VEGFR3 modulates brain microvessel branching in a mouse model of 22q11.2 deletion syndrome

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ABSTRACT

The loss of a single copy of *TBX1* accounts for most of the clinical signs and symptoms of 22q11.2 deletion syndrome (22q11.2DS), a common genetic disorder that is characterized by multiple congenital anomalies and brain-related clinical problems, some of which likely have vascular origins. *Tbx1* mutant mice have brain vascular anomalies, thus making them a useful model to gain insights into the brain disorders associated with the human disease. Here, we found that *Tbx1* has a dynamic expression pattern in brain endothelial cells (ECs), including tip cells, during early vascularization, but it is not expressed in EC progenitors. Its main morphogenetic function in the brain is to regulate negatively filopodia biogenesis and vessel branching. Because of similar phenotypes reported for *Vegfr3* loss of function, we pursued a mouse genetic approach to test TBX1-VEGFR3 interaction through gain and loss of function experiments. *Vegfr3* is expressed in brain ECs with extensive overlap with *Tbx1* expression. We demonstrate that inactivating *Vegfr3* in the *Tbx1* expression domain in a *Tbx1* mutant background enhances vessel branching and filopodia formation to a greater extent than that observed in the individual mutants. Furthermore, using a mouse transgenic line, we show that increasing *Vegfr3* expression in the *Tbx1* expression domain fully rescued the vessel branching and filopodia phenotypes caused by *Tbx1* loss of function. Similar results were obtained using an *in vitro* model of endothelial tubulogenesis. Overall, these results provide genetic evidence that *Vegfr3* is a regulator of early vessel branching and filopodia formation in the brain, and is a likely critical effector of the brain vascular phenotype caused by *Tbx1* loss of function.
INTRODUCTION

Seminal studies in rodents have described the process by which the brain is vascularized through angiogenic sprouting from the perineural vascular plexus. In the embryonic mouse brain, vascularization initiates in the hindbrain around E10 and proceeds in a caudal-rostral direction. Within the brain parenchyma, the number of blood vessels increases rapidly to form a dense vascular network that is able to support neurogenesis, which in the mouse initiates around E11.5.

Tbx1 mutant mice are a model of 22q11.2 deletion syndrome (22q11.2DS), a relatively common chromosome microdeletion disorder, for which most of the clinical problems result from TBX1 haploinsufficiency (Ogata et al., 2014; Paylor et al., 2006; Torres-Juan et al., 2007; Yagi et al., 2003). In practical terms, the most vexing aspect of the clinical phenotype is the brain-related clinical problems, some of which might have vascular origins. TBX1 is a member of the family of T-box transcription factors that is widely expressed in the embryonic pharynx, and it is also expressed in the endothelial lining of a subset of brain vessels in pre-term mouse embryos (Paylor et al., 2006). We have previously shown that Tbx1 plays a critical cell-autonomous role in brain endothelial cells (ECs) that determines the correct density and functionality of brain vessels (Cioffi et al., 2014). We have proposed that TBX1 exerts these effects through interactions, direct or indirect, with Vegfr3 and Dll4 in ECs, both of which have anti-angiogenic functions in the mouse brain (Sucting et al., 2007; Tammela et al., 2011) and are regulated by TBX1 in ECs, including brain ECs, in vivo and in vitro (Chen et al., 2010; Cioffi et al., 2014). Endothelial-specific inactivation of each of these genes causes brain vessel hyperbranching (Cioffi et al., 2014; Sucting et al., 2007; Tammela et al., 2011). We have previously shown that the Notch agonist JAG1 is not sufficient to rescue fully endothelial microtubule hyperbranching in cultured ECs silenced for TBX1 (Cioffi et al., 2014), suggesting that Vegfr3 and/or Notch-independent pathways are involved.

In this study, we investigated the TBX1-VEGF-C/VEGFR3 axis. We demonstrate that Tbx1 expression is activated in vascular endothelial cells, rather than in their mesodermal precursors, during early brain vascularization. Expression initiates in the hindbrain neuroepithelium at E10.5, and then spreads to most of the brain by E15.5. We found a broad overlap between TBX1 and VEGFR3 in brain vessels and, importantly, genetic experiments determined that Tbx1-driven enhanced expression of Vegfr3 is sufficient to rescue the brain vasculature phenotype of Tbx1 mutants. Overall, our results indicate that Vegfr3 is a key effector of the modulatory function of TBX1 in brain vessel branching.
MATERIALS AND METHODS

Mouse lines and genotyping

Mouse studies were performed according to the animal protocol 257/2015-PR reviewed by the local IACUC committee and by the Italian Istituto Superiore di Sanità and approved by the Italian Ministero della Salute, according to Italian law and European guidelines. The following mouse lines were used: Tbx1\textsuperscript{lacZ/+} (Lindsay et al., 2001), Tbx1\textsuperscript{Cre/+} (Huynh et al., 2007), Vegfr3\textsuperscript{flox/+} (Zarkada et al., 2015), Vegfr3\textsuperscript{+/-} (Martucciello et al., 2020), Rosa\textsuperscript{mTmG} (Muzumdar et al., 2007), TgVegfr3 (Martucciello et al., 2020). Genotyping of mice was performed as in the original reports.

Immunofluorescence on brain sections

Cryosections: E9.5, E10.5, E11.5 embryos were fixed in 4% PFA/1x PBS at 4°C overnight, washed in 1x PBS and incubated for 12 h in serial dilutions of sucrose/1x PBS (10%, 20%, 30% sucrose) at 4°C. Brains were then incubated for 2 h at 4°C in 50:50 v/v 30% sucrose/1x PBS/OCT prior to embedding in OCT. Ten micron coronal sections were cut along the rostral–caudal brain axis on a cryotome. Alternatively, specimens were stored at −80°C. Experiments were performed on serial sections 200 µm apart (5 embryos/genotype). Sections were briefly microwaved to boiling point in 10 mM sodium citrate (pH 6.0) 3 times, for antigen enhancement, cooled, rinsed in 1x PBS, permeabilized with 0.1% Triton x-100 blocked in 10% goat serum (GS)/1x PBS/0.1% Triton x-100 for 1h at RT.

Thick brain sections: E13.5, E15.5, E18.5 mouse brains were fixed in 4% PFA at 4°C overnight and subsequently embedded in 4% low melting agarose in 1x PBS. Serial thick coronal sections of 50 µm (E13.5) or 100 µm (E15.5, E18.5) were cut on a vibratome at 4°C. Brains (fixed) that were not used immediately were stored at 4°C in 0.0025% NaNO3.

Immunofluorescence was performed on serial sections along the rostral–caudal brain axis using the following antibodies: mouse monoclonal anti-GLUT1 (Abcam, ab40084), chicken polyclonal anti-GFP (Abcam, ab13970), anti-mouse monoclonal VEGFR3/FLT4 (eBioscience, clone AFL4, Cat. #14-5988-85), rabbit polyclonal anti-VEGFR3/FLT4 (Elabscience, #36398), rat anti-mouse monoclonal VEGFR2/FLK1 (BD Pharmingen, Avas 12α1, Cat. #550549). Secondary antibodies used were goat anti-mouse Alexa Fluor 594 and 488, goat anti-rabbit Alexa Fluor 488 and 594, goat anti-rat Alexa Fluor 594, goat anti-chicken Alexa Fluor 488. Sections were then incubated with primary antibodies overnight at 4°C in the same blocking solution reducing the GS to 5%, rinsed, and incubated in secondary antibodies for 1h 30 min at RT. Fluorescence was observed with an epifluorescence microscope (Leica DMI6000B, acquisition software LAS AF 2.6 or Nikon Confocal.
Microscope A1, mounted on Nikon ECLIPSE Ti, acquisition software NiS element. Images were digitally documented with a camera and computer processed using Adobe Photoshop® version 6 for Windows.

**Quantitative analysis of vascular anomalies**

All quantitative analyses were performed on three brain sections per embryo in rostral, medial and caudal positions that correspond visually to the following stereotaxic levels (Paxinos G, and Franklin K., 2012). Rostral: Bregma 0.74 mm; Medial: Bregma -2.54 mm; Caudal: Bregma -4.84 mm, where R: encompasses the dorsal and ventral sub-pallium; M: the dorsal and ventral thalamus; C: the dorsal and ventral midbrain.

*Vessel branch point and filopodia counts:* We analyzed a minimum of 5 embryos per genotype in all experiments. Flattened images were digitally reconstructed from confocal z stacks of 2.5 µm representing three different brain regions (rostral, medial and caudal). For each flattened image, we manually counted all the branch points in a single field per section, and thus three fields per embryo. The area counted per field was 1.444 mm² and the total length of the vascular network per field was measured. For filopodia, we counted all the filopodia in two sub-fields per section (rostral, medial and caudal) for a total of six fields per embryo. The area counted per field was 0.045 mm² and counts are expressed as the mean number of filopodia per 100 µm of vessel length. Counts were performed using the ImageJ cell counter plugin.

**Statistical analysis**

The statistical analysis of the data pertaining to the brain vessel density was performed using a likelihood ratio test for Negative Binomial generalized linear models. We first calculated the mean number of branchpoints / field / embryo. We then calculated the mean for the group (embryos / genotype). The latter value was used for the statistical analysis. For the analysis of data pertaining to filopodia we performed the Kruskal Wallis test followed by the unpaired Mann Whitney U test.

**Cell manipulations**

HUVECs (Lonza) were transfected by Lipofectamine™ 2000 (Invitrogen). For *TBX1* or *VEGFR3* knockdown (kd) in HUVECs, RNA interference was performed using a commercial siRNA for *TBX1* or *VEGFR3* (ON-TARGETplus SMARTpool, Thermo Fisher Scientific) (40 nM) or a control (non-targeted) siRNA (Thermo Fisher Scientific) as previously described (Cioffi et al., 2014). mRNA expression was evaluated by qReal Time PCR. For *Vegfr3* overexpression, HUVECs were transfected with 1µg of pcDNA3 plasmid vector containing a full-length mouse *Vegfr3* cDNA
Protein expression was evaluated by western blotting with anti-VEGFR3 antibody. Twentyfour hours after transfection, cells were collected and processed for matrigel assay.

For Vegfr3 over-expression in Tbx1<sup>KD</sup> endothelial cells, 24 h after transfection of HUVECs with a TBXI or control siRNAs, cells were transfected with pcDNA3-Vegfr3. Twentyfour hours after the second transfection, cells were collected and processed for matrigel assays. Tbx1 and Vegfr3 mRNA expression was evaluated by qReal Time PCR.

**Matrigel assays**

200 ml of Matrigel (BD Bioscience) was plated onto chilled 15-mm wells and incubated at 37°C for 30 min, as per the manufacturer’s instructions. HUVECs in 6-well plates, previously transfected as described above, were trypsinized and counted. 1.5 × 10<sup>5</sup> treated cells in EGM-2 media (Lonza) were added to each well containing Matrigel. After 16 h at 37°C, the formation of microtubules was analyzed using an Olympus CKX41 Image Analyzer. The quantification of microtubule branch points was performed after dividing each large image into nine sub-images. The number of branch points was calculated as the sum of counts made in all nine sub-images. Quantitative analysis was performed using the Image J software.

**RESULTS**

*Tbx1 expression is activated in ECs rather than in EC progenitors*

Many of the TBX1-dependent functions in the cardiovascular system are linked to progenitors rather than to differentiated cells. As Tbx1 is widely expressed in the head mesoderm, we addressed the question of whether the gene is activated in EC precursors before the neuroepithelium is vascularized. To this end, we crossed Tbx1<sup>Cre/+</sup> mice with Rosa<sup>mTmG</sup> reporter mice (Muzumdar et al., 2007) and evaluated the distribution of Tbx1-fated cells in serial coronal brain sections of Tbx1<sup>Cre/+</sup>; Rosa<sup>mTmG</sup> embryos between stages E9.5 and E18.5, where these cells are marked by Tbx1<sup>Cre</sup>-activated expression of GFP. Sections were co-immunostained with anti-KDR (E9.5 - E11.5) or anti-Glut1 (E13.5 - E18.5) to identify ECs. Overall, results showed that Tbx1-expressing cells and their progeny populate the developing brain following caudal to rostral and ventral to dorsal trajectories, as shown in the cartoon in Fig. 2M. Specifically, at E9.5, a few ECs (KDR+) in the perineural vascular plexus (PNVP) were GFP+, while the surrounding head mesenchyme was heavily populated with GFP+
cells (Fig. 1A). The first GFP+ cells localized to blood vessels (KDR+) within the hindbrain neuroepithelium at E10-E10.5 (Fig. 1B). Between E10.5 and E11.5, the number of GFP+ cells increased dramatically in the hindbrain neuroepithelium, such that by E11.5, co-expression of GFP and KDR was almost 100% (Fig. 1C). However, at this stage, the midbrain (Fig. 1D) and forebrain (Fig. 1E) contained only a few isolated GFP+;KDR+ cells. At E13.5 (Fig. 2A-F), the presence of GFP+;GLUT1+ cells increased in the ventral forebrain (Fig. 2ab', 2cd') and ventral midbrain (Fig. 2ef'), but the dorsal forebrain (Fig. 2ab, 2cd) and dorsal midbrain (Fig. 2ef) remained largely free of GFP+ cells. The contribution to and distribution of GFP+ cells in the aforementioned brain structures were maintained at E15.5 (Fig. 2G-L) in the forebrain (boxed areas in Fig. 2G and 2I) and in the midbrain (boxed area Fig. 2K), and at E18.5 (Supplementary Fig. S1, A-F), suggesting that Tbx1 is not activated significantly in dorsal brain regions during embryonic development (Supplementary Fig. S1, A, C, E). At no developmental stage were GFP+/KDR-negative or GFP+/GLUT1-negative cells seen within the brain parenchyma, confirming that Tbx1-fated cells are endothelial cells.

**Tbx1 and VEGFR3 co-localize in brain ECs, including tip cells**

In order to determine the extent of overlap between TBX1 and VEGFR3, we first determined whether the anti-VEGFR3 antibody used in this study showed pan-endothelial expression in the mouse brain as reported (Watanabe et al., 2019). For this, and for the subsequent analysis of TBX1-GFP and VEGFR3 co-expression, we used the same sectional series of Tbx1Cre/+; Rosa26mTmG embryos. Co-immunostaining with anti-GLUT1 and anti-VEGFR3 antibodies revealed overlapping expression of the two proteins in virtually all brain vessels along the rostral-caudal brain axis at E13.5 (Supplementary Fig. S1, G-L). We next co-immunostained an adjacent series of sections with anti-GFP and anti-VEGFR3 antibodies. This revealed extensive co-localization of the proteins in brain vessels along the rostral-caudal brain axis at the stages analyzed, namely E13.5 (Fig. 3A-F, 3ab, 3cd, 3ef, 3ef'), E15.5 (Fig. 3G-L, 3gh, 3ij, 3kl) and E18.5 (Supplementary Fig. S1, M - R). We then asked whether the co-localization of TBX1-GFP and VEGFR3 in brain vessels included endothelial tip cells, which are distinguished from stalk cells by the presence of filopodia. Anti-GFP immunostaining of brain sections of Tbx1Cre/+; Rosa26mTmG embryos at E13.5 and E15.5 labelled endothelial tip cells, including their filopodial extensions (Fig. 3M-P). We found that many but not all GFP+ tip cells expressed VEGFR3, but two different anti-VEGFR3 antibodies failed to label the filopodia (Fig. 3N, 3P). This may be due to an inability of the antibodies to bind to the receptor in this territory, or to a lack of the receptor at this location in these mutants.
Vegfr3 haploinsufficiency enhances the angiogenic sprouting phenotype of Tbx1 mutants.

We next tested whether Tbx1 and Vegfr3 interact genetically. For this, we intercrossed Tbx1Cre/+ (Tbx1Cre is a null allele) and Vegfr3flox/+ mice and quantified brain vessel branchpoint density and angiogenic sprouting (filopodial density) in thick (100 μm) brain cryosections of conditional compound heterozygous embryos at E18.5 immunostained with anti-GLUT1 (Fig. 4A-C, Supplementary Fig. S2). Results revealed an increased density of endothelial filopodia in Tbx1Cre/+;Vegfr3flox/+ embryos compared to Tbx1Cre/+ embryos (Fig. 4B, 4C, 4f, P = 0.0317 or WT controls P = 0.0079), but there was no difference in branchpoint density (Fig. 4F). Thus, Vegfr3 heterozygosity enhances the brain vasculature phenotype in Tbx1 heterozygous (Tbx1Cre/) mutants, albeit mildly. Interestingly, a similar result was obtained in Vegfr3+/− (Fig. 4E) embryos (germline heterozygous) versus WT embryos (Fig. 4F', 4f'), i.e., increased filopodial density (P = 0.0079) but unaltered branchpoint density (P = 0.117), indicating that there might be non-productive angiogenic sprouting in Vegfr3+/− embryos. As far as we know, this is the first time that Vegfr3 has been reported to be haploinsufficient.

The extensive co-localization of Tbx1-GFP and VEGFR3 in brain vessels, together with the predominantly ventral distribution of Tbx1-fated cells (GFP+), suggest that ventral rather than dorsal brain structures may be the preferred sites of TBX1-VEGFR3 interaction, and in particular the ventral hindbrain, where TBX1 is highly expressed. VEGFR3 was expressed in most brain vessels already at E13.5, (this study and (Watanabe et al., 2019)) but, brain vessel density was normal in Tbx1 homozygous embryos at this stage (P = 0.07, not shown), suggesting that the critical interaction between the two genes likely occurs between E13.5 and E15.5.

Enhanced expression of Vegfr3 in Tbx1-depleted ECs rescues microtubule hyperbranching

Because reduced dosage of Vegfr3 enhances the Tbx1 mutant phenotype, we reasoned that VEGFR3 may be an effector of TBX1 in regulating brain vessel density. If this were the case, supplemental Vegfr3 expression should rescue the phenotypic consequences of Tbx1 mutation. The likelihood of success of this approach depends upon the effects of altered Vegfr3 dosage on EC proliferation and vessel growth, but in vivo studies indicate that VEGFR3 has both pro- and anti-angiogenic potential (Karkkainen et al., 2000; Karkkainen et al., 2001; Mäkinen et al., 2001). Therefore, prior to initiating in vivo rescue experiments, we first tested whether isolated ECs respond to Vegfr3 overexpression in a way that would predict in vivo rescue. We tested this in a functional assay that contains only ECs (HUVECs) cultured in Matrigel™ (Fig. 5). We have shown previously that TBX1 knockdown in HUVECs causes microtubule hyperbranching in Matrigel™ endothelial cultures (Cioffi et al., 2014). Thus, results obtained in the cell-based assay were consistent with in
vivo data obtained in the same study in Tbx1 mutant mice, which showed brain vessel hyperplasia (Cioffi et al., 2014). In Matrigel assays, we found that knockdown of Vegfr3 by transfection with a VEGFR3 targeting siRNA led to an approximately 60% increase in microtubule branching compared to HUVECs transfected with a control siRNA (Fig. 5A, \( P = 0.0138 \)), while over-expression of Vegfr3 in HUVECs, achieved by transfection with a plasmid containing a full-length murine Vegfr3 cDNA (pcDNA3-Vegfr3), led to microtubule hypobranching; reduced by approximately 24% compared to HUVECs transfected with the empty plasmid vector (Fig. 5B, \( P = 0.0027 \)). We then tested whether over-expression of Vegfr3 would rescue microtubule hyperbranching in HUVECs after TBX1 knockdown. For this, we co-transfected HUVECs with pcDNA3-Vegfr3 and siRNA-TBX1 or siRNA-CTR (control) and measured microtubule branching 12 h after plating in Matrigel™ and 24 h after the second transfection. Results showed that over-expression of Vegfr3 partially rescued microtubule hyperbranching in HUVECs after TBX1 knockdown, although it did not restore branching to wild type levels (Fig. 5C, \( P = 0.0148 \)).

Transgenic expression of Vegfr3 rescues brain vessel hyperplasia caused by loss of Tbx1 function in vivo

We asked whether over-expression of Vegfr3 in the Tbx1 expression domain would rescue the brain vessel abnormalities of Tbx1 mutant embryos. To this end, we crossed Tbx1\textsuperscript{Cre/+} mice with mice carrying a single copy of a Cre-activatable murine Vegfr3 transgene, TgVegfr3 mice (Martucciello et al., 2020). We then intercrossed mice with the genotypes Tbx1\textsuperscript{Cre/+}, TgVegfr3 and Tbx1\textsuperscript{lacZ/+} and measured brain vessel density and filopodial density in E18.5 embryos with the following genotypes: Tbx1\textsuperscript{+/+}, Tbx1\textsuperscript{Cre/+}, Tbx1\textsuperscript{Cre/lacZ}, TgVegfr3; Tbx1\textsuperscript{Cre/+} and TgVegfr3; Tbx1\textsuperscript{Cre/lacZ} that were immunostained with anti-GLUT1 (Fig. 6, Supplementary Fig. 3). Results revealed the presence of brain vessel hyperplasia (+17%, \( P = 0.0365 \)) and increased filopodial density (+85%, \( P = 0.0079 \)) in Tbx1 heterozygous (Tbx1\textsuperscript{Cre/+}) embryos (Fig. 6B - 6b', 6F, 6f) and in Tbx1 homozygous embryos (+35.5% branchpoints, \( P = 2.126e-09 \) and +58% filopodia, \( P = 0.0079 \) respectively (Fig. 6D - 6d', 6F, 6f). More importantly, Tbx1\textsuperscript{Cre/+}-induced activation of the Vegfr3 transgene fully rescued both brain vessel phenotypes in Tbx1\textsuperscript{Cre/+}; TgVegfr3 (heterozygous for Tbx1) embryos (Fig. 6C - 6c') and Tbx1\textsuperscript{Cre/lacZ}; TgVegfr3 (homozygous for Tbx1) embryos compared to controls (6E - 6e', 6F), i.e., they returned to wild type levels. As Tbx1\textsuperscript{Cre}-driven recombination in the mouse brain is limited to ECs (shown in Fig. 2), these results indicate that Tbx1-driven activation of Vegfr3 transgene in brain vessels is sufficient to compensate for the loss of function of Tbx1 and rescues the normal brain vessel density in this mouse model.
In conclusion, the data from our loss and gain of function genetic experiments are consistent with a Tbx1 > Vegfr3 gene cascade that establishes the correct brain vessel density in the developing mouse brain. As full rescue of the brain vascular anomalies caused by Tbx1 mutation was achieved by activating Vegfr3 exclusively in Tbx1-expressing ECs, we propose that critical Tbx1 functions in brain angiogenesis are cell-autonomous.

**DISCUSSION**

Tbx1 positively regulates three genes that suppress angiogenesis, Vegfr3, Dll4 and Unc5b (Cioffi et al., 2014); the inactivation of any one of which leads to increased brain vessel density, similar to that observed in Tbx1 mutants (Lu et al., 2004; Suchting et al., 2007; Tammela et al., 2008). We have shown that a JAG1 peptide only partially rescued microtubule hyperbranching in Tbx1-depleted cultured HUVECs (Cioffi et al., 2014), suggesting that Notch-independent pathways contribute to the pathological process. Here we investigated the TBX1-VEGF-C/VEGFR3 axis. Because TBX1 can regulate Vegfr3 expression at a transcriptional level (Chen et al., 2010), we hypothesize that this is the mechanistic basis of the observed interactions, but is this enough? There are several points to consider, i) the increased brain vessel density in Tbx1−/− embryos is not severe, thus TBX1 appears to have only a limited, modulatory/suppressor function over vessel branching, ii) Tbx1 positively regulates at least three genes that suppress angiogenesis, Vegfr3, Dll4 and Unc5b (Cioffi et al., 2014); the inactivation of any one of which leads to increased brain vessel density, similar to that observed in Tbx1 mutants (Lu et al., 2004; Suchting et al., 2007; Tammela et al., 2011), iii) a JAG1 peptide only partially rescued microtubule hyperbranching in cultured Tbx1-depleted HUVECs (Cioffi et al., 2014), suggesting that Notch-independent pathways are involved (hence investigating the VEGF-C-VEGFR3 pathway). Thus, while it is possible that the loss of Tbx1 perturbs a genetic circuit modulating vessel branching through multiple mechanisms, our data demonstrate that enhanced expression of Vegfr3 is sufficient to rebalance or override it and re-establish normal branching. Thus, we conclude that VEGFR3 is a critical target of Tbx1 in brain microvessels.

We have gained insights into the timing of the critical Tbx1 - Vegfr3 interaction. We found VEGFR3 to be expressed in most brain vessels between E13.5 and E18.5. Thus, TBX1 and VEGFR3 co-localize in a subpopulation of brain ECs from the timepoint in which the brain vessel hyperbranching phenotype was first identifiable in Tbx1 mutant embryos, namely at E15.5, suggesting that the co-presence of the two proteins may be part of the pathogenetic mechanism. The
time course analysis of Tbx1 cell fate mapping performed here showed that at E10.5, isolated GFP+ cells (Tbx1-expressing and their descendents) were within hindbrain vessels and in the PNVP, but we did not observe entire blood vessels that were GFP+. The latter would be expected if Tbx1 were activated in EC progenitors. After E11.5, the number of GFP+ cells increased progressively in more rostral and dorsal brain regions, as illustrated in the cartoon in Fig. 1. Thus, the distribution of Tbx1-fated cells likely reflects a wave of activation that expands in different regions of the growing brain vascular network, rather than the deployment of Tbx1-expressing EC progenitors and their progeny. The fact that the brain vascular phenotype was only detectable after E13.5, is consistent with the interpretation that critical TBX1 functions are in differentiated ECs and not in EC progenitors, in contrast, for example to the role of TBX1 in cardiac lymphatic vessels (Martucciello et al., 2020).

There are no published studies of VEGFR3 function specifically in brain vessels. Some studies have suggested that VEGFR3 does not play a primary role in angiogenesis, and that the phenotypic consequences of its inactivation upon this process are due to modulation of VEGFR2. For example, targeted inactivation of the tyrosine kinase or the ligand-binding domain of VEGFR3 did not affect angiogenesis in mouse embryos or yolk sacs (Zhang et al., 2010). As VEGFR3 and VEGFR2 form heterodimers, inactivation of the Vegfr3 gene might affect signalling through the VEGFR2 receptor. Our study does not exclude this possibility as inactivation of Tbx1 in cultured HUVECs and in brain ECs isolated from Tbx1 mutants led to increased expression of Vegfr2 (Cioffi et al., 2014). Another intriguing possibility is that the bi-modal functions of VEGFR3 in ECs (pro- or anti-angiogenic) depend upon local levels of Notch (Benedito et al., 2012). Several studies have contributed to the definition of an autoregulatory loop in which VEGF-C-VEGFR3 signalling activates Dll4-Notch, which promotes the conversion of endothelial tip cells into stalk cells, and subsequently suppression of the VEGF receptors, including VEGFR3 (Benedito et al., 2012; Jakobsson et al., 2009; Tammela et al., 2008; Tammela et al., 2011), thereby preventing excessive angiogenesis. We have proposed that TBX1 operates upstream of this autoregulatory loop (Cioffi et al., 2014), because genetic inactivation of Vegfr3 and Dll4, which are regulated by TBX1, and of Tbx1 itself, all result in brain vessel hyperbranching (Cioffi et al., 2014; Suchting et al., 2007; Tammela et al., 2011), due presumably to the dominance of tip cells over stalk cells. The results of our current study sustain this hypothesis, because reduced expression of Vegfr3 enhances the Tbx1 mutant phenotype, while increased expression suppresses it. In addition, Vegfr3 and Tbx1 are co-expressed in tip cells, consistent with an interaction that represses vessel branching.
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FIGURE LEGENDS

Figure 1. Distribution of Tbx1-fated cells in the brain of Tbx1<sup>Cre/+; Rosa<sup>mTmG</sup></sup> embryos.
(A - E) Representative coronal brain sections of embryos at E9.5 (A), E10.5 (B) and E11.5 (C, caudal section), (D, medial section), (E, rostral section) immunostained for GFP (green) and KDR (red). White arrowheads in A and B indicate the perineural vascular plexus and white arrows cells co-expressing GFP and KDR. White arrows in C-E (merge) are shown as enlarged, single color channels to the right. (F) the cartoon shows the position of the coronal brain sections shown in panels C (caudal, C), D (medial, M) and E (rostral, R). Green shaded arrows indicate the relative density of GFP+ cells (<i>Tbx1</i>-fated) along the rostral --> caudal brain axis at E11.5. Scale bars, A, 250 µm, B-E, 500 µm. Abbreviations: hm, head mesenchyme, hb, hindbrain, Cx, cortex, LV, lateral ventricle, BG, basal ganglia, mb, midbrain, Thal, thalamus, Hyp, hypothalamus.

Figure 2. Distribution of Tbx1-fated cells in the brain of Tbx1<sup>Cre/+; Rosa<sup>mTmG</sup></sup> embryos.
Representative coronal brain sections of embryos at E13.5 (A - F) and E15.5 (G - L) immunostained for GFP (green) and GLUT1 (red). Boxed areas in A, C, E, G, I, K are enlarged in the adjacent panels. (M) cartoon showing the relative density of GFP+ cells along the rostral --> caudal and dorsal --> ventral axes. Abbreviations: R, rostral, M, medial, C, caudal, Cx, cortex, GE, ganglionic eminences, Thal, thalamus, Hyp, hypothalamus, Pn, pons, sc, superior colliculus.

Figure 3. TBX1 and VEGFR3 colocalize in brain endothelial cells.
Representative coronal brain sections of Tbx1<sup>Cre/+; Rosa<sup>mTmG</sup></sup> embryos at E13.5 (A - F) and E15.5 (G - P) immunostained for TBX1-GFP (green) and VEGFR3 (red). Boxed areas in A, C, E, G, I, K are enlarged in the adjacent panels. (M-P) High magnification images show that endothelial filopodia are labelled by anti-GFP (M, O) but not anti-VEGFR3 (N, P, MN, OP) antibodies. Abbreviations: R, rostral, M, medial, C, caudal, Cx, cortex, GE, ganglionic eminences, Thal, thalamus, Hyp, hypothalamus, Pn, pons, sc, superior colliculus.

Figure 4. Tbx1 and Vegfr3 interact genetically to regulate brain vessel and filopodial density.
(A - E) Representative coronal brain sections (medial) of E18.5 embryos immunostained for GLUT1. The cartoon indicates the position of the counting boxes (M1, M2), shown at high magnification in panels M1 (a - e) and M2 (a' - e'), used to quantify vessel branchpoint (F, F') and filopodial (f, f') density in embryos with the indicated genotypes. *** P value <0.001, ** P value <0.01, * P value <0.05. Error bars ± SD. Abbreviations: Cx, cortex, hp, hippocampus, Thal, thalamus.
Figure 5. Vegfr3 overexpression represses formation of endothelial microtubules.
A) microtubule hypobranching after transfection with pcDNA3-Vegfr3, B) hyperbranching after VEGFR3-targeted siRNA, C) pcDNA3-Vegfr3 rescues hyperbranching caused by TBX1 knockdown. *** P value <0.001, ** P value <0.01, * P value <0.05. Error bars ± SD. Scale bar, 200 µm. Abbreviations: EV, empty vector.

Figure 6. Vegfr3 over-expression rescues brain vessel abnormalities in Tbx1 mutants.
(A - E) Representative coronal brain sections (medial) of embryos at E18.5 immunostained for GLUT1. The cartoon indicates the position of the counting boxes (M1, M2), shown at high magnification in panels M1 (a - e) and M2 (a' - e'), that were used to quantify vessel branchpoint (F) and filopodial (f) density in embryos with the indicated genotypes. *** P value <0.001, ** P value <0.01, * P value <0.05. Error bars ± SD. Abbreviations: Cx, cortex, hp, hippocampus, Thal, thalamus.

SUPPLEMENTARY FIGURES

Supplementary Figure S1. Distribution of Tbx1-fated cells
(A - F) Tbx1-fated cells in rostral (R), medial (M) and caudal (C) coronal brain sections of Tbx1Cre/+; Rosa<sup>mTmG</sup> embryos at E18.5 immunostained with antibodies to GFP (green) and GLUT1 (red). Boxed areas in A, C, E, are enlarged in the adjacent panels. (G - L) Co-localization of GLUT1 and VEGFR3 in brain vessels in rostral (R), medial (M) and caudal (C) coronal brain sections of Tbx1<sup>Cre/+</sup>; Rosa<sup>mTmG</sup> embryos at E13.5. Boxed areas in G, I, K are enlarged in the adjacent panels. (M - R) Expression of GFP and VEGFR3 in brain vessels in rostral (R), medial (M) and caudal (C) coronal brain sections of Tbx1<sup>Cre/+</sup>; Rosa<sup>mTmG</sup> embryos at E18.5. Boxed areas in M, O, Q are enlarged in the adjacent panels. Abbreviations: Cx, cortex, GE, ganglionic eminences, Thal, thalamus, Hyp, hypothalamus, Pn, pons, sc, superior colliculus.

Supplementary Figure S2. Tbx1 and Vegfr3 interact genetically to regulate brain vessel and filopodial density.
Representative coronal brain sections, in rostral (A - E) and caudal (F - J) positions of E18.5 embryos immunostained for GLUT1. The cartoon of a rostral brain section (R) indicates the position of the counting boxes R1 and R2, which are shown at high magnification in panels R1, a - e and R2, a' - e'. The cartoon showing a caudal brain section (C) indicates the position of the counting boxes C1 and
C2, shown at high magnification in panels C1, a - e and C2, a' - e'. Abbreviations: Cx, cortex, GE, ganglionic eminences, sc, superior colliculus, Pn, pons.

Supplementary Figure S3. Vegfr3 over-expression rescues brain vessel abnormalities in Tbx1 mutants.

Representative coronal brain sections, in rostral (A - E) and caudal (F - J) positions, of embryos at E18.5 immunostained for GLUT1. The cartoon showing a rostral brain section (R) indicates the position of the counting boxes R1 and R2, which are shown at high magnification in panels R1, a - e and R2, a' - e'. The cartoon showing a caudal brain section (C) indicates the position of the counting boxes C1 and C2, shown at high magnification in panels C1, a - e and C2, a' - e'. Abbreviations: Cx, cortex, GE, ganglionic eminences, sc, superior colliculus, Pn, pons.