**Tbx1 regulates brain vascularization**

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The transcription factor **TBX1** is the major gene involved in 22q11.2 deletion syndrome (22q11.2DS). Using mouse models of these diseases, we have previously shown that TBX1 activates **VEGFR3** in endothelial cells (EC), and that this interaction is critical for the development of the lymphatic vasculature. In this study, we show that TBX1 regulates brain angiogenesis. Using loss-of-function genetics and molecular approaches, we show that TBX1 regulates the **VEGFR3** and **DLL4** genes in brain ECs. In mice, loss of **TBX1** causes global brain vascular defects, comprising brain vessel hyperplasia, enhanced angiogenic sprouting and vessel network disorganization. This phenotype is recapitulated in EC-specific Tbx1 conditional mutants and in an EC-only 3-dimensional cell culture system (matrigel), indicating that the brain vascular phenotype is cell autonomous. Furthermore, EC-specific conditional Tbx1 mutants have poorly perfused brain vessels and brain hypoxia, indicating that the expanded vascular network is functionally impaired. In EC-matrigel cultures, a Notch1 agonist is able to partially rescue microtubule hyperbranching induced by TBX1 knockdown. Thus, we have identified a novel transcriptional regulator of angiogenesis that exerts its effect in brain by negatively regulating angiogenesis through the DLL4/Notch1–VEGFR3 regulatory axis. Given the similarity of the phenotypic consequences of **TBX1** mutation in humans and mice, this unexpected role of **TBX1** in murine brain vascularization should stimulate clinicians to search for brain microvascular anomalies in 22q11.2DS patients and to evaluate whether some of the anatomical and functional brain anomalies in patients may have a microvascular origin.

**INTRODUCTION**

**TBX1** encodes a T-box transcription factor that has a major role in 22q11.2 deletion syndrome (22q11.2DS). This common microdeletion disorder is characterized by diverse congenital abnormalities, including structural brain anomalies, cognitive disorders and an increased risk of psychiatric diseases, especially schizophrenia. The most consistently found structural brain abnormalities are reduced cerebral and cerebellar volumes, increased ventricular volume, altered gyral morphology and reduced grey and white matter volumes in the parietal and temporal lobes (reviewed in ¹). The key role played by the **TBX1** gene in the disease has been determined by studies conducted in humans and mice that demonstrate that **TBX1** mutation is sufficient to recapitulate most of the complex physical phenotype caused by the chromosome 22 microdeletion (²). Whether it also contributes to the anatomical brain anomalies and psychiatric diseases is not known because these have not been evaluated in the majority of patients with **TBX1** point mutations. Inactivation of Tbx1 in the mouse phenocopies most of the features of the human disease, while heterozygous gene inactivation causes mild isolated cardiac defects at reduced penetrance. Tbx1+/− mice also display behavioral anomalies that are relevant to the human disease. In 22q11.2DS patients and in Tbx1+/− mice, the pathogenetic basis of the brain phenotypes is unknown. In mice, Tbx1 is expressed in brain microvessel ECs (³) but not in other brain cell types, thus the cellular origin of the behavioral defects in Tbx1+/− mice is not obvious. We have proposed that reduced expression of endothelial Tbx1 may impact brain development or function by impairing the transcriptional regulation of as yet unidentified molecules that act on neurons.

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In order to understand how endothelial Tbx1 may affect embryonic development generally and brain development in particular, we inactivated the gene specifically in ECs. We have previously reported the effect of this on the development of the lymphatic system (4). Specifically, Tbx1 promotes lymphangiogenesis and loss of Tbx1 causes widespread loss of the lymphatic vessels soon after the onset of their development. We also showed that Tbx1 supports growth and maintenance of lymphatic vessels through its ability to activate Vegfr3 directly. In the present study, we show that in the mouse, Tbx1 negatively regulates brain angiogenesis. Loss of endothelial Tbx1 causes global hyperplasia of brain vessels and functional brain vascular defects that lead to brain hypoxia. In order to identify the molecular pathway in which Tbx1 functions in ECs we searched the literature for mouse mutants that display similar brain vessel hyperplasia. Among those identified were mutations of several genes in the Notch signaling pathway, including Unc5b, Dll4 and Vegfr3, which is negatively regulated by Dll4-Notch1. We found that these and other Notch signaling molecules are indeed regulated by Tbx1 in vivo and in cultured ECs.

The Delta-Notch and VEGF signaling pathways are key regulators of angiogenesis in mammals and fish. Studies in mouse have shown that specification of endothelial cells (ECs) to tip cells and stalk cells is controlled by Delta-Notch signaling (5), whereas VEGF-VEGFR2 signaling is involved in angiogenic sprouting (6). However, the two pathways are intimately related because VEGF induces expression of Delta-like ligand 4 (DLL4) in a subset of ECs. Ligand binding to the cognate Notch receptor on adjacent ECs suppresses the tip cell phenotype in those cells and they become stalk cells. Thus, VEGF induces DLL4 as a negative feedback regulator of the tip cell phenotype and of vascular sprouting (5,7–9). Through these interactions, a delicate balance is established that favors the formation of a mature vascular network of appropriate density. Many additional molecules have been shown to modulate angiogenesis by their interaction with VEGF and with DLL4-Notch, reviewed in 10. Thus, the coordinated action of pro- and anti-angiogenic molecules allows organisms to finely tune angiogenesis and thereby, meet the widely different oxygen and nutritional requirements of different tissues and organs. Nowhere is this truer than in the mammalian brain with its high demand for oxygen and nutrition.

How might Tbx1 loss of function cause brain vessel hyperplasia? We propose a model in which Tbx1 exerts a bimodal regulation of brain angiogenesis through the transcriptional regulation of DLL4 and Vegfr3 in ECs.

RESULTS

Most brain vessels derive from Tbx1-expressing cells

We have previously shown that in mouse brain Tbx1 is expressed in some (<10%) microvessel ECs (3). In order to evaluate the extent to which Tbx1-expressing cells contribute to the brain vascular network, we fate mapped these cells. The experiments were performed on Tbx1<sup>cre</sup>+/R26RYFP embryos at E18.5 (n = 5) that were generated by crossing transgenic mice that express Cre recombinase from the endogenous Tbx1 promoter, Tbx1<sup>cre</sup> (11) with mice carrying a YFP reporter gene under the control of a ubiquitous promoter, R26R (12). To label all brain vessels, we perfused the embryos with FITC-conjugated Isolectin B4, which binds to ECs and then immunostained sections with anti-GFP to detect expression of the Tbx1-driven reporter (Fig. 1 and Supplementary Material, Fig. S1). Thus, co-localization of isolectin B4 and reporter expression indicates that the vessel derives from Tbx1-expressing ECs. Results showed that in all five animals analyzed the majority of brain vessels along the rostral caudal axis were double labeled. Quantitative analysis performed on rostral, medial and caudal brain sections showed that 79.1% ± 2.55% (SEM) of Tbx1-traced ECs (yellow) to brain vessels along the rostral caudal axis.

Loss of Tbx1 causes brain hypervascularization and vessel branching defects

To study the importance of Tbx1 for brain vascularization, we immunostained thick (100 µm) brain sections with anti-Glut1 antibody, which labels ECs, and then used confocal microscopy to generate 3D reconstructions of different brain regions along the rostral-caudal axis of the embryonic and adult brain of Tbx1 mutants. This revealed an increase in the number of vessel branch points, i.e. vessel density, of 33.5% throughout the brain of Tbx1<sup>lacZ/lacZ</sup> animals at E18.5 (Fig. 2A’–D’). Similar defects were evident at E13.5 and E15.5 (Supplementary Material, Fig. S2). Strikingly, Tbx1<sup>lacZ/lacZ</sup> brain vessels also showed a 37.2% increase in the number of filopodial bursts (Fig. 2C’) compared with controls (P < 0.001), indicating
increased angiogenesis. Filopodia bursts are a morphological characteristic of specialized ECs (tip cells) that are present at the leading edge of vascular sprouts, and that integrate directional cues from their surroundings and hence define the direction in which new sprouts grow. Angiogenic sprouts fuse periodically, become lumenized and therefore generate a functional vascular network. The brain vessel network in 

\textit{Tbx1}^{lacz/lacz} \hspace{1em} \text{animals} \hspace{1em} \text{was} \hspace{1em} \text{also highly disorganized (Fig. 2D')} \hspace{1em} \text{and characterized by an increased number of microcirculatory loops.} \hspace{1em} *P < 0.01, \hspace{1em} **P < 0.001. \hspace{1em} \text{Error bars (SEM).}

\textbf{TBX1 knockdown in cultured ECs increases microtubule branching}

To test whether \textit{Tbx1} modulates endothelial tube formation \textit{in vitro}, we performed a functional assay using human umbilical vein ECs (HUVECs) cultured in Matrigel\textsuperscript{TM} (BD Bioscience). This assay is based on the ability of cells to migrate, to establish cell–cell and cell–ECM contacts and to regulate cell survival.
density in ECs early in development (14). In Tie2Cre transgenic mice, the promoter driving Cre is EC-specific. For these experiments, we first bred the Cre mice with mice carrying a conditionally recombining allele (15), the promoter driving Cre is EC-specific. We then compared brain vessel density in ECs recapitulates the hypervascularization phenotype in adult Tbx1+/- animals. (A and B) Brain vessels identified by anti-Glut1 immunostaining in control (A and B, n = 5) and Tbx1lacz/+ (A’ and B’, n = 5) mice at 8 months of age. Insets in A and A’ indicate the area magnified in B and B’.

We analyzed the effect of Tbx1 knockdown on the capacity of HUVECs to form microtubules. For this, we transfected HUVECs with a Tbx1-specific small-interfering RNA (4). Inhibition of Tbx1 expression was achieved after 48 h as shown by a quantitative real-time (RT) PCR and western blotting, respectively (Fig. 4A and B). A strong knockdown of Tbx1 protein was also demonstrated by immunofluorescence (Fig. 4C and D). After treatment with the Tbx1 siRNA, we observed a 30% increase (P < 0.001) in microtubule branch points (Fig. 4E’ and F) compared with HUVECs treated with a control (non-targeting) siRNA in five independent experiments. This result suggests that the vascular phenotype caused by Tbx1 dosage reduction is cell autonomous.

Loss of Tbx1 in ECs recapitulates the hypervascularization phenotype

Mature ECs arise from mesodermal precursors. In order to obtain in vivo confirmation of the cell autonomous basis of the brain vascular phenotype in Tbx1 mutants, we deleted the gene using two mesodermal Cre drivers. MesP1Cre/+ mice (13) express Cre recombinase under the control of a mesodermal promoter, and it has been shown to induce recombination in brain ECs early in development (14). In Tie2Cre transgenic mice (15), the promoter driving Cre is EC-specific. For these experiments, we first bred the Cre mice with mice carrying a conditional (floxed) Tbx1 allele (16). We then compared brain vessel density in MesP1Cre/+; Tbx1flox/flox and Tie2Cre; Tbx1flox/flox embryos with that of Tbx1flox/+ embryos (controls) at E18.5. In MesP1Cre/+; Tbx1flox/flox embryos, we found a 32.7% increase in vessel density (Fig. 5A’ and A’’) compared with controls (P = 0.004), which is comparable with that seen in Tbx1lacz/lacz embryos (Fig. 2). In Tie2Cre; Tbx1flox/flox embryos (Fig. 5B’ and B’’), there was a 32% increase in brain vessel density (P = < 0.001). Thus, we conclude that loss of Tbx1 in ECs is sufficient to cause brain vessel hyperplasia.

EC-specific inactivation of Tbx1 disrupts brain vessel perfusion

Hypervascularity phenotypes have been associated with vascular insufficiencies that compromise vessel function. For example, in a mouse tumor model, blocking DLL4 with a neutralizing antibody resulted in increased tumor vessel density, but the vessels were poorly perfused and hypoxic, which, paradoxically, stimulated further angiogenesis (17). To establish whether Tbx1 loss of function causes brain vascular insufficiency, we evaluated the competence of the blood–brain barrier (BBB) in Tbx1lacz/lacz and wild-type embryos at E18.5. For this, embryos were perfused with a low molecular weight (443 D) molecule, primary amine-reactive Sulpho NHS-biotin (Pierce). The biotinylated tracer was revealed on brain cryosections stained with FITC-conjugated streptavidin. No leakage of the tracer into the brain parenchyma was seen in mutants or controls (Supplementary Material, Fig. S3), indicating that Tbx1 loss of function does not affect BBB function. We then evaluated brain vessel patency. For this, E18.5 embryos were perfused with FITC-isoflectin B4 (labels patent vessels). Subsequently, all brain vessels were labeled in thick (100 μm) sections by immunostaining with an anti-Glut1 antibody (Fig. 6C and D). We performed these experiments on EC-specific Tbx1 null mutants (Tie2Cre; Tbx1flox/flox) and controls (Tie2cre; Tbx1flox/flox), rather than in Tbx1lacz/lacz embryos, because the latter have cardiac defects that might affect the distribution of the tracer. Tie2Cre; Tbx1flox/flox mutants have normal hearts (4). Results showed that in Tie2cre; Tbx1flox/flox embryos there were multiple brain regions with unperfused vessels (red arrows in Fig. 6B). The poorly perfused tissue was located in different brain regions in the five mutant embryos analyzed, while no such regions were observed in five control embryos. These results suggest that the increased vascular network in these mutants is functionally impaired and may not efficiently deliver oxygen and nutrients to the brain. We tested this by immunostaining adjacent brain sections with an antibody against carbonic anhydrase IX (CAIX), a hypoxia-inducible enzyme. In the regions identified as poorly perfused by FITC-isoflectin B4 staining, we observed expression of CAIX (Fig. 6C). In contrast, no CAIX staining was observed in well-perfused brain tissue in control brains (Fig. 6C).

Tbx1 regulates DLL4 expression in cultured ECs in a dosage-dependent manner

The inactivation of genes involved in angiogenesis in mice and zebrafish has been instrumental in revealing their role in this complex developmental process. Only a few of these mutants show blood vessel hyperplasia similar to that seen in Tbx1 mutant embryos. Two such mutants are DLL4 and Vegfr3 (18,19). Given that Vegfr3 is regulated by DLL4-Notch in ECs (19,20), we hypothesized that genes in these signaling pathways would be Tbx1 targets in ECs. Indeed, we have already shown that Tbx1 regulates Vegfr3 in lymphatic ECs (4). DLL4 encodes a transmembrane ligand for the receptor Notch1 that is expressed in arterial blood vessels and in sprouting ECs. DLL4 expression in tip cells induces the stalk cell phenotype in adjacent ECs, and...
thus, regulates tip cell numbers and thereby angiogenesis (5). We first performed a qRT-PCR on HUVECs transfected with a TBX1-specific or control (non-targeting) siRNA. Results showed that DLL4 mRNA levels increased after TBX1 overexpression, \( P = 0.029 \), while they were reduced after TBX1 knockdown, \( P = 0.001 \) (Fig. 7A). We next tested whether TBX1 regulates the expression of genes that are known targets of Notch signaling in HUVECs knocked down for TBX1 (Fig. 7B) and in brain ECs isolated from adult Tbx1lacz/+ mice (Fig. 7D, data from two independent experiments using five animals/genotype). In HUVECs, we detected a significant down regulation of UNCS5B, \( P = 0.022 \), NRARP, \( P = 0.05 \) and VEGFR3, \( P = 0.0006 \), but not of HEY1. In isolated Tbx1lacz/+ brain ECs, we detected a significant down regulation of Dll4, \( P = 0.033 \), Hey1, \( P = 0.047 \), Narp, \( P = 0.044 \) and Vegfr3, \( P = 0.042 \), but not of Unc5B. The lower response of Notch targets to Tbx1 dosage reduction in isolated brain ECs compared with HUVECs with siRNA knockdown is likely to reflect the differences in the amount of residual TBX1 expression in the two experimental settings, namely, 50% in Tbx1lacz/+ brains versus <20% in TBX1-siRNA treated HUVECs. As some of these genes may be regulated by TBX1 independently of the Dll4-Notch pathway, we also quantified expression of activated Notch in the same experimental setting. Results showed that TBX1 knockdown reduced expression of activated Notch1 protein in TBX1-siRNA-treated HUVECs (Fig. 7C). Together, these results suggest that loss of TBX1 significantly affects DLL4-Notch signaling.

We next asked whether TBX1 contributes directly to the regulation of the DLL4 gene. To this end, we carried out computational searches of the gene sequence to identify putative T-box binding elements (TBEs). We found three sites that matched the TBX5 consensus (the TBX1 consensus sequence is not yet available), two in intron 3 (TBEs1 and 2) and one in intron 5 (TBE3) (Fig. 4A, Supplementary Material, Fig. S4A). Next, we carried out chromatin immunoprecipitation (ChIP) assays using an anti-TBX1 antibody and found that the TBE3 region is occupied by TBX1 in HUVECs (Supplementary Material, Fig. S4A). However, an electrophoretic mobility shift assay suggested that immuno-purified TBX1 protein is not able to bind the sequence of this TBE (Supplementary Material, Fig. S4B). Thus, the occupation of this region of the DLL4 gene by TBX1 appears not to be related to the presence of the computationally detected TBE. We searched for other potential TBE sites nearby (+600 bp from the primer pair used for ChIP), but found none. It is possible that TBX1 is recruited to this region by other factors known to interact directly with it, for example through the chromatin remodelling component Baf60a (21) or other protein interactors. Tbx1 can also operate without binding directly to the DNA (22). We also carried out luciferase assays using a
540-bp fragment of DNA containing the TBE3 (TBE3_LUC). We found that TBX1 can transactivate, albeit weakly, the luciferase construct containing this fragment (Supplementary Material, Fig. S4C). This result is consistent with our ChIP results.

**Rescue of microtubule branching defects by a Notch agonist**

Our data suggest that TBX1-Dll4/Notch1 signaling in ECs suppresses angiogenic sprouting and microtubule formation. To test this hypothesis in a gain-of-function experiment, we used a synthetic peptide corresponding to the δ/serrate/Lag-2 domain of Jagged1 (Jag1) that has proven Notch agonistic activity (23). We transfected HUVECs with a TBX1-specific siRNA or a control (non-targeting) siRNA. Twenty-four hours later, transfected HUVECs were incubated with Jag1 peptide (1 μg/ml) for 30 min prior to plating in Matrigel. In four independent experiments, in HUVECs knocked down for TBX1 and treated with Jag1, we observed a 9.15% decrease in the microtubule formation of these cells compared to controls. This result is consistent with our ChIP results and supports the hypothesis that TBX1-Dll4/Notch1 signaling suppresses angiogenic sprouting and microtubule formation.
branch points compared with TBX1-siRNA treated HUVECs, P = 0.03 (Fig. 8A and B) demonstrating a partial rescue of the microtubule branching defect. We also analyzed expression of the Notch target genes cited previously in the same cell cultures. We found that in HUVECs silenced for TBX1, reduced expression of UNC5B and NRARP, but not of HEY1 and VEGFR3, was partially rescued by Jag1 treatment (Fig. 8C). Together, these data indicate that the TBX1-Dll4/Notch interaction contributes to the signaling ‘milieu’ required for microtubule branching but that other Notch-independent pathways are also involved.

DISCUSSION

In this study, we have identified for the first time an unexpected vascular phenotype in the brain of Tbx1 mouse mutants. Tissue-specific deletion demonstrated that the elimination of the Tbx1 gene from ECs is sufficient to recapitulate fully the brain vessel hyperplasia phenotype observed in germ-line Tbx1 null mutants. We exclude that the vascular phenotype is secondary to heart defects because in Tie2Cre;Tbx1<sup>lacz/lacz</sup> animals, which have normal hearts, the brain vascular phenotype was neither quantitatively nor qualitatively different to that observed in Tbx1<sup>lacz/lacz</sup>;Mesp1<sup>cre/+</sup>, Tbx1<sup>lacz/lacz</sup> mice, both of which have severe heart abnormalities.

Tbx1 is a transcription factor, and therefore, the origin of the brain vascular phenotype is due to misregulation of one or more of its’ target genes. We have identified two target genes in ECs, Vegfr3 (4) and Dll4 (this study). Interestingly, the down regulation of either of these genes leads to brain vessel hyperplasia, similar to that of Tbx1 mutants. Thus, our data establish for the first time a transcriptional link between these genes in ECs and expand the repertoire of genes controlling brain vascularization. Our rescue experiments using Jag1 demonstrated that Notch signaling has a role in the Tbx1 endothelial phenotype, at least in the cell-based model, but they also indicate that acting on Notch signaling alone is insufficient to fully rescue the microtubule hyperbranching phenotype. Thus, Tbx1 behaves as a higher level regulator over multiple pathways. Links between Dll4 and Vegfr3 have been described in the literature, indeed recent studies indicate that their relationship in angiogenic ECs is unexpectedly complex (19,24). Tammela et al. showed that genetic inactivation of Vegfr3 in ECs causes excessive angiogenic sprouting and branching and vessel hyperplasia in the retina and hindbrain (19). Furthermore, while retinal vessel hyperplasia was only partially rescued by the administration of a Vegfr2 blocking antibody, administration of the Jag1-related peptide used in our study (23) fully rescued the phenotype, suggesting that the primary effect of Vegfr3 on angiogenic ECs is determined by Dll4-Notch signaling rather than Vegfr2-Vegfr2 signaling. This was confirmed by Benedito et al. in a study that showed that Notch regulates Vegfr3 in ECs and induces angiogenesis independently of Vegfr-Vegfr2 signaling (24). The authors of the latter study proposed that Vegfr3 can be pro-angiogenic or anti-angiogenic depending upon the local level of Notch signaling. Furthermore, this switch is ligand independent. In Tbx1<sup>lacz/lacz</sup> animals, endothelial expression of Vegfr3, Dll4 and activated Notch1 was reduced while Vegfr2 expression was upregulated. Together, these molecular changes are predicted to enhance angiogenesis either via the classical ligand-dependent Vegf-Vegfr2-MAPK pathway, or via a ligand-independent Vegfc/Vegfr3-Dll4/Notch pathway, as proposed by Tammela et al.

What do these new data tell us about Tbx1-induced brain hypervascularization? First, they suggest that Tbx1, Vegfr3 and Dll4 are linked in a regulatory pathway that controls sprouting angiogenesis in brain. Indeed, dosage reduction of any of the
three genes causes brain vessel hyperplasia. Second, they indicate that Tbx1 could intervene in the Vegfr3-Dll4/Notch1 pathway at two different points by positive regulation of Vegfr3 and Dll4. In both cases, the effect would be to reinforce Notch signaling and its anti-angiogenic effect on ECs. A model of the proposed interactions is shown in Figure 7E.

Finally, and perhaps most importantly, we have shown that the anatomical brain vascular anomalies associated with EC-specific inactivation of Tbx1 are linked to functional defects. Specifically, the expanded vascular network is not properly perfused and does not deliver oxygen efficiently to the affected brain region.

How might these data contribute to our understanding of 22q11.2DS? Our finding of brain microvascular damage in Tbx1 mutants opens an unpredicted and novel hypothesis concerning the structural brain anomalies, cognitive defects and psychiatric diseases found in 22q11.2DS patients, which are still of unknown origin and yet of major clinical importance. To date, discussion of a possible vascular contribution to these diverse brain phenotypes has centered on cardiovascular defects, which occur commonly in 22q11.2DS patients, and large vessel anomalies, such as constriction or absence of vessels emanating from the Circle of Willis and tortuous carotid arteries (reviewed in 1). Interestingly, we have not observed carotid artery tortuosity in EC-specific Tbx1 conditional null mutants, which also do not have congenital heart defects (our unpublished data). While our data obtained in mice cannot establish a link between brain vascular insufficiency and brain phenotypes in patients, we are convinced that a search for similar microvascular defects in

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**Figure 7.** TBX1 regulates Dll4 and Notch targets in ECs. (A) Quantitative Real Time (qRT) PCR shows that Dll4 mRNA levels are increased in HUVECs after TBX1 overexpression and reduced after TBX1 knockdown by siRNA treatment. Western blotting shows that Dll4 protein levels are reduced after TBX1 knockdown. (B and D) qRT-PCR reveals altered expression of Notch targets in HUVECs knocked down for TBX1 by siRNA (B) and in ECs freshly isolated from adult Tbx1lacZ/+ brains (D). Experiments were performed in triplicate and repeated three times. The expression of NRP1 and VEGFR1 was not significantly affected by TBX1 knockdown, data not shown. (C) Cleaved NOTCH1 receptor protein levels are reduced after TBX1 knockdown by siRNA. (E) Schematic of Tbx1 functions in ECs. The dashed lines indicate interactions between the Vegfr3 and Dll4/Notch signaling pathways that have been proposed by others (refs 19 and 24). See discussion for details.
22q11.2DS patients is now justified, given the generally high similarity between the phenotypic consequences of Tbx1 mutation in mouse and man.

**MATERIALS AND METHODS**

**Mice and tissues**

The following mouse lines were used: Tbx1lacz/+(25), Tbx1 flox/+(16), Tie2Cre (15), R26R (9), Tbx1 Cre/+ (11) and Mesp1Cre (13). Genotyping was performed as in the original reports. All animal experiments were performed in compliance with Italian laws and institutional guidelines and were approved by the local animal ethics committee.

**Immunohistochemistry on brain sections**

E18.5 and adult brains at 8 months of age were fixed in 4% PFA at 4°C overnight and subsequently embedded in 4% agarose. 100 μm coronal sections were cut on a freezing microtome and were post-fixed in 4% PFA at room temperature for 10 min prior to immunostaining. Immunohistochemistry was performed on serial sections along the rostral–caudal brain axis using the following antibodies: mouse monoclonal anti-Glut1 (Abcam), rabbit anti-GFP (Invitrogen), CAIX (Novus Biologicals). Secondary antibodies used were goat anti-mouse Alexa Fluor 594 and 488 (Invitrogen) and anti-rabbit Alexa Fluor 488. Fluorescence was observed with an epifluorescence microscope (Leica) and digitally documented with a camera. Images were computer processed using Adobe Photoshop® version 6 for windows.

For analysis of the brain microvasculature sections were immunostained with anti-Glut1. For experiments using tracer molecules, deeply anesthetized embryos were perfused through the heart (left ventricle) with specific tracer molecules; an incision to the left atria was made to release blood/tracer during perfusion. A peristaltic pump was used to maintain even perfusion pressure, set at the rate of 1 ml/min and for a total volume of 3 ml/embryo. For analysis of the BBB, embryos were perfused with primary amine-reactive Sulpho NHS-biotin (Pierce), The biotinylated tracer was revealed on brain sections stained with FITC-conjugated streptavidin (Zymed).

For the analysis of brain vessel perfusion, embryos were perfused with FITC-conjugated isolectin B4 (Sigma-Aldrich, labels patent vessels). Histological sections of perfused brains were then immunostained with anti-Glut1 (labels all vessels).

For cell fate mapping experiments, embryos were perfused with FITC-conjugated isolectin B4, isolated brains were fixed in 4% PFA at 4°C overnight, followed by a sucrose gradient and inclusion in OCT. Coronal brain cryosections of 20 μm were immunostained with anti-GFP antibody (identifies YFP reporter expression).

**Quantitative analysis of vascular anomalies**

We analyzed 5 animals per genotype in all experiments.
Vessel branch point and filopodia counts: Three-dimensional images were digitally reconstructed from confocal z stacks representing three different brain regions (frontal, medial and caudal). For each flattened image, we manually counted all the vessels or filopodia in 2 fields, one from each side of the midline, for a total of 6 fields per animal. The area counted per field was 1896 mm².

Vessel counts in cell fate mapping experiments: This was performed on fluorescence micrographs, without image compression. We manually counted all the vessels in 2 fields, one from each side of the midline, in one frontal, one medial and one caudal section per animal. The percentage of vessels derived from Tbx1-expressing ECs was calculated as follows: number of double positive vessels (FITC-isolecandin B4 and anti-GFP)/by the number of FITC-isolecandin B4 positive only vessels. Quantitative analyses were performed using the ImageJ software (www.uhnresearch.ca/facilities/wcif/imagej).

Statistical analysis
The statistical analysis of the brain vessel phenotype was performed using a likelihood ratio test for Negative Binomial generalized linear models. This test was selected because the data analyzed were raw counts and therefore do not conform to a continuous distribution. We first calculated the mean number of vascular features (branchpoints, filopodia, double stained vessels) per field/animal. We then calculated the mean for the group (5 animals/genotype). The latter value was used for the statistical analysis.

Cell manipulations
HUVECs (Lonza) were transfected with 50 ng of Tbx1 expression vector or with empty vector and with a GFP control vector (Amaza GmbH) to monitor transfection efficiency. Cells were collected after 24 h and fixed in 4% PFA for immunocytofluorescence or lysed for RNA isolation.

RNA interference was performed as previously described (4) using a commercial siRNA for Tbx1 (ON-TARGETplus SMARTpool, Thermo Fisher Scientific) (40 nm) and a control non-targeting siRNA (Thermo Fisher Scientific). Transfection efficiency was tested in control cultures transfected with a fluorescent siRNA (Siglo red transfection indicator, Thermo Fisher Scientific). 24 h after transfection, cells were transfected again at the same conditions and collected 24 h after the second transfection. mRNA expression was evaluated by qRT-PCR.

Human primer sequences used for qReal Time PCR:
- m Tbx1_R : 5′-CTGACCAAATACCTGCTGAGTA-3′
- m Tbx1_F : 5′-GGCTGATATCTGATGCTGG-3′
- m Dll4_F : 5′-GATGAATGGCTGCTCAAAGTCTC-3′
- m VEGFR2_F : 5′-ATAGAAGGTGCCCAGGAAG-3′
- m NOTCH1_F : 5′-TGCCCTGAGAATGATCATG-3′
- m NOTCH1_R : 5′-CAGGTTGATGGTGGTGC-3′

Murine primer sequences used for qReal Time PCR:
- m Tbx1_F : 5′-TCGACCAAATACCTGCTGAGTA-3′
- m Tbx1_R : 5′-GGCTGATATCTGATGCTGG-3′
- m Dll4_F : 5′-GATGAATGGCTGCTCAAAGTCTC-3′
- m VEGFR2_F : 5′-ATAGAAGGTGCCCAGGAAG-3′
- m NOTCH1_F : 5′-TGCCCTGAGAATGATCATG-3′
- m NOTCH1_R : 5′-CAGGTTGATGGTGGTGC-3′

Treatment of HUVECs with Jag1 peptide
HUVECs in 6-well plates were treated with the Tbx1 siRNA or non-targeting siRNA for 48 h. They were then incubated with or without Jag1 peptide (Anaspec) (aa 188–204; CDDYYYGFGCNKFCRPR) or with a scrambled peptide (RCGPDFDNYGKYRF) as a control at a concentration of 1 μg/ml. mRNA expression was evaluated by qRT-PCR.

Matrigel assay
200 μl 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 were treated with the Tbx1 siRNA or non-targeting control siRNA (40 nM) for 48 h. They were then trypsinized, and counted. 1.5 × 10⁵ treated cells in EGM-2 media (Lonza) were added to each well of Matrigel assay. After 16 h, the formation of microtubes was analyzed using an Olympus CKX41 Image Analyzer. For some experiments, trypsinized HUVECs were incubated with Jag1 peptide, or a scrambled peptide, after trypsinization and prior to plating in Matrigel. The quantification of branch points was performed after dividing each large image into nine sub-images. The number of branch points was calculated as the total number of spots in all nine sub-images. Quantitative analysis was performed using the Image J software.

Preparation of primary mouse brain ECs
Mouse brain microvessels were prepared as previously described (26,27). Briefly, brains were removed from 5 wild...
type and 5 Tbx1lacz/+ mice of 8 weeks and transferred into Dulbecco’s modified Eagle’s medium (DMEM) on ice. The meninges were removed and the cortex was dissected away from the surrounding tissue. The cortical tissue was homogenized, then digested for 1 h at 37°C with 0.7 mg/ml type 2 collagenase and 39 U/ml DNase I in DMEM (Worthington). The digested material was diluted with DMEM and centrifuged at 1000 g for 10 min at 4°C. Pellets were resuspended in a 20% w/v bovine serum albumin (Sigma Aldrich) and DMEM and centrifuged for 20 min at 1000 g and 4°C. While capillaries and blood cells form a pellet, myelin floats as a thick band and is removed. The pellets were further digested in 1 mg/ml collagenase/dispase mix (Roche) and 39 U/ml DNase I in DMEM for 1 h at 37°C. This helps to remove contaminating pericytes and astrocytes. The digested microvesSEL solution was diluted with DMEM and centrifuged at 1000 g and 4°C for 10 min. The pellet was washed with PBS prior to RNA extraction.

**ChIP assay**

ChIP experiments were performed as previously described (28). Chromatin from crosslinked HUVECs was immunoprecipitated with rabbit anti-Tbx1 (Abcam) or with rabbit IgG as a negative control (Santa Cruz). DNA purified from chromatin was quantified by PCR using the following primers: h DLL4TBE_intron5F: 5′-CCTGAGTTGGCTTTACCTT-3′ h DLL4TBE_intron5R: 5′-CCGAAAAGTCGGTTAGATGC-3′ GAPDHCHIP_F: 5′-CTCTGGCTCTCTCTTGTCGAC-3′ GAPDHCHIP_R: 5′-ACGACAAAATCCCGTTGACTC-3′

**TBX1 immunoprecipitation**

Human chorionicarcoma-derived placental JEG3 cells were maintained in MEM (SIGMA) supplemented with 10% FBS. Cells at 60–70% confluence were transfected with X-tremeGENE 9 DNA Transfection Reagent (Roche) following the manufacturer protocol. 48 h after transfection, cells were harvested and the cell pellet resuspended in 5 pellet volumes of cold CE buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% v/v NP40, 1 mM DTT and 1× protease inhibitors, adjusted to pH 7.6), and centrifuged at 1400 rpm for 4 min. 2× pellet volume of NE buffer (20 mM TrisHCl, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% Glycerol and 1× protease inhibitors, pH 8.0) was added to the nuclear pellet and incubated on ice for 30 min. Prior to IP, 100 μl of protein-G Dynabeads (Life Technology, #10004D) were cross-linked to anti-Tbx1 antibody (Abcam, #ab18530) following the manufacturer’s instructions. Rabbit IgG (Abcam, #ab46540) served as a negative control for proteins interacting nonspecifically with the beads. Following cross-linking, nuclear extracts were added to the beads and incubated overnight at 4°C, then washed six times with PBS/0.1% Tween-20. Two consecutive elutions were performed with 0.1 M Glycine pH 2.5 and immediately neutralized with Tris-HCl, pH 8.0. Samples were subjected to SDS-polyacrylamide gel electrophoresis and proteins were stained with colloidal coomassie or transferred into PVDF membrane for western blotting analysis.

**Electrophoretic mobility shift (EMS) assays**

EMS assays were carried out in reaction volumes of 20 μl containing 5 ng of immunopurified TBX1 protein. The 3′ ends of the fragments of probes were labeled with DIG-11-ddUTP using the 2nd-generation DIG gel shift kit (Roche), according to the manufacturer’s protocol. The labeled mixtures were incubated at 20°C for 30 min, loaded onto 6% polyacrylamide gels in 0.5 × Tris-borate-EDTA buffer, and then separated by gel electrophoresis at 80 V for 120–150 min. Subsequently, the DIG-labeled DNA was transferred to a nylon membrane (Hybond-N; GE Healthcare) at 400 mA for 20 min and then visualized by an enzyme immunosay using the DIG gel shift kit. The palindromic T or M oligonucleotides 5′-CTAGATTTTACACCTAGGTGTT-3′ (T) or 5′-CTAGATTTTCACCTAGGTaT-3′ (M) were used as positive control (29), 5′-CTCTGGCTCTCTCTTGTCGAC-3′ GAPDHCHIP_F: 5′-CTCTGGCTCTCTCTTGTCGAC-3′ GAPDHCHIP_R: 5′-ACGACAAAATCCCGTTGACTC-3′

**Luciferase assays**

For the luciferase construct, a 524-bp DNA fragment surrounding a conserved TBE in intron 5 of the DLL4 genomic sequence was PCR amplified with primers 5′-GATTCTCACCTGG ATCTTT-3′ and 5′-ATAGCCAGAGTGACTACC-3′. The amplicon, named TBE3_LUC, was first cloned into T-vector, PCRII-TOPO (Invitrogen), and after KpnI–XhoI digestion, was subcloned into a pGL3 luciferase expression vector (Promega). A TBX1 expression vector was co-transfected with the enhancer reporter into JEG3 cells, which were harvested 48 h later. JEG3 cells transfected with empty expression vector constituted the control sample. Experiments were performed in triplicate and repeated twice.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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