in the developing vasculature and in pericytes/smooth muscle cells. Tfeb-mediated deletion gave rise to embryonic lethality accompanied by vascular abnormalities. Postnatal Cdh5-mediated deletion of Tfeb showed a relatively strong phenotype in retinal and kidney vascular development. A dominant phenotype was the reduced proliferation of endothelial cells in the retina and kidney vasculature. In contrast, migration in vitro was not affected by Tfeb deletion. Through transcript analyses on Tfeb-silenced cells, the authors identified changes in GO:s related to angiogenesis, cell cycle etc. ChiP-Seq analyses from cells overexpressing an active variant of Tfeb showed associatation of Tfeb with promoters of transcription factors including Jun, Fos, Myc, Gata2, Max, of which the majority but not all contained the canonical Tfeb-binding site. One primary target gene was VEGFR2 which however lacked the canonical Tfeb promoter binding site. Expression of VEGFR2 in Tfeb-deficient cells increased considerably in vitro and in vivo, at least in part due to direct Tfeb-regulation of miRNAs 15a-5p and 16-5p. The relative phosphorylation of VEGFR2 was decreased in Tfeb-deficient cells, as was downstream signalling in the PLCg and Erk pathways. The authors moreover identified Myo1c as a Tfeb target gene and showed upregulation of Myo1c in Tfeb-

deficient cells, leading to increased clearance of VEGFR2. Thus TFEB regulate VEGFR2 expression levels in many different ways, through other transcription factors, miRNA and internalization/trafficking in a Myo1C-dependent manner. This is an ambitious study with novel information on the many aspects of regulation of VEGFR2 expression and trafficking and the role in vessel biology.

Major comments
1. Throughout please define n. Now for example in Fig. 2B and in Fig. 4D, the authors give n=6. Is this 6 vessels or 6 mice?
2. Page 9, the authors perform comparative transcript analysis on siRNA-treated cells. Why not instead isolate endothelial cells from their mouse models to do these analyses? This would allow a global analysis to replace the siRNA-cell analysis + manual annotation shown in Figs. 3 and EV6 (if the authors keep Fig. EV6 please see to that all the gene names can be read).
3. On page 10, please better explain TFEBS142A when it is introduced for the first time. In Fig. 5B explain which are the ECs used and what was the level of TFEBS142A expression relative to the endogenous TFEB level.
4. It is interesting that the increased VEGFR2 expression was associated with apparently dysfunctional signalling. Is the pVEGFR2 blot shown in Fig. 6A done on the surface-enriched pool or the total pool? Please show data on both pools.
5. In Fig. 7F, please show the Rab4/VEGFR2 colocalization stainings.
6. The schematic illustration in Fig. 8 and the legend indicate increased VEGFR2 on the surface as a consequence of Tfeb regulation of Myo1c. This is confusing - Tfeb-deletion results in increased Myo1c which should lead to increased VEGFR2 internalization and reduced cell surface localization. Please go through.

Minor
7. Page 7, tamoxifen not tamoxifene.
8. In Fig. 6Aiii, it would be more relevant to show pYVEGFR2/cell surface VEGFR2
9. For Fig 6A, the authors describe that the PM levels of VEGFR2 were more slowly internalized in response to VEGF in the sh-TFEB cells than in wt and that the "peak" of clearance was delayed. It's unclear to me how the authors define the peak - I see a gradual disappearance of VEGFR2.
10. Page 15, last line; here the authors have to write "surface VEGFR2" and not "membrane VEGFR2" as obviously total VEGFR2 is also membrane-bound.

Referee #2:

In this study, the authors examine a non-canonical role of TFEB on VEGFR2 expression and function. The authors identify TFEB in vitro as a negative regulator of 1) VEGFR2 expression via modulating DLEU2 and mir-15a/16-1 expression and 2) VEGFR2 externalization via inhibiting Myo1C expression.

The study has interesting novel aspects to it, in particular the regulation of VEGFR2 by TFEb. Some key data are lacking however, and some technical aspects are murky.

Main points

1. The statement in the abstract 'Using loss of function TFEB mutants, we show defects in fetal and newborn mouse vasculature caused by increased VEGFR2 levels and impaired signaling, resulting in reduced endothelial proliferation' is not supported - ie there is no way of knowing from the data whether increased VEGFR2 had anything to do with the vascular defects (data is correlative).

2. Is elevated VEGFR2 normalized by miR inhibition in vivo ? This would also help point #1

3. Glaringly, no data is provided on the canonical role of TFEB, ie activation of the CLEAR pathway and of lysosome biogenesis. The authors should provide data on what happens to lysosome when knocking down or overexpressing TFEB. The more externalization of VEGFR2 data could all be due, for example, to less lysosome biogenesis in shTFEB condition (which, incidently, would be interesting).

4. The authors should provide the data on how cell cycle genes are regulated by TFEB.

5. Figure 2C, EV4D, EV8A-B. Is the impedence recording by XCelligence real time cell analyzer a well established proliferation assay ? Little to no description of the assay is shown. To this reviewer, this assay appears more to be measuring cell confluence and barrier function than proliferation. Authors should provide regular proliferation assay data with growth curve by cell counting.

6. Figure 4A and 5A. In Figure 4A, shTFEB leads to elevated VEGFR2. But in Figure 5D, blocking miRs doesn't elevate VEGFR2 at baseline. At face value, this indicates that TFEB is regulating VEGFR2 via a different mechanism than miR (since blocking miR does not recapitulate blocking TFEB). This problem needs to be addressed.

7. The TFEB regulating VEGFR2 via miRs seems to be true only when the authors aberrantly activate TFEB by overexpressing constitutive active mutant TFEB. Can authors show for example whether Torin treatment in normal culture leads to the same phenotype as TFEB S142A OE on DLEU2, miRs, VEGFR2 expression ? Otherwise, much of the data may reflect non-physiological consequences of super-high activation.

8. Figure 5. Can authors show miR-15a/16-1 are regulated by DLEU2 by knocking down DLEU2 ?

Further issues

1. Figure 1C. Quantification on vascular defect is not provided.

2. Figure 1D. are CDH5-CreERT2 TFEB mice viable growing to adulthood ? Data should be provided on viability, health, etc - critical components to interpreting the reported findings.

3. Figure 2A. How do we know the ki67+ cells are actually endothelial cells ? can the authors show Ki67+ ECs by co-staining with for example ERG ?

4. Figure 4. Not clear what the authors mean by topology of VEGFR2 in the legend?

5. Figure 5A. Description on each group 'IgG NI, TFEB NI, IgG I, TFEB I' is missing.

Referee #3:

This study uses TFEB loss-of-function mutant mice to address the role of this transcription factor in the vasculature of embryos and new-born animals. The authors show that TFEB regulates VEGFR2 expression and trafficking. Specifically, TFEB transactivates the miR-15a/16-1 cluster, causing a reduction in the stability of the VEGFR2 transcripts. TFEB also downregulates the expression of MYO1C, preventing delivery of VEGFR2 to the cell surface. Absence of TFEB results in increased accumulation of VEGFR2 at the plasma membrane and abnormal VEGF-induced signaling.

This study is relevant because it provides mechanistic information to explain the previously reported observation that the embryonic vasculature is unable to invade the placenta in TFEB KO mice, causing early embryonic lethality.

Comments and suggested improvements:

Major points:

1) One concern with this study is that the consequences of TFEB depletion on VEGFR2 signaling in vivo remain unclear. In addition, the increased VEGFR2 levels at the cell surface in shTFEB cells is inconsistent with the reduced signaling. The authors suggest that this may be due to the reduced co-localization of VEGFR2 with caveolin in TFEB-depleted cells, but the data are not convincing. The quality of the immunofluorescences shown in Figure 6C is low, the images do not reflect the proposed increase in surface and total VEGFR2 levels in shTFEB cells, and it is difficult to observe distinct puncta, making it difficult to determine the degree of co-localization between CAV1 and VEGFR2. The degree of co-localization between CAV1 and VEGFR2 in control and TFEB-depleted cells could be better assessed by performing OptiPrep cellular fractionation.

Finally, the differences in VEGF-induced signaling in control and shTFEB cells seems minimal. Despite the quantification shown in Fig EV7A, the amount of phospho-PLC and phospho-Erk1/2 is very comparable after 20-30 min stimulation. It is, therefore unclear whether these small differences will have a physiological relevance in vivo or if the contribution of TFEB to EC proliferation may play a much more important role.

2) As mentioned by the authors in the discussion, the role of TFEB in VEGFR2 trafficking is poorly characterized. It is suggested that increased MYO1C levels in TFEB-depleted cells increases VEGFR2 recycling to the plasma membrane. However, there are problems with this interpretation: 1) VEGFR2 recycling is only monitored by measuring co-localization with Rab4 and the corresponding images are not shown; 2) internalization experiments done in the presence of primaquine (an inhibitor of recycling) showed reduced VEGFR2 endocytosis in shTFEB cells (Fig 6D) but MYO1C silencing does not affect VEGFR2 internalization (Fig EV7G); 3) VEGFR2 total levels are the same in shTFEB and shTFEB/shMYO1C cells (Fig 7C), suggesting that the effect of TFEB depletion on VEGFR2 levels is not due to altered MYO1C-mediated trafficking.

3) The authors performed transcriptome analysis of control and shTFEB ECs but it would have been more informative to analyze gene expression in these cells in response to VEGF.

4) Several experiments compare different parameters in control versus TFEB142A-expressing cells. However, TFEB142 expression requires induction with doxycycline for 24h. Were the control cells also treated with doxycycline as control? Were the control cells also transduced with a control pTRIPZ lentivirus?

5) The study does not provide any information on the mechanism of regulation of TFEB in ECs. Does endogenous TFEB constitutively localizes to the nucleus in ECs? Is TFEB activated in response to angiogenic factors? Also, does TFEB regulates VEGFR2 and MYO1C expression in other cell types, for example in response to starvation or exercise?

Additional points:

- According to Fig 2C, TFEB silencing completely inhibits proliferation of lung ECs. What is the consequence of this inhibition in vivo?

- In Fig 3B, please specify which GO categories are up-regulated and which ones down-regulated

- How efficient is TFEB depletion after Cre induction? Please confirm TFEB depletion in mouse ECs by western-blot.

- It is difficult to see the increased VEGFR2 levels in the images shown in Figure 4E, please show separate colors (VEGFR2 in green and iB4 in red).

- Is the trafficking of other MYO1C-dependent cargo affected in TFEB-depleted cells?

- Shouldn't miR-inhb15a-5p and miR-inhb16-5p induce VEGFR2 upregulation in control cells (Fig 5D)?

- Include a table showing the 502 differentially expressed genes in shTFEB ECs as Supplementary Material, including p values and p adjusted values.

- It was reported that VEGF stimulation induces transport of VEGFR2 from Golgi to CAV1-enriched domains at the plasma membrane (Tiwari et al., 2013). Is this process impaired in TFEB-depleted cells? Also, is Src phosphorylation in response to VEGF reduced in TFEB negative cells?

Author Correspondance 30th October 2017

Attached you will find a document explaining what we can do to reply to the referees' criticisms. Most of them are very useful and addressable in a fine time frame. Some others are questionable and I have explained my opinions.

If possible I'd appreciate to know if the proposed experiments and replies could render the MS suitable for a further submission.

Editor Correspondance 3rd November 2017

Many thanks again for your message and the constructive outline provided. I think many of the points the referees raised will be well addressed by what you propose. However, I think that the causality issue remains the weak point, even if the work is revised as outlined. If you nevertheless achieve to better address this aspect, I'll be happy to look at a revised manuscript in the future and to involve the same referees again. I would need strong support from them on such a revised version though.

Referee #1:

Major comments

Doronzo et al describe the features of Tfeb, a transcription factor expressed in the developing vasculature and in pericytes/smooth muscle cells Tfeb-mediated deletion gave rise to embryonic lethality accompanied by vascular abnormalities. Postnatal Cdh5-mediated deletion of Tfeb showed a relatively strong phenotype in retinal and kidney vascular development. A dominant phenotype was the reduced proliferation of endothelial cells in the retina and kidney vasculature. In contrast, migration in vitro was not affected by Tfeb deletion. Through transcript analyses on Tfeb-silenced cells, the authors identified changes in GO:s related to angiogenesis, cell cycle etc. ChiP-Seq analyses from cells overexpressing an active variant of Tfeb showed associatation of Tfeb with promoters of transcription factors including Jun, Fos, Myc, Gata2, Max, of which the majority but not all contained the canonical Tfeb-binding site. One primary target gene was VEGFR2 which however lacked the canonical Tfeb promoter binding site.
Expression of VEGFR2 in Tfeb-deficient cells increased considerably in vitro and in vivo, at least in part due to direct Tfeb-regulation of miRNAs 15a-5p and 16-5p. The relative phosphorylation of VEGFR2 was decreased in Tfeb deficient cells, as was downstream signalling in the PLCg and Erk pathways. The authors moreover identified Myo1c as a Tfeb target gene and showed upregulation of Myo1c in Tfeb-deficient cells, leading to increased clearance of VEGFR2. Thus TFEB regulate VEGFR2 expression levels in many different ways, through other transcription factors, miRNA and internalization/trafficking in a Myo1C-dependent manner. This is an ambitious study with novel information on the many aspects of regulation of VEGFR2 expression and trafficking and the role in vessel biology.

I thank this referee for his/her precise and proper analysis of our results. As noted, the dominant phenotype observed in vitro and in vivo was the reduced proliferation of endothelial cells, without any significant effects on cell motility. This observation together other helpful criticisms/suggestions, suggested to re-consider some points of our results. In particular we better interrogated the data sets of gene expression and ChiPseq performed in TFEB manipulated endothelial cells. This data reassessment, demonstrated that TFEB directly promoted the expression of CDK4, which is a master gene of the molecular machinery regulating G1-S transition of cell-cycle and thus explains the observed proliferative defects. Of note the literature data demonstrate that the in vivo deletion of the cell-cycle genes down-modulated in TFEB silenced endothelial cells do not result in any vascular defects. I postulated that the effect on VEGFR2 reported in Tfeb null mice could represent a compensatory response, even if futile, to the block of cell proliferation. The integrated effect between the reduced cell proliferation and the impairment of VEGFR2 behaviour results in the vascular phenotype observed.

1. Throughout please define n. Now for example in Fig. 2B and in Fig. 4D, the authors give n=6. Is this 6 vessels or 6 mice?

When required we carefully defined in the text “n=”.

2. Page 9, the authors perform comparative transcript analysis on siRNA-treated cells.
Why not instead isolate endothelial cells from their mouse models to do these analyses?

We decide to use human cells instead of murine cells from KO mice to obtain enough amount of mRNA for Illumina microarray and to validate the transcripts of interest by qPCR. Having used tiny mice (P17) for each assay, the gene expression analysis would require approximately 30 mice per each genotype and additional animals would be needed for validations. Furthermore the transcriptional analysis performed on silenced human EC allowed us to define candidates, which are well conserved across species. Moreover the main targets (including TFEB, cell cycle related genes, DLEU2, MYO1C, VEGFR2) identified by genome analyses in human cells were validated in lung ECs isolated from control and Tfeb KO mice (*Tfeb*$^{iEC-/-}$) (Figures EV2, 5A, 5B, 5C, 5D, 7E, 7F, 8B, EV4D, EV8B).

This would allow a global analysis to replace the siRNA-cell analysis + manual annotation shown in Figs. 3 and EV6 (if the authors keep Fig. EV6 please see to that all the gene names can be read).

According to the new paper setting, we decided to delete this figure because it was less relevant.

3. On page 10, please better explain TFEBS142A when it is introduced for the first time.

We specify it in the text-body (page 10, lines 24-26) and also in material and methods paragraph " Cell, genetic manipulation and biological assay" page 29, lines 25-26 and page 30, lines 1-5.

In Fig. 5B (probably EV5B) explain which are the ECs used and what was the level of TFEBS142A expression relative to the endogenous TFEB level.

We specify in the text-body that we overexpressed TFEBS142A in human ECs (in the text-body page 10, lines 24-26; material and methods paragraph page 29, lines 25-26 and page 30, lines 1-5). In Figure EV5B we quantify the cytosolic and nuclear TFEB quantity as ratio between control and TFEBS142A ECs.

4. It is interesting that the increased VEGFR2 expression was associated with apparently dysfunctional signalling. Is the pVEGFR2 blot shown in Fig. 6A done on the surface-enriched pool or the total pool? Please show data on both pools.

As reported in material and methods (section "Biochemical analysis of PM distribution of VEGFR2") (pages 38-39) and in the results (page 14, lines 5-6*)* PM protein pool was separated from total protein pool using streptavidin-agarose beads as previously described (Napione et al., 2012a).
As show in the present Figure 6C (Figure 6A in the previous version of the manuscript) we analysed the abundance of VEGFR2 both in cell-PM-enriched pool and in total pool while pY1175 VEGFR2 was evaluated only in the total pool as indicated in the figure legend and in the text page 19, lines 16-17.

As suggested by the referee, we tried to evaluate the amount of pY1175 VEGFR2 in PM (biotinylated fraction). As shown in the representative figure of immunostaining (Figure 1 of this letter: panel A, experiments n=5) we detected only a weak and not quantifiable signal of pY1175 VEGFR2 in PM samples respect the signal that we obtained with total pool samples. Therefore it seems very difficult to present consistent results.
To improve these results and avoid putative technical issues intrinsic to biotin assay, we repeated the experiments by using a commercial kit ("Plasma Membrane, Protein extraction kit", Abcam) able to separate PM from whole cells. In our hands this technique did not improve the quality of the experiment.

As evidenced in the representative immunoblotting (Figure 1 of this letter: panel B, experiments n=5), this approach was unable to give clear signals. The provided buffers have an unknown composition and I can speculate that they do not protect proteins from the phosphatase activity.
Because we aren't confident on these results despite the number of the replicates (n=5), we decided to not insert this result in the new version of the manuscript. However I'm convinced that the observed alteration of pY1175-VEGFR2 in the total pool together the other signalling alterations reported (See Figure 6C) in sh-TFEB ECs, support the concept that in absence of TFEB signalling activities of VEGFR2 are impaired.
I respectfully note that many other studies have used this technical strategy.

5. In Fig. 7F, please show the Rab4/VEGFR2 colocalization stainings.

According to thus suggestion, we introduced the Rab4/VEGFR2 colocalization staining (Figure EV7C) (page 16, lines 10-11). The data indicate that TFEB silencing greatly increased the colocalization of VEGFR2 with Rab4 positive vesicles. This effect is mediated by TFEB-MYO1C modulation. As described in the same section, MYO1C-silencing decreased the amount of VEGFR2 co-localized with $Rab4^+$ vesicles (Page 16, lines 25-26, Figure EV7C). This data suggested that MYO1C deletion reduces the transport of the receptor to $Rab4^+$ endosome involved in its exocytosis. This result matches the observation that MYO1C silencing in sh-TFEB ECs reduced specifically the amount of PM VEGFR2 (see Figures 7D,7G and EV7D) (page 17, lines 1-5).

6. The schematic illustration in Fig. 8 and the legend indicate increased VEGFR2 on the surface as a consequence of Tfeb regulation of Myo1c. This is confusing - Tfeb-deletion results in increased Myo1c which should lead to increased VEGFR2 internalization and reduced cell surface localization. Please go through.

On the basis of the new results obtained we decided to delete this figure.

Minor

7. Page 7, tamoxifen not tamoxifene.
We modified in the text body.

8. In Fig. 6Aiii, it would be more relevant to show pYVEGFR2/cell surface VEGFR2.
We discussed this issue in point 4.

9. For Fig 6A, the authors describe that the PM levels of VEGFR2 were more slowly internalized in response to VEGF in the sh-TFEB cells than in wt and that the "peak" of clearance was delayed. It's unclear to me how the authors define the peak - I see a gradual disappearance of VEGFR2.

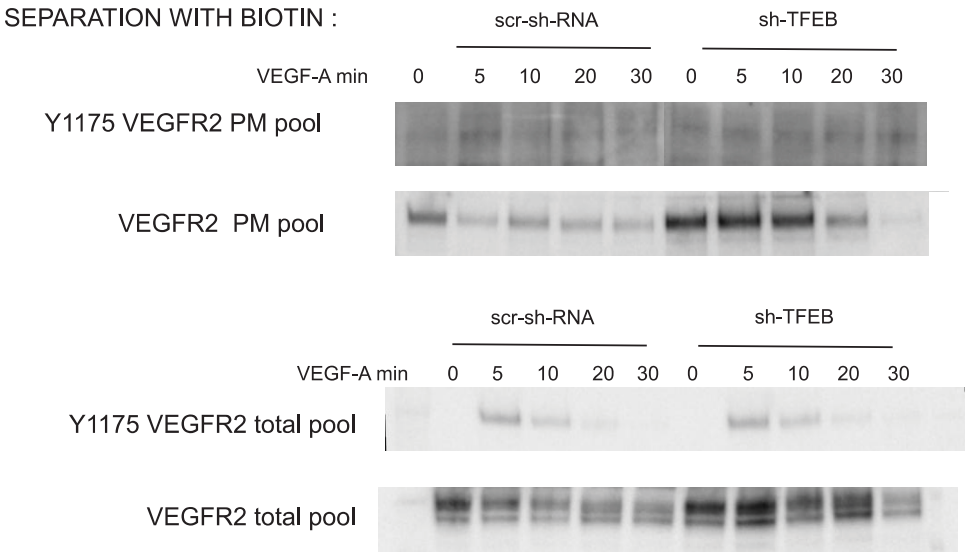
I agree with the referee's note and this noun was deleted in the description of Figure 6C (page 15, lines 4-12).

10. Page 15, last line; here the authors have to write "surface VEGFR2" and not "membrane VEGFR2" as obviously total VEGFR2 is also membrane-bound.

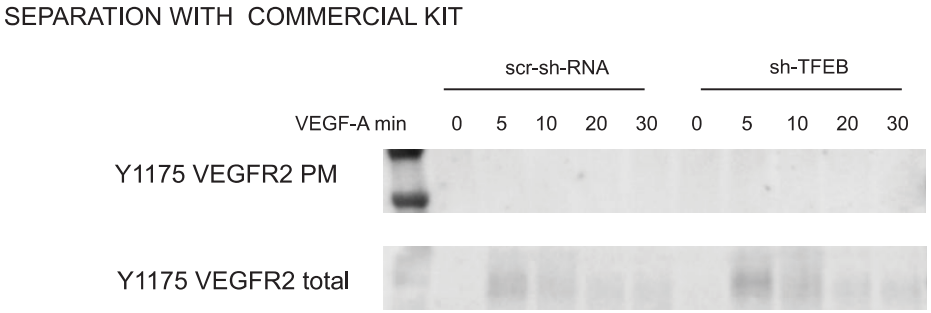
In the present version of the MS we well considered this point through the text.

Referee #1: Figure 1

Panel A



Panel B



Panel A. Representative figure of immunostaining (experiments n=5) of pY1175 VEGFR2 in PM samples and total pool samples obtained with biotin separation from scr-shRNA and sh-TFEB ECs after VEGF-A stimulation (30 ng/ml, 0-30 min).

Panel B. Representative figure of immunostaining (experiments n=5) of pY1175 VEGFR2 in PM samples and total pool samples obtained with commercial kit (“Plasma Membrane, Protein extraction kit”, Abcam) from scr-shRNA and sh-TFEB ECs after VEGF-A stimulation (30 ng/ml, 0-30 min).

Referee #2

In this study, the authors examine a non-canonical role of TFEB on VEGFR2 expression and function. The authors identify TFEB in vitro as a negative regulator of 1) VEGFR2 expression via modulating DLEU2 and mir-15a/16-1 expression and 2) VEGFR2 externalization via inhibiting Myo1C expression.
The study has interesting novel aspects to it, in particular the regulation of VEGFR2 by TFEB. Some key data are lacking however, and some technical aspects are murky.

I thank this referee for his/her useful comments. The current version has tried to clarify the technical murkiness and to add the required new data. Stimulated by referees' comments and by the observation that the dominant phenotype observed in vitro and in vivo was the reduced proliferation of endothelial cells, without any significant effects on cell motility, I have re-considered some meanings of our results. In particular we better interrogated the data sets of gene expression and ChiPseq performed in TFEB manipulated endothelial cells. This data reassessment, demonstrated that TFEB directly promoted the expression of CDK4, which is a master gene of the molecular machinery regulating G1-S transition of cell-cycle and thus explains the observed proliferative defects. Of note, the literature data demonstrate that the in vivo deletion of the cell-cycle genes down-modulated in TFEB silenced endothelial cells do not result in any vascular defects. I postulated that the effect on VEGFR2 reported in Tfeb null mice could represent a compensatory response, even if futile, to the block of cell proliferation. The integrated effect between the reduced cell proliferation and the impairment of VEGFR2 behaviour results in the vascular phenotype observed. Therefore, I kindly ask you to read the replies to your comments according to this new set of the paper.

Main points

1. The statement in the abstract 'Using loss of function TFEB mutants, we show defects in fetal and newborn mouse vasculature caused by increased VEGFR2 levels and impaired signaling, resulting in reduced endothelial proliferation' is not supported - ie there is no way of knowing from the data whether increased VEGFR2 had anything to do with the vascular defects (data is correlative).
The abstract has been completely re-written.

4. The authors should provide the data on how cell cycle genes are regulated by TFEB.

According to the indication of the referee we investigated the role of TFEB in the regulation of cell cycle genes related to support the alteration of endothelial proliferation both in vivo than in vitro.
As described in "TFEB modifies the transcriptional landscape in ECs" and "The CDK4 gene is a direct target of TFEB" paragraphs and in figures 3 and 4 we improved the transcriptomic analysis on human sh-TFEB ECs and we evaluated a strong correlation between TFEB silencing and the down regulation of cell cycle genes. In particular we described that after the silencing of TFEB in ECs both in vivo than in vitro, there is a down-regulation of genes involved in the transition of G1-S phase of the cell cycle (Figures 4A, 4B, 4C and 4D). TFEB is also able to directly regulate CDK4 transcription and expression through the binding of its promoter (Figures 4E, 4F and 4G). Consistently to the down regulation of CDK4, we demonstrated a reduction of the phosphorylation of its substrate, the Rb protein (Figure 4H).

2. Is elevated VEGFR2 normalized by miR inhibition in vivo ? This would also help point #1

We demonstrated that TFEB regulates the expression of DLEU-2 and its intragenic miR-15a/16-5p, which negatively control VEGFR2 mRNA. Correctly, the referee suggests to perform an in vivo rescue experiment with exogenous miR16 in Tfeb KO mice or to mimic the effect of Tfeb deletion by treating wild-type mice by an antagomir.
We think that these experiments are in principle feasible in mouse eye by intravitreal injection. This approach excludes the bio-distribution problems related to i.v. injection but it's limited by the narrow temporal window related to the post-natal maturation of the retinal vasculature as well as by the pharmacokinetics of the antagomir. Therefore this experiment requires a huge effort for setting doses and times and in my opinion is far from the principal meaning of the present version of the manuscript.
However experiments performed in our laboratory by Alessio Noghero and Stefania Rosano related to another project can be useful to reply to this comment and the data are here confidentially reported. By analysing the transcriptome by RNAseq in a 3D model of sprouting angiogenesis, in which spheroid of endothelial cells are embedded in fibrin and stimulated by VEGF (Figure 1 of this letter), we demonstrate that the expression of miR-15a/16-1 declined after 6 hours. This is consistent with a hypothesis that at the early phase of angiogenesis more VEGFR2 transcript is required. Therefore we performed experiments by treating the VEGF-stimulated 3D spheroids with mir16 agonist or inhibitor without any modification of the phenotype (Figure 2 of this letter). We hypothesize that compensatory effects occur when this miR is exogenously modulated. This hypothesis is supported by the whole analysis of differentially expressed miRs and gene coding RNAs in VEGF stimulated and unstimulated-spheroids (Figure 3 of this letter). This figure shows that many miRs (179) are modulated in sprouting angiogenesis and poorly linked to the 600 genes differentially expressed and belonging the following GO: Cell migration, cell proliferation and cell metabolism.

However, we tried to reproduce in vitro the suggested experiment focusing on the possibility to rescue the effect of TFEB deletion on EC proliferation. As show in figures 8E and EV8, the transduction of the specific mir-mimic-15a-5p or mir-mimic-16-5p correlated with the down-regulation of VEGFR2 transcription and expression in scr-shRNA and in sh-TFEB ECs (Figures 8D and 8E). According to the more general effect of TFEB deletion on genes regulating cell growth, the delivery of both mir-mimics was unable to overcome the effect deletion on VEGFA-mediated cell proliferation (see paragraph "TFEB deletion up-regulates VEGFR2 by inhibiting mir15a/16-1-dependent post-transcriptional regulatory mechanisms" and figure EV8C).

3. Glaringly, no data is provided on the canonical role of TFEB, ie activation of the CLEAR pathway and of lysosome biogenesis.

4. The authors should provide data on what happens to lysosome when knocking down or overexpressing TFEB. The more externalization of VEGFR2 data could all be due, for example, to less lysosome biogenesis in shTFEB condition (which, incidently, would be interesting).

As described in "TFEB deletion alters VEGFR-2 membrane trafficking" paragraph and in figures 6D and 6E we investigated the role of TFEB in the regulation of CLEAR pathway and autophagic/lysosome biogenesis.

5. Figure 2C, EV4D, EV8A-B. Is the impedence recording by XCelligence real time cell analyzer a well established proliferation assay ? Little to no description of the assay is shown. To this reviewer, this assay appears more to be measuring cell confluence and barrier function than proliferation. Authors should provide regular proliferation assay data with growth curve by cell counting.

We remove the proliferation experiments executed by XCelligence real time cell analyzer. ECs proliferation has now been evaluated as proliferation rate and DNA content (page 9, lines 10-19; page 30 lines 21-25 and page 31 lines 1-2) by using Click-iT® EdU Flow Cytometry Cell Proliferation Assay (ThermoFisher Scientific) and propidium iodide (PI) staining according to manufacturer protocol. Data were acquired with a CyAn ADP flow cytometer (Dako) and analysed with FlowJo software (Tree Star, Ashland, OR, USA).

6. Figure 4A and 5A. In Figure 4A, shTFEB leads to elevated VEGFR2. But in Figure 5D, blocking miRs doesn't elevate VEGFR2 at baseline.
At face value, this indicates that TFEB is regulating VEGFR2 via a different mechanism than miR (since blocking miR does not recapitulate blocking TFEB). This problem needs to be addressed.

We improved the number of the experiments (from n=3 to n=6) to reduce their variability. As shown in figures 8F-8G after the transduction of mir-inibitor 15a-5p and mir-inhibitor 16-5p we evaluated a significant increase of the transcription/protein expression of VEGFR2 both in control ECs than in TFEBS142A ECs (page 19, lines 3-7).

7. The TFEB regulating VEGFR2 via miRs seems to be true only when the authors aberrantly activate TFEB by overexpressing constitutive active mutant TFEB. Can authors show for example whether Torin treatment in normal culture leads to the same phenotype as TFEBS142A OE on DLEU2, miRs, VEGFR2 expression ? Otherwise, much of the data may reflect non-physiological consequences of super-high activation.

I believe that the suggested experiments with Torin could give confusing results because this compound is not specific for TFEB but it is a mTOR modulator, which a has wide spectrum of activities. However, I agree with this referee that the loss- (LOF) and gain-of-function (GOF) approaches here used not necessarily reflect a physiologic conditions. However, I respectfully underline that the combined interpretation of GOF and LOF data supports a physiologic role of this post-transcriptional regulatory system. This interpretation is supported by the following data: i) the expression of miR 15a-5p and 16-5p as well as DLEU-2 is respectively up- down- and up-regulated in shTFEB- ECs as well as in lung ECs isolated from Tfeb null mice and in TFEBS142A ECs (Figures 8B, 8C, EV8B); ii) the experiments performed with miR-mimic (Figures 8D, 8E) and miR-inhibitor (Figures 8F, 8G) indicate that VEGFR2 modulation by these compounds occurs both in shTFEB ECs and in TFEBS142A-ECs; iii) TFEB binds DLEU2 promoter and presumably has a physiologic role on the expression of this genes and that of intragenic miR 15a-5p and 15-5p.
In my opinion the referee's comments would be right if the VEGFR2 modulation by miRs had occurred only in TFEBS142A cells, but our data indicate that a specular event occurs also in silenced cells.

However, to be more careful in the data interpretation the sentence in the old version: *These data indicated a direct role for these miRs in the regulation of VEGFR2 by TFEB* has been changed to "These data suggest a direct role for these miRs in the regulation of *VEGFR2* by TFEB" (page 19, lines 8).

8. Figure 5. Can authors show miR-15a/16-1 are regulated by DLEU2 by knocking down DLEU2 ?

As suggested by the referee we analyzed the effect of DLEU2 silencing on the transcription of miR-15a-5p and miR-16-5p in human ECs. We performed a new experiment in which we silenced *DLEU2* by specific sh-RNA both in control and in TFEBS142A ECs (pag 18, lines 18-25). The data reported clearly indicate that

the absence of DLEU2 blocks the activity of TFEBS142A to increase the expression of mir-15a-5p and mir-16-5p .

Further issues

1. Figure 1C. Quantification on vascular defect is not provided.

As we known, there are not available current tools to quantify in vivo vascular alterations that recognize specific morphological patterns. For this reason, we measured the "% point prevalence" of these alterations (similarly to the proportion of a population with a particular condition related to the total population at a specific point in time). At E10.5 (our point time), after endomucin immunostaining of the vessels, embryos with genotypes blindly analysed (n=25) were divided in two groups: "positive embryos" showing vascular alterations and "negative embryos" with absence of vascular defects. Following that, we separated the embryos of the two groups accordingly to their genotype (control n=13; *Tfeb*$^{EC-/-}$ n=12) and the % point prevalence in control or *Tfeb*$^{EC-/-}$ embryos was calculated as :

% Point prevalence = (number of positive embryos/ number positive embryos + number of negative embryos) x100

The data obtained are reported at page 6, lines 10-12).

2. Figure 1D. Are CDH5-CreERT2 TFEB mice viable growing to adulthood ? Data should be provided on viability, health, etc - critical components to interpreting the reported findings.

A specific sentence was added at page 28 (lines 1-7). "The systemic effect of *Tfeb* deletion was evaluated by analyzing hematological and biochemical parameters in blood after 1 month from the Cre induction (p0-p8) in *Tfeb*$^{iEC-/-}$mice. Mice survival was of 32.4±5.9% (mice n=24, p=0.003 vs *control mice*), without any significant modification of renal and hepatic functions. However, the *Tfeb*$^{iEC-/-}$ mice only presented an increase of % reticulocytes (950.5± 200.8% *versus* 516.2 ± 107.1% in control mice; mice n=10; p=0.04) and % platelets (98.25± 27.24% *versus* 31.75 ± 15.3% in control mice; mice n=10; p=0.006).

3. Figure 2A. How do we know the ki67+ cells are actually endothelial cells ? can the authors show Ki67+ ECs by co-staining with for example ERG ?

We better explained that Ki67 expression was just considered in endothelial cells. This result was obtained by considering Ki67+ nuclei only in cells recognized by isolectin iB4, which is a lectin specific for endothelial cells (Legend of figure 2).

4. Figure 4. Not clear what the authors mean by topology of VEGFR2 in the legend?
We modified the term in the text legend.

5. Figure 5A. Description on each group 'IgG NI, TFEB NI, IgG I, TFEB I' is missing.

We modified the legend of the figures 4, 5, 7 and 8. We described that "ChIP was performed using digested chromatin from control ECs and TFEBS142A ECs incubated with IgG (indicated in the bar graph as "+IgG") or with Ab anti-TFEB (indicated in the bar graph as "+Ab anti-TFEB") , followed by qPCR for ..." Also we

specified in the “Material and methods” paragraph “ChIP” that “Samples treated with IgG were used as a negative control” (page 36, lines 5-6).

Referee #2: Figure 1

Figures for Referees not shown.

Referee #2: Figure 2

Figures for Referees not shown.

Referee #2: Figure 3

Figures for Referees not shown.

Referee #3

This study uses TFEB loss-of-function mutant mice to address the role of this transcription factor in the vasculature of embryos and new-born animals. The authors show that TFEB regulates VEGFR2 expression and trafficking. Specifically, TFEB transactivates the miR-15a/16-1 cluster, causing a reduction in the stability of the VEGFR2 transcripts. TFEB also downregulates the expression of MYO1C, preventing delivery of VEGFR2 to the cell surface. Absence of TFEB results in increased accumulation of VEGFR2 at the plasma membrane and abnormal VEGF-induced signaling. This study is relevant because it provides mechanistic information to explain the previously reported observation that the embryonic vasculature is unable to invade the placenta in TFEB KO mice, causing early embryonic lethality.

I thank this referee for his/her useful and positive comments. Stimulated by referees' comments and by the observation that the dominant phenotype observed in vitro and in vivo was the reduced proliferation of endothelial cells, without any significant effects on cell motility, I have re-considered some meanings of our results. In particular we better interrogated the data sets of gene expression and ChiPseq performed in TFEB manipulated endothelial cells. This data reassessment, demonstrated that TFEB directly promoted the expression of CDK4, which is a master gene of the molecular machinery regulating G1-S transition of cell-cycle and thus explains the observed proliferative defects. Of note, the literature data demonstrate that the in vivo deletion of the cell-cycle genes down-modulated in TFEB silenced endothelial cells do not result in any vascular defects. I postulated that the effect on VEGFR2 reported in Tfeb null mice could represent a compensatory response, even if futile, to the block of cell proliferation. The integrated effect between the reduced cell proliferation and the impairment of VEGFR2 behaviour results in the vascular phenotype observed.

Therefore, I kindly ask you to read the replies to your comments according to this new set of the paper.

Major points:

1) One concern with this study is that the consequences of TFEB depletion on VEGFR2 signaling in vivo remain unclear. In addition, the increased VEGFR2 levels at the cell surface in shTFEB cells is inconsistent with the reduced signaling.

The authors suggest that this may be due to the reduced co-localization of VEGFR2 with caveolin in TFEB-depleted cells, but the data are not convincing. The quality of the immunofluorescences shown in Figure 6C is low, the images do not reflect the proposed increase in surface and total VEGFR2 levels in shTFEB cells, and it is difficult to observe distinct puncta, making it difficult to determine the degree of co-localization between CAV1 and VEGFR2. The degree of co-localization between CAV1 and VEGFR2 in control and TFEB-depleted cells could be better assessed by performing OptiPrep cellular fractionation.

To improve the quality of the pictures we repeated the immunofluorescence experiments and acquired the relative images with the Leica TCS SP8 Confocal, confocal laser scanning microscopy platform with high photon efficiency. I think that the quality of the images are improved and are now showed in figure EV6B. Therefore we didn't perform experiments with OptiPrep cellular fraction technique.

Finally, the differences in VEGF-induced signaling in control and shTFEB cells seems minimal. Despite the quantification shown in Fig EV7A, the amount of phospho-PLC and phospho-Erk1/2 is very comparable after 20-30 min stimulation. It is, therefore unclear whether these small differences will have a

physiological relevance in vivo or if the contribution of TFEB to EC proliferation may play a much more important role.

Of note, several studies performed with VEGFR2 inhibitors clearly demonstrate that an evident reduction of EC proliferation triggered by VEGF-A does not require the complete inhibition of VEGFR2 phosphorylation (i.e. Cancer Res 62, 381,2002; Mol Canc Ther 6, 2012, 2007; Cancer Res 60, 4152, 2000; Cancer Res 60, 2178,2000; Cancer Res 66,9134,2006). For instance the VEGFR2 inhibitor AZD2171 shows a IC50 of VEGF-A-stimulated receptor phosphorylation and VEGF-A stimulated EC proliferation respectively of 5 and 4 nM (Cancer Res 65, 4389, 2005) roughly indicating that when the receptor is still phosphorylated, proliferation is clearly reduced. As indicated in figure 6, in sh-TFEB ECs the decrease of VEGFR2 phosphorylation (Figure 6C) is also correlated with a decrease of its internalization (Figure 6F) that is strongly involved in the activation of VEGFR2 downstream signalling. So the defect of VEGFR2 signalling after *TFEB* silencing is the result of the combination of different alterations.

2) As mentioned by the authors in the discussion, the role of TFEB in VEGFR2 trafficking is poorly characterized. It is suggested that increased MYO1C levels in TFEB-depleted cells increases VEGFR2 recycling to the plasma membrane. However, there are problems with this interpretation:

2.1. VEGFR2 recycling is only monitored by measuring co-localization with Rab4 and the corresponding figure was not shown.

In the present version of the MS we showed the VEGFR2/Rab4 colocalization (Figure EV7C, page 16, lines 10-11). The analysis of VEGFR2 recycling here reported is in agreement with other studies (e.g. Jopling et al., Cell 2014) which clearly report that Rab4+ vesicles represent the main rout of the VEGFR2 exocytosis.
Furthermore, I respectfully underline that exocytic trafficking was further analysed by the co-staining between CAV-1 (page 15, lines 13-20; Figure EV6B) and TGN46 (page 16, lines 6-11; Figures EV6B, EV6D), which is a Golgi marker.

2.2 Internalization experiments done in the presence of primaquine (an inhibitor of recycling) showed reduced VEGFR2 endocytosis in shTFEB cells (Fig 6D) but MYO1C silencing does not affect VEGFR2 internalization (Fig EV7G);

As previously described (Valdembri, Caswell et al., 2009) we performed the internalization experiments in the presence of primaquine, a specific inhibitor of exocytosis, to overcame the recycling of the receptor via exocytosis after its internalization. So, in this way it is possible to analyse just the “entry” of the protein.

To accomplish the referee’s comments we analysed endocytosis with a second technique (Antibody feeding assay , Gourlaouen M et al. M, 2013), which excludes the use of primaquine (see page 40, lines 1-6). This system also confirms the inhibition of VEGFR2 internalization in sh-TFEB ECs stimulated by VEGF-A (Figure EV6C).

About the observation that the MYO1C silencing did not affect VEGFR2 internalization (Figure EV7F), our data confirmed a previous observation done on EGFR (doi:10.4161/15548627.2014.984272). In this report

the authors demonstrated that the silencing of MYO1C correlated with the inhibition of autophagosome-lysosome fusion, but it did not inhibit EGFR induce internalization and trafficking.

2.3. VEGFR2 total levels are the same in shTFEB and shTFEB/shMYO1C cells (Fig 7C), suggesting that the effect of TFEB depletion on VEGFR2 levels is not due to altered MYO1C-mediated trafficking.

As reported in the text, Tiwari and colleagues (doi:10.1152/ajpheart.00744.2012) suggested that in the presence of VEGF-A stimulation, MYO1C modified the surface and the total level of VEGFR2 protein, without affecting the gene transcription. They reported that after VEGF stimulation (30 min) and in the absence of MYO1C, VEGFR2 appeared to move from plasmamembrane to lysosomes where it was processed for degradation.
I respectfully invite to note that this experimental setting differs from that here exploited. In our experiments we analyzed the effect of MYO1C silencing in sh-TFEB ECs cultured in normal conditions (20% FCS without any starving procedure). In this condition, the silencing of MYO1C was able to reproduce the reduction of VEGFR2 at plasmamembrane (Figures 7D, 7G, EV7D) as reported by Tiwari and colleagues. However the lack of effect of MYO1C silencing in sh-TFEB ECs on the total amount of VEGFR2 is not surprising because the impairment lysosome pathway in these cells results in an reduced protein degradation (see Figures 6C, 6D, 6E). Another possible explanation for the difference between our paper and Tiwari's results could be the different endothelial stimulation (see Figure 4 of Tiwari paper) and the time of the analysis after stimulation.

3) The authors performed transcriptome analysis of control and shTFEB ECs but it would have been more informative to analyze gene expression in these cells in response to VEGF.

In this work we aimed at evaluating the TFEB mediated gene modulation in vasculature to explain the vascular phenotype observed in EC-Tfeb mutant mice, where ECs are stimulated through the cooperation of more angiogenic inducers. Therefore we compared control and TFEB mutant cells. Stimulation with VEGF would have caused the activation of genes not directly modulated by TFEB itself.

4) Several experiments compare different parameters in control versus TFEB142A-expressing cells. However, TFEB142 expression requires induction with doxycycline for 24h. Were the control cells also treated with doxycycline as control? Were the control cells also transduced with a control pTRIPZ lentivirus?

This issue was better explain in the text-body (page 29, lines 25,26; page 30, lines 1-5). In particular control cells were also infected with the pTripz-TFEBS142A virus but they were not stimulated with doxycycline. Control experiments were carried out on not infected cells to evaluate non-specific effects due to stimulation with doxycycline. These data are reported in figure 1 of this letter.

5) The study does not provide any information on the mechanism of regulation of TFEB in ECs.
Is TFEB activated in response to angiogenic factors?

I agree that our study does not provide any information on the mechanisms of regulation of TFEB in ECs. We are actively working on this topic, but I believe that these experiments are not necessary for the major

aim of this paper. For this referee we evaluated the effect of VEGFA in inducing TFEB nuclear translocation in ECs, without any evident effect. We attached the figure 2.

Does endogenous TFEB constitutively localizes to the nucleus in ECs?
At page 9, lines 5-10 we reported that in EC standard culture conditions TFEB showed a cytosolic and nuclear localization. In the presence of Torin, a mTor inhibitor mimicking starving conditions , we observed an increase of its nuclear translocation (Figure EV4A).

Also, does TFEB regulates VEGFR2 and MYO1C expression in other cell types, for example in response to starvation or exercise?
I respectfully observe that this kind of analysis is very interesting but far of the principal goal of the MS focused on the role of Tfeb in vascular development .

Minor criticism:

- According to Fig 2C, TFEB silencing completely inhibits proliferation of lung ECs. What is the consequence of this inhibition in vivo?
We investigated the phenotype of the lung vessels in control and *Tfeb*$^{iEC-/-}$ mice (P17) after Cre induction at p1. As shown in figure 3 of this letter, the number of the vessels stained by CD31 is modestly reduced in *Tfeb*$^{iEC-/-}$ mice respect the control mice. No other alterations were observed.

- In Fig 3B, please specify which GO categories are up-regulated and which ones down-regulated
In figure 3B we introduced this data.

- How efficient is TFEB depletion after Cre induction? Please confirm TFEB depletion in mouse ECs by western-blot.
These data were confirmed by staining Tfeb in renal tissue after Cre induction (Figure EV2D). After Cre activation, TFEB protein was present in vessels and in other tissues of control mice. On the contrary in *Tfeb*$^{iEC-/-}$ mice TFEB protein was maintained in all tissue but not in vasculature confirming the efficiency and the cell specificity of the induction (Figure EV2E) (page 7, lines 10-12).

- It is difficult to see the increased VEGFR2 levels in the images shown in Figure 4E, please show separate colours (VEGFR2 in green and iB4 in red).

Figure 5B was modified according to this suggestion.

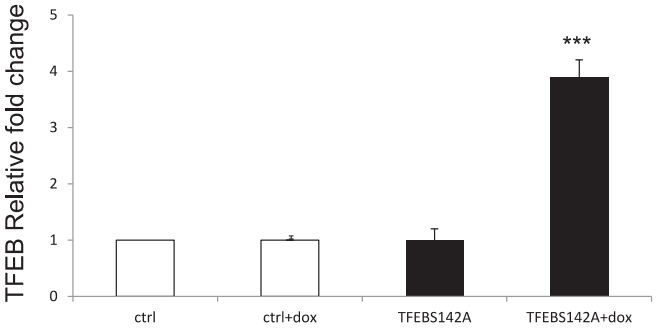
- Is the trafficking of other MYO1C-dependent cargo affected in TFEB-depleted cells?
We investigated the cell distribution of CAV-1 and GLUT-1 protein after TFEB silencing. We studied the quantity of PM and total CAV-1 and GLUT-1 protein in scr-shRNA and sh-TFEB ECs. Immunoblotting analysis shown in Figure 4 of this letter evidenced that the PM and total quantity of these proteins were not modified in sh-TFEB respect scr-shRNA ECs .

- Shouldn't miR-inhb15a-5p and miR-inhb16-5p induce VEGFR2 upregulation in control cells (Fig 5D)?
As shown in figure 5D and in the revised manuscript figures 8F and 8G the mir-inhibitors increased both transcript and protein of VEGFR2 gene .

- Include a table showing the 502 differentially expressed genes in shTFEB ECs as Supplementary Material, including p values and p adjusted values.
The data were included as "Table 1".

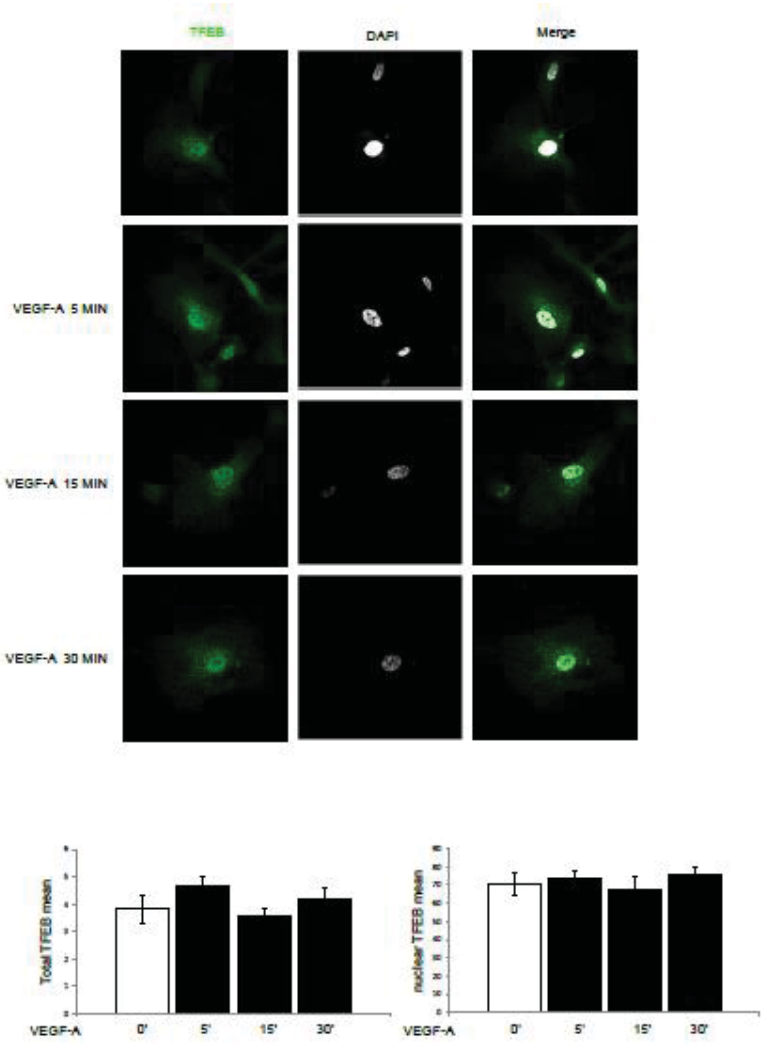
- It was reported that VEGF stimulation induces transport of VEGFR2 from Golgi to CAV1-enriched domains at the plasma membrane (Tiwari et al., 2013). Is this process impaired in TFEB-depleted cells? Also, is Src phosphorylation in response to VEGF reduced in TFEB negative cells?
As decribed in point 2.1 we checked the modulation of the localization of VEGFR2 at Golgi compartment and in CAV-1-enriched domains after the silencing of TFEB. These data are reported in Figures EV6B (page 15, lines 13-20) and EV6D (page 16, lines 6-9).
In figures 6C and EV6A we added the experiments about the modulation of Src and p-Src in scr-shRNA and sh-TFEB ECs. We evidenced a down-regulation of the phosphorylation of the protein in sh-TFEB vs scr-shRNA ECs.

Referee #3: Figure 1



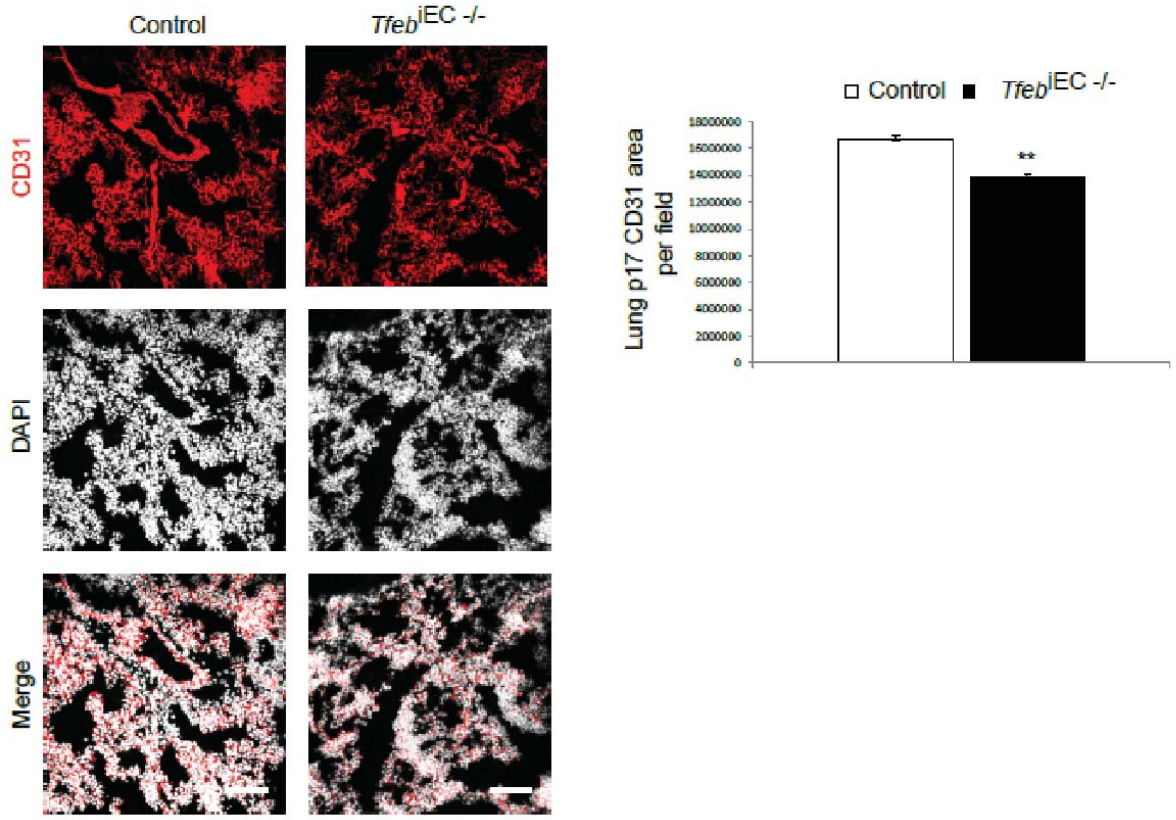
Modulation of TFEB expression in control ECs (Ctrl) and in pTRIPZ-TFEBS142A (TFEBS142A) ECs treated with vehicle and in control ECs (Ctrl+dox) and pTRIPZ-TFEBS142A (TFEBS142A+dox) treated with doxycycline (0.5 ug/ml 24h). Data are expressed as relative fold change compared with the expression in Ctrl ECs after normalization to the housekeeping gene TBP (n=3, mean±SEM; ***p<0.0001, by Student's *t*-test).

Referee #3: Figure 2



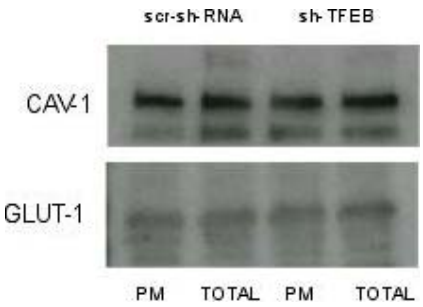
Representative image of human ECs after VEGF-A stimulation (30 ng/ml, 0-30 min) stained with anti-TFEB Ab and DAPI. Bar graphs indicate the nuclear and total mean of TFEB (n=3, ANOVA=ns versus unstimulated cells by Bonferroni post-test).

Referee #3: Figure 3



EC Tfeb deletion compromise lung vessel development at p17. Representative images of immunostaining of vessels of control and Tfeb$^{iEC-/-}$ mice with a anti-CD31 Ab (scale bar: 50 µm). Bar graph indicates the CD31+ vascular area per field in control and Tfeb$^{iEC-/-}$ mice (mice n=5, mean±SEM; **p<0.001 versus control mice by Student's *t*-test).

Referee #3: Figure 4



Representative figures of immunostaining (experiments n=3) of CAV-1 and GLUT-1 in PM samples and total pool samples obtained with biotin separation from scr-shRNA and sh-TFEB ECs.

Thanks for submitting your revised manuscript to The EMBO Journal. My colleague who is the handling editor on your manuscript is away at the moment I have stepped in as 2nd editor on the paper to help move things forward. We have now received the input from the three referees and the comments are provided below. As you can see, the referees appreciate that the analysis has been strengthened, but still have some concerns in particular about the link between TFEB and cell cycle regulation.

I have carefully looked at everything and would like to ask you to submit a revised version that addresses the points raised.

Here is my take on the concerns raised and what should be done.

Ref #1

Point 1: "....I strongly recommend to remove the comments on the lack of a vessel phenotype in mice gene targeted for different cell cycle genes...."

I agree with the arguments that the referee is making and I think that you should provide a more balanced discussion regarding this point and it is OK to say that we don't know if there is a phenotype as this aspect was not looked at in the cited studies.

Point #2: "My main recommendation on this ambitiously revised paper is to better validate the different siRNAs..."

Please address this point, the requested controls and specify which siRNAs were used in the different experiments

Ref #3

Point 1: "...One of the main problems is the low quality of some of the immunofluorescences..."

Please address this point and improve the quality of some of the immunofluorescences (Fig EV6B, EV6C, EV6D and EV7C).

Point 2: "...a role of TFEB in cell cycle control..."

This is a point that can be addressed in the discussion

Point 3: " Additional controls..."

Please add data to address these points.

REFEREE REPORTS

Referee #1:

The paper by Doronzo et al. represents a major effort to elucidate the role of the transcription factor TFEB. I appreciate the authors' ambition to dissect the several-layered effects of Tfeb deletion on endothelial cell proliferation. The revised paper has in my mind made several new interesting findings, on the other hand, as a consequence of the revision, the manuscript has become considerably more complex. I note however that my criticisms on the primary submission have been taken care of.

The authors find that TFEB regulates cell cycle progression by regulating the CDK4/Rb pathway by direct binding of TFEB to the CDK4 promoter. This is all good. However, the authors doubt however that this explains the strong effect of Tfeb deletion as mice lacking cell cycle genes do not show vascular defects. This conclusion is backed up with references to a number of papers (Geng, et al., 2003, Lu, Wood et al., 2008, Murphy, et al., 1997, Roa, Avdievich et al., 2008, Lincoln, et al., 2002, Liu, et al., 1998, Murga, et al., 2001, Rane et al., 1999, Rempel et al., 2000, Spruck et al., 2001). I looked through these references (briefly) and the conclusion on cell cycle genes not regulating vascular development I think cannot be made as the mice either die before onset of vasculogenesis or they die later/survive but may be stunted. In none of these references have endothelial cells/vessels been examined.
I strongly recommend to remove the comments on the lack of a vessel phenotype in mice gene targeted for different cell cycle genes. Simply describe the effect of CDK4 on endothelial cell proliferation and go on to describe the effect on Tfeb deletion on Myo1c/miR15a/16-1 and the consequence for an essential regulator of endothelial function; VEGFR2. It's not clear to me why the effect on VEGFR2 is compensatory as Myo1C and miR15a/16-1 are directly regulated by TFEB?

My main recommendation on this ambitiously revised paper is to better validate the different siRNAs that form a very important basis for the conclusions. A number of siRNAs for Tfeb, Myo1c and Dleu2 are listed in the Methods and in Fig. EV4, the effects of different Tfeb siRNAs are shown. But which ones were used and could their effects be rescued by reintroduction of Tfeb? I could not find controls for Myo1c or Dleu2.

Referee #2:

The authors have addressed my comments satisfactorily

Referee #3:

In the revised version of the manuscript entitled "TFEB controls vascular development by regulating the proliferation of endothelial cells" the authors have put a great deal of effort in answering the referees' criticisms and have improved the manuscript by further addressing the contribution of TFEB to cell cycle regulation. Unfortunately, some major concerns remain.

1) While it seems clear that TFEB depletion results in increased VEGFR2 expression and plasma membrane accumulation, the consequences of this on VEGFR2 trafficking and signaling are much less convincing. One of the main problems is the low quality of some of the immunofluorescences. In particular, I am not convinced that the staining for Cav and Rab4 shown in Fig EV6B and Fig EV7C, respectively, are specific. This may be due to the low quality of the available antibodies; however, it is unclear why the TGN46 staining shown in Fig EV6D does not show a typical TGN pattern or why the authors chose what appears to be a random cytosolic area for the inset. In addition, the expected co-localization between Rab5 and VEGFR2 in VEFGA-treated cells is not at all apparent in Fig EV6C. Finally, it is proposed but not probed that the very modest reduction in VEGFR2 signaling contributes to the vascular phenotype seen in TFEB KO mice.

2) The new data showing a role of TFEB in cell cycle control are intriguing and interesting but raise many new questions. Is this an unique role of TFEB in endothelial cells or might it be relevant in other cell types? Conditional knockouts of TFEB in different tissues have been generated (e.g. liver, muscle) but no obvious proliferative phenotype has been reported. Furthermore, the best characterized mechanism of TFEB activation is nutrient deprivation. It is, therefore, counterintuitive that TFEB would promote proliferation under conditions in which nutrients are scarce and mTORC1 remains inactive.

Additional controls are needed:

- Include negative control primers in Fig 4F

- Given that Rb levels are slightly elevated in sh-TFEB cells, the authors should show phospho-Rb/total Rb ratios in Fig 4H

- Is TFEB over-expression sufficient to cause increased CDK4 protein levels and proliferation in endothelial cells?

- Confirm that TFEB re-expression in sh-TFEB endothelial cells rescues VEGFR2 levels and VEGFA-mediated signaling.

- Fig EV4 shows that approximately a 20% of human ECs have TFEB in the nucleus in basal conditions. However, this number increases to 70% in Referee #3: Figure2, what is the reason for this discrepancy?

2nd Revision - authors' response 13th September 2018

I appreciated the constructive and useful comments of the referees about our revision. According to referees' suggestions I carefully revised the manuscript and in particular I considered the referees' comments you highlighted. The point-to-point reply is enclosed.

Referee #1:

I thank this referee for her/his useful comments and suggestions

1. *I strongly recommend to remove the comments on the lack of a vessel phenotype in mice gene targeted for different cell cycle genes*.

I agree with the observation of the referee that the quoted papers do not contain a deep analysis of the vascular phenotype, but they just mentioned the lack of vascular defects. Accordingly, these references lines 10-15 pag 21 "Actually, deletion mouse mutants of cell-cycle genes down- modulated in TFEB silenced-ECs did not show any obvious vascular phenotype (Geng et al., 2003; Lu et al., 2008; Murphy et al., 1997; Roa et al., 2008; Lincoln et al., 2002; Liu et al., 1998; Murga et al., 2001; Rane et al., 1999; Rempel et al.; 2000, Spruck et al., 2001)" were removed.

2. *"My main recommendation on this ambitiously revised paper is to better validate the different siRNAs...". Please address this point, the requested controls and specify which siRNAs were used in the different experiments.*

   ….I could not find controls for Myo1c or Dleu2

In the paragraph "Cells, genetic manipulation and biological assays" of Material and Methods (page 30 lines 3-10) we specified the shRNAs used in the different experiments.

In figures Appendix figure S2 and Appendix figure S3 we added the results of the validation of the different shRNAs against MYO1C and DLEU2. In particular figure Appendix figure S2C shows the level of MYO1C protein expression in ECs after the treatment with a scr-shRNA and with 3 different sh-MYO1Cs. We also confirmed the efficacy of these compounds by quantifying MYO1C mRNA by RT- PCR in scr-shRNA and shMYO1C ECs (Figure Appendix figure S2D) (page 16, line 25).
DLEU2 is a RNA Gene and is affiliated with the non-coding RNA class. For this reason we tested the efficacy of 2 different specific sh-RNAs by quantifying DLEU2 mRNA in scr- shRNA and sh-DLEU2 ECs (Figure Appendix figure S3C) (page 18, line 24).

… 3. About the effects of the specific siRNAs (DLEU2, MYO1C) tested, this referee writes
"*could their effects be rescued by reintroduction of Tfeb?"*

Silencing experiments were performed with commercial sh-RNAs that targeted the CDS of human *TFEB*. These sh-RNA recognize also the sequence of TFEBS142A that we introduced by lentivirus infection in ECs. As shown the immunoblotting images and the bar graph of qPCR analysis of the following figure the overexpression of TFEBS142A in the presence of two different TFEB shRNA (shTFEB#1 and shTFEB#5) failed to reduce VEGFR2 and MYO1C expression and to increase DLEU2 expression which are similar to that evaluated after TFEB silencing.

The negative results of the proposed rescue experiments can be differently explained.
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I underline that the meaning of the suggested experiment can be indirectly inferred by the mirrored results obtained by analyzing different key TFEB target genes (VEGFR2, DLEU2, MYO1C, CDK4, miRNAs) (see figures 2, 4, 8, EV5, 7 and Appendix figure S2) in silenced or overexpressing TFEB ECs.

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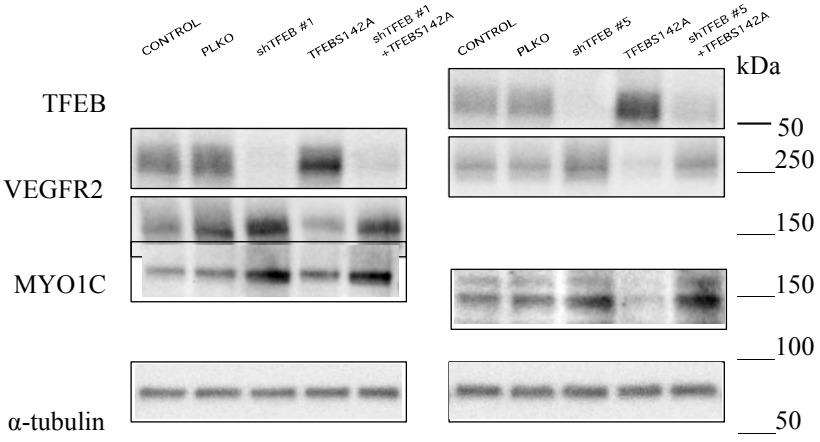
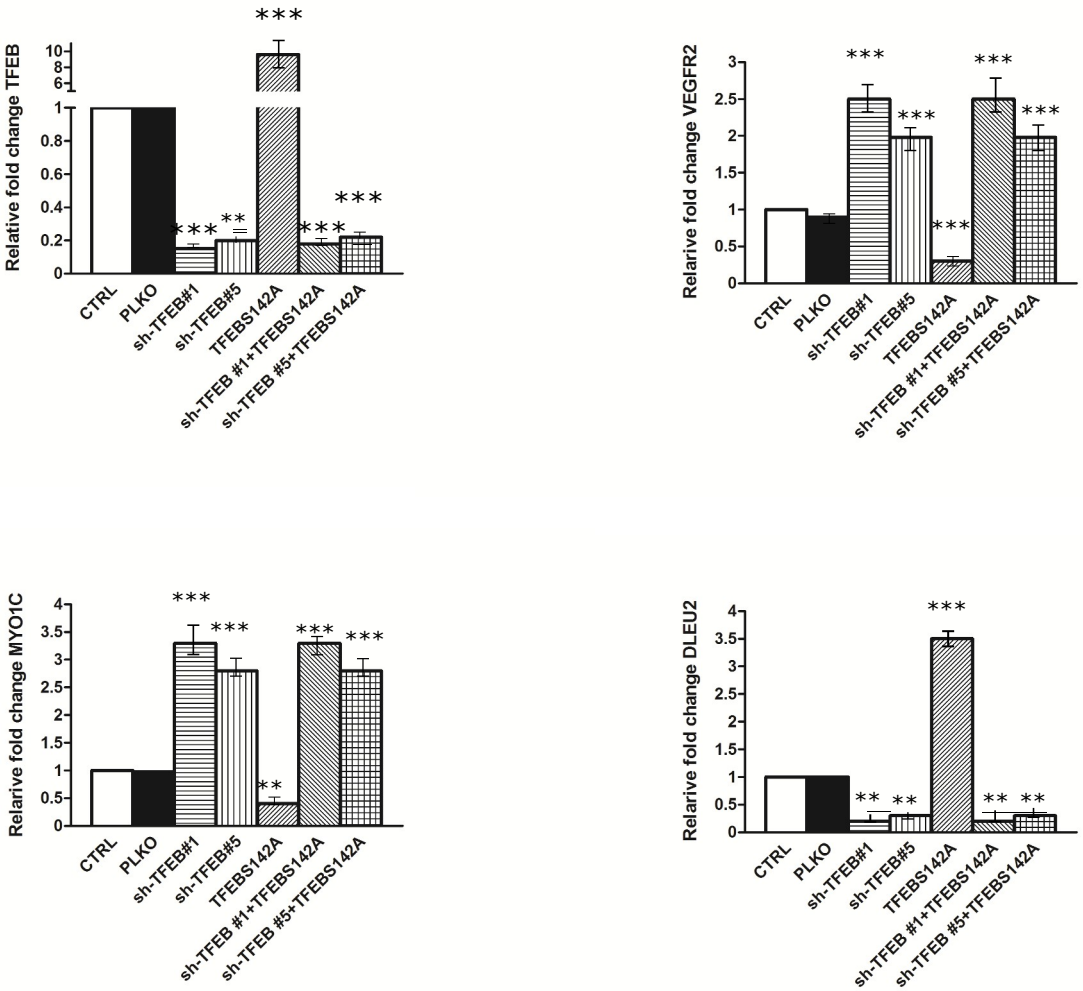


Representative western blots (1 of 3 experiments) of TFEB, VEGFR2 and MYO1C. Analyses were performed on control and TFEBS142A human ECs, scr-shRNA and sh-TFEB (sh-TFEB #1 or #5) human ECs and in ECs in which TFEB was reintroduced after the silencing (sh-TFEB+TFEBS142A)



qPCR of TFEB, VEGFR2, MYO1C and DLEU2 performed on control and TFEBS142A human ECs, scr- shRNA and sh-TFEB (sh-TFEB #1 or #5) human ECs and in ECs in which TFEB was reintroduced after the silencing (sh-TFEB+TFEBS142A). Data are expressed as relative fold change compared with control ECs after normalization for housekeeping gene TBP (n=3, mean±SEM; **p<0.001, ***p<0.0001 by Student's t-test)

Referee #3:

I thank this referee for her/his comments and suggestions that help to better clarify some issues of the manuscript.

> 1. *While it seems clear that TFEB depletion results in increased VEGFR2 expression and plasma membrane accumulation, the consequences of this on VEGFR2 trafficking and signaling are much less convincing. One of the main problems is the low quality of some of the immunofluorescences. In particular, I am not convinced that the staining for Cav and Rab4 shown in Fig EV6B and Fig EV7C, respectively, are specific. This may be due to the low quality of the available antibodies; however, it is unclear why the TGN46 staining shown in Fig EV6D does not show a typical TGN pattern or why the authors chose what appears to be a random cytosolic area for the inset. In addition, the expected colocalization between Rab5 and VEGFR2 in VEFGA-treated cells is not at all apparent in Fig EV6C.*

I am afraid that this referee continues to be sceptic on the revised figures, which are not yet able to support the results about VEGFR2 trafficking. The referee suggested that the low quality of the immunofluorescences could be due to the low quality of available antibodies.
In preliminary setting experiments we tested the following antibodies anti-Cav-1 and Rab4: rabbit polyclonal antibody to Caveolin-1 ab2910 from Abcam, anti-cav-1 monoclonal 7C8 from ThermoFisher Scientific and anti-cav-1 (N20) from Santa Cruz Biotechnology; Anti-Rab4 antibody (ab13252) from Abcam and Purified Mouse Anti-Rab4 from BD Biosciences. For our experiments we selected the antibodies anti-cav-1 (N20) from Santa Cruz and the purified Mouse Anti-Rab4 from BD Biosciences on the basis of the best ratio between specific and unspecific signals. These antibodies were successfully exploited by others to analyze the dynamics of these molecules (DOI: 10.1161/ATVBAHA.112.250621; DOI:10.1038/s41598-018-25640-0; DOI: 10.4161/cc.9.17.12928; doi: 10.1242/jcs.02850;doi: 10.1091/mbc.E10-01-0074).

Furthermore, I would like to underline that our experiments always compared scr-shRNA and sh-TFEB ECs and we immunostained at the same time the two cell types. The same reagents (fixative buffer, saturation buffer and antibody dilution) and the same microscope settings (laser power, gain and off settings, magnification) (see page 33, lines 22-24) were always used for the two cell lines. By the use of these experimental procedures I'm extremely confident that the eventually unspecific signal was quantified both in scr-shRNA and in sh-TFEB ECs but it does not alter the effect mediated by TFEB silencing. Then, our considerations about the alteration of VEGFR2 trafficking in sh-TFEB ECs are not derived just from a qualitative and subjective analysis of the immunofluorescence images but are supported by a quantitative analysis of the same.
However according to the referee comments and to improve the quality of the images new figures are provided. In particular we show the staining for Cav-1, TGN46, Rab4 and Rab5 at higher magnification (Figures Appendix figure S1B, Appendix figure S1C, Appendix figure S1D and Appendix figure S2E).

As suggested by the referee, TGN46 staining and quantification was performed again to exclude unspecific cytosolic signal and to better identify the VEGFR2 signal in Golgi area. Now I show the staining of TGN46 at higher magnification (Figures Appendix figure S1D). We re-quantified the VEGFR2/TGN46 colocalization rate and we confirmed that TFEB silencing did not altered the Golgi localization of VEGFR2.

About the expected colocalization between Rab5 and VEGFR2 in VEGFA-treated cells I agree with the referee that in Figure Appendix figure S1C there is a not complete colocalization between Rab5 and VEGFR2 and that same Rab5 endosomes are free of VEGFR2 molecules. It is possible to suppose that this result is consistent with the rapid and complex physiological trafficking of VEGFR2 molecules that cannot exclude a different localization of the receptor in the same time in different vesicles as previously described (Nat Rev Mol Cell Biol. 2016;17(10):611-25 doi: 10.1038/nrm.2016.87). To improve the quality of the images new figures are provided. In particular we show the staining for Rab5 and VEGFR2 at higher magnification (Figure Appendix figure S1C).

2. *The new data showing a role of TFEB in cell cycle control are intriguing and interesting but raise many new questions. Is this a unique role of TFEB in endothelial cells or might it be relevant in other cell types? Conditional knockouts of TFEB in different tissues have been generated (e.g. liver, muscle) but no obvious proliferative phenotype has been reported. Furthermore, the best characterized mechanism of TFEB activation is nutrient deprivation. It is, therefore, counterintuitive that TFEB would promote proliferation under conditions in which nutrients are scarce and mTORC1 remains inactive.*

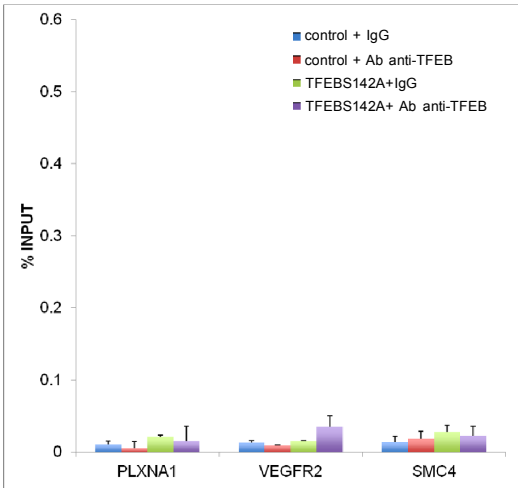
I respectfully disagree with the interpretation that the well demonstrated positive role of TFEB on autophagy contradicts the positive effect on cell cycle here reported. Actually it is likely simplistic to affirm that "*it's counterintuitive that TFEB would promote proliferation under conditions in which nutrients are scarce and mTORC1 remains inactive*". A recent review summarizes the complex circuits occurring between cell cycle and autophagy (doi: 10.3389/fonc.2017.00051) showing that in some circumstances the two processes can simultaneously occur.
About the statement that "Conditional knockouts of TFEB in different tissues have been generated (e.g. liver, muscle) but no obvious proliferative phenotype has been reported" , I carefully read these papers, but their aim was not to investigate the effect of TFEB on proliferation and therefore this investigation was not specifically addressed.

However, to better clarify this point the following sentences was added in discussion (Page 21, lines 12-18). "Interestingly, E2F is able to activate autophagy genes (Polager et al., 2008) supporting the emerging concept that autophagy and cell-cycle are not mutually exclusive processes (Mathiassen et al., 2017). Therefore the dual positive effects of TFEB on autophagy and cell-cycle are not necessarily a paradox but they may therefore depend on the temporal context and stimuli. It is possible to speculate that when TFEB is active to trigger the clearance of senescent cells by autophagy it can initiate the machinery involved in the cell renewal."

3. " *Additional controls are needed".*

3a) Include negative control primers in Fig 4F.

As indicated in the text “page 36, lines 3-4” and in the legend of figures 4F, 5G and 7B Chip analysis was performed using digested chromatin from control ECs and TFEBS142A ECs incubated with IgG or with Ab anti-TFEB, following by qPCR. The absence of any signal in the sample with IgG indicate a specific amplification signal mediated by TFEB. The specificity of the Chip results was also confirmed by the absence of the amplification signal when we performed the assay on genes that are not a target gene for TFEB such as VEGFR2 or SMC4. To underline these results we also repeated the Chip analysis on PLXA1 that Chip-seq analysis did not predicted as target of TFEB. As shown in the following figure no amplification signal was detected.



Analysis of TFEB binding on PLXNA1, VEGFR2 and SMC4. Chip was performed usind digested chromatin from control ECs and TFEBS142A ECs incubated with IgG (indicated in the bar graph as "+IgG") or with Ab anti-TFEB (indicated in the bar graph as "+Ab anti-TFEB"), followed by qPCR for *PLXNA1, VEGFR2 and SMC4*. Bar graph shows the percent enrichment (n=3, mean±SEM, p=ns by Student's *t*-

3b) Given that Rb levels are slightly elevated in sh-TFEB cells, the authors should show phospho-Rb/total Rb ratios in Fig 4H.

We introduced the quantification of the ratio phospho-Rb/total Rb in figure 4H and we indicated it in page 12 line 25.

3c) Is TFEB over-expression sufficient to cause increased CDK4 protein levels and proliferation in endothelial cells?

We quantified the modulation of CDK4 transcription (Figure EV5M) and its protein expression (Figure EV5N) after TFEB overexpression and we confirmed the positive role of TFEB in the regulation of this gene. We also investigated ECs proliferation by the evaluation of EdU incorporation and we show that this was increased after TFEBS142A overexpression (Figure EV5L). These results confirmed the effect of TFEB overexpression on cell proliferation that was extensively observed in cancer cells (Calcagnì et al., 2016, Haq & Fisher, 2011).

3d) *Confirm that TFEB re-expression in sh-TFEB endothelial cells rescues VEGFR2 levels and VEGFA-mediated signaling***.**

Silencing experiments were performed with commercial sh-RNAs that targeted the CDS of human *TFEB*. These sh-RNA recognize also the sequence of TFEBS142A that we introduced by lentivirus infection in ECs. As shown the immunoblotting images and the bar graph of qPCR analysis of the attached figure, the overexpression of TFEBS142A in the presence of two different TFEB shRNA (shTFEB#1 and shTFEB#5) failed to reduce VEGFR2 and MYO1C expression and to increase DLEU2 expression which are similar to that evaluated after TFEB silencing.

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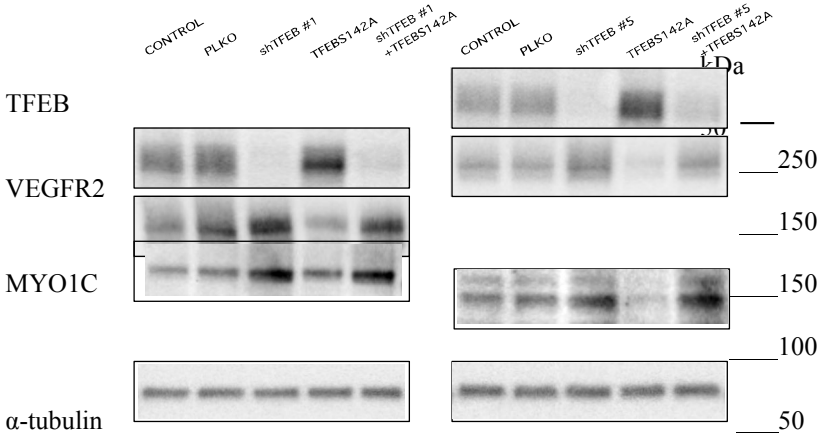

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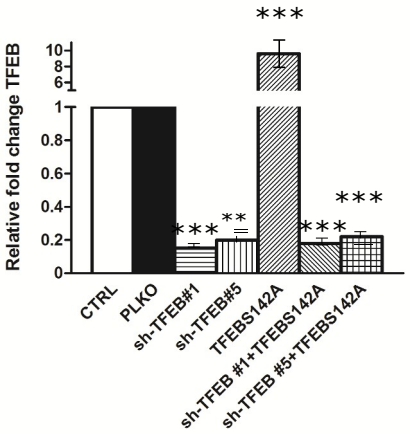

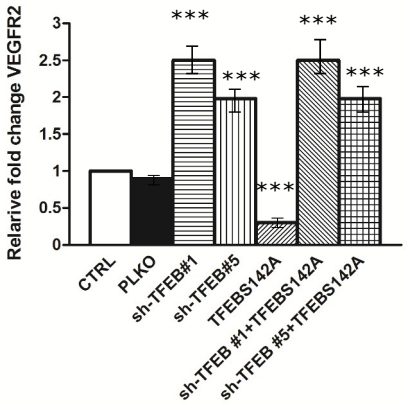

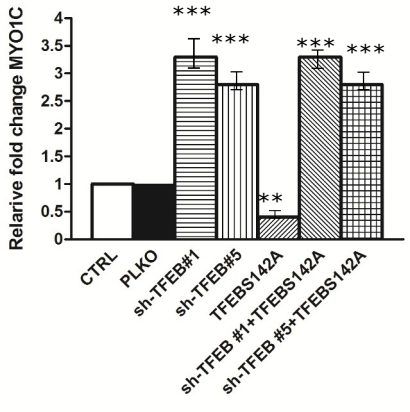

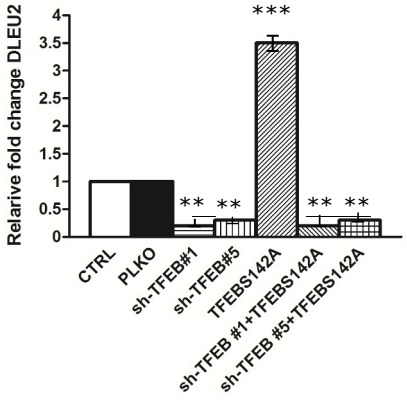

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housekeeping gene TBP (n=3, mean±SEM; **p<0.001, ***p<0.0001 by Student's t-test)

3e) Fig EV4 shows that approximately a 20% of human ECs have TFEB in the nucleus in basal conditions. However, this number increases to 70% in Referee #3 Figure 2: what is the reason for this discrepancy?

The experiment shown in figure 2 for referee #3 provided the in the old version of the manuscript, was performed in ECs starved overnight with MEM without FCS, to selectively evaluate the role of VEGF-A on TFEB translocation without any confounding effects from other stimuli. On the contrary, Figure EV4A shows the distribution of TFEB in ECs under physiological conditions such as MEM with FCS 20%.

3rd Editorial Decision 6th November 2018

Thank you for submitting a revised version of your manuscript and my apologies again for the extended duration of the reassessment period, partly brought on by extensive travelling on my side. I have now had the chance to go through the revised manuscript and your response to the remaining referee concerns and I am pleased to inform you that your manuscript is now in principle ready for acceptance in The EMBO Journal.

However, before we can officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address in a final revised version.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: GABRIELLA DORONZO, FEDERICO BUSSOLINO
Journal Submitted to: EMBO JOURNAL
Manuscript Number: EMBOJ-2017-98250R2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

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B- Statistics and general methods

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1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Paragraph "Statistical analysis" of matherial and metods section
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Paragraph "Statistical analysis" of matherial and metods section
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Paragraph "Statistical analysis" of matherial and metods section
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.	Paragraph "Statistical analysis" of matherial and metods section
For animal studies, include a statement about randomization even if no randomization was used.	Paragraph "Mice" of matherial and metods section
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.	Paragraph "Mice" of matherial and metods section
4.b. For animal studies, include a statement about blinding even if no blinding was done	Paragraph "Mice" of matherial and metods section
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.	Paragraph "Statistical analysis" of matherial and metods section
Is there an estimate of variation within each group of data?	Paragraph "Statistical analysis" of matherial and metods section
Is the variance similar between the groups that are being statistically compared?	Paragraph "Statistical analysis" of matherial and metods section

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Paragraph "Mice" of matherial and metods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Paragraph "Cell, genetic manipulation and biological assays" of matherial and metods section

* 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.	Paragraph "Mice" of matherial and metods section

9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Paragraph "Cell, genetic manipulation and biological assays" of matherial and metods section
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

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	Paragraph "Statistical analysis" of matherial and metods section
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).	Paragraph "Statistical analysis" of matherial and metods section
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).	N/A
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.	All computational code used to analyse the data is available from the corresponding author

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.	N/A