IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

TFEB controls vascular development by regulating the proliferation of endothelial cells

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1685458 since 2019-01-01T16:13:37Z

Published version:

DOI:10.15252/embj.201798250

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

27 November 2022

TFEB CONTROLS VASCULAR DEVELOPMENT BY REGULATING THE PROLIFERATION OF ENDOTHELIAL CELLS

Gabriella Doronzo[1,2], Elena Astanina[1,2], Davide Corà[3], Giulia Chiabotto[8], Valentina Comunanza[1,2], Alessio Noghero[1,2], Francesco Neri[4], Alberto Puliafito[1,2], Luca Primo[1,2], Carmine Spampanato[5,6,7], Carmine Settembre[5,6,7], Andrea Ballabio[5,6,7], Giovanni Camussi[8], Salvatore Oliviero[9] and Federico Bussolino[1,2].

[1] Department of Oncology; University of Turin; Candiolo, 10060, Italy
[2] Candiolo Cancer Institute-FPO-IRCCS; Candiolo, 10060, Italy
[3] Department of Translational Medicine; Piemonte Orientale University Amedeo Avogadro; Novara, 28100, Italy
[4] Leibniz Institute on Aging – Fritz Lipmann Institute; Jena, 07745, Germany
[5] Telethon Institute of Genetics and Medicine (TIGEM); Pozzuoli (Naples), 80078, Italy
[6] Department of Translational Medicine, Federico II University; Naples, 80121, Italy
[7] Ian and Dan Duncan Neurological Research Institute and Department of Molecular and Human Genetics, Baylor College of Medicine; Houston, TX 77030, USA
[8] Department of Medical Sciences; University of Turin; Turin, 10129, Italy
[9] Department of Life Sciences and Systems Biology; University of Turin; Turin, 10123, Italy

Corresponding authors:
gabriella.doronzo@unito.it
federico.bussolino@unito.it
Department of Oncology
University of Turin
s.p. 142, Km 3.95
10060 Candiolo-Italy

SUMMARY

The role of the transcription factor EB (TFEB) in the control of cellular functions, including in vascular bed, is mostly thought to be the regulation of lysosomal biogenesis and autophagic flux. While this is its best-known function, we report here the ability of TFEB to orchestrate a non-canonical program involved in the control of cell-cycle and VEGFR2 pathway in the developing vasculature. In endothelial cells, TFEB deletion halts proliferation by inhibiting the CDK4/Rb pathway, which regulates the cell cycle G1-S transition. In an attempt to overcome this limit, cells compensate by increasing the amount of VEGFR2 on the plasma membrane through a microRNA-mediated mechanism and the control of its membrane trafficking. TFEB transactivates the miR-15a/16-1 cluster, which limits the stability of the VEGFR2 transcript, and negatively modulates the expression of *MYO1C,* which regulates VEGFR2 delivery to the cell surface. In TFEB knocked-down cells, the reduced and increased amount respectively of miR-15a/16-1 and MYO1C result in the overexpression on plasmamembrane of VEGFR2, which however shows low signaling strength.

Using endothelial loss-of-function Tfeb mouse mutants, we present evidence of defects in fetal and newborn mouse vasculature caused by the reduced endothelial proliferation and by the anomalous function of VEGFR2 pathway. Thus, this study revealed a new and unreported function of TFEB that expands its role beyond the regulation of autophagic pathway in the vascular system.

Keywords: angiogenesis/ embryo/ membrane trafficking/ proliferation/ miRNA transcription

INTRODUCTION

Transcription factor EB (TFEB) belongs to the microphthalmia family of bHLH-leucine zipper molecules. It is involved in the biogenesis and function of the endo-lysosomal compartment, including membrane trafficking and autophagy (Napolitano & Ballabio, 2016; Raben & Puertollano, 2016; Settembre, et al., 2013b). Furthermore, TFEB mutation characterizes a subset of renal cell carcinoma carrying the t(6;11)(p21:q13) translocation, which leads to a *TFEB* promoter substitution with the 5’ upstream regulatory sequence of the *alpha* intronless gene (Calcagnì, et al., 2016).

TFEB recognizes E-box-type DNA sequences (Palmieri, et al., 2011) and resides in the cytosol, moving to the nucleus when lysosomes and autophagy are required for cell activities (Martina, et al., 2012; Settembre, et al., 2012). The functions of TFEB are mainly regulated by mTOR complex1 (mTORc1), which integrates energy availability with cellular demand. In the presence of nutrients, mTORc1 phosphorylates TFEB and inhibits its transport into the nucleus. Conversely, under starvation conditions, when mTORc1 is inactive, unphosphorylated TFEB rapidly accumulates in the nucleus. In general terms, TFEB is involved in the pathogenesis of lysosomal storage diseases (Xu & Ren, 2015), and through its connection with mTOR pathway, in the control of energy expenditure both in physiology (Mansueto et al., 2017) and in pathology (Di Malta et al., 2017; Perera et al., 2015).

An increasing number of observations suggests a pivotal role of TFEB in vascular biology. Targeted inactivation of TFEB in mice results in impaired placental vascularization and inhibits the expression of VEGF-A in labyrinthine cells (Steingrímsson, Tessarollo et al., 1998). The embryonic vasculature is unable to invade the placenta, halting the exchange of nutrients and causing lethal hypoxia and embryonic lethality. During atherogenesis, the lysosomal stress induced by the accumulation cholesterol activates a TFEB response,

which triggers an anti-inflammatory (Lu et al., 2017) and anti-atherogenic response (Emanuel et al., 2014). Finally, the overexpression of Tfeb in endothelial cells (ECs) promotes post-ischemic angiogenesis through the activation of autophagic flux (Fan et al., 2018).

Using EC-specific loss-of-function mouse mutants (*Tfeb*$^{EC-/-}$) and cellular models, we investigated the effect of *Tfeb* deletion on the vasculature in the embryo and in new-born mice. We found that TFEB positively controls the expression of Cyclin-Dependent Kinase 4 (CDK4) and its deletion results in the block of cell growth and in a futile attempt to recover this process by targeting Vascular-Endothelial Growth Factor (VEGF) Receptor (R)-2.

RESULTS

Tfeb is expressed in embryonic and post-natal vessels.

To analyze *Tfeb* expression in the vasculature, we used constitutive knock-in *Tfeb-EGFP* mice. Tfeb was expressed very early in developing vessels and persisted in newborn pups. The vascular expression was heterogeneous and not generalized to all ECs (Figures 1A, 1B and EV1A), suggesting a dynamic role in the vasculature. At E9.5, Tfeb-EGFP co-localized with endothelial endomucin in head and in the intersomitic vessels as well as yolk-sac capillaries (Figure EV1A). We then examined the expression of Tfeb in retina and kidney, whose vascular beds undergo post-natal development (Gariano & Gardner, 2005, Little & McMahon, 2012). At p5, Tfeb-EGFP was present in both large and small retinal vessels at the vascular front and vascular plexus (Figure 1A). The analysis of renal vessels at p17 showed that Tfeb was present in glomerulus, capillaries and some small arteries (Figure 1B). As reported by the whole mRNA expression analysis (Steingrímsson et al., 1998), Tfeb-EGFP was present in alpha-smooth muscle actin (SMA) positive cells and pericytes of embryo tissues (E9.5) (Figures EV1B, EV1C) and retina (p5) (Figure EV1D) as well as in renal podocytes (p17) (Figure EV1E), as inferred by the use of specific antibodies anti-SMA, neural/glial antigen-2 (NG2) and podocin.

Tfeb expression in ECs is essential for vascular development.

To investigate the role of Tfeb in embryonic vessel development, we interbred *Tfeb*^{floxed} mice (Settembre,et al., 2013a) with *Tie2-Cre* mice, which allows EC-specific gene targeting from E8.5 (Kisanuki, et al., 2001). *Tie2-Cre*$^{-}$*/Tfeb*^{floxed} (control) and *Tie2-Cre*$^{+}$*/Tfeb*$^{-/+}$ (*Tfeb*$^{EC-/+}$) embryos survived, while *Tie2-Cre*$^{+}$*/Tfeb* $^{-/-}$ (*Tfeb*$^{EC-/-}$) mice were absent among the different progenies (n=8), indicating embryonic lethality.

At E9.5, analysis of control, *Tfeb*$^{EC-/+}$ and *Tfeb*$^{EC-/-}$ embryos showed indistinguishable phenotypes from normal vasculature (Figure EV2A). At E10.5, *Tfeb*$^{EC-/+}$ mice were similar to control, while *Tfeb*$^{EC-/-}$ presented an altered vascular phenotype (Figure 1C). Indeed, embryos and yolk-sacs from *Tfeb*$^{EC-/-}$ mice were smaller and paler than controls (Figure 1C), with evident hypoxic areas (Figure EV2B). Whole-mount (i, ii) and endomucin staining of the head (iii), ocular (iv) and intersomitic regions (v) displayed vascular defects characterized by excessive fusion into irregular dilated vessels, reduced branching and failure to follow the normal anatomical patterns seen in control mice. Furthermore, the intersomitic region was characterized by reduced vascular invasion into somitic tissues and the presence of avascularized zones (Figure 1C). The percentage of point prevalence of vascular defects in *Tfeb*$^{EC-/-}$ (n=12) and control embryos (n=13) at E10.5 was respectively 83% and 16% .

Since Tie2 is also expressed by hematopoietic precursors at E8.5 (Takakura, et al., 1998), we evaluated the effects of *Tfeb* deletion on the maturation of this system. The expression of markers characterizing the hematopoietic and endothelial lineages, together with Tie2, was analyzed in yolk sacs at E9.5. The percentage of Tie2^{+} cells in the control was similar to that of *Tfeb*$^{EC-/-}$ yolk sacs as well as the abundance of early endothelial precursors identified as Tie2^{+}/Flk^{+} and Tie2^{+}/Flk^{+}/CD31^{+}. Interestingly we observed a slight reduction in the Tie2^{+}/CD117^{+}/CD41^{+} cells and Tie2^{+}/CD117^{+}/CD71^{+} cells that represent early erythroid precursors (Figure EV2C).

These data support the concept that TFEB acts in a later phase of vascular development, mostly characterized by the remodeling of the primitive vascular plexus.

Tfeb is involved in retinal and renal vascular maturation after birth.

To overcome early embryonic lethality, we generated inducible EC-specific mutants by crossing *Tfeb*^{floxed} mice with *Cdh5-CreERT2* mice (Wang, et al., 2010), thus allowing generating *Cdh5-CreERT2*$^{+}$*/Tfeb*$^{-/+}$ (*Tfeb*$^{iEC-/+}$) and *Cdh5-CreERT2*$^{+}$*/Tfeb*$^{-/-}$ (*Tfeb*$^{iEC-/-}$) and *Cdh5-CreERT2*$^{-}$*/ Tfeb*^{floxed} (control) mice.

The successful deletion of *Tfeb* after in vivo Cre induction by tamoxifen was established by detecting the Tfeb delta allele in genomic DNA after lox site recombination. In *Tfeb*$^{iEC-/+}$ and *Tfeb*$^{iEC-/-}$ mice, *Tfeb* deletion specifically occurred in endothelium as evidenced by the decrease of the exons 5 and 6 transcript only in ECs but not in epithelial cells isolated from the lungs (Figure EV2D). The recombination efficiency and specificity were further demonstrated by the marked Tfeb reduction in the renal vasculature of *Tfeb*$^{iEC-/-}$ mice (p17) but not in surrounding tissues (Figure EV2E).

According to the post-natal maturation of retinal and renal vasculature, we examined the consequences of *Tfeb* deletion in the ECs of these organs. At p5, retinas from *Tfeb*$^{iEC-/-}$ mice showed impaired outgrowth of the superficial capillary network (Figure 1D). The retinal vascular mesh was wider with a reduction of the vascular area, vascular density, branching points and vessel size (Figure 1D). The vascular front did not show any differences in the number and length of filopodia (Figure 1D). The detrimental effect of *Tfeb* deletion persisted up to p15, when vascular retinal net reaches the maturity. At p10 the net features were similar to those reported at p5. From p10 to p15, the vascular area reached the size observed in control mice but the alterations in vascular density, vessel diameters and branching points were still present (Figure EV3A).

Correct assembly of the glomerular vasculature is required for filtration barrier function (Bartlett, et al., 2016). At p17, *Tfeb*$^{iEC-/-}$ kidneys were smaller (% area of *Tfeb*$^{iEC-/-}$ mice *versus* control 72.6±7.9 %; mice n=3, $p<0.01$), characterized by poorly developed glomeruli (Figure EV3B) and reduced volumes (20.53 ± 2.50 x 10^4 μm^3 for control mice

and 14.43 ± 3.27 x 10^4 μm^3 for *Tfeb*$^{iEC-/-}$; mice n=3, p<0.002). The altered glomerular development paralleled the reduced CD31$^+$ endothelial area in *Tfeb*$^{iEC-/-}$ kidneys (p17) (Figure EV3C), which persisted up to p27 (Figure EV3D). Transmission electron microscopy showed deep alterations in glomerular structure, with expansion of the mesangium by deposition of extracellular matrix, focal loss of podocyte foot processes and endothelial fenestration (Figure 1E). According to the increased deposition of extracellular matrix in *Tfeb*$^{iEC-/-}$ (Figure 1E), we observed a significant accumulation of collagen IV along capillaries, and collagen I in the interstitium, while collagen V was only moderately increased (Figure EV3E), supporting the hypothesis that *Tfeb* deletion could be instrumental in renal fibrosis.

In *Tfeb*$^{iEC-/-}$ mice we did not observed any alterations of the ratio between vascular muscle cells and ECs both in retina (ratio NG2 area/ IB4$^+$ vascular area 1.2 ± 0.1 in control mice and 1.2 ± 0.3 in *Tfeb*$^{iEC-/-}$; mice n=4, p=ns) and in renal glomeruli (ratio podocytes/ CD31$^+$ vascular area 1± 0.1 in control mice and 0.9± 0.1 in *Tfeb*$^{iEC-/-}$; mice n=4, p=ns). These data support the role of the endothelial dysfunction in the histological alterations of the retina and kidney observed after *Tfeb* deletion.

Tfeb deletion reduces the proliferation of ECs.

The main processes characterizing vascular development are the proliferation and the migration of ECs and their relationships with extracellular matrix (Carmeliet & Jain, 2011). Therefore, to understand the vascular defects observed in vivo, we studied ECs lacking TFEB by analyzing their growth, motility and morphogenetic capability when layered on extracellular matrix.

The in vivo analysis of Ki-67$^+$ EC nuclei at p5 and p17 indicated a marked reduction in proliferating ECs in both the retina (37% in vascular front and 50% in vascular plexus) and kidney vessels (30%) of *Tfeb*$^{iEC-/-}$ mice (Figures 2A, 2B). Of note, in kidney the total

number of Ki-67$^+$ cells per field was not modified in *Tfeb*$^{iEC-/-}$ mice compared to control (Figure 2B) indicating that the proliferation rate of other cell types was not modified and suggesting that the impaired EC growth is instrumental in the alteration of renal maturation.

Before evaluating the effect of TFEB deletion in vitro, we experimentally verified whether TFEB mechanism of action in ECs was similar to that described in other cell types. In EC standard culture conditions, we showed a cytosolic and nuclear endogenous expression of TFEB (Figure EV4A). Furthermore, when ECs were treated with Torin, an mTor inhibitor that mimics starvation conditions and activates TFEB (Settembre et al., 2012), we observed an increase of its nuclear translocation (Figure EV4A). We silenced TFEB via specific short-hairpin RNA (Figures EV4B, EV4C) and the in vitro ECs proliferation rate was evaluated by the count of 5-ethynyl-2'-deoxyuridine (EdU) positive cells. The proliferative effect of Fetal Calf Serum (FCS) and Vascular Endothelial Growth Factor-A (VEGF-A) was significantly impaired in TFEB-silenced human ECs (sh-TFEB ECs) (Figure 2C) and in ECs from lung of *Tfeb*$^{iEC-/-}$ mice (Figure EV4D). TFEB silencing in ECs specifically restrained the G_1–S cycle transition, as assessed by propidium iodide staining (Figure 2D). In sh-TFEB ECs stimulated by VEGF-A or FCS we evidenced an increased percentage of cells blocked in the G1 phase and a decreased percentage of those progressing in S phase (Figure 2D).

Then we studied the effect of TFEB deletion on EC migration. An indirect in vivo evidence of EC motility is the analysis of filopodia, which characterize migrating cells. As shown in Figure 1D, the number of filopodia at the front of retinal plexus was similar in control and in *Tfeb*$^{iEC-/-}$ mice, suggesting that Tfeb deletion did not affect EC motility. Accordingly, sh-TFEB and scr-shRNA ECs in vitro chemotactic response to VEGF-A was similar suggesting that TFEB down-regulation did not interfere with EC motility (Figure EV4E).

Finally, the effect of TFEB deletion was further examined in a morphogenetic assay. As shown in Figure 2E the absence of TFEB dampened ECs to form tube-like structures on reduced growth factor Matrigel only after VEGF-A stimulation.

TFEB modifies the transcriptional landscape in ECs.

To describe the genetic program triggered by TFEB in ECs, we investigated the transcriptome modifications induced by TFEB silencing and the DNA promoter regions to which TFEB is recruited. The comparative transcriptome analysis of scr-shRNA and sh-TFEB ECs by LIMMA (Smyth, 2002) defined a subset of 502 differentially expressed genes (DEGs)(133 up-regulated and 369 down-regulated, $|log_2FC|> 0.5$ and FDR< 0.1) (Figure 3A). A volcano plot showed the changes in the log_2 fold-change and p-values for all the genes in sh-TFEB ECs compared to scr-shRNA cells (Figure EV5A). The enriched biological functions of up- and down-regulated genes were determined by DAVID analysis. In sh-TFEB ECs, down-regulated genes characterized processes related to “Cell Cycle”, “Cell Division”, “ Mitotic cell cycle process”, “ G1/S transition of mitotic cell cycle”, “DNA replication” and “DNA metabolic process”. This result is consistent with the observation of the arrest of cell cycle and proliferation in vivo and in vitro after *Tfeb* silencing. On the other hand, up-regulated gene enrichment was limited to “Positive regulation of protein catabolic process” and “Endocytosis” categories (Figure 3B). Gene Sets Enrichment Analysis (GSEA) revealed the enrichment of the “negative regulation of cellular process”, “negative regulation of proliferation” and “vesicle-mediated transport” categories, supporting the DAVID analysis (Figure 3C).

To identify direct TFEB gene targets potentially supporting the transcriptional modulation described above, we performed Chromatin-immunoprecipitation sequencing (ChIP-Seq) in ECs overexpressing a TFEB mutant (S142A) protein, which is constitutively translocated to the nucleus and biologically active (Settembre et al., 2012) (Figure EV5B).

TFEB ChIP-Seq showed several distinct binding events compared with IgG ChIP-Seq (Figure EV5C). TFEB binding regions on DNA correlated with open chromatin regions (DNAse) and with transcription factors known to be associated with active gene promoters (FOS, JUN, RNA PolII, MYC) (Figure EV5D).

In particular, we defined a set of 1066 Ref/Seq protein-coding genes showing strong TFEB binding enrichment on their core promoter. Of these, 71% contained the canonical TFEB binding sequence CACGTG (Palmieri et al., 2011)(Figure EV5E).

Gene-expression profiling of ECs revealed that TFEB was principally bound to highly expressed genes (Figure EV5F), at hypomethylated regions (Figure EV5G) and that almost 15% of the peaks (p-value<$10e^{-8}$) were located on gene promoters (Figure EV5H).

GSEA was used to investigate the correlation between the regulated genes and the list of TFEB-bound genes. An enrichment plot showed that both up-regulated and down-regulated genes can be TFEB targets (Figure EV5I).

DAVID analysis on ChiP-seq data set confirmed the known role of TFEB in lysosome/autophagic pathway but also underlined its involvement in cell cycle regulation, angiogenesis, blood vessel development and morphogenesis (Figure 3D).

Taken together, these data support the in vivo and in vitro results showing that TFEB is involved in EC proliferation by regulating the expression of genes directly involved in the control of cell cycle.

The CDK4 gene is a direct target of TFEB.

As evidenced by transcriptome analysis (Figure 4A), qPCR (Figure 4B) and immunoblotting analysis (Figure 4C), TFEB silencing in human ECs negatively regulated the expression of genes involved in cell proliferation, including Cyclin-Dependet Kinase 4 (CDK4), cyclins (CCNA1, CCNA2), E2F transcription factors (E2F1, E2F2, E2F4) and their targets (MCM5, MCM6, CDC25B, CDCA4, CDCA7, PLK1, PCNA), which are involved in

the control of S phase and mitosis. These data were further validated in murine lung ECs isolated from control and *Tfeb*$^{iEC-/-}$ mice (Figure 4D). Therefore we interrogated the ChiP-seq data set to identify the direct TFEB targets within these modulated genes and we found that the promoter of CDK4 contains a binding site for TFEB (Figure 4E).

To further confirm the direct activity of TFEB on CDK4 expression, ChIP and promoter-luciferase assay were performed. As shown in Figure 4F, the chromatin immunoprecipitated with an antibody anti-TFEB contained the *CDK4* promoter identified by PCR using two CDK4-specific primers. We further investigated the *CDK4* promoter activity by luciferase reporter assay in ECs overexpressing TFEBS142A, an active constitutive variant that localizes preferentially in the nucleus (Settembre, et al., 2012). TFEBS142A-carrying luciferase reporter vectors respectively containing CDK4 full length promoter and a deleted form lacking 100 bps encompassing the putative TFEB binding site were analyzed. TFEBS142A overexpression resulted in a significant increase of *CDK4* promoter activity, which was completely blunted by the deletion of TFEB binding site (Figure 4G).

One of the most important substrate of CDK4 is Retinoblastoma (Rb) protein, which associates with E2F transcription factor in quiescent cells. When cells progress in G1 phase, CDK4 phosphorylates Rb, allowing the release of E2F, which migrates into the nucleus and activates the transcription of genes required for S-phase (Malumbres & Barbacid, 2009). Transcriptomic analysis of shTFEB ECs did not indicate Rb as a DEG and immunoblotting analysis showed that its expression was not altered by TFEB silencing (Figure 4H). On the contrary we evidenced in shTFEB ECs a down-regulation of phospho-Rb (Figure 4H), which paralleled the reduced expression of CDK4 (Figures 4B, 4C).

These data support the rationale that CDK4-Rb-E2F axis is a target of TFEB in ECs, and suggest that its impairment could explain the in vivo alteration of vascular development described in *Tfeb*$^{iEC-/-}$ mice. Interestingly, deletion mouse mutants of cell-cycle genes down-modulated in TFEB silenced-ECs (Figure 4A) died at very early stage (Geng, et al.,

2003, Lu, Wood et al., 2008, Murphy, et al., 1997, Roa, Avdievich et al., 2008) or were alive (Lincoln, et al., 2002, Liu, et al., 1998, Murga, et al., 2001, Rane et al., 1999, Rempel et al., 2000, Spruck et al., 2001) but none of them, including *Cdk*$^{-/-}$, showed evident vascular defects.

VEGFR2 gene is an indirect target gene of TFEB in ECs.

The above considerations stimulated us to study other possible mechanisms sustaining the vascular phenotype. We investigated a possible role for VEGF-A/VEGFR2 pathway, which is definitely a key machinery involved in EC migration, proliferation and morphogenesis (Simons et al., 2016). We were also intrigued by the observation that full *Tfeb*$^{-/-}$ mice exhibited a reduced placenta expression of VEGF-A (Steingrímsson et al., 1998). Furthermore, while the reduced transcription of *CDK4* in sh-TFEB ECs could justify the observed proliferative block, this defect was not necessarily accountable for the morphogenetic alterations observed in vivo (Figure 1D) and in vitro in the Matrigel morphogenetic assay (Figure 2E), which is independent from the proliferation even in the presence of VEGF-A (Serini et al., 2003).

For these reasons we investigated whether TFEB deletion could interfere on VEGFR2 expression. The expression of *Vegfr2* was increased in the vessels of *Tfeb*$^{EC-/-}$ embryos (E10.5) (Figure 5A) and of retina and kidney of *Tfeb*$^{iEC-/-}$ mice compared with that in control mice (Figures 5B, 5C). Since neural cells (Robinson et al., 2001) and podocytes (Bartlett et al., 2016) both express *Vegfr2,* we examined the amount of receptor co-localized with the specific endothelial markers iB4 or CD31 in retina and kidney and confirmed the increased level of the receptor in the ECs of *Tfeb*$^{iEC-/-}$ mice (Figures 5B, 5C).

Transcriptome data validated by qPCR (Figures 5D, 5E) and immunoblotting (Figure 5F) showed the up-regulation of VEGFR2 in murine lung ECs isolated from *Tfeb*$^{iEC-/-}$ mice and sh-TFEB ECs. However this transcriptional effect is independent from a direct binding of

TFEB on *VEGFR2* promoter, as demonstrated by ChIP-Seq (not shown) and ChIP-qPCR (Figure 5G) performed in sh-TFEB ECs.

In sh-TFEB ECs, the analysis of VEGFR2 localization by FACS (Figure 6A), total internal reflection fluorescence (TIRF) microscopy (Figure 6B) and biotinylation of endothelial plasma membrane (PM) (Napione et al., 2012) (Figure 6C) indicated a strong accumulation of the receptor at the PM.

In the absence of a direct effect of TFEB on *VEGFR2* transcription (Figure 5G), we hypothesized that the altered expression of VEGFR2 and its increased localization on PM might be the result of the combined activity of an altered membrane trafficking and a miR-dependent post-transcriptional mechanism.

TFEB deletion alters VEGFR-2 membrane trafficking.

On the basis of the described results, we investigated whether the accumulation of VEGFR2 in PM (Figures 6A, 6B, 6C) could be related to a specific TFEB-mediated mechanism orchestrating the VEGFR2 membrane trafficking (Simons et al., 2016).

Actually TFEB deletion negatively modulated the genetic program sustaining lysosome biogenesis, autophagy, vesicles trafficking and exocytosis (Medina et al., 2011, Napolitano & Ballabio, 2016, Raben & Puertollano, 2016) in many cell types including ECs (Lu et al., 2017). Transcriptomic analysis indicated that the expression of some genes characterized by the presence of the Coordinated Lysosomal Expression and Regulation (CLEAR) element (Palmieri et al., 2011) were modified in shTFEB-ECs (DEGs down-regulated: ATP6V0D1, CLCN7, CTSB; DEGs up-regulated: RRAG). Similarly TFEB silencing reduced markers of autophagic flux. In particular, the ratio between Microtubule-associated proteins 1A/1B light chain (LC) 3-II and LC3-I, the expression of Unc-51 like autophagy activating kinase (ULK-1) (Martina et al., 2012, Settembre et al., 2012) and

Autophagy related gene (ATG)9A (Rao et al., 2016) were reduced in sh-TFEB ECs (Figures 6D, 6E).

Because lysosomal and autophagy pathways are interconnected with endosomal system (Pavel & Rubinsztein, 2017) we analyzed the dynamics of VEGFR2 trafficking in sh-TFEB ECs. Whereas PM VEGFR2 started to decrease immediately after VEGF-A challenge in scr-shRNA ECs (~ 30% reduction after 5 min), knocking down TFEB consistently altered VEGFR2 PM clearance that was slower (~ 20% reduction) and started only after 10 min from stimulus. Concomitantly, the reduction of total amount of VEGFR2, correlated with its degradation, in scr-shRNA ECs became evident after 5 min of VEGF-A challenge (~14% *versus* unstimulated ECs), while this drop was evident later (10 min) in sh-TFEB ECs (~17% *versus* unstimulated ECs) (Figure 6C).

The perturbing effect of TFEB silencing was further analyzed by studying the co-localization of VEGFR2 with caveolin-1 (CAV-1)-rich membrane rafts, which represent specific domains that favor the signaling properties of the receptor (Cho et al., 2004; Labrecque et al., 2003). As inferred from colocalization analysis, TFEB silencing did not significantly alter the expression and localization of CAV-1 on the PM, but interestingly, it modified the spatial relationship between VEGFR2 and CAV-1. sh-TFEB ECs exhibited an approximately 25% reduction in CAV-1-associated VEGFR2 compared with scr-shRNA ECs (Figure EV6B).

The effect of TFEB silencing on VEGFR2 distribution was further evaluated by the specific quantification of receptor endocytosis by a receptor internalization assay (Valdembri et al., 2009). TFEB silencing moderately but significantly altered VEGFR2 internalization time-course. Whereas VEGFR2 internalization started immediately (5 min) after VEGF-A challenge in control cells (~12% *versus* unstimulated ECs), a 10 minutes delay was seen

in sh-TFEB ECs suggesting an impairment of internalization process (~5% *versus* unstimulated ECs, p=ns after 5 min of VEGF-A incubation and ~7% *versus* unstimulated ECs, p<0.01 after 10 min of VEGF-A incubation) (Figure 6F). The inhibition of internalization of VEGFR2 in sh-TFEB ECs was confirmed by the reduction of colocalization with $Rab5^+$ endosomes after VEGF-A stimulation (Figure EV6C).

Because VEGFR2 exocytosis from endosomal compartment to PM participates to properly maintain its signaling properties (Simons et al., 2016), we investigated the colocalization of the receptor with the Golgi marker Trans-Golgi Network 46 (TGN46) and $Rab4^+$ exocytic vesicles (Jopling, Odell et al., 2014). TFEB silencing did not modify the amount of VEGFR2 localized in Golgi compartment (Figure EV6D), but increased that accumulated in $Rab4^+$ vesicles (170,2±9 % compared to scr-shRNA ECs; n=4, p<0,01) (Figure EV7C).

These observations prompted us to interrogate the enriched gene set belonging to the Gene Ontology “protein targeting to membrane” in TFEB-silenced ECs to identify a mechanism responsible for the observed accumulation of VEGFR2 in the PM.

Within the modulated genes (ADORA1, ARL6, CACNB1, ICMT, ATG3, SDCBP, SEC63, ATG4C, MGEA5, MICALL1, MYO1C, TAOK2, NCF1, PRKCI), we focused our attention on Myosin 1c (MYO1C), which was involved in VEGFR2 exocytosis (Tiwari et al., 2013). ChIP-Seq (Figure 7A) and ChIP-qPCR (Figure 7B) indicated that TFEB can bind to the *MYO1C* promoter. ECs overexpressing *TFEBS142A* showed reduced activation of *MYO1C* promoter (Figure EV7A) and consequently a decreased transcription (Figure EV7B). On the contrary in sh-TFEB ECs MYO1C was up-regulated (Figures 7C, 7D). This phenotype was also observed in the vasculature of retina (p5) and kidney (p17) of $\textit{Tfeb}^{iEC-/-}$ mice (Figures 7E, 7F).

Interestingly, MYO1C-silencing decreased the amount of VEGFR2 co-localized with $Rab4^+$ vesicles (Figure EV7C). This data suggested that MYO1C deletion reduces the transport

of the receptor to Rab4$^+$ endosomes involved in its exocytosis. This result matches the observation that MYO1C silencing in sh-TFEB ECs reduced specifically the amount of PM VEGFR2, as demonstrated by immunoblotting (Figure 7D), the ratio of the VEGFR2 signal recorded in TIRF and epifluorescence modes (Figure 7G), and the ratio of VEGFR2 on the PM to total expression measured by FACS (Figure EV7D).

On the contrary MYO1C silencing both in scr-shRNA than in sh-TFEB ECs did not affect VEGFR2 transcription (Figure EV7E), total protein expression (Figure 7C; densitometric analysis as % of scr-shRNA: 220.7±5.2 % in sh-TFEB ECs $p<0.0001$; 114.7±7.7% in sh-MYO1C p=ns; 230.3±3.7 % in sh-TFEB+sh-MYO1C ECs $p<0.0001$, n=3) and receptor internalization (Figure EV7F).

The inhibitory effect of TFEB deletion on ECs proliferation was not counteracted by MYO1C silencing in human ECs (Figure EV7G). This result is not surprising in view of the deep influence of TFEB on genes related to cell proliferation (Figures 3B, 3C).

TFEB deletion up-regulates VEGFR2 by inhibiting a miR-15a/16-1-dependent post-transcriptional regulatory mechanism.

Because TFEB-silencing up-regulated VEGFR2 mRNA independently from a direct action on the promoter (Figure 5G), we further speculated a role for a miR-dependent post-transcriptional mechanism, which is widely involved in the control of vascular functions, including VEGFR2 (Chamorro-Jorganes et al., 2013; Dang et al., 2013; Park et al., 2013).

By crossing data of literature about the genomic location of miRs (Chamorro-Jorganes et al.; 2013, Dang et al., 2013) directly involved in the angiogenic process (Figure EV8A) with our Chip-Seq data set, we identified the “structural maintenance of chromosome 4” (SMC4) and the “deleted in leukemia-2” (DLEU2) genes (Figure EV8A) as two putative miR host genes involved in VEGFR2 regulation by TFEB. ChIP-Seq and ChIP-qPCR indicated that TFEB bound to the *DLEU2* but not the *SMC4* promoter (Figure 8A). For

these reasons, we focused on *DLEU2*, which is a tumor suppressor gene that is lost early in chronic lymphatic leukemia (Klein et al., 2010). *DLEU2* encodes a sterile transcript as well as the miR-15a/16-1 cluster, which is located intronic to the gene. This cluster encodes mature miR-15a-3p/5p and miR-16-1-3p/5p (Yue & Tigyi, 2010), which regulate the cell cycle and apoptosis and influence vascular function (Jackstadt & Hermeking, 2015; Sun et al., 2013), including VEGFR2 expression (Chamorro-Jorganes et al., 2011; Chan, Yue et al., 2013).

DLEU2 was down- and up-regulated in sh-TFEB and TFEBS142A ECs respectively (Figure 8B). The deletion of *Tfeb* resulted also in the down-modulation of *Dleu2* in lung ECs isolated from mutant mice (Figure 8B).

Next, we analyzed the expression of isoforms of the miR-15a/16-1 cluster in ECs (Figures 8C, EV8B). miR-15a-5p and miR-16-5p were expressed at high levels, whereas miR-15a-3p and miR-16-1-3p levels were negligible and were therefore not considered further.

In parallel with *DLEU2*, miR-15a-5p and miR-16-5p were down and up-regulated in sh-TFEB and TFEBS142A ECs, respectively (Figure 8C). Similarly, the in vitro Cre-mediated *Tfeb* deletion resulted in a marked reduction of miR-15a-5p and miR-16-5p in lung ECs isolated from mutant mice (Figure EV8B).

The functional connection between DLEU2 and the intragenic mir-15a-5p and mir-16-5p was further validated in sh-DLEU2 ECs, in which expression of TFEBS142A was unable to increase their expression. In particular the fold change expression of mir-15a-5p and mir-16-5p was respectively increased of 2.8±0.6 and 4.8±0.7 in ECs carrying TFEBS142A compared to control cells ($n=3$, $p<0.0001$). The co-expression of shDLEU2 and the constitutively active TFEB mutant blocked the expression of both miRs (mir-15a-5p: 0.2±0.2 relative fold change; and mir-16-5p: 0.4±0.2 relative fold change $n=3$, $p<0.0001$ *versus* TFEBS142A ECs).

To determine the possible regulatory role for miR-15a-5p and miR-16-5p in *VEGFR2* expression, we employed gain- and loss-of-function approaches.

The up-regulation of VEGFR2 transcription and protein synthesis observed in sh-TFEB ECs was rescued by transducing the specific mimic of miR-15a-5p and miR-16-5p (Figures 8D, 8E). On the contrary the down-regulation of VEGFR2 transcription and protein synthesis observed in TFEBS142A ECs was rescued by transduction of the specific inhibitors of miR-15a-5p and miR-16-5p (Figures 8F, 8G).

These data suggest a direct role for these miRs in the regulation of *VEGFR2* by TFEB.

On the contrary in sh-TFEB ECs, the rescue of the level of VEGFR2 similar to that presented in scr-shRNA ECs was not able to overcome the reduced proliferation stimulated by VEGF-A (Figure EV8C), in agreement with the direct effect of TFEB on CDK4 (Figures 4A-4F) .

VEGFR2 signal is reduced in TFEB-silenced ECs

The above described alterations of VEGFR2 behavior in shTFEB-ECs prompted us to investigate its signaling properties by analyzing VEGFR2 phosphorylation at Y1175 in total lysates, which is a discrete docking site for PLCγ and one of the most important effector of activated VEGFR2 (Simons et al., 2016). In sh-TFEB ECs compared with control cells, the degree of receptor phosphorylation after VEGF-A challenge was smaller relative to the larger amount of total VEGFR2 (Figures 6C, EV6A). We also observed a concomitant decrease in the phosphorylation of PLCγ and ERK-1/2, which are implicated in EC proliferation in response to VEGF-A (Takahashi et al., 2001) (Figures 6C, EV6A). The activation of c-Src, another tyrosine kinase substrate of VEGFR2 involved in cytoskeletal rearrangements and cell-matrix adhesion (Ferrando, Chaerkady et al., 2012) (Figures 6C, EV6A) was reduced in sh-TFEB challenged with VEGF-A.

These data indicate that the altered mRNA dynamics connected with a defect of receptor trafficking result in a reduced function of VEGFR2 in ECs lacking TFEB.

DISCUSSION

Here, we demonstrate that the EC-targeted deletion of Tfeb alters the mid and late phase of vascular development in mouse. By combining gene expression and ChiPseq analyses in genetically modified ECs where TFEB is either silenced or overexpressed, we found that this transcription factor positively regulates the expression of *CDK4* and miR-15a/16-1 cluster, while it exerts a repressor activity on *MYO1C*. The absence of TFEB results in a reduction of CDK4 and the consequent block of cell-cycle in ECs. At the same time, we found that VEGFR2 trafficking and its compartment localization is profoundly modified. Such alterations are mediated by the up-regulation of *MYO1C* and the abrogation of miR-15a/16-1-mediated post-transcriptional control of VEGFR2 expression, respectively. While the abnormal VEGFR2 behavior could be a potential compensation mechanism with respect to the cell-cycle hindrance, we demonstrated that the overall signaling activity is impaired.

The effect of TFEB on cell proliferation has been extensively investigated and primarily connected with its effect on autophagic flux. Knockdown of TFEB decreased proliferation of prostate (Blessing et al., 2017) and pancreatic cancer cell lines (Perera et al., 2015). On the contrary, TFEB up-regulation in renal and in cancer cells resulted in an increased proliferation rate (Calcagnì et al., 2016; Di Malta et al., 2017). The necessary role of TFEB in cell growth is further indirectly supported by the enhanced cell proliferation observed in renal cell carcinoma carrying the t(6;11)(p21:q13) translocation, which leads to a *TFEB* promoter substitution with the 5’ upstream regulatory sequence of the *alpha* intronless gene (Calcagnì et al., 2016). The lack of promoter-mediated physiological control of TFEB

expression promotes clonogenic cell growth (Haq & Fisher, 2011). In ECs, besides controlling autophagic flux (Fan et al., 2018) (Figures 6D,6E) our data indicate that TFEB exerts a more direct effect on cell proliferation. TFEB deletion reduced the expression of genes belonging to the “regulation cell cycle” GO and most importantly it directly bound the promoter of *CDK4*, an activator of G1-S transition of cell-cycle. The reduced availability of CDK4 impaired the phosphorylation of Rb, which relieves the Rb-mediated inhibition of the transcription factor E2F involved in the expression of cell cycle related genes (Malumbres & Barbacid, 2009).

However, the vascular phenotype observed in Tfeb mutants cannot be simply explained by the down modulation of CDK4 and the other cell-cycle related genes. 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).

Commented [1]: Parte da eliminare

VEGFR2 is considered the master gene of vascular development and angiogenesis in adult life (Simons et al., 2016). Therefore the alterations of VEGFR2 biology here observed can contribute to explain the vascular phenotype of Tfeb mouse mutants. We speculate that in an attempt to compensate the cell cycle defect triggered by TFEB deletion, ECs increased the amount of PM VEGFR2, which, however, showed signaling limitations. In murine and human ECs lacking TFEB, we demonstrated the down-modulation of the expression of intragenic miR-15a/16-1 cluster, which specifically targets the VEGFR2 3’UTR (Chamorro-Jorganes et al., 2011; Chan et al., 2013) and the subsequent post-transcriptional stabilization of *VEGFR2* mRNA. We also show that *TFEB* silencing increased the expression of the motor protein MYO1C, which functions as a cargo transporter (Greenberg & Ostap, 2013) and it has been reported to deliver VEGFR2

to PM (Tiwari et al., 2013). Actually, MYO1C silencing restored the effect of T*FEB* deletion on the co-localization between VEGFR2 and Rab4^{+} vesicles, which are involved in the receptor exocytosis. This data is consistent with previous observations that the amount of membrane VEGFR2 is reduced by *MYO1C* depletion, whereas *MYO1C* overexpression rescues VEGFR2 at the PM (Jopling et al., 2014; Simons et al., 2016, Tiwari et al., 2013). However the increased expression of VEGFR2 and its localization at PM was not paralleled by increased receptor signaling.

Actually, in sh-TFEB ECs we reported the decrease of the phosphorylation of VEGFR2 in Y1175, which represents a docking site for PLC© (Simons et al., 2016) and of the down-stream signal molecules c-Src and Erk1/2.

This discrepancy can be explained by the effect of TFEB on VEGFR2 membrane localization and trafficking. First in sh-TFEB ECs, the PM accumulated VEGFR2 defectively co-localized with CAV-1-rich domains, which are hotspots for signaling activity and required for VEGFR2 activation (Cho et al., 2004). Second, when TFEB was knocked-down, we observed an alteration of receptor trafficking analyzed by biotinylated VEGFR2 internalization, FACS and TIRF. Of note, the accumulation of VEGFR2 induced by the inhibition of endocytic (Gourlaouen et al., 2013; Sawamiphak et al., 2010) and recycling (Ballmer-Hofer et al., 2011; Lanahan et al., 2013) pathways leads to the inhibition of its signaling including the activation of PLCγ and MAP kinases.

The existence of extensive cross-talks of autophagic and lysosome pathways with the mechanisms fueling endocytosis is well demonstrated (Pavel & Rubinsztein, 2017). Therefore it is intriguing to speculate that the canonical effect of TFEB on endothelial autophagic flux (see Figures 6D,6E) negatively influence the VEGFR2 endocytosis which, is known to be important in its signaling (Simons et al., 2016).

Altogether these observations shed light on the TFEB-mediated cellular mechanisms regulating VEGFR2 expression and allow a better understanding of the observed vascular phenotype in Tfeb mutants.

The in vivo vascular defects observed in *Tfeb*$^{EC-/-}$ and *Tfeb*$^{iEC-/-}$ mice validated the concept that one of the major consequences of *TFEB* deletion is the reduction of EC proliferation and the impairment of VEGFR2 activity. After the establishment of the primitive vascular plexus, the formation of the vascular tree is promoted by sprouting angiogenesis, which is characterized by the presence of either migratory tip cells or proliferating stalk cells. Tip cells guide network expansion, while stalk cells proliferate behind the tip cells to extend the vascular lumen as the sprouts elongate. The crucial role of VEGFR2 in this scenario has been demonstrated by experiments performed with ECs derived from embryonic stem cells, in which cells that are heterozygous for the *Vegfr2* allele show defects in stalk-tip dynamics (Jakobsson et al., 2010).
EC *Tfeb*-deficient embryos died between E10.5 and E11.5, displaying defects in the patterning of several regions of the vascular tree, with a reduction in larger caliber vessels and the capillary network. The effect of TFEB presumably begins after the formation of primitive vascular plexus because *Tfeb* deletion did not affect the appearance of hemangioblasts. Furthermore, post-natal vascular maturation in the retina and kidney was altered in *Tfeb* mutants, with reduced proliferation. In *Tfeb* mutants, the retinal vasculature presented a delayed expansion of the vascular plexus and a significant reduction in vessel density. However, the ability of tip cells to extend filopodia was unaffected. This phenotype resembles that caused by the concomitant endothelial ablation of *Vegfr2* and *Vegfr3*, in which the vascular hyperplasia caused by *Vegfr3* deletion is counterbalanced by the absence of *Vegfr2* (Zarkada et al., 2015), or by enhanced Notch activity in stalk cells (Guarani et al., 2011; Phng et al., 2009).

The deletion of *Tfeb* in renal ECs impairs the maturation of the glomerulus, a process that is strictly dependent of the VEGF-A / VEGFR2 axis (Esser et al., 1998). We observed a deep alteration of the cellular structures with the fusion of podocyte foot processes and the disappearance of endothelial fenestrae. Generally, the formation of the filtration barrier is regulated by a paracrine mechanism involving VEGF-A released by podocytes and VEGFR2 expressed on glomerular ECs. The phenotype observed in *Tfeb* mutants is similar to that described in whole-body postnatal deletion of *Vegfr2* and podocyte-specific *Vegfa* knockout (Eremina et al., 2003; Sison et al., 2010). Interestingly, pre- or post-natal *Vegfa* deletion in podocytes promotes nephrotic syndrome or thrombotic microangiopathy, respectively (Eremina et al., 2008; Eremina et al., 2003).

Altogether, these data bring new insight in the regulatory effect of TFEB in vascular cells (Fan et al., 2018; Lu et al., 2017) by activating a genetic program regulating cell proliferation and VEGFR2 functions. We speculate that TFEB represents a powerful cellular tool that connects vascular needs with the metabolic state. Recent data indicate that cellular nutrient levels regulate TFEB concentrations through an autoregulatory feedback loop, in which TFEB binds to its own promoter in a starvation-dependent manner and induces its own expression (Settembre et al., 2013a) and that TFEB negatively regulates the level of hypoxia-inducible factor-1 (Hubbi et al., 2013). Furthermore, TFEB controls lipid breakdown, and its overexpression activates fatty acid oxidation (Settembre et al., 2013a), which is necessary for sprouting angiogenesis, by fueling de novo nucleotide synthesis for DNA duplication(Schoors, Bruning et al., 2015).

Furthermore, the recent observation that shear stress up-regulates TFEB (Lu et al., 2017) allows hypothesizing its role in regulating the optimal PM amount of VEGFR2, which, through a multimeric complex with VE-cadherin, PECAM-1 and VEGFR3 (Baeyens et al.,

2016), transduces the frictional force from blood flow into biochemical signals that regulate gene expression and cell behavior.

MATERIALS AND METHODS

Reagents

Wizard SV Genomic DNA Purification System, GoTaq G2 Hot Start Polymerase and The Dual-Luciferase® Reporter (DLR™) Assay System (Promega Corporation); Dynabeads Protein G, Power SYBR Green PCR Master Mix, Phusion Site-Directed Mutagenesis Kit, Lipofectamine® RNAiMAX Reagent, Click-iT® EdU Flow Cytometry Cell Proliferation Assay, Illumina TotalPrep RNA Amplification Kit, pMCS-CypridiLuc_AC167_MYO1C_promoter, pMCS-CypridiLuc_AC167_MYO1C_promoter_d1 pMCS-CypridiLuc_AC167_MYO1C_promoter_d2, pMCV-GreenReLuc, Lipofectamine® RNAiMAX Reagent, Cypridina Luciferase Glow Assay Kit, Pierce Renilla Luciferase Glow Assay Kit, High Capacity cDNA Reverse Transcription kit, Taqman PCR Universal MasterMix, Taqman assays, SYBR GreenER kit, EZ-Link™ Sulfo-NHS-SS-Biotin, Maxisorp 96-well plates, phalloidin-555 and DAPI (Thermo Fisher Scientific); shRNA against *DLEU2* (Dharmacon); mirVanaTM miRNA control and inhibitors or mimics (Ambion); hypoxyprobe-1-FITC-conjugated antibody (Chemicon); collagenase/dispase (Roche); miRNeasy Mini Kit and QIAQuick PCR Purification Kit (Qiagen); NextSeq 500 Illumina sequencer and HumanHT-12 v4.0 Expression Bead Chip GenomeStudio software V2011.01 (Illumina); NEBNext® ChIP-Seq Library Prep Reagent Set for Illumina (New England Biolabs); CDK4-luciferase full length and CDK4-luciferase fragment A (Addgene); IntraPrep kit (Beckman Coulter); IgG (12-370, Millipore); human VEGF-A (R&D System); streptavidin-agarose beads (Upstate Biotechnology), Growth Factor Reduced Matrigel (Corning). Other reagents, if not indicated, were from Sigma Aldrich.

Antibodies

Anti-GFP and anti-Ki67(SP6) from Thermo Fisher Scientific; Isolectin-B4, anti-MYO1C and anti-α-Tubulin (B-5-1-2), anti-LAMP1, anti-PCNA (PC10) from Sigma Aldrich; anti-CD31, anti-FLK1-APC-coniugated (Avas12alpha1), anti-CD71-FITC-coniugated(C2), anti-CD117(2B8)-PE-cy7- coniugated, anti-CD31-FITC-coniugated(Mec 13.3), anti-CD102, anti-IgG_{2a} k Isotype APC-conjugated (R3595), anti-IgG2bk Isotype-PE-Cy7 conjugated(2B8), anti-CD326, anti-Rab4 and anti-Rab5 from BD Biosciences; anti-VEGFR2 (55B11), anti-p-Tyr-1175-VEGFR2(D5B11), anti-PLCγ-1, anti-p-Tyr-783-PLCγ-1, anti-ERK1/2, anti-p-ERK-1/2 (T202/Y204)(E10), anti-Src(36D10), anti-p-Src (Tyr416)(D49G4), anti-CDK4(D9G3E), anti-Rb(4H1), anti p-Rb (Ser780)(C84F6), and anti-p-Rb (Ser807/811)(D20B12), anti-PCNA(PC10), anti-ULK1 (D8H5), anti-ATG9A(D409D) from Cell Signaling Technology; anti-endomucin (V.7C7), anti-Flk-1(A-3), anti-Podocin(G-20), anti-Caveolin-1(N-20) and anti-E2F2(TFE-25), anti-E2F1 from Santa Cruz Biotechnology; anti-human VEGFR2 (89109) from R&D System; anti-TFEB from MyBiosource; hypoxyprobe-1-FITC-conjugated antibody (Chemicon); anti-LC3 from Novus Biologicals; anti-Cyclin D1(SP4), anti-E2F1 and anti-TGN46(2F7.1) from Abcam. For

Mice

All animal procedures were approved by the ethics committee of the University of Turin and by the Italian Ministry of Health (Protocol approval no. 864/2015-PR).

To generate a transgenic mice expressing Tfeb-GFP, the sequence for the open reading frame of EGFP was inserted between the last amino acid and the translation termination codon in exon 9 (NCBI transcript NM_001161722.1). The positive selection marker (Puromycin resistance - PuroR) was flanked by FRT sites and was inserted into intron 8. The targeting vector was generated using BAC clones from the C57BL/6J RPCIB-731 BAC library and was transfected into the TaconicArtemis C57BL/6N Tac ES cell line. Homologous recombinant clones were isolated using positive (PuroR) and negative (Thymidine kinase - Tk) selection. The constitutive KI allele was obtained after Flp-mediated removal of the selection marker. The Tfeb-EGFP fusion protein was expressed from the endogenous Tfeb promoter. The remaining recombination site was located in a non-conserved region of the genome.

Endothelium-specific silencing of Tfeb was achieved by crossing $\mathrm{Tfeb}^{\mathrm{flox}}$ mice (Settembre et al., 2013a) with the following: i) transgenic mice expressing Cre-recombinase driven by the Tie2 promoter (*Tie2-Cre*) (The Jackson Laboratory) to obtain a line with constitutive silencing of Tfeb in the endothelium ($Tfeb^{EC}$); and ii) transgenic mice expressing tamoxifen-inducible Cre-recombinase ($\mathrm{Cre\text{-}ER}^{\mathrm{T2}}$) driven by the vascular endothelial cadherin promoter ($Cdh5\text{-}Cre\text{-}\ ER^{T2}$) ($Tfeb^{iEC}$). $Tfeb^{floxed}/Cre^{+}$ mice (indicated respectively as $Tfeb^{EC-/+}$ or $Tfeb^{EC-/-}$ and $Tfeb^{iEC-/+}$ or $Tfeb^{iEC-/-}$ in the consideration of *Tfeb* deletion in heterozygosis or homozygosis) were compared with $Tfeb^{floxed}/Cre^{-}$ (without Cre) mice of the same progenies (indicated as control). Inducible Cre was activated by daily tamoxifen administration from p1 to p3 (1 mg/ml, 50 µl by intragastric injection) or from p5 to p8 (2 mg/ml, 50 µl by i.p. injection)(Pitulescu, Schmidt et al., 2010). To exclude tamoxifen pathological effects both control mice that $Tfeb^{iEC-/+}$ and $Tfeb^{iEC-/-}$ mice were treated.

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).

% point prevalence of vascular alterations was evaluated at E10.5 (our point time). Briefly, 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 = (n positive embryos/ n positive embryos+ n negative embryos) x100

Mouse genotyping

Mouse genotypes were analyzed by PCR with genomic DNA from yolk sacs, embryos and tail samples. DNA was extracted using the Wizard SV Genomic DNA Purification System. PCR reactions were performed using appropriate primers for specific PCR samples with a commercial kit (GoTaq G2 Hot Start Polymerase). The following primers used for genotyping:

Tfeb: 5’-CAGCCCCTTACCAGCGTCCC-3’ and 5’-GGGTGCAATCTAATCAGGGGGC-3’;

Tfeb delta allele: 5’-GTAGAACTGAGTCAAGGCATACTGG-3’ and 5’-GGGTGCAATCTAATCAGGGGGC-3’;

Cdh5-Cre-ER^{T2}: 5′-GCCTGCATTACCGGTCGATGCAACGA-3′ and

5′-GTGGCAGATGGCGCGGCAACACCATT-3′;

Tie2-Cre: 5'-GCCTGCATTACCGGTCGATGCAACGA-3' and

5'-GTGGCAGATGGCGCGGCAACACCATT-3';

Tfeb-EGFP: 5'-CTGTATAGCACTGGCTCTGTAGACC-3' and

5'-GGTGGTGGGAATGGAAACC-3'.

Cells, genetic manipulation and biological assays

Lung ECs were isolated from control and *Tfeb*^{iEC} mice by positive selection with anti-CD31 and anti-CD102 Abs conjugated to Dynabeads and maintained in vitro up to passage 2 in M199 with 20% FCS. Lung epithelial cells were isolated with the same procedure by using anti-CD326 Ab and maintained up to passage 2 in DMEM with 10% FCS. When *Tfeb* silencing was not induced in vivo (as previously described) but in vitro, lung ECs were incubated with 4-OH-tamoxifen (5 μM, 48h or 72h) to allow *Cre* activation and *Tfeb* silencing. The correct recombination of the *Tfeb* allele was verified by real-time PCR (Power SYBR Green PCR Master Mix). PCR was performed using specific primers (5'-GACTCAGAAGCGAGAGCTAACAG-3' and 5'-TGGCCTTGGGGATCAGCATT-3') recognizing the exon 5-6 region of *Tfeb.*

In vitro experiments were carried out on human endothelial cells (indicated as ECs) isolated from umbilical-cord veins maintained as described previously (Napione, Strasly et al., 2012b). To minimize cell variability, pools of 5 different donors were used. The isolation of primary human ECs was approved by the Office of the General Director and Ethics Committee of the Azienda Sanitaria Ospedaliera Ordine Mauriziano di Torino hospital (protocol approval no. 586, Oct 22 2012 and no. 26884, Aug 28 2014) and informed consent was obtained from each patient. Cells were tested for mycoplasma contamination by means of Venor GeM Mycoplasma Detection Kit.

The *TFEBS142A* mutant was generated from TFEB cDNA (Origene, cod. SC122773) by inserting a single point mutation using the Phusion Site-Directed Mutagenesis Kit.

TFEBS142A was cloned into the pTRIPZ inducible vector, and the transgene was induced by doxycycline addition (0.5 µg/ml) for 3h (for ChIP-Seq experiments) or 24 h (other experiments): these cells were indicated as TFEBS142A. ECs infected with pTRIPZ-TFEBS142A but not treated with doxycycline were used as control and indicated as "control". To exclude unspecific doxycycline effects, ECs no infected were also treated.

Loss-of-function experiments were carried out with shRNA against TFEB (Catalog number TRCN0000013111, TRCN0000437429, TRCN0000440038, TRCN0000413524, TRCN0000013110 and TRCN0000437246 NM_007162.2) or *MYO1C* (Catalog number TRCN0000122925, TRCN0000122927, TRCN0000122928 NM_033375) and *DLEU2* (Catalog number TRCN0000072484, TRCN0000072485, NR_002612) cloned into the pLKO.1-puro non-Mammalian vector. In particular, in the different experiments we used shRNA TRCN0000013111 against TFEB, shRNA TRCN0000122927 against MYO1C and shRNA TRCN0000072484 against DLEU2. ECs were transduced with specific lentiviral particles (MOI=1) prepared according to (Follenzi, Ailles et al., 2000) in the presence of 8 µg/ml polybrene. The medium was replaced after 24 h, and cells stably expressing the lentivirus were selected on puromycin (1 µg/ml) for 24 h. ECs were transfected with the appropriate control (miR-inhibitor/ miR-mimic control) has-miR-15a-5p and has-miR-16-5p mirVanaTM miRNA inhibitors or mimics (90 nmol/L, 24 h) using Lipofectamine® RNAiMAX Reagent. We verified the degree and the specificity of miRNA inhibition or up-regulation and the eventually off-target effects using qPCR (see specific section).

Proliferation rate and DNA content were evaluated by using Click-iT® EdU Flow Cytometry Cell Proliferation Assay and propidium iodide (PI) staining according to manufacturer protocol. Briefly, human or lung murine ECs ($5x10^6$) were starved overnight and then stimulated with 20% FCS or VEGF-A (30 ng/ml) for 24h. After 1 hr incubation with 10 µM thymidine fluorescent analog 5-ethynyl-2'-deoxyuridine (EdU), cell suspensions were processed for EdU detection and cell DNA was labelled by a 3 hrs treatment with 50 µg/ml

PI and 100 µg/ml Ribonuclease A. Data were acquired with a CyAn ADP flow cytometer (Dako) and analysed with FlowJo software (Tree Star, Ashland, OR, USA).

EC chemotaxis and morphogenesis were assayed and analyzed as described previously (Serini, Ambrosi et al., 2003). In particular, in morphogenesis assay ECs (2 x 10^4) were re-suspended with poor MEM and loaded on the top of the growth factor reduced Matrigel. Each conditional group contained 6 wells. Following incubation at 37°C for 6h with VEGF-A (30 ng/ml), each well was fixed with 4% paraformaldehyde, permeabilized in PBS 1% Triton X-100 and stained with phalloidin-555 and DAPI.

Yolk sac cells analysis

Yolk sacs from control or *Tfeb*^{EC} embryos were collected at E9.5-E10.5 and genotyped as described above. Each yolk sac was treated separately to obtain single-cell suspensions by incubation with collagenase/dispase at 37°C for 1 h with occasional trituration. The samples were then subjected to flow cytometry analysis (see specific section).

Tissue and cell staining and analysis

Whole-mount samples were prepared (Yokomizo et al., 2012) by fixing tissues in 4% paraformaldehyde for 1 h on ice. Tissues were incubated overnight at 4°C in DPBS, 1% Triton X-100, containing the indicated Abs. After washes, tissues were incubated for 1 h in the same buffer containing the appropriate Alexa fluor secondary Abs. After fixation with 4% paraformaldehyde for 30 min at RT, samples were flat-mounted onto glass coverslips.

Tissue sections were frozen in OCT compound and cut into 6-10-µm thick sections. Embryo kidney and lung slices were fixed in 4% paraformaldehyde and Zinc fixative, respectively, for 10 min at room temperature, permeabilized, stained as indicated, and then incubated with the appropriate fluorescently labeled secondary Ab.

Hypoxia was detected by the formation of pimonidazole adducts 1 h after intraperitoneal (i.p.) injection of 60 mg/kg pimonidazole hydrochloride in pregnant females. Mice were then sacrificed, and the embryos were harvested and immunostained with hypoxyprobe-1-FITC-conjugated Ab.

Immunofluorescence staining of vessels was manually quantified using ImageJ software after excluding any small-sized, not-interconnected objects. Immunoreactivity was calculated as the surface area of each Ab staining colocalized with the EC markers endomucin, isolectin B4 or CD31 and normalized to the total vascular surface area visualized by the same molecules. The number of branching points per field was quantified as described previously (Samarelli et al., 2014).

To quantify differences in the average length and total number of filopodia between control and *Tfeb*^{iEC} mice, we used custom-made image analysis algorithms written in MATLAB. (MATLAB and Image Processing Toolbox, The MathWorks, Inc., Natick, Massachusetts, United States.) Briefly, confocal stacks were maximum projected and then processed to obtain filopodia or the main vascular network. To extract main vascular network, images were median filtered, thresholded and then the external perimeter of the vasculature was measured.As for filopodia, images were filtered with a linear rotating kernel filter (Lee Y-K et al., 1990) and then processed with a multiscale vessel enhancement filter (Frangi, AFet al., 1998). Quantifications were based on the total length of the segmented filopodia divided by the total perimeter of the main vascular network and average length of Major Axis of each of the filopodial structure identified.

For transmission electron microscopy, mice were perfused with fixative buffer (2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M sodium cacodylate buffer). Kidneys were immediately placed into fresh fixative and then cut into small cubes. The analysis was performed at the Department of Cellular and Molecular Medicine, UCSD (La Jolla, CA).

For cell immunofluorescence staining, ECs ($7x10^4$), grown up on cover slides, were fixed in 4% paraformaldehyde, permeabilized (PBS 0,1% Triton X-100) and then incubated with the indicated primary Abs and the appropriate Alexa fluor secondary Abs.

FACS

Cell suspensions from dissociated yolk sacs were incubated with indicated specific Abs and then analyzed on a CyAn ADP Analyzer (Beckman Coulter). DAPI positive dead cells were excluded from analysis. Cytosolic and PM VEGFR2 staining was performed using an IntraPrep kit (Beckman Coulter) and the data were analyzed using Summit 4.3 software (Beckman Coulter). The following Abs were used: anti-VEGFR2-PE-coniugated (89106); anti-IgG_1-PE-coniugated(11711); anti-CD202b(Tie2)-PE-conjugated(Tek4); anti-IgG_1 k Isotype-PE-conjugated(RTK2071); anti-FLK1-APC-coniugated(Avas12alpha1); anti-IgG_{2a} k Isotype-APC-conjugated(R3595); anti-CD71-FITC-coniugated(C2); anti-IgG_1 k Isotype-FITC-conjugated(R3-34); anti-CD117-PE-Cy7-conjugated(2B8) and anti-IgG2bk Isotype-PE-Cy7 conjugated.

TIRF microscopy

TIRF microscopy was performed using a Leica AM TIRF MC system mounted on a Leica AF 6000LX workstation with a 63X oil-immersion objective and a laser penetration depth of 110 nm. ECs were fixed, saturated and permeabilized, and then treated with Ab anti-VEGFR2 (A3) and appropriate Alexa fluor secondary Ab.

Quantification of immunofluorescence analysis

Immunofluorescence images were acquired on a TCS SPE or TCS SP8 STED confocal laser-scanning microscopes (Leica Microsystems). Different fields (tissue samples:15-20; cell samples: 5-8) per sample section were randomly chosen for analysis. When the same

molecule was evaluated in different samples, laser power, gain and offset settings were maintain. Images were quantified using ImageJ software or TCS SP8 quantification software. For each analysis, at least 3 different experiments were performed.

Microarray experiments

RNA extracted using a miRNeasy Mini Kit was amplified and labeled using an Illumina TotalPrep RNA Amplification Kit, and 750 ng of cRNA probe was hybridized to a HumanHT-12 v4.0 Expression Bead Chip. All experiments were performed in triplicate. Cubic spline-normalized probe intensity data, together with detection p-values, were obtained using GenomeStudio software V2011.01. We selected probes with a detection p-value < 0.05. For each gene, we retained the associated probe with the largest mean expression value across all samples. For each probe, the $\log_2$ signal was converted to the $\log_2$ ratio against the global average expression of that probe in all samples. Data were clustered using Gene Expression Data Analysis Suite (GEDAS) (www://gedas.bizhat.com) and LIMMA (Smyth, 2002) was used to identify the modulated genes. A threshold $|\log_2 FC|$ of >0.5 and an adjusted p-value < 0.1 were used to select differentially expressed genes. Statistical analyses were performed in the R environment (http://www.R-project.org/).

GSEA was performed using the public platform at http://www.broadinstitute.org/gsea/msigdb/downloads.jsp. In particular, after gene filtering for all the datasets, probes were collapsed on Gene Symbols, again selecting the probe with the largest mean expression across all the experiments for each gene. GSEA statistics were calculated with the default settings based on a Pearson metric. P-values and FDRs were calculated by repeating sample permutations 1000 times. The data were further analyzed for enrichment in biological themes (GO – Biological Processes, Molecular Functions, Cellular Components) by using the DAVID resource (http://david.abcc.ncifcrf.gov).

miRs annotation

We defined a set of miRs associated with angiogenesis on the basis of published literature with evidence based on experimental data (Chamorro-Jorganes et al., 2013). For each miR, we considered the corresponding pre-miR based on the annotations provided by the Ensembl database, version Human GRCh37 (GENCODE 19, miRBase 18). In case the miR was intragenic, we identified the host gene and selected only those originating from the same DNA strand. Of these, we further chose only miRs with VEGFR2 as a validated target (Chamorro-Jorganes et al., 2011, Chan et al., 2013). Finally, for this short list of candidates, we checked significant TFEB binding peak(s) in the putative core promoter region of the host gene.

ChIP-Seq

For genome-wide analysis of TFEB binding, sequencing libraries were constructed using the NEBNext® ChIP-Seq Library Prep Reagent Set for Illumina and a NextSeq 500 Illumina sequencer. ChIP-Seq reads were aligned to the hg19 genome assembly using Bowtie v0.12.7 with the following parameters: -q --max /dev/null -v 1 -S –sam-nohead -m 1. Data were filtered using the following specifications: duplicate reads were filtered out. BedGraph files were generated by using MACS tool. Peak calling was performed as described previously (Krepelova, Neri et al., 2014) using a p-value cutoff = 1E-08. TFEB target genes were defined as those having a peak between -500 and +100 from the annotated transcription start site.

ChIP

Chromatin immunoprecipitation of TFEB was performed as described previously (Krepelova et al., 2014). Briefly, approximately $2x10^7$ crosslinked ECs were resuspended in 250 µl of SDS lysis buffer (10 mM EDTA, 1% SDS, 16.7 mM Tris, pH 8) with protease

inhibitors and incubated for 10 min on ice. After sonication, the cell lysate was centrifuged at 12,000 g for 10 min at 4°C. The supernatant was diluted ten-fold with ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1% Triton) before immunoprecipitation.

The supernatant was incubated with 5 μg of Ab anti-TFEB or IgG with rotation at 4°C for 16 h. Samples treated with IgG were used as a negative control. Afterwards, previously BSA-saturated beads (Dynabeads® Protein G) were added for 2h. Immunoprecipitated complexes were extensively washed before adding SDS elution buffer 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, DTT 5 mM, NaCl 150 mM) for 30 min at room temperature. After decrosslinking, DNA was purified using a QIAQuick PCR Purification Kit according to the manufacturer's instructions.

Luciferase reporter assay

Cells were seeded in 24 well plates at a density of 4 × 10^4 cells per well. After ChIP-Seq analysis, to identified the relative TFEB MACS peak on the different promoter gene, ECs cells were transfected with CDK4-luciferase full length promoter (from J. Modiano, Addgene plasmid #86656) and CDK4-luciferase fragment A (from J. Modiano, Addgene plasmid #86658) in which the predicted ChIP-Seq TFEB peak near TSS on CDK4 promoter is deleted. ECs cells were transfected with pMCS-CypridiLuc_AC167_MYO1C_promoter (sequences -1500/+200 from TSS of the longest isoform of MYO1C gene according to Ensembl GRCh37, transcript ID: ENST00000359786), pMCS-CypridiLuc_AC167_MYO1C_promoter_d1 (in which the putative TFEB binding site, has been deleted), pMCS-CypridiLuc_AC167_MYO1C_promoter_d2 (in which 100 bps around the same putative TFEB binding site have been deleted) synthesized Geneart support (Thermo Fisher

Scientific) and with pMCV-GreenReLuc using Lipofectamine® RNAiMAX Reagent according to the manufacturer's instructions.

Luciferase activities were analyzed with Pierce Cypridina Luciferase Glow Assay Kit and Pierce Renilla Luciferase Glow Assay Kit or The Dual-Luciferase® Reporter (DLR™) Assay System using a Glomax 20/20 luminometer (Turner Biosystems, Sunnyvale, CA, USA). The relative reporter activity was calculated by normalizing the luciferase activity with renilla luciferase activity.

Real-time PCR

Extracted RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription kit. Real-time PCR was performed on a CFX96 system (Bio-Rad) using Taqman PCR Universal MasterMix and specific Taqman assays. Experiments were performed in triplicate. TBP, RNU44 and U6 were used as reference genes.

The following Taqman assays were used: TFEB (human, Hs00292981_m1, mouse, Mm00448968_m1); CDK4 (human Hs00364847_m1, mouse Mm00726334_s1); E2F2 (human Hs00918090_m1, mouse Mm01192124_m1); E2F4 (human Hs00608098_m1, mouse Mm01247763_m1), E2F1 (human Hs00153451_m1, mouse Mm00432939_m1); CCNA1 (human Hs00171105_m1); CCNA2 (human Hs00996788_m1); CCNE2 (human Hs00180319_m1); CDCA4 (human Hs00937497_s1); CDCA7 (human Hs00230589_m1); CDT1 (human Hs00925491_g1, mouse Mm00466006_m1); CDC25B (human Hs01582335_m1); CDCA5 (human Hs00969392_g1); PCNA (human Hs00427214_g1, mouse Mm00448100_g1); VEGFR2 (human Hs00911700_m1, mouse Mm01222421_m1); DLEU2 (human Hs00863925_m1, mouse Mm01319189_m1); MYO1C (Hs00300761_m1); miR-15a-3p (002419); miR-15a-5p (000389); miR-16-1-3p (002420); miR-16-5p (000391); TBP (human Hs00427620, mouse Mm_00446971); RNU44 (001094); and U6 (001973).

For the ChIP experiments, immunoprecipitated DNA was analyzed by qPCR using a SYBR GreenER kit. The oligonucleotide sequences of the gene promoters were as follows: VEGFR2 5'- CCAACGAAGAGCCCTAGTGA-3' and 5'-ACCAGAAGGAACGAATGTGG-3'; DLEU2 5'-TTTGTGCAGTTTCAGCAAAG-3' and 5'-CTGTGTACTTAGGTCGTGTG-3'; and SMC4 5' CTGAAGAGGCGTTTCTGGAC-3' and 5'-GGAAGAGCGAGGGATTCTTT-3'; CDK4 5'- TGTGATAGCAACAGATCACG-3' and 5'-GTTCCTACGGCCCCATAC-3'.

Western blot

Western blotting and quantitative analysis using a ChemiDoc Touch Imaging System (Bio-Rad) and Image Lab software 5.2.1 (Bio-Rad) were performed as described (Napione et al., 2012a) with the use of specific Abs above indicated. The following Abs were used: anti-TFEB; anti-VEGFR2 (55B11), anti-p-Tyr-1175-VEGFR2 (D5B11), anti-PLCγ-1, anti-p-Tyr783-PLCγ-1, anti-ERK-1/2, anti-p-ERK-1/2 (T202/Y204) (clone E10), anti-Src (36D10), anti-p-Src (Tyr416)(D49G4), anti-CDK4 (D9G3E), anti-Rb (4H1), anti p-Rb (Ser780)(C84F6) and anti-p-Rb (Ser807/811)(D20B12) anti-PCNA (PC10), anti-ULK1 (D8H5), anti-ATG9A (D409D); anti-LC3; anti-CD31; anti-MYO1-C, anti-LAMP1 and anti-α-tubulin (B-5-1-2); anti-Cyclin D1 (SP4), anti-E2F1 and anti-E2F2 (TFE-25).

Biochemical analysis of PM distribution of VEGFR2

ECs were starved in M199 for 3 h and then stimulated with VEGF-A at 37°C (30 ng/ml) to allow VEGFR2 internalization. Cells were placed at 4°C, and the remaining receptors on PM were labeled with 0.15 mg/ml of impermeant sulfo-NHS-SS-biotin in PBS for 10 min. The unreacted biotin was quenched with TBA (25 mM Tris, pH 8, 137 mM NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM Na_2HPO_4) and ECs were solubilized in lysis buffer (20 mM Tris, pH 7.5, 125 mM NaCl, 10% glycerol, 1% NP40, protease inhibitor

cocktail). This fraction represents the total cellular VEGFR2. PM biotin-VEGFR2 complexes were separated from total VEGFR2 (biotin-free) using streptavidin-agarose beads (Napione et al., 2012). After protein extraction from the beads, equivalent volumes of the PM and total samples were resolved by SDS-PAGE and blotted with specific Abs. Loading controls were performed with an anti-CD31 or α-tubulin Abs.

VEGFR2 internalization assay

VEGFR2 internalization assays were performed as previously described (Valdembri et al., 2009). ECs were PM-labeled at 4°C with 0.5 mg/ml sulfo-NHS-SS-biotin in PBS for 30 min on ice. Labeled cells were washed with cold MEM 1% FBS and cold PBS, and endocytosis was induced using prewarmed MEM, 1% FBS, containing VEGF-A (30 ng/ml) and 0.1 M primaquine. At the indicated times, ECs were transferred to ice, and biotin was removed from the PM by incubation with 20 mM sodium 2-mercaptoethanesulfonate (MesNa) in 50 mM Tris-HCl (pH 8.6), 100 mM NaCl, and 0.015 N NaOH for 1 h at 4°C. MesNa was quenched by the addition of 20 mM iodoacetamide for 10 min. Cells were lysed in a specific buffer at 4°C (25 mM Tris-HCl, pH 7.6, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM Na_3VO_4, 0.5 mM EGTA, 1% Triton X-100, 5% glycerol, protease inhibitor cocktail). The levels of biotinylated VEGFR2 were determined by capture-ELISA. Briefly, Maxisorp 96-well plates were coated overnight with 5 μg/ml of anti-VEGFR2 (89109) Ab at 4°C and were blocked in PBS containing 0.1% Tween-20 with 5% BSA for 1 h at RT. VEGFR2 was captured by overnight incubation of the cell lysate at 4°C. Unbound material was removed by washing, and the wells were incubated with streptavidin-conjugated horseradish peroxidase for 1 h at 4°C. Biotinylated VEGFR2 was detected with a chromogenic reaction.

The percentage of internalization of VEGFR2 was calculated as the percent of biotin-VEGFR2 complexes in cell lysates after VEGF-A stimulation with respect to all the biotin-VEGFR2 complexes in unstimulated cells.

“Antibody feeding assay” was performed as previously described (Gourlaouen et al., 2013). ECs were incubated for 3h in serum-free M199 medium at 37 °C, then PM VEGFR2 was labeled with anti-C-Terminal-VEGFR2 Ab (R&D System) for 30 min at 4 °C and then cells were fixed or transferred to prewarmed serum-free M199 medium containing VEGF-A (30ng/ml) at 37 °C for 15 min to permit internalization. Cells were then incubated with the anti-Rab5 Ab and the appropriate Alexa fluor secondary Abs.

Statistical analysis

Sample sizes were not selected according to a specific power analysis but just in agreement with similar experiments done in other laboratories working in vascular development and quoted in the specific references. No statistical methods was used to predetermine sample size.

We did not randomize sample/animals because our experimental design did not require this type of strategy. The investigators were not blinded to allocation during experiments and outcome assessment. The data are indicated as the mean ± SEM. Statistical analyses were performed using unpaired Student’s *t*-test (two-tailed) or two-way ANOVA, followed by post-hoc pairwise analysis tests, as indicated. A p-value < 0.05 was considered significant.

Data availability

The datasets generated during the current study are available in The Gene Expression Omnibus of the National Center for Biotechnology Information (accession number GSE88896).

DEGs list is available in Table 1.

ACKNOWLEDGMENTS

This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) grants: 12182, 14284,11982,17639); Ministry of University and Research (RBAP11BYNP, RBFR08F2FS-002), Fondazione Cassa di Risparmio and Compagnia di San Paolo (Torino) and Italian Telethon Foundation. The authors thank L. Tamagnone, L. Napione, G. Serini (University of Torino, Italy), S. Sigismund (IFOM, Milano, Italy) for helpful discussions, T. Meerloo (UCSD, San Diego, US) and S. Giove (Candiolo Cancer Institute, Candiolo, Italy) for technical support.

AUTHOR CONTRIBUTIONS

Conceptualization, GD, SO, FB, LP, GC, EA; Methodology, GD, EA, AB; Formal Analysis, DC, AP, FN; Investigation, GD, GC, AN, VC, FN; Resources, AB; Writing-Original Draft, GD, FB; Writing-Review & Editing, FB; Supervision, FB; Funding acquisition, FB, SO, AB.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

REFERENCES

Baeyens N, Bandyopadhyay C, Coon BG, Yun S, Schwartz MA (2016) Endothelial fluid shear stress sensing in vascular health and disease. *J Clin Invest* 126: 821-828

Ballmer-Hofer K, Andersson AE, Ratcliffe LE, Berger P (2011) Neuropilin-1 promotes VEGFR-2 trafficking through Rab11 vesicles thereby specifying signal output. *Blood* 118: 816-826

Bartlett CS, Jeansson M, Quaggin SE (2016) Vascular Growth Factors and Glomerular Disease. *Annu Rev Physiol* 78: 437-461

Blessing AM, Rajapakshe K, Reddy Bollu L, Shi Y, White MA, Pham AH, Lin C, Jonsson P, Cortes CJ, Cheung E, La Spada AR, Bast RC, Merchant FA, Coarfa C, Frigo DE (2017) Transcriptional regulation of core autophagy and lysosomal genes by the androgen receptor promotes prostate cancer progression. *Autophagy* 13: 506-521

Calcagnì A, Kors L, Verschuren E, De Cegli R, Zampelli N, Nusco E, Confalonieri S, Bertalot G, Pece S, Settembre C, Malouf GG, Leemans JC, de Heer E, Salvatore M, Peters DJ, Di Fiore PP, Ballabio A (2016) Modelling TFE renal cell carcinoma in mice reveals a critical role of WNT signaling. *Elife* 5, pii:e17047

Carmeliet P, Jain RK (2011) Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473: 298-307

Chamorro-Jorganes A, Araldi E, Penalva LO, Sandhu D, Fernández-Hernando C, Suárez Y (2011) MicroRNA-16 and microRNA-424 regulate cell-autonomous angiogenic functions in endothelial cells via targeting vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1. *Arterioscler Thromb Vasc Biol* 31: 2595-2606

Chamorro-Jorganes A, Araldi E, Suárez Y (2013) MicroRNAs as pharmacological targets in endothelial cell function and dysfunction. *Pharmacol Res* 75: 15-27

Chan LS, Yue PY, Wong YY, Wong RN (2013) MicroRNA-15b contributes to ginsenoside-Rg1-induced angiogenesis through increased expression of VEGFR-2. *Biochem Pharmacol* 86: 392-400

Cho CH, Lee CS, Chang M, Jang IH, Kim SJ, Hwang I, Ryu SH, Lee CO, Koh GY (2004) Localization of VEGFR-2 and PLD2 in endothelial caveolae is involved in VEGF-induced phosphorylation of MEK and ERK. *Am J Physiol Heart Circ Physiol* 286: H1881-1888

Dang LT, Lawson ND, Fish JE (2013) MicroRNA control of vascular endothelial growth factor signaling output during vascular development. *Arterioscler Thromb Vasc Biol* 33: 193-200

Di Malta C, Siciliano D, Calcagni A, Monfregola J, Punzi S, Pastore N, Eastes AN, Davis O, De Cegli R, Zampelli A, Di Giovannantonio LG, Nusco E, Platt N, Guida A, Ogmundsdottir MH, Lanfrancone L, Perera RM, Zoncu R, Pelicci PG, Settembre C et al. (2017) Transcriptional activation of RagD GTPase controls mTORC1 and promotes cancer growth. *Science* 356: 1188-1192

Emanuel R, Sergin I, Bhattacharya S, Turner J, Epelman S, Settembre C, Diwan A, Ballabio A, Razani B (2014) Induction of lysosomal biogenesis in atherosclerotic macrophages can rescue lipid-induced lysosomal dysfunction and downstream sequelae. *Arterioscler Thromb Vasc Biol* 34: 1942-1952

Eremina V, Jefferson JA, Kowalewska J, Hochster H, Haas M, Weisstuch J, Richardson C, Kopp JB, Kabir MG, Backx PH, Gerber HP, Ferrara N, Barisoni L, Alpers CE, Quaggin SE (2008) VEGF inhibition and renal thrombotic microangiopathy. *N Engl J Med* 358: 1129-1136

Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, Gerber HP, Kikkawa Y, Miner JH, Quaggin SE (2003) Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. *J Clin Invest* 111: 707-716

Esser S, Wolburg K, Wolburg H, Breier G, Kurzchalia T, Risau W (1998) Vascular endothelial growth factor induces endothelial fenestrations in vitro. *J Cell Biol* 140: 947-959
Fan Y, Lu H, Liang W, Garcia-Barrio MT, Guo Y, Zhang J, Zhu T, Hao Y, Chen YE (2018) Endothelial TFEB (Transcription Factor EB) Positively Regulates Postischemic Angiogenesis. *Circ Res* 122: 945-957

Ferrando IM, Chaerkady R, Zhong J, Molina H, Jacob HK, Herbst-Robinson K, Dancy BM, Katju V, Bose R, Zhang J, Pandey A, Cole PA (2012) Identification of targets of c-Src tyrosine kinase by chemical complementation and phosphoproteomics. *Mol Cell Proteomics* 11: 355-369

Gariano RF, Gardner TW (2005) Retinal angiogenesis in development and disease. *Nature* 438: 960-966

Geng Y, Yu Q, Sicinska E, Das M, Schneider JE, Bhattacharya S, Rideout WM, Bronson RT, Gardner H, Sicinski P (2003) Cyclin E ablation in the mouse. *Cell* 114: 431-443

Gourlaouen M, Welti JC, Vasudev NS, Reynolds AR (2013) Essential role for endocytosis in the growth factor-stimulated activation of ERK1/2 in endothelial cells. *J Biol Chem* 288: 7467-7480

Greenberg MJ, Ostap EM (2013) Regulation and control of myosin-I by the motor and light chain-binding domains. *Trends Cell Biol* 23: 81-89

Guarani V, Deflorian G, Franco CA, Krüger M, Phng LK, Bentley K, Toussaint L, Dequiedt F, Mostoslavsky R, Schmidt MH, Zimmermann B, Brandes RP, Mione M, Westphal CH, Braun T, Zeiher AM, Gerhardt H, Dimmeler S, Potente M (2011) Acetylation-dependent regulation of endothelial Notch signalling by the SIRT1 deacetylase. *Nature* 473: 234-238

Haq R, Fisher DE (2011) Biology and clinical relevance of the micropthalmia family of transcription factors in human cancer. *J Clin Oncol* 29: 3474-3482
Housden BE, Muhar M, Gemberling M, Gersbach CA, Stainier DY, Seydoux G, Mohr SE, Zuber J, Perrimon N (2016) Loss-of-function genetic tools for animal models: cross-species and cross-platform differences. *Nat Rev Genet* 18: 24-40

Hubbi ME, Hu H, Kshitiz, Ahmed I, Levchenko A, Semenza GL (2013) Chaperone-mediated autophagy targets hypoxia-inducible factor-1α (HIF-1α) for lysosomal degradation. *J Biol Chem* 288: 10703-10714

Jackstadt R, Hermeking H (2015) MicroRNAs as regulators and mediators of c-MYC function. *Biochim Biophys Acta* 1849: 544-553

Jakobsson L, Franco CA, Bentley K, Collins RT, Ponsioen B, Aspalter IM, Rosewell I, Busse M, Thurston G, Medvinsky A, Schulte-Merker S, Gerhardt H (2010) Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. *Nat Cell Biol* 12: 943-953

Jopling HM, Odell AF, Pellet-Many C, Latham AM, Frankel P, Sivaprasadarao A, Walker JH, Zachary IC, Ponnambalam S (2014) Endosome-to-Plasma Membrane Recycling of

VEGFR2 Receptor Tyrosine Kinase Regulates Endothelial Function and Blood Vessel Formation. *Cells* 3: 363-385

Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M (2001) Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol* 230: 230-242

Klein U, Lia M, Crespo M, Siegel R, Shen Q, Mo T, Ambesi-Impiombato A, Califano A, Migliazza A, Bhagat G, Dalla-Favera R (2010) The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. *Cancer Cell* 17: 28-40

Labrecque L, Royal I, Surprenant DS, Patterson C, Gingras D, Béliveau R (2003) Regulation of vascular endothelial growth factor receptor-2 activity by caveolin-1 and plasma membrane cholesterol. *Mol Biol Cell* 14: 334-347

Lanahan A, Zhang X, Fantin A, Zhuang Z, Rivera-Molina F, Speichinger K, Prahst C, Zhang J, Wang Y, Davis G, Toomre D, Ruhrberg C, Simons M (2013) The neuropilin 1 cytoplasmic domain is required for VEGF-A-dependent arteriogenesis. *Dev Cell* 25: 156-168

Lincoln AJ, Wickramasinghe D, Stein P, Schultz RM, Palko ME, De Miguel MP, Tessarollo L, Donovan PJ (2002) Cdc25b phosphatase is required for resumption of meiosis during oocyte maturation. *Nat Genet* 30: 446-449

Little MH, McMahon AP (2012) Mammalian kidney development: principles, progress, and projections. *Cold Spring Harb Perspect Biol* 4: pii: a008300

Liu D, Matzuk MM, Sung WK, Guo Q, Wang P, Wolgemuth DJ (1998) Cyclin A1 is required for meiosis in the male mouse. *Nat Genet* 20: 377-380

Lu H, Fan Y, Qiao C, Liang W, Hu W, Zhu T, Zhang J, Chen YE (2017) TFEB inhibits endothelial cell inflammation and reduces atherosclerosis. Sci Signal 10. pii: eaah4214

Lu LY, Wood JL, Minter-Dykhouse K, Ye L, Saunders TL, Yu X, Chen J (2008) Polo-like kinase 1 is essential for early embryonic development and tumor suppression. *Mol Cell Biol* 28: 6870-6

Malumbres M, Barbacid M (2009) Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer* 9: 153-166

Mansueto G, Armani A, Viscomi C, D'Orsi L, De Cegli R, Polishchuk EV, Lamperti C, Di Meo I, Romanello V, Marchet S, Saha PK, Zong H, Blaauw B, Solagna F, Tezze C, Grumati P, Bonaldo P, Pessin JE, Zeviani M, Sandri M et al. (2017) Transcription Factor EB Controls Metabolic Flexibility during Exercise. *Cell Metab* 25: 182-196

Martina JA, Chen Y, Gucek M, Puertollano R (2012) MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. *Autophagy* 8: 903-14

Medina DL, Fraldi A, Bouche V, Annunziata F, Mansueto G, Spampanato C, Puri C, Pignata A, Martina JA, Sardiello M, Palmieri M, Polishchuk R, Puertollano R, Ballabio A (2011) Transcriptional activation of lysosomal exocytosis promotes cellular clearance. *Dev Cell* 21: 421-430

Murga M, Fernández-Capetillo O, Field SJ, Moreno B, Borlado LR, Fujiwara Y, Balomenos D, Vicario A, Carrera AC, Orkin SH, Greenberg ME, Zubiaga AM (2001) Mutation of E2F2 in mice causes enhanced T lymphocyte proliferation, leading to the development of autoimmunity. *Immunity* 15: 959-970

Murphy M, Stinnakre MG, Senamaud-Beaufort C, Winston NJ, Sweeney C, Kubelka M, Carrington M, Bréchot C, Sobczak-Thépot J (1997) Delayed early embryonic lethality following disruption of the murine cyclin A2 gene. *Nat Genet* 15: 83-86

Napione L, Pavan S, Veglio A, Picco A, Boffetta G, Celani A, Seano G, Primo L, Gamba A, Bussolino F (2012) Unraveling the influence of endothelial cell density on VEGF-A signaling. *Blood* 119: 5599-5607

Napolitano G, Ballabio A (2016) TFEB at a glance. *J Cell Sci* 129: 2475-81
Palmieri M, Impey S, Kang H, di Ronza A, Pelz C, Sardiello M, Ballabio A (2011) Characterization of the CLEAR network reveals an integrated control of cellular clearance pathways. *Hum Mol Genet* 20: 3852-3866

Park C, Kim TM, Malik AB (2013) Transcriptional regulation of endothelial cell and vascular development. *Circ Res* 112: 1380-1400

Pavel M, Rubinsztein DC (2017) Mammalian autophagy and the plasma membrane. *FEBS J* 284: 672-679

Perera RM, Stoykova S, Nicolay BN, Ross KN, Fitamant J, Boukhali M, Lengrand J, Deshpande V, Selig MK, Ferrone CR, Settleman J, Stephanopoulos G, Dyson NJ, Zoncu R, Ramaswamy S, Haas W, Bardeesy N (2015) Transcriptional control of autophagy-lysosome function drives pancreatic cancer metabolism. *Nature* 524: 361-365
Phng LK, Potente M, Leslie JD, Babbage J, Nyqvist D, Lobov I, Ondr JK, Rao S, Lang RA, Thurston G, Gerhardt H (2009) Nrarp coordinates endothelial Notch and Wnt signaling to control vessel density in angiogenesis. *Dev Cell* 16: 70-82

Raben N, Puertollano R (2016) TFEB and TFE3: Linking Lysosomes to Cellular Adaptation to Stress. *Annu Rev Cell Dev Biol* 32: 255-278

Rane SG, Dubus P, Mettus RV, Galbreath EJ, Boden G, Reddy EP, Barbacid M (1999) Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. *Nat Genet* 22: 44-52

Rao Y, Perna MG, Hofmann B, Beier V, Wollert T (2016) The Atg1-kinase complex tethers Atg9-vesicles to initiate autophagy. *Nat Commun* 7: 10338

Rempel RE, Saenz-Robles MT, Storms R, Morham S, Ishida S, Engel A, Jakoi L, Melhem MF, Pipas JM, Smith C, Nevins JR (2000) Loss of E2F4 activity leads to abnormal development of multiple cellular lineages. *Mol Cell* 6: 293-306

Roa S, Avdievich E, Peled JU, Maccarthy T, Werling U, Kuang FL, Kan R, Zhao C, Bergman A, Cohen PE, Edelmann W, Scharff MD (2008) Ubiquitylated PCNA plays a role in somatic hypermutation and class-switch recombination and is required for meiotic progression. *Proc Natl Acad Sci U S A* 105: 16248-16253

Robinson GS, Ju M, Shih SC, Xu X, McMahon G, Caldwell RB, Smith LE (2001) Nonvascular role for VEGF: VEGFR-1, 2 activity is critical for neural retinal development. *FASEB J* 15: 1215-1217

Samarelli AV, Riccitelli E, Bizzozero L, Silveira TN, Seano G, Pergolizzi M, Vitagliano G, Cascone I, Carpentier G, Bottos A, Primo L, Bussolino F, Arese M (2014) Neuroligin 1 induces blood vessel maturation by cooperating with the α6 integrin. *J Biol Chem* 289: 19466-19476

Sawamiphak S, Seidel S, Essmann CL, Wilkinson GA, Pitulescu ME, Acker T, Acker-Palmer A (2010) Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. *Nature* 465: 487-91

Schoors S, Bruning U, Missiaen R, Queiroz KC, Borgers G, Elia I, Zecchin A, Cantelmo AR, Christen S, Goveia J, Heggermont W, Goddé L, Vinckier S, Van Veldhoven PP, Eelen G, Schoonjans L, Gerhardt H, Dewerchin M, Baes M, De Bock K et al. (2015) Fatty acid carbon is essential for dNTP synthesis in endothelial cells. *Nature* 520: 192-197

Serini G, Ambrosi D, Giraudo E, Gamba A, Preziosi L, Bussolino F (2003) Modeling the early stages of vascular network assembly. *EMBO J* 22: 1771-1779

Settembre C, De Cegli R, Mansueto G, Saha PK, Vetrini F, Visvikis O, Huynh T, Carissimo A, Palmer D, Klisch TJ, Wollenberg AC, Di Bernardo D, Chan L, Irazoqui JE, Ballabio A (2013a) TFEB controls cellular lipid metabolism through a starvation-induced autoregulatory loop. *Nat Cell Biol* 15: 647-658

Settembre C, Fraldi A, Medina DL, Ballabio A (2013b) Signals from the lysosome: a control centre for cellular clearance and energy metabolism. *Nat Rev Mol Cell Biol* 14: 283-296

Settembre C, Zoncu R, Medina DL, Vetrini F, Erdin S, Huynh T, Ferron M, Karsenty G, Vellard MC, Facchinetti V, Sabatini DM, Ballabio A (2012) A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO J* 31: 1095-1108

Simons M, Gordon E, Claesson-Welsh L (2016) Mechanisms and regulation of endothelial VEGF receptor signalling. *Nat Rev Mol Cell Biol* 17: 611-625

Sison K, Eremina V, Baelde H, Min W, Hirashima M, Fantus IG, Quaggin SE (2010) Glomerular structure and function require paracrine, not autocrine, VEGF-VEGFR-2 signaling. *J Am Soc Nephrol* 21: 1691-1701

Smyth GK (2002) *Limma: linear models for microarray data. In: Bioinformatics*. Springer, New York

Spruck C, Strohmaier H, Watson M, Smith AP, Ryan A, Krek TW, Reed SI (2001) A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. *Mol Cell* 7: 639-50

Steingrímsson E, Tessarollo L, Reid SW, Jenkins NA, Copeland NG (1998) The bHLH-Zip transcription factor Tfeb is essential for placental vascularization. *Development* 125: 4607-4616

Sun CY, She XM, Qin Y, Chu ZB, Chen L, Ai LS, Zhang L, Hu Y (2013) miR-15a and miR-16 affect the angiogenesis of multiple myeloma by targeting VEGF. *Carcinogenesis* 34: 426-35

Takakura N, Huang XL, Naruse T, Hamaguchi I, Dumont DJ, Yancopoulos GD, Suda T (1998) Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. *Immunity* 9: 677-686

Tiwari A, Jung JJ, Inamdar SM, Nihalani D, Choudhury A (2013) The myosin motor Myo1c is required for VEGFR2 delivery to the cell surface and for angiogenic signaling. *Am J Physiol Heart Circ Physiol* 304: H687-696

Valdembri D, Caswell PT, Anderson KI, Schwarz JP, Konig I, Astanina E, Caccavari F, Norman JC, Humphries MJ, Bussolino F, Serini G (2009) Neuropilin-1/GIPC1 signaling regulates alpha5beta1 integrin traffic and function in endothelial cells. *PLoS Biol* 7: e25

Wang Y, Nakayama M, Pitulescu ME, Schmidt TS, Bochenek ML, Sakakibara A, Adams S, Davy A, Deutsch U, Lüthi U, Barberis A, Benjamin LE, Mäkinen T, Nobes CD, Adams RH (2010) Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature* 465: 483-486

Xu H, Ren D (2015) Lysosomal physiology. *Annu Rev Physiol* 77: 57-80
Yokomizo T, Yamada-Inagawa T, Yzaguirre AD, Chen MJ, Speck NA, Dzierzak E (2012) Whole-mount three-dimensional imaging of internally localized immunostained cells within mouse embryos. *Nat Protoc* 7: 421-431

Yue J, Tigyi G (2010) Conservation of miR-15a/16-1 and miR-15b/16-2 clusters. *Mamm Genome* 21: 88-94

Zarkada G, Heinolainen K, Makinen T, Kubota Y, Alitalo K (2015) VEGFR3 does not sustain retinal angiogenesis without VEGFR2. *Proc Natl Acad Sci U S A* 112: 761-766

FIGURE LEGENDS

Figure 1. Mouse genetic ablation of endothelial *Tfeb* leads to vascular defects.

(A,B) *Tfeb* expression in the vasculature (mice n=10). (A) Representative images of retinal vessels (p5) (A) (scale bar: 50 μm) and (B) glomerular, arterial (scale bar: 10 μm) and interstitial vessels (scale bar: 50 μm) of kidney (p17) of *Tfeb-EGFP* mice stained with anti-iB4 (A), anti-CD31 (B) and anti-GFP (A,B).

(C) Alterations in the embryonic vasculature in *Tfeb*$^{EC-/-}$ mice at E10.5 (mice n=25). Representative images of whole-mount embryos and yolk sacs (i, ii) (scale bar: 0.5 mm). Vessels of the head (iii), ocular (iv) and intrasomitic regions (v) were stained with anti-endomucin Ab (scale bar: 100 μm).

(D) Alteration of retinal vascular maturation in *Tfeb*$^{iEC-/-}$ at p5. Representative images of whole mounts of retina and immunostaining of vascular front and vascular plexus with an anti-iB4 Ab (scale bar: 50 μm). Bar graphs indicate the percent of vascular area versus total area of the retina, the percent of vascular density, the number of branches per field, vessel diameter, the number of filopodia per field and the length of filopodia (mice n=10, mean±SEM; ***p< 0.0001 *versus* control mice by Student's *t*-test).

(E) Alteration of the glomerular ultrastructure in *Tfeb*$^{iEC-/-}$ mice at p17 (mice n=5). Representative transmission electron micrographs of renal tissue. Magnification: i) x6000, ii) x20000), iii-iv) x10000, v-vi) x25000; scale bar: 1μm). Asterisks indicate the accumulation of extracellular matrix in mesangium; white and black triangles indicate the fusion of podocyte foot processes and the lack of endothelial fenestrae, respectively.

Figure 2. *Tfeb* deletion reduces EC proliferation in vivo.

(A,B) Reduced EC proliferation in the retina (p5) and kidney (p17) of in *Tfeb*$^{iEC-/-}$ mice.

(A) Representative images of vessels of the vascular front and vascular plexus in the

retina of control and *Tfeb*$^{iEC-/-}$ mice stained with anti-iB4 and Ki-67 antibodies (scale bar: 50 μm). Bar graph indicates the percentage of EC Ki-67^{+} nuclei *versus* total nuclei co-localized with iB4^{+} vessels (mice n=6, mean±SEM; **p<0.001 and *p< 0.01 *versus* control mice by Student's *t*-test).

(B) Representative images of vessels of the kidney in control and *Tfeb*$^{iEC-/-}$ mice stained with anti-CD31 and Ki-67 antibodies (scale bar: 50 μm). Bar graph indicates the percent of EC Ki-67^{+} nuclei *versus* total nuclei co-localized with CD31^{+} vessels (mice n=6, mean±SEM; **p<0.001 *versus* control mice by Student's *t*-test). Podocin staining is shown to glomerular localization.

(C)(D) *TFEB* silencing reduced EC proliferation. Representative graph of scr-shRNA and sh-TFEB ECs treated for 24 hours with FCS (20%) or VEGF-A (30 ng/ml). (C) DNA incorporation of EdU was detected by flow cytometry. The percentage of proliferating cells is indicated (n=4, mean±SEM; ***p<0.0001 *versus* scr-shRNA ECs by Student's *t*-test).

(D) DNA content was determined by propidium iodide staining and assessed by fluorescences-activated cell sorter analysis (representative experiment out of 4 with similar results).

(E) *TFEB* silencing reduced human EC morphogenesis. Representative images of tube-like-structure of scr-shRNA and sh-TFEB human ECs stained with phalloidin-555. Bar graph indicates the percentage of phalloidin^{+} area in sh-TFEB and scr-shRNA ECs (scale bar: 0.25 mm; n=6, mean±SEM; ***p<0.0001 *versus* scr-shRNA by Student's *t*-test).

Figure 3. TFEB gene regulation in human ECs.

(A) Heatmap showing unsupervised hierarchical clustering of human differentially expressed genes between human scr-shRNA and sh-TFEB ECs. Red: up-regulated genes; green: down-regulated genes.

(B) Selection of enriched functional GO categories by DAVID analysis in differentially expressed genes between human sh-TFEB and scr-shRNA ECs. GO analyses were performed individually on down- or up-regulated genes using DAVID tool (biological process). GO terms are ranked by *P*-value corrected by Benjamini Hockberg method, and the number of genes is indicated.

(C) Selection enriched of Molecular Pathways by GSEA analysis of microarray data comparing human sh-TFEB and scr-shRNA ECs. Normalized enrichment scores (NESs) and p-values are reported.

(D) Selection of enriched functional GO categories by DAVID analysis on ChiP-seq data set performed in human ECs overexpressing TFEBS142A. GO analyses were performed using DAVID tool (biological process). GO terms are ranked by *P*-value corrected by BH method, and the number of genes is indicated.

Figure 4. TFEB regulates cell cycle genes.

(A-C) (A) Heatmap, (B) qPCR and (C) immunoblots showing the differentially expressed cell cycle related genes between scr-shRNA and sh-TFEB ECs. (A) Red: up-regulated genes; green: down-regulated genes. (B) Data are expressed as relative fold-change compared with the expression in scr-shRNA cells after normalization to the housekeeping gene TBP (n=3, mean±SEM; **p<0.001 and ***p<0.0001 by Student's *t*-test). (B) Immunoblots of total lysates from scr-shRNA and sh-TFEB ECs probed with specific Abs. The bar graph shows the densitometric analysis expressed as the ratio between the cell cycle genes and α-tubulin (n=3, mean±SEM; ***p<0.0001 *versus* scr-shRNA by Student's *t*-test).

(D) Modulation of cell cycle genes expression in the lung ECs derived from control and *Tfeb*$^{iEC-/-}$ mice. Data are expressed as relative fold-change compared with the expression

in ECs of control mice after normalization to the housekeeping gene TBP (n=3, mean±SEM; **p<0.001 and ***p<0.0001, by Student's *t*-test).

(E-G) Analysis of TFEB binding and modulation of the *CDK4* promoter in human ECs. (E) Representative snapshot of TFEB binding on *CDK4* in human ECs. (F) 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 *CDK4.* Bar graph shows the percent enrichment (n=3, mean±SD). (G) Analysis of TFEB modulation of *CDK4* promoter in human ECs. Bar graph shows the relative luciferase activity % evaluated in control and TFEBS142A human ECs after transfection of *CDK4* full promoter and *CDK4* promoter deleted of TFEB binding site (n=3, mean±SEM; *p<0.01 and **p<0.001 by Student's *t*-test).

(H) Modulation of Rb protein in sh-TFEB ECs. Immunoblots of total lysates from scr-shRNA and sh-TFEB ECs probed with phospho-Rb and total Rb Abs. The bar graph shows the densitometric analysis expressed as the ratio between phospho-Rb and total Rb (n=3, mean±SEM; **p<0.001, ***p<0.0001 *versus* scr-shRNA by Student's *t*-test).

Figure 5. Regulation of VEGFR2 by TFEB in ECs.

(A-C) Increase of *Vegfr2* expression in the vasculature of *Tfeb*$^{EC-/-}$ and *Tfeb*$^{iEC-/-}$ mice. (A) Representative immunostaining images of *Tfeb*$^{EC-/-}$ embryonic vessels (E10.5) stained with anti-endomucin and anti-Vegfr2 Abs (scale bar: 50 µm). Bar graph indicates the Vegfr2 mean intensity only in endomucin^{+} vessel areas (embryos n=6, mean±SEM; ***p< 0.0001 *versus* control embryos by Student's *t*-test). (B) Representative immunostaining images (i) and detail (ii) of the vascular front and vascular plexus of the retina (p5) of control and *Tfeb*$^{iEC-/-}$ mice with anti-iB4 and anti-Vegfr2 Abs (scale bar: 50 µm). Bar graph indicates the Vegfr2 mean intensity only in iB4^{+} vessel areas (mice n=6, mean±SEM; ***p<0.0001

versus control mice by Student's *t*-test). (C) Representative immunostaining images of the glomerulus (p17) of control and *Tfeb*$^{iEC-/-}$ mice with anti-podocin, anti-CD31 and anti-Vegfr2 Abs (scale bar: 50 μm). Bar graphs indicate the Vegfr2 mean intensity in CD31^{+} vessel areas (mice n=6, mean±SEM; **p<0.001 *versus* control mice by Student's *t*-test).

(D-F) *VEGFR2* expression is regulated by TFEB.

(D-E) qPCR of VEGFR2 in lung ECs obtained from control and *Tfeb*$^{iEC-/-}$ mice (D) and in human sh-TFEB. (E) Data are expressed as relative fold-change compared with the expression in control cells after normalization to the housekeeping gene TBP (n=3, mean±SEM; **p<0.001 and ***p<0.0001, by Student's *t*-test). (F) Immunoblots of total lysates from scr-shRNA and sh-TFEB ECs probed with anti-VEGFR2 and α-tubulin Abs. The bar graph shows the densitometric analysis expressed as the ratio between VEGFR2 and α-tubulin (n=3, mean±SEM; ***p<0.0001 *versus* scr-shRNA by Student's *t*-test).

(G) Analysis of TFEB binding to the *VEGFR2* promoter in human ECs. ChIP was performed using digested chromatin from human 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 *VEGFR2.* Bar graph shows the percent enrichment (n=3, mean±SD).

Figure 6. Role of TFEB in regulating VEGFR2 localization and activity in human ECs.

(A,B) Silenced *TFEB* alters the localization of VEGFR2. (A) FACS analysis of surface VEGFR2 expression on human scr-shRNA and sh-TFEB ECs. Bar graph shows the ratio between total and PM VEGFR2 (n=6, mean±SEM; **p<0.001 *versus* scr-shRNA by Student's *t*-test). (B) Representative TIRF and epifluorescence images of human scr-shRNA and sh-TFEB ECs after staining with anti-VEGFR2 Ab (scale bar: 10 μm). Bar graphs show the ratio of VEGFR2 analyzed in epifluorescence and TIRF mode analyzed by TIRF (n=40, mean±SEM; ***p<0.0001 *versus* scr-shRNA by Student's *t*-test).

(C) Silenced *TFEB* alters the localization and the phosphorylation state of VEGFR2 and its signal transduction. Representative immunoblot of PM biotinylated portion and total cell lysates of scr-shRNA and sh-TFEB ECs after VEGF-A stimulation (30 ng/ml). Blots of total or PM cell lysates were probed with anti-VEGFR2. Blots of total cell lysates were probed with anti-p-Y1175-VEGFR2, anti-PLCγ, p-PLCγ, anti-ERK-1/2, anti-pERK1/2, anti-p-Src, anti-Src, anti CD31 and α-tubulin Abs. The bar graphs (i,ii) show densitometric analysis of stimulated versus unstimulated scr-shRNA and sh-TFEB ECs expressed as: (i) % of VEGFR2 on PM fraction (n=3, mean±SEM; ANOVA p<0.02; **p<0.001 *versus* scr-shRNA by Bonferroni post-test), (ii) % of VEGFR2 total (n=3, mean±SEM; ANOVA p>0.05; *p<0.05 and **p<0.001 *versus* scr-shRNA by Bonferroni post-test).

(D,E) Regulaton of autophagy and lysosome pathway by TFEB silencing. (D) qPCR and (E) immunoblots showing the differentially expressed autophagy and lysosome related genes between human scr-shRNA and sh-TFEB ECs. (D) Data are expressed as relative fold-change compared with the expression in control cells after normalization to the housekeeping gene TBP (n=3, mean±SEM; ***p<0.0001 by Student's *t*-test). (E) Immunoblots of total lysates from human scr-shRNA and sh-TFEB ECs probed with anti-ULK-1, anti-ATG9, anti-LC3-I/II, anti-LAMP-1 and α-tubulin Abs. The bar graph shows the densitometric analysis expressed as the ratio between scr-shRNA and sh-TFEB ECs (n=3, mean±SEM; **p<0.001, ***p<0.0001 *versus* scr-shRNA by Student's *t*-test).

(F) *TFEB* silencing inhibits VEGFR2 internalization. Bar graphs of VEGFR2 internalization expressed as the percent of internalized VEGFR2 *versus* PM VEGFR2 after VEGF-A stimulation (n=6, mean±SEM, ANOVA p<0.0001; ***p<0.0001 *versus* scr-shRNA by Bonferroni post-test).

Figure 7. Role of MYO1C in the localization of VEGFR2 in *TFEB*-silenced ECs.

(A-C) Analysis of TFEB binding and modulation of the *MYO1C* promoter in human ECs. (A) Representative snapshot of TFEB binding on *MYO1C* in human ECs. (B) 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 *MYO1C.* Bar graph shows the percent enrichment (n=3, mean±SD). (C) qPCR of *MYO1C* expression in scr-shRNA, sh-TFEB, sh-MYO1C and sh-TFEB+sh-MYO1C ECs. Data are expressed as relative fold change compared with the expression in scr-shRNA ECs after normalization to the housekeeping gene TBP (n=3, mean±SEM; **p<0.001 and ***p <0.0001 by Student's *t*-test).

(D) *MYO1C* silencing reverses the effect of *TFEB* silencing on the up-regulation of PM VEGFR2. Analyses were performed on human ECs carrying appropriate scr-shRNA or sh-TFEB in the presence or absence of sh-MYO1C. Representative western blot of MYOC1, total and PM biotinylated VEGFR2 (representative experiment out of 4 with similar results).

(E,F) Representative immunostaining images of the vascular plexus of the retina (p5) (E) and glomerulus (p17)(F) of control and *Tfeb*$^{iEC-/-}$ mice with anti-CD31 and anti-MYO1C Abs (scale bar: 50 µm). Bar graph indicates the Myo1C mean intensity only in vessel areas CD31^{+} (n=6, mean±SEM; ***p<0.0001 *versus* control mice by Student's *t*-test).

(G) Representative TIRF and epifluorescence images of VEGFR2 in scr-shRNA, sh-TFEB, sh-MYO1C and sh-TFEB+sh-MYO1C human ECs (scale bar: 10 µm). Bar graph shows the ratio between PM and total VEGFR2 (n=40, mean±SEM; ***p<0.0001 *versus* scr-shRNA by Student's *t*-test).

Figure 8. Indirect regulation of VEGFR2 via miR-15a/16 by TFEB in ECs.

(A) Analysis of TFEB binding to *DLEU2* and *SMC4* promoters in human ECs. 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 *DLEU2 and SMC4.* Bar graph shows the percent enrichment (n=3, mean±SD).

(B) *DLEU2* expression is regulated by TFEB. qPCR of *DLEU2* in human scr-shRNA, sh-TFEB or control and TFEBS142A ECs (left panel) and lung ECs obtained from control and *Tfeb*$^{iEC-/-}$ mice (right panel). Data are expressed as relative fold-change compared with the expression in scr-shRNA and control cells after normalization to the housekeeping gene TBP (n=3, mean±SEM; *p<0.01, and ***p<0.0001 by Student's *t*-test).

(C) Human miR-15a-5p and miR-16-5p are regulated by TFEB. qPCR of miR-15a-5p (left panel) and miR-16-5p (right panel) in sh-TFEB or TFEBS142A ECs. Data are expressed as relative fold-change compared with the expression in scr-shRNA and control cells after normalization to the housekeeping gene RNU44 (n=3, mean±SEM; ***p<0.0001 by Student's *t*-test).

(D-G) VEGFR2 expression is regulated by TFEB through a miR-dependent mechanism. (D,F) qPCR of VEGFR2 in human scr-shRNA and sh-TFEB ECs (D,E) and in control and TFEBS142A ECs (F,G) treated with a specific miR-control, miR-15a-5p and miR-16-5p mimics or inhibitors. Data are expressed as relative fold-change compared with the expression in control cells after normalization to the housekeeping gene TBP (n=3, mean±SEM; **p<0.001 and ***p<0.001 *versus* control or scr-shRNA plus miR-control and ##p<0.001 and ###p<0.001 *versus* sh-TFEB and TFEBS142A plus miR-contol by Student's *t*-test). (E,G) Representative western blot of VEGFR2 expression under the same experimental conditions previously reported. The bar graph shows the densitometric analysis expressed as the ratio between VEGFR2 and α-tubulin (n=3, mean±SEM; **p<0.001 and ***p<0.0001 *versus* control alone and ##p<0.001 *versus* TFEBS142A alone by Student's *t*-test).

EXPANDED VIEW FIGURE LEGENDS

Figure EV1. Different cells types express Tfeb in embryos, yolk sac, retina and kidney.

(A)*Tfeb* expression in embryos at E9.5 (embryos n=6). Representative images of head (details a, b, c), intrasomitic region (details a, b, c) and yolk sac of *Tfeb-EGFP* embryos stained with anti-GFP and anti-endomucin Abs (scale bar: 100 µm).

(B,C,D) Tfeb expression in ECs and smooth muscle cells in embryos, retina and kidney. Representative images of *Tfeb-EGFP* embryos (E9.5) (B), yolk sac (E9.5) (C), retina (p5) (D) and renal glomerulus (E)(embryos and mice n=6) stained with anti-CD31, anti-alpha SMA, anti-NG2,anti-podocin and anti-GFP Abs (scale bar: 100 µm).

Figure EV2. *Tfeb* deletion in embryos and pups.

(A) EC *Tfeb* deletion does not induce alterations in the embryonic vasculature at E9.5 (embryos n=6). Representative images of whole-mount embryos (i) (scale bar: 0.5 mm). Vessels of the head (ii), ocular (iii) and intrasomitic regions (iv) were stained with anti-endomucin Ab (scale bar: 100 µm).

(B) EC *Tfeb* deletion induces embryonic hypoxia at E10.5 (embryos n=6). Representative images of vessels and tissues of the head (i) and intrasomitic region (ii) of control and *Tfeb*$^{EC-/-}$ embryos stained with anti-endomucin (red) and the hypoxic marker pimonidazole (green) (scale bar: 100 µm).

(C) Expression of Tie2 and markers of the endothelial and hematopoietic lineages in Tfeb$^{EC-/-}$ yolk sacs at E9.5. Bar graphs indicate the percentage of yolk sac Tie2^{+} cells or Flk^{+} or CD31^{+} or CD117^{+}/CD41^{+} and CD117^{+}/CD71^{+} within the Tie2^{+} population (n=3, mean±SEM; *p<0.01 *versus* control mice by Student's *t*-test).

(D) Genotype of *Tfeb*^{iEC} mice. Schematic representation of murine *Tfeb,* the targeted allele and the knockout allele (delta allele) and qPCR analysis of mRNA encoded by *Tfeb* exon

5-6 in lung ECs and epithelial cells isolated from control, *Tfeb*$^{iEC-/+}$ and *Tfeb*$^{iEC-/-}$ mice. Data are expressed as relative fold-change compared with the expression in control mice after normalization to the housekeeping gene TBP (mice n=3, mean±SEM; *p<0.01 and **p<0.001 *versus* control mice by Student's *t*-test).

(E) Tfeb expression in renal tissue of control and *Tfeb*$^{iEC-/-}$ mice (p17). Representative images of renal glomerulus, artery, interstitial vessels and the surrounding tissue of control and *Tfeb*$^{iEC-/-}$ mice (mice n=6). Tissue were stained with anti-CD31 and anti-GFP Abs (scale bar: 100 μm).

Figure EV3. Post-natal maturation of retinal and renal vasculature after endothelium *Tfeb* silencing.

(A) EC *Tfeb* deletion compromises retinal vascular maturation at p10 and p15. Representative images of immunostaining of vascular front and vascular plexus of retina of control and *Tfeb*$^{iEC-/-}$ mice (p10, p15) with an anti-iB4 Ab (scale bar: 50 μm).

(B,C,D) EC *Tfeb* deletion compromises glomerular development and renal vascularization at p17. (B) Representative images of renal trichrome staining of control and *Tfeb*$^{iEC-/-}$ mice at p17 (original magnification x10, scale bar: 25 μm). (C) Bar graph indicates the CD31^{+} vascular area in control and *Tfeb*$^{iEC-/-}$ mice (p17) (mice n=5, mean±SEM; **p<0.001 *versus* control mice by Student's *t*-test). (D) EC *Tfeb* deletion compromises renal vascularization at p27. Bar graph indicates the CD31^{+} vascular area in control and *Tfeb*$^{iEC-/-}$ mice (p27) (mice n=5, mean±SEM; **p<0.001 *versus* control mice by Student's *t*-test).

(E) EC *Tfeb* deletion correlates with collagen deposition in kidney at p27. Representative images of immunostaining of kidney of control and *Tfeb*$^{iEC-/-}$ mice (p27) with anti-collagen I, -collagen V and -collagen IV antibodies (scale bar: 50 μm). Quantification area per field: collagen I: 26107.2±3200.82 μm^2 in control mice vs 43069±9754.7 μm^2 in *Tfeb*$^{iEC-/-}$ mice, mice n=4, p=0.02; collagen V: 33500.4±4201 μm^2 in control mice vs 47007.9±6287.7 μm^2

in *Tfeb*$^{iEC-/-}$ mice, mice n=4, p=0.04; collagen IV: 10972.12±1856.8 μm^2 in control mice vs 17525.07±2106.58 μm^2 in *Tfeb*$^{iEC-/-}$ mice, mice n=4, p=0.03)

Figure EV4. TFEB expression and regulation in human ECs. Additional effects of *Tfeb* deletion on human ECs.

(A) mTOR inhibition activates TFEB nuclear translocation. Representative images of human ECs under basal conditions or after Torin stimulation (100 nM, 1 h) stained with anti-TFEB Ab and DAPI (scale bar: 20 μm). Bar graphs indicate the percentage of TFEB positive nuclei *versus* total nuclei after Torin challenge (n=3, mean±SEM; **p< 0.001 *versus* control cells by Student's *t*-test).

(B,C) Characterization of TFEB shRNA (n=3). (B) Western blot of TFEB in human ECs carrying five different commercial sh-TFEB RNAs or the appropriate control (scr-shRNA) transduced by pLKO lentivirus vector. (C) *TFEB* mRNA expression analyzed by qPCR in ECs carrying TFEB shRNA. Data are expressed as relative fold-change compared with scr-shRNA after normalization to the housekeeping gene TBP (n=3, mean±SEM; ***p<0.0001 by Student's *t*-test).

(D) *TFEB* silencing reduced murine EC proliferation. Representative graph of lung ECs isolated from control and *Tfeb*$^{iEC-/-}$ mice ECs treated for 24 hours with FCS (20%) or VEGF-A (30 ng/ml). DNA incorporation of EdU was detected by flow cytometry. The percentage of proliferating cells is indicated (n=4, mean±SEM; ***p<0.0001 *versus* ECs derived from control mice by Student's *t*-test).

(E) *TFEB* silencing does not affect human EC migration. Bar graph indicates the percentage of migrated sh-TFEB and scr-shRNA ECs stimulated with VEGF-A (30 ng/ml, 5 h) (n=6, mean±SEM, p=ns).

Figure EV5. TFEB gene regulation in human ECs.

(A) Volcano plots of human gene expression showing fold-change and p-value data comparing human sh-TFEB and scr-shRNA ECs. Red: up-regulated genes; green: down-regulated genes.

(B) Characterization of TFEBS142A overexpression in human ECs. Representative images of human ECs transduced with pTRIPZ-TFEBS142A inducible lentivirus (scale bar: 20 µm) after transgene induction with doxycycline (0.5 µg/ml, 3 h)(TFEBS142A) or not (control) and stained with anti-TFEB Ab and DAPI. Bar graph indicates the quantity of cytosolic and nuclear TFEB as ratio between TFEBS142A and control ECs (n=10, mean±SEM, **p<0.0001 and ***p<0.0001 by Student's *t*-test).

(C) Representative genomic view of the TFEB ChIP-Seq analysis. The TFEB ChIP-Seq plot shows distinct binding peaks with respect to the IgG ChIP-Seq.

(D) Comparison of the TFEB-bound genomic regions with respect to the other indicated ChIP-Seq regions taken from the literature. TFEB correlates with open chromatin (DNAse) and with transcription factors known to be associated with active gene promoters (FOS, JUN, RNA PolII, MYC).

(E) Enrichment of the Ebox DNA-binding sequence of TFEB (CACGTG) on 70.9% of TFEB target genes in human ECs.

(F) Comparison of expression values in human ECs between TFEB-bound and unbound genes.

(G) DNA methylation analysis of the TFEB-bound regions in human ECs using 450K Illumina microarray data from the literature (methylation status of 6461 CpG has been analyzed).

(H) Genomic localization of TFEB-bound regions in human ECs.

(I) GSEA analysis of TFEB-bound genes comparing human sh-TFEB and scr-shRNA ECs microarray data. Enrichment plot shows modulated TFEB-bound genes.

Figure EV6. TFEB silencing and VEGFR2 activity and localization in human ECs.

(A) Densitometric analysis of immunoblot of total lysates from human scr-shRNA and sh-TFEB ECs (after VEGF-A stimulation, 30 ng/ml) probed with anti-PLCγ, anti-p-PLCγ, anti-ERK-1/2, anti-p-ERK1/2, anti-p-Src, anti-Src and anti-α-tubulin Abs. The bar graphs show the densitometric analyses after normalization with α-tubulin as ratio of p-PLCγ (n=3, mean±SEM; ANOVA p<0.0001; **p<0.001 *versus* scr-shRNA by Bonferroni post-test), p-ERK-1/2 (n=3, mean±SEM; ANOVA p<0.0001; ***p<0.0001 *versus* scr-shRNA by Bonferroni post-test), p-Src (n=3, mean±SEM; ANOVA p<0.0001; **p<0.0001 and ***p<0.0001 *versus* scr-shRNA by Bonferroni post-test) *versus* total PLCγ, ERK-1/2 and Src.

(B) *TFEB* silencing alters VEGFR2 localization into caveolae in human ECs. Representative immunofluorescence images of human scr-shRNA and sh-TFEB ECs after staining with anti-VEGFR2 and anti-CAV-1 Abs (scale bar: 10 µm). Bar graphs show the colocalization rate of VEGFR2 and CAV-1 (n=40, mean±SEM; ***p<0.0001 *versus* scr-shRNA by Student's *t*-test).

(C) *TFEB* silencing alters VEGFR2 localization into Rab5+ endosomes in human ECs. Representative immunofluorescence images of antibody feeding assay with anti-VEGFR2 and anti-Rab5 Abs (scale bar: 10 µm) in absence or presence of VEGF-A (30 ng/ml) stimulation. Bar graphs show the colocalization rate of VEGFR2 and Rab5 in human scr-shRNA and sh-TFEB ECs (n=15, mean±SEM; **p<0.0001 *versus* scr-shRNA by Student's *t*-test).

(D) *TFEB* silencing and VEGFR2 localization into Golgi in human ECs. Representative immunofluorescence images of VEGFR2 and Golgi colocalization in human scr-shRNA and sh-TFEB ECs obtained by staining with anti-VEGFR2 and anti-TGN46 Abs (scale bar:

10 μm). Bar graphs show the colocalization rate of VEGFR2 and TGN46 (n=15, mean±SEM; p=ns *versus* scr-shRNA by Student's *t*-test).

Figure EV7. Role of *MYO1C* silencing on TFEB effects in ECs.

(A) Analysis of TFEB modulation of *MYO1C* promoter in human ECs. Bar graph shows the relative luciferase activity % evaluated in control and TFEBS142A human ECs after transfection of MYO1C promoter, MYO1C promoter d1 and MYO1C promoter d2 (n=3, mean±SEM; ***p<0.0001 by Student's *t*-test).

(B) qPCR of *MYO1C* expression in control and TFEBS142A human ECs. Data are expressed as relative fold change compared with the expression in control ECs after normalization to the housekeeping gene TBP (n=3, mean±SEM; ***p <0.0001 by Student's *t*-test).

(C) *TFEB* silencing and VEGFR2 localization into Rab4+ endosome in human ECs. Representative immunofluorescence images of VEGFR2 and Rab4 colocalization obtained by scr-shRNA, sh-TFEB, sh-MYO1C and sh-TFEB+sh-MYO1C human ECs staining with anti-VEGFR2 and anti-Rab4 Abs (scale bar: 10 μm). Bar graphs show the colocalization rate of VEGFR2 and Rab4 (n=15, mean±SEM; **p <0.001, ***p <0.0001 *versus* scr-shRNA by Student's *t*-test).

(D) Cell distribution of VEGFR2. FACS analysis with anti-VEGFR2 antibody was performed in scr-shRNA, sh-TFEB, sh-MYO1C and sh-TFEB+sh-MYO1C human ECs. (1 representative experiment out of 3; % of the extracellular and intracellular protein is indicated).

(E) qPCR of *VEGFR2* expression in scr-shRNA, sh-TFEB, sh-MYO1C and sh-TFEB+sh-MYO1C human ECs. Data are expressed as relative fold change compared with scr-shRNA ECs after normalization for housekeeping gene TBP (n=3, mean±SEM; **p<0.001 and ***p<0.0001 by Student's *t*-test).

(F) *MYO1C* silencing does not modify VEGFR2 internalization. Bar graph of VEGFR2 internalization expressed as the percent of internalized VEGFR2 *versus* PM VEGFR2 after VEGF-A stimulation in scr-shRNA, sh-TFEB, sh-MYO1C and sh-TFEB+sh-MYO1C human ECs (n=6, mean±SEM, ANOVA p<0.0001; ***p<0.0001 *versus* scr-shRNA by Bonferroni post-tests).

(G) *MYO1C* silencing does not modified EC proliferation. Representative graph of scr-shRNA, sh-TFEB, sh-MYO1C and sh-TFEB+sh-MYO1C human ECs treated for 24 hours with VEGF-A (30 ng/ml). DNA incorporation of EdU was detected by flow cytometry. The percentage of proliferating cells is indicated (n=4, mean±SEM; **p<0.001, ***p<0.0001 *versus* scr-shRNA ECs by Student's *t*-test).

Figure EV8. *TFEB* indirectly regulates VEGFR2 expression

(A) Table of angiogenesis-associated miRs and their correlation with TFEB in ECs.

(B) Expression of miR-15a and 16 isoforms in by qPCR (n=3, mean±SEM). *Tfeb* deletion in murine ECs down-regulates miR-15a-5p and miR-16-5p. qPCR of miR-15a-5p and miR-16-5p in lung ECs obtained from control and $Tfeb^{iEC-/-}$ mice in which *Tfeb* was silenced for 48h or 72h by in vitro treatment with 4-OH-tamoxifene (5 µM for 48 or 72 h). Data are expressed as relative fold-change compared with the expression in ECs derived from control mice after normalization to the housekeeping gene U6 (n=3, mean±SEM; ***p<0.0001 by Student's *t*-test).

(C) miR15a-5p and miR-16-5p not modified EC proliferation after TFEB silencing. Representative graph of human scr-shRNA, sh-TFEB ECs proliferation after transduction with miR-control, miR-15a-5p and miR-16-5p mimics and after stimulation with VEGF-A (30 ng/ml, 24h). DNA incorporation of EdU was detected by flow cytometry. The percentage of proliferating cells is indicated (n=4, mean±SEM; **p<0.0001,***p<0.0001 *versus* scr-shRNA ECs by Student's *t*-test).