Loss of the transcription factor RBPJ induces disease-promoting properties in brain pericytes

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Sufficient vascular supply is indispensable for brain development and function, whereas dysfunctional blood vessels are associated with human diseases such as vascular malformations, stroke or neurodegeneration. Pericytes are capillary-associated mesenchymal cells that limit vascular permeability and protect the brain by preserving blood-brain barrier integrity. Loss of pericytes has been linked to neurodegenerative changes in genetically modified mice. Here, we report that postnatal inactivation of the Rbpj gene, encoding the transcription factor RBPJ, leads to alteration of cell identity markers in brain pericytes, increases local TGFβ signalling, and triggers profound changes in endothelial behaviour. These changes, which are not mimicked by pericyte ablation, imperil vascular stability and induce the acquisition of pathological landmarks associated with cerebral cavernous malformations. In adult mice, loss of Rbpj results in bigger stroke lesions upon ischemic insult. We propose that brain pericytes can acquire deleterious properties that actively enhance vascular lesion formation and promote pathogenic processes.
The brain vasculature has unique characteristics strongly related to the metabolic and physiologic properties of the surrounding tissue. As the brain consumes large amounts of energy on demand without the possibility of energy storage, regional and dynamic differences in brain activity are matched by changes in blood flow. At the same time, exchange between the circulation and brain tissue is tightly controlled by the blood–brain barrier (BBB), which prevents neuronal damage and is critical for brain homeostasis. Pericytes are capillary-associated vessel wall (mural) cells and, together with neural cells and endothelial cells (ECs), part of the so-called neurovascular unit (NVU), the structure underlying the BBB. Pericyte deficiency in mice increases BBB permeability, which involves changes in EC gene expression and enhanced transport of vesicles across the EC monolayer (i.e., endothelial transcytosis) without compromising endothelial cell–cell junctions. One of the changes in brain ECs that results from the loss of pericytes is the downregulation of Mfsd2a, which encodes a transmembrane transport protein. Interestingly, endothelial transcytosis is increased in Mfsd2a-deficient mice leading to BBB defects. Pericyte contractility has been implicated in the regulation of cerebral blood flow, whereas other data attributes this function to arteriolar smooth muscle cells (SMCs). Pericyte loss in mutant mice has been also associated with a number of neuropathologies such as white matter disease, neurovascular uncoupling, Alzheimer’s disease, and age-dependent memory impairment.

Here, we report that loss of the transcription factor RBPJ alters fundamental aspects of brain pericyte identity, which involves increased contractility, capillary obstruction and the formation of aneurysms, increased TGFβ activation, haemorrhaging, and NVU dysfunction. Our data argue that these processes are triggered by the loss of RBPJ repressor activity and not because of its important and well-known role as a transcriptional activator in the Notch signalling pathway. Strikingly, Rbpj deficiency in pericytes induces the acquisition of vascular lesions resembling cerebral cavernous malformations (CCMs) and has other detrimental effects, which are not recapitulated by mice lacking pericytes. We therefore propose that pericytes can acquire disease-promoting properties, which lead to vascular malformations in the brain and increased tissue damage after ischaemic injury.

**Results**

**Inducible gene targeting in mouse brain pericytes.** In order to genetically target pericytes in vivo, we have recently developed Pdgfrb-CreERT2 transgenic mice, which were shown to work efficiently in mural cells of the postnatal retina, an extracranial part of the central nervous system (CNS). Analysis of the cerebral vasculature from these mice in combination with the Rosa26-mTmG Cre reporter allele showed efficient and mural cell-specific recombination (i.e., GFP expression) throughout the brain after tamoxifen administration during embryonic or early postnatal development (Supplementary Fig. 1a, b). Recombination efficiency in cortical regions of the cerebrum of young pups (P10) was around 80% (Supplementary Fig. 1c) and no obvious differences were found among distinct regions of the brain (Supplementary Fig. 1d). Likewise, efficient targeting of mural cells was achieved after tamoxifen induction in juvenile and adult animals (Supplementary Fig. 1e).

**Rbpj-deficient pericytes impair brain vascular morphogenesis.** To address the role of RBPJ in mural cells, we generated tissue-specific Rbpj conditional knockouts (RbpjppC; Fig. 1a) by interbreeding of Pdgfrb-CreERT2 and Rbpjfox/fox animals. Inducible inactivation of Rbpj mediated by Pdgfrb-CreERT2-driven recombination resulted in widespread focal haemorrhaging in the brain with higher prevalence in cortical regions of the cerebrum and cerebellum, and less prevalent or completely absent in the hippocampal formation, brain stem (thalamus and hypothalamus), and olfactory bulb (Fig. 1b–d and Supplementary Fig. 1f, g). Notably, despite early and efficient deletion of Rbpj with tamoxifen injection from postnatal day 1 (P1) to P3, the vascular lesions were restricted to the CNS (Supplementary Fig. 1h), started to develop at P7, and were prominent by P10. Moreover, animals with hemizygous deletion of Rbpj showed no phenotypic alteration, were undistinguishable from Cre-negative littermates (Supplementary Fig. 2a–h), and were therefore used as controls in experiments that required Cre-induced expression of reporter alleles.

**Microscopic and quantitative analysis of the brain cortical vasculature revealed an increase in vascular area together with a reduction of vascular density in RbpjppC animals (Fig. 1e). This was due to a significant enlargement of veins and capillaries together with a decline in the number of side branches, whereas arteries remained mostly unaffected in terms of size and morphology (Fig. 1f, g). In particular, veins, venules, and the surrounding capillaries were highly tortuous and showed local constrictions leading to the formation of aneurysms in adjacent vessel segments (Supplementary Fig. 3a). Furthermore, analysis of the vasculature in the pial surface of the brain cortex by systemic perfusion of coloured dyes revealed the existence of arteriovenous malformations, which bypass capillaries and allow direct shunting of arterial blood into veins (Fig. 1h and Supplementary Fig. 3b).

It has been previously reported that the vascularization of the CNS occurs mainly via sprouting angiogenesis, which is initiated in the embryo and continues during postnatal stages in order to expand and remodel the brain vasculature. Mural cell-specific Rbpj mutant animals showed a remarkable reduction in the number of sprouts and instead of the usual tip-cell morphology, characterized by extension of long filopodia, emerging capillaries were blunt-ended and often dilated resembling microaneurysms. In addition, the total number and density of EC nuclei, identified by expression of the transcription factor ERG, was strongly increased both in capillaries and veins from early stages (P7) onward (Fig. 1k and Supplementary Fig. 3c, d). EdU administration revealed a >2-fold increase in EC proliferation (Fig. 1l and Supplementary Fig. 3e), leading to the abnormal accumulation of ECs with superimposed nuclei within the twisted and tangled RbpjppC capillaries (Fig. 1m). At the molecular level, RT-qPCR gene expression analysis of freshly sorted ECs from brain cortex revealed a strong downregulation in the expression of Dll4 and Esmt1, known markers of sprouting endothelial tip cells, and a significant upregulation of Myc, a powerful driver of EC proliferation. Transmission electron microscopy confirmed the strong EC hyperplasia together with the emission of intraluminal protrusions, enlargement of the sub-endothelial basement membrane, and formation of intra-cytoplasmic canaliculi in the RbpjppC endothelium. In contrast, the ultrastructure of EC junctions was maintained in line with normal expression of tight junction and adherens junction proteins. Likewise, vascular ensheathment by astrocyte endfeet was maintained even in areas with haemorrhages. Noteworthy, degenerative changes in the endothelium of RbpjppC mice, such as accumulation of pinocytotic vesicles and cytoplasmic vacuolization, were obvious already at P7 and were substantially worse by P10. The changes may compromise EC integrity and can be related to the increased EC apoptosis detected at P10 (Supplementary Fig. 3i, j).

The changes in blood vessel organization and, in particular, the enlargement of capillaries and veins described above induce a
strong reduction in blood flow velocity in these vessels, as revealed by two-photon in vivo imaging of the brain vasculature, without strong alterations in the arterial side of the network (Fig. 2e, f).

Remarkably, the Rbpj<sup>iPC</sup> vascular anomalies were not related to changes in pericycle coverage, which remained unaltered (Fig. 2g, h), nor to differences in recombination efficiency between homozygous mutants and heterozygous controls (Supplementary Fig. 3k). In addition, Pdgfrb-CreERT2-controlled acute ablation of mural cells by induced expression of diphtheria toxin in ROSA-DTA knock-in mice failed to cause similar phenotypic outcomes despite high efficiency of mural cell depletion.
**Fig. 1** *Pdgfrb-CreERT2*-mediated *Rbpj* deletion compromises brain vessels. a *Pdgfrb-CreERT2* transgene and Cre-mediated recombination of *Rbpj*loxP allele. b Whole brains and coronal sections from control and *Rbpj*loxP mice at P7 or P10 after tamoxifen administration (P1–P3). Scale bar, 2 mm. c Haemorrhagic index. p-values, Mann-Whitney U-test, n = 8. d Red blood cell (RBC) extravasation in brain parenchyma. HPF, hippocampal formation. p-values, one-way ANOVA and Tukey’s test, n = 5. e Vascular area and density in brain cortices. p-values, Student’s t-test, n = 6–7. f Confocal images of blood vessels (CD31, white) in brain cortices. Arteries (A) and veins (V) are indicated. Scale bar, 100 μm. g Quantitation of vessel diameter in cortical blood vessels. p-values, Brown-Forsythe and Welch one-way ANOVA with Tamhane’s T2 test, n = 4, n.s., not statistically significant. h Arteriovenous malformation in brain superficial vasculature as revealed by the presence of latex in the caudal rhinal vein (arrowhead). Scale bar, 1 mm. i Confocal images of cortical blood vessels stained for GLUT1 (red, white) and PDGFRβ (white). Normal sprouts (dashed circles) replaced by blunt-ended capillaries (yellow arrowheads) in *Rbpj*loxP mice. Scale bar, 50 μm. j Quantitation of sprouting in brain cortices. p-values, Welch’s t-test, n = 4. k Confocal images of blood vessels (ICAM2, red) and EC nuclei (ERG1, white) in brain cortices. Scale bar, 100 μm. l Quantitation of EC proliferation in blood vessels of P7 mice. p-values, Welch’s t-test, n = 7–8. m Confocal images of iPSC capillary network stained for ICAM2 (red), ERG (white), and GFP (green, recombined mural cells). Right panel shows single optical sections of boxed inset in *Rbpj*loxP (left column). Recombined pericytes (white arrows) around tangled capillaries with superimposed EC nuclei (white arrowheads). Scale bar, 50 μm (left panels) and 10 μm (high magnifications). n RT-qPCR analysis in sorted ECs from brain cortices of P7 and P10 mice. p-values, Brown-Forsythe and Welch one-way ANOVA with Sidak’s test for Esml, n = 4–8. All images correspond to P10 mice unless otherwise indicated. Error bars represent s.e.m. Source data are provided as a Source Data file (Supplementary Fig. 4a–e). Likewise, chronic paucity of pericyte coverage in mice lacking the retention motif of the growth factor PDGF-B (*Pdgfrb*Ret/Ret mutants19) did not phenocopy the *Rbpj*loxPC vascular architecture, endothelial sprouting defects, and haemorrhaging (Supplementary Fig. 4f–j).

Secondary to the *Rbpj*loxPC vascular defects, noticeable changes in other cellular components of the NVU were observed. Reactive astrogliosis, a typical response to CNS injury, was detected by immunodetection of microtubule-associated protein 2 (MAP2), a dendritic marker, showed signal-depleted foci around severely astrogliotic areas in *Rbpj*loxPC iPC brains (Supplementary Fig. 5c, d), and oedema, a common finding after focal cerebral ischaemia20, was evident in the *Rbpj*loxPC cortex and found in association with collapsed capillaries (Supplementary Fig. 5e). Neurons are especially sensitive to deprivation of oxygen or glucose and therefore to damages in vascular integrity21. Immunodetected of microtubule-associated protein 2 (MAP2), a dendritic marker, showed signal-depleted foci around severely affected *Rbpj*loxPC blood vessels (Supplementary Fig. 5f). Likewise, NeuN and neurofilament-H (NF-H) immunostaining, markers for postmitotic neurons and axons, showed similar irregularities around *Rbpj*loxPC vascular lesions (Supplementary Fig. 5g).

Together, these results show that *Rbpj* inactivation in mural cells induces severe vascular abnormalities that compromise NVU integrity without affecting pericyte coverage and through mechanisms that are not recapitulated by chronic or acute pericyte ablation.

**Rbpj maintains the molecular identity of brain pericytes.** To gain insight into the molecular alterations elicited by the inactivation of *Rbpj* in brain mural cells, we interbred the conditional *Rbpj*loxPC mutant with Rpl22tm1.IPsm knock-in animals22, which enable Cre-controlled hemagglutinin-tagging of the ribosomal protein Rpl22 (RiboTag) and thereby immunoprecipitation of actively translating, polyribosome-bound transcripts. Following tamoxifen administration from P1–P3, RNA from P7 and P10 control and *Rbpj*loxPC brain cortices was isolated and sequenced in triplicates. Unsupervised hierarchical clustering and principal component analysis (PCA) of all RNA-seq datasets (Supplementary Fig. 6a, b) showed high reproducibility of the gene expression profiles among samples of the same group and a bigger difference between control and *Rbpj*loxPC animals as the phenotype worsens at P10. Differential gene expression analysis with a false discovery rate (FDR)-adjusted p-value < 0.05 and an absolute log fold change > 0.5 identified 450 differentially expressed genes (DEGs) at P7, of which 234 were upregulated and 216 downregulated, and 2551 DEGs at P10, 1402 of which were upregulated and 1149 downregulated (Fig. 3a, b and Supplementary Data 1 and 2). Given the drastic increase in DEGs at P10, we reasoned that secondary effects elicited by bleeding and inflammation could be important contributors at this stage and we therefore first focused on the incipient and more discrete changes detected at P7 for gene ontology (GO) analysis. GO analysis for biological processes linked the gene expression changes in P7 *Rbpj*loxPC PDGFRβ+ mural cells to vascular development, blood vessel morphogenesis and angiogenesis, while the extracellular matrix (ECM) and cell surface were the overrepresented cellular components (Fig. 3c). Next, we classified groups of genes with similar expression profiles across the different genotypes and stages analyzed. To this end, we applied model-based hybrid-hierarchical clustering23 to 14,742 transcripts and focused on clusters that group together genes which are consistently up- or downregulated both at P7 and P10 (Fig. 3d). Interestingly, the group of downregulated genes included several commonly used pericyte markers, including *Pdgfrb*, *Anpep*, *Rgs5*, *Csgp4*, and *Notch3*, which were also reduced in freshly sorted *Rbpj*loxPC brain mural cells analyzed by RT-qPCR (Fig. 3e and Supplementary Fig. 6c). Moreover, 30 out of 49 recently proposed pericyte markers based on single-cell RNA-sequencing were downregulated in *Rbpj*loxPC pericytes (Fig. 3f). At the same time, the cluster of consistently upregulated genes included numerous markers such as *Acta2* (ɑSM) and *Tgfn* (SM22a), which were recently shown to be at least 10-fold enriched in arterial/arteriolar SMCs relative to pericytes24 (Fig. 3g, h).

Given that the *Pdgfrb-CreERT2* transgene triggers recombination in all mural cell types and to rule out that the increase in SMC markers might reflect a larger abundance of this mural cell type, we analyzed vessel-associated cells around arteries and arterioles. Remarkably, the coverage of vascular SMCs was reduced and irregular in *Rbpj*loxPC brains (Fig. 4a, b), whereas mutant pericytes showed increased immunostaining for the contractility-related cytoskeletal proteins ɑSM, SM22a, Vimentin, Desmin, and Nestin (Fig. 4c–g and Supplementary Fig. 3g). Moreover, phosphorylation of myosin light chain 2, which reflects the contractile activity of actomyosin, was strongly increased in *Rbpj*-deficient pericytes, whereas it was almost undetectable in control brain microvasculature (Fig. 4h). Increased contractility of pericytes was also seen in a collagen gel contraction assay and, likewise, the overexpression of *Acta2* and *Tagfn* was confirmed in vitro after *Rbpj* inactivation in cultured pericytes (either by lentivirus-mediated Cre expression or treatment of cells with TAT-Cre protein) (Fig. 4i–k and Supplementary Fig. 6d). Consistent with the aforementioned changes in the expression...
of cytoskeletal proteins, we noticed that mutant pericytes showed profound morphological changes with a marked increase in cellular projections and membrane protrusions (Fig. 4).

Taken together, these data argue that the loss of Rbpj compromises pericyte identity, leading to increased contractility and the upregulation of vascular SMC markers.

Notch signalling reduction fails to phenocopy Rbpj deletion. Given the notorious differences in the phenotype elicited by Rbpj deletion in pericytes with respect to artery-associated SMCs, we next investigated the activation level of canonical Notch signalling by analyzing two different reporter mouse lines (CBF:H2B-Venus and Hey1-EGFP) in which fluorescent protein expression is
controlled by RBPJ binding or by expression of the Notch downstream gene Hey1 (http://www.gensat.org/), respectively. Interestingly, both lines fail to show evidence for Notch signalling in brain pericytes during early stages of postnatal development (from P1 to P15) (Supplementary Fig. 7a, b), whereas SMCs covering penetrating arterioles in the cortex or pial arteries in the brain surface show reporter expression (Supplementary Fig. 7c, d). These results suggest that the phenotypic changes seen in RbpjPC pericytes are probably unrelated to the lack of Notch activation. In order to further characterize the relevance of canonical Notch signalling in pericytes, we studied the role of Notch family receptors in mural cells. Our RiboTag RNA-seq analysis had shown that Notch3, Notch2, and Notch1, in this order, are the most abundant receptors expressed in brain PDGFRβ+ cells during early postnatal stages (Supplementary Fig. 8a). Notch3 constitutive and ubiquitous knockout mice are viable and, despite of arterial SMC degeneration and focal disturbances in the BBB, pericytes remain unaffected. Notch1 is not very active in mural cells, as reflected by very low frequency of recombination in mouse brain pericytes of the Notch1tm3(cre)Rosa26Cre reporter in the Rosa26-mTomG background (Supplementary Fig. 8b). Pdgfrb-CreERT2-mediated inactivation of Notch2 in a global Notch3−/− background had also no effect on pericytes and brain vascular morphogenesis (Supplementary Fig. 8c–e). Furthermore, Pdgfrb-CreERT2 controlled expression of dominant negative Mastermind-like-1 in Rosa26dnMaml1 mice (hereafter, Maml1dnPC), which blocks all Notch-mediated activation of the RBPJ-associated transcriptional activator complex, impaired expression of the Notch target gene Hey1 in freshly sorted brain mural cells but failed to induce obvious changes in vascular organization, pericyte abundance, EC density, or endothelial sprouting (Supplementary Fig. 8f–l). The expression of tip cell markers and Myc was also not significantly altered in sorted Maml1dnPC brain ECs. Moreover, the expression of known pericyte markers, strongly compromised in RbpjPC mutants, was not significantly changed in Maml1dnPC brains (Supplementary Fig. 8m, n). Finally, we tested the effects of Pdgfrb-CreERT2-mediated expression of constitutively active Notch1 intracellular domain in Gt(Rosa)26Sortm1(Notch1)Dam mice. The resulting NICDPC gain-of-function mutants showed no appreciable alteration in the expression of the mural cell markers PDGFRβ, SM22α, Desmin, and CD13. Vascular area, pericycle coverage, or the frequency of endothelial sprouting were comparable in NICDPC and control littermates (Supplementary Fig. 9a–g).

These data establish that alterations in canonical Notch signalling do not impair proper vascular morphogenesis in the postnatal brain, indicating that the deleterious effects of RbpjPC inactivation in mural cells arise independently from Notch.

### Rbpj-deletion alter ECM composition and TGFβ signalling

In addition to its role in Notch signalling, RBPJ can also repress gene expression. As Notch pathway mutants or mural cell ablation failed to reproduce the RbpjPC phenotype, we hypothesized that the functional changes in RBPJ-deficient pericytes are caused by the upregulation of transcripts that are normally expressed at low levels. Gene set enrichment analysis (GSEA) of consistently upregulated genes in the P7 and P10 RiboTag RNA-seq data revealed significant upregulation of TGFβ signalling targets in RbpjPC PDGFRβ+ cells compared to controls (Fig. 5a). Immuno-staining of RbpjPC brain sections confirmed a striking increase in phosphorylated SMAD3 (Fig. 5b, c), the intracellular signal transducer and transcriptional modulator activated by TGFβ, relative to control. Increased phosphorylation of SMAD1/5 was also detected (Fig. 5d, e) indicating elevated signalling through bone morphogenetic proteins (BMPs). These changes were already evident at early stages of phenotypic alteration (Supplementary Fig. 10a), suggesting that augmented SMAD activation is not a secondary consequence of disrupted cerebrovascular morphogenesis. Moreover, the anatomical localization of vascular lesions coincides with areas of higher phospho-SMAD immunoreactivity (Supplementary Fig. 10b).

Analysis of single optical sections from confocal images revealed that increased SMAD3 phosphorylation is not restricted to pericytes of RbpjPC animals but also affects ECs, whereas SMAD1/5 phosphorylation was only detected in the mutant endothelium (Fig. 5f, g). Accordingly, sorted ECs from P7 mutant brain cortices showed a significant increase in the expression levels of Id1 and Id3 (Supplementary Fig. 10c), two well-characterized downstream targets of BMP-mediated signalling. Noteworthy, RNA-seq analysis of RbpjPC showed an upregulation of Tgfβ3 gene expression starting from P7 and aggravated at P10, a result that was confirmed by RT-qPCR interrogation of freshly sorted brain mural cells (Fig. 5h), and which is consistent with the increased phospho-SMAD3 levels seen both in mutant ECs and pericytes. At the same time, Bmp2 and Bmp4 transcripts were significantly augmented in freshly isolated P7 and P10 RbpjPC ECs from brain cortex (Fig. 5i), implying that the increased phosphorylation of SMAD1/5 is a consequence of excessive BMP production by ECs. Furthermore, endothelial expression of Nrp1, a co-receptor for vascular endothelial growth factor (VEGF) and semaphorins, which promotes tip cell function and limits SMAD2/3 activation, was significantly downregulated in the RbpjPC brain vasculature (Fig. 5i).

It is well established that ECM proteins play a central role in shaping TGFβ and BMP signalling gradients by controlling the storage, localization, and activation of ligands34, 35. GSEA of consistently upregulated transcripts demonstrated a significant
enrichment of genes encoding ECM and ECM-associated proteins including several TGFβ-related molecules in Rbpj\textsuperscript{iPC} PDGFR\textsuperscript{β} cells (Supplementary Fig. 10d, e). Among these genes, matrix glycoproteins (i.e., Fibrillin-1, Fibrillin-2, Fibronectin, and Perlecan) responsible for binding, targeting, and concentrating the large latent complex of TGFβ in specific locations\textsuperscript{36–38} were strongly upregulated in Rbpj\textsuperscript{iPC} PDGFR\textsuperscript{β} cells (Fig. 5j), potentially facilitating the storage of latent TGFβ in the basement membrane shared by ECs and pericytes. At the same time, thrombospondin-1 (Thbs1), an important activator of latent TGFβ that disrupts the non-covalent interactions between the latency associated peptide (LAP) and mature TGFβ\textsuperscript{34}, was 

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upregulated, as confirmed by RNA-seq, qPCR of FACS-isolated mural cells, immunostaining, and in vitro after TAT-Cre-mediated Rbpj depletion (Fig. 5k, l).

TGFβ activation regulates ECM remodelling and has been associated with the induction of fibrosis by promoting the expression of basement membrane proteins, cell adhesion protein receptors, and metalloproteinases as well as their inhibitors. In this regard, several ECM proteins induced by TGFβ, such as plasminogen activator inhibitor 1 (Serpin1), connective tissue growth factor (Ctgf), TGFβ-induced (Tgibi), tissue inhibitor of metalloproteinase 1 (Timp1), osteopontin (Postn), tenasin (Tnc), and matrix metalloproteinases (i.e., Mmp10 and Mmp25), were upregulated in RbpjPC brain pericytes (Supplementary Fig. 10e, f). Likewise, strong changes in the expression pattern of integrin α subunits in pericytes together with increased activation of endothelial integrin β1 were obvious in the RbpjPC vasculature at P10, arguing further for extensive changes in cell–ECM interactions (Supplementary Fig. 11a–c). Importantly, no such changes were triggered by the inhibition of Notch signalling in brain mural cells (Supplementary Fig. 11d–g).

In order to address whether the gene expression changes associated to increased TGFβ signalling are mediated by direct binding of RBPJ, we performed ChIP-seq in cultured primary brain pericytes using an antibody targeted against the transcription factor (Supplementary Fig. 12a and Supplementary Table 1). As expected, the DNA binding motif for RBPJ was significantly enriched in the 11,094 peaks identified (Fig. 6a, Supplementary Fig. 12b, c and Supplementary Data 3). Moreover, GREAT analysis (Genomic Regions Enrichment of Annotations Tool) was able to identify the Notch signalling pathway as an overrepresented functional term amongst the genes associated to RBPJ binding sites (Fig. 6b), further validating the specificity of the approach. In addition, ChIP-seq experiments using antibodies against the chromatin marks H3K4me3 and H3K4me1 revealed that RBPJ peaks are either enriched in promoters (H3K4me3+) or distal enhancer regions (H3K4me1+) of the bound genes (Fig. 6c). Among the RBPJ-bound genes, we identified 122 that are significantly upregulated in both P7 and P10 pups (Fig. 6d and Supplementary Data 4). Interestingly, this list includes several molecules that control TGFβ signalling (Tgfβ3, Thbs1, Fln1, and Fbn2), drive fibrotic changes (Tgibi, Serpin1) or are responsible for cell–ECM interactions (Igα3, Igα5, Igα8) (Fig. 6d and Supplementary Fig. 12d). In addition, we also found transcription factors, such as Atf3 and Runx1 (Fig. 6d and Supplementary Fig. 12d), which have been proposed to promote fibrosis in connection to increased TGFβ signalling.

Altogether, our data shows that the loss of RBPJ in pericytes triggers a complex response involving excessive production of TGFβ3 and alterations in ECM composition, which lead to increased activation of SMAD3 together with non-cell-autonomous changes, such as increased phosphorylation of SMAD1/5, in the adjacent endothelium. Furthermore, characterization of the genomic landscape in cultured pericytes revealed that RBPJ binds the transcriptional domains of genes controlling TGFβ signalling. Many of these genes are significantly overexpressed after the loss of RBPJ function, further suggesting a critical repressor role of RBPJ in pericytes during early postnatal development.

Pathological implications of Rbpj inactivation in pericytes. Tamoxifen administration over 3 consecutive days starting from P3 or P5 produced RbpjPC mutants with prominent vascular lesions in the cerebellum (Supplementary Fig. 13a), which recapitulate defects in the cerebellar white matter of mouse models for CCMs. Common features include dilated, haemorrhagic venules in the white matter with secondary inflammatory reaction (Fig. 7a). Another hallmark of CCMs is the cleavage of the matrix proteoglycan versican by the metalloproteinase ADAMTS-4, leading to the exposure of the neo-epitope DPEAAE. Abnormal presence of DPEAAE was obvious in RbpjPC cerebellar lesions (Supplementary Fig. 13b). CCMs arise from mutations affecting the genes encoding KRIT1/CCM1, CCM2, or PDCDC10/CCM3 proteins in the endothelium, but the corresponding transcripts were only slightly reduced in the RbpjPC brain endothelium (Fig. 7b). Activation of the MEKK3-MEK5-ERK5-MEF2 signalling axis leading to increased expression of the Kruppel-like family transcription factors Klf2 and Klf4 is a key process in CCM pathogenesis. In addition, integrin β1 activation has been reported as a signal triggering elevated Klf2 expression. Strikingly, Klf2 and Klf4 were significantly upregulated in the RbpjPC endothelium, and both Klf4 protein and activated integrin β1 were strongly increased in mutant cerebellar white matter lesions (Fig. 7b–e).

Next, we further evaluated the effect of Rbpj deletion in adult animals. Surprisingly, tamoxifen-induced recombination after weaning (3 weeks of age), did not induce any overt phenotype in the CNS of RbpjPC mutants, which showed normal vascular organization, proper expression of mural cell differentiation markers, and no BBB defects (Supplementary Fig. 13c, d). Given that Rbpj deletion did not affect neurovascular homeostasis in this setting, we used the permanent distal middle cerebral artery occlusion (dMCAO) model to induce ischaemic stroke in the adult brain cortex. Angiogenesis is a primary neurovascular response during stroke recovery and pericytes play important, yet not fully understood roles in different stages of ischaemic stroke. As Pdgfrb-CreERT2 transgenic mice enable efficient targeting of mural cells in adult mice, we first performed lineage tracing analysis in combination with the Rosa26RtTA reporter allele in animals with one or two functional copies of Rbpj. Tamoxifen was administered 1 week before dMCAO surgery and samples were collected 7 days post-operation. As expected, the ischaemia-affected brain hemisphere showed strong neuronal depletion and dilation of vascular structures together with significant upregulation of PDGFRβ expression.
Interestingly, ischaemia-induced PDGFRβ expression was not restricted to perivascular locations and clearly exceeded the area covered by recombined (GFP+) pericytes (Fig. 7f), suggesting post-ischaemic recruitment of PDGFRβ+ cells or de novo expression of this receptor in previously negative cells. GFP-labelled pericytes showed drastic morphological changes in comparison to mural cells of the contralateral hemisphere. Typically, pericytes are tightly attached to ECs, but after stroke they emit long protrusions that bridge adjacent capillaries (Fig. 7f and Supplementary Fig. 13e, f).

In order to assess whether Rbpj is necessary for pericytes during the vascular response to ischaemic stroke, adult control
and RbpjPC mice were subjected to dMCAO. RbpjPC mutants developed bigger cortical lesions with larger space-occupying effect due to brain oedema and increased corrected lesion volumes (Fig. 7g, h). Histologic analysis revealed higher phospho-SMAD3 immunoreactivity in RbpjPC than in control stroke samples (Fig. 7i), an effect that partially resembles the phenotype seen in postnatal mutants. In addition, a stronger inflammatory response was detected in RbpjPC animals together with localized vascular aberrations such as vessel tortuosity and ballooning (Fig. 7j, j). Pericytes have been proposed to have an important role in neuroinflammation, mostly through secretion of cytokines49. In this regard, our RNA-seq analysis of mutant pericytes from young pups revealed strong overexpression of pro-inflammatory chemokines (Fig. 7k) together with upregulation of inflammation-related adhesion molecules, MMPs, and phagocytic receptors (Supplementary Fig. 13g). Interestingly, (C–C motif) ligand 2 (CCL2) is induced upon Rbpj deletion since early stages (P5 and P7), is strongly upregulated shortly after Rbpj deletion in vitro (Supplementary Fig. 13h), and is bound by RBPJ (Fig. 7i), suggesting a potential direct role of RBPJ in controlling a pro-inflammatory genetic program in pericytes, which, if activated, is likely to worsen stroke outcome.

Discussion

The critical role of pericytes in BBB development50 and maintenance4 is well appreciated and, conversely, pericyte loss can trigger brain vascular damage that ultimately leads to secondary neurodegenerative changes8. Altogether, the current view of pericytes portrays them as protective cells that safeguard neurovascular homeostasis. Here, we provide evidence that the loss of RBPJ generates dysfunctional pericytes that compromise neurovascular homeostasis and induce severe vascular lesions that are not present in animals with acute or chronic pericyte depletion. Rbpj-deficient pericytes induce pathogenic transformation of the vasculature resembling CCMs at the morphological and molecular level, and result in bigger stroke lesions upon ischaemic insult.

Single-cell molecular profiling of brain mural cells has suggested that pericytes and vein-associated SMCs represent a continuum that is distinct from the SMCs covering arteries and arterioles24. Consistent with this finding, Pdgfrb-CreERT2-mediated Rbpj inactivation prominently affects the morphogenesis and integrity of capillaries and veins, and drives a profound change in mural cell identity characterized by reduced expression of pericyte-specific markers and upregulation of genes characteristic for arterial SMCs. This indicates that proper control of the molecular and functional properties of pericytes is critical for correct vascular patterning, function, and BBB integrity. Moreover, our data indicate that RBPJ functions in a Notch-independent fashion in postnatal brain pericytes. Previous reports highlighting activation of Notch targets such as Hey1 in retinal pericytes associated to the retinal angiogenic front12 may reflect tissue-specific programs, alternative signalling mechanisms driving Hey1 expression, or differential control of pericyte behaviour in the context of angiogenic growth into avascular (retinal) tissue.

Based on our in vivo data, we also propose that RBPJ helps to repress SMC differentiation, which is consistent with a recent in vitro study showing that RBPJ depletion in human aortic SMCs enhances the differentiation of these cells in a similar way as TGFβ1 stimulation51. The same study also found that RBPJ knockdown results in increased association of active transcription marks with a large number of TGFβ signalling and matrix-associated genes, which is consistent with our own results. Apart from enhancing vascular SMC differentiation and contractility, TGFβ signalling has been implicated in the pathogenesis of tissue fibrosis39 and CCMs44, and may induce a pro-inflammatory state in pericytes through the expression of chemokines49. In addition, excessive activation of SMAD2/3 in ECs has been shown to limit tip cell specification30, potentially explaining the strong reduction of sprouting angiogenesis in RbpjPC mutants. Moreover, Rbpj-mutant pericytes may directly promote inflammatory processes in the brain by excessive production of CCL2, an important mural cell-derived mediator of neuroinflammation52. Interestingly, BMP expression in ECs is increased after exposure to proinflammatory stimuli53 and once up-regulated, BMP2 and BMP4 induce pro-inflammatory effects34 and activation of SMAD1/5, which has been shown to drive cell cycle progression leading to excessive cell proliferation55.

The proposed Notch-independent role of RBPJ in pericyte biology are particularly important during scenarios where physiological or regenerative angiogenesis is involved, presumably reflecting its relevance for fate commitment and intercellular communication in environments associated to increased cellular plasticity. Moreover, the severe defects on vascular integrity induced by mural cell-specific deletion of Rbpj strongly argue for potential involvement of pericytes in pathogenic transformation. On this regard, it should be noted that the molecular mechanisms responsible for CCM lesions development (namely increased RhoA activity56, augmented MEK3/ERK2,4 signalling45, and abnormal activation of the TGFβ/BMP/SMAD pathway44) may be influenced by additional mechanisms such as the behaviour of pericytes. Likewise, the recently appreciated relevance of mural cells in the elicitation of neuroinflammation52 further highlights the important implications of pericytes in pathogenic responses and recovery after insult.

Based on the sum of our findings, we propose that improper intercellular communication elicited by dysfunctional pericytes...
can lead to profound changes in the adjacent endothelium and the surrounding tissue, which together drive neurovascular pathogenesis.

**Methods**

**Mutant mouse models and inducible genetic experiments.** *Pdgfrb-CreERT2* were interbred with *Rosa26mTomT* Cre-reporter mice in order to monitor recombination efficiency and specificity. Mural cell-specific *Rbpj* conditional knockouts (*Rbpj<sup>IPC</sup>*) were generated by crossing *Pdgfrb-CreERT2* with *Rbpj<sup>lox/lox</sup>* mice. For some experiments the *Rosa26mTomT* reporter allele was included in the *Pdgfrb-CreERT2* *Rbpj<sup>lox/lox</sup>* background. In such cases, *Pdgfrb-CreERT2<sup>+/+</sup>*/*Rbpj<sup>lox/lox</sup>* double heterozygous animals were used as controls, whereas Cre-negative littermates were used as controls in other experiments. Animals with a single-allele inactivation of *Rbpj* were indistinguishable from Cre-negative littermates.

For quantitative transcriptome analysis, *Pdgfrb-CreERT2* *Rbpj<sup>lox/lox</sup>* mice were interbred with *Rpl22<sup>tm1.Psam</sup>* (RiboTag) animals. As control for RNA-seq analysis, age and weight-matched pups bearing the *Pdgfrb-CreERT2* transgene and two copies of the RiboTag allele, but wildtype for *Rbpj*, were used.
Fig. 5 Increased TGFβ signalling in the RbpjPC cerebral vasculature. a GSEA of overexpressed genes (RNA-seq model-based clustering analysis) reveals upregulation of TGFβ targets in RbpjPC. ES, enrichment score; NES, normalized enrichment score; FDR, false discovery rate. b Confocal images showing increased phosphorylation of SMAD3 (phSMAD3, white) in P10 RbpjPC brain cortex vasculature (tdt−, red). Scale bar, 100 μm. c phSMAD3 immunosignal intensity quantitation in P10 brain vasculature. p-values, Welch’s t-test, n = 6. d Confocal images showing increased phosphorylation of SMAD1/5 (phSMAD1/5, white) in P10 RbpjPC brain cortex vasculature (tdt−, red). Scale bar, 100 μm. e phSMAD1/5 immunosignal intensity quantitation in P10 brain vasculature. p-values, Welch’s t-test, n = 6. f Confocal single optical sections showing localization of phSMAD3 (f, white) in the nuclei (DAPI+, blue) of ECs (ICAM2−, red, yellow arrowheads) or pericytes (GFAP+, green; white arrowheads) in P10 cortical vessels. Note phSMAD3 presence in ECs and pericytes, while phSMAD3/5 is only detectable in ECs. Scale bar, 25 μm. f Tgfb3 expression in cortical mural cells from P7 or P10 mice analyzed by RNA-seq (top) or RT-qPCR (bottom). p-adjusted values (RNA-seq) or p-values, Student’s t-test, n = 3–4. i RT-qPCR analysis in sorted ECs from P7 and P10 brain cortices. p-values, Brown-Forsythe and Welch one-way ANOVA with Tamhane’s T2 test (Bmp2 and Bmp4), or one-way ANOVA (Thbs1−/−) with Sidak’s post-hoc test, n = 5. j Gene expression fold change based on RNA-seq counts in P7 and P10 brain cortex mural cells. p-adjusted values. n = 3. k Thrombospondin-1 (Tb1s3) expression analysis in vivo (RNA-seq and RT-qPCR for FACS-sorted mural cells) and in vitro in cultured pericytes. p-adjusted values (RNA-seq), p-values, Welch’s t-test (in vitro), or Brown-Forsythe and Welch one-way ANOVA with Holm-Sidak’s test (RT-qPCR), n = 3–4. l Confocal images showing increased expression of thrombospondin-1 (Tb1s3, white) in mural cells (PDGFRβ+, green) of P10 RbpjPC PC cortical vasculature (ICAM2−, red). Scale bar, 50 μm. Error bars represent s.e.m. Source data are provided as a Source Data file.
Scientific, A21447). Streptavidin AF405 (1:200, Invitrogen S32351) was used for detection of biotinilated-IB4 stained samples. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, D9542) diluted at 1 μg ml⁻¹ together with the secondary antibodies.

Processing of samples and staining conditions were kept identical and performed in parallel on samples from mutant mice and their respective controls for each experiment.

**Proliferation assay in vivo.** For in vivo analysis of cell proliferation, pups were injected intraperitoneally with 5-ethyl-2-deoxyuridine (EdU, Life Technologies, A10044; 30 mg kg⁻¹ dissolved in DMSO-PBS 1:10 at 2 mg ml⁻¹) 4 h before dissection. Brains were collected and processed as described before. After secondary antibody staining, EdU labelling was detected by means of a Click-it EdU Alexa Fluor-647 Imaging Kit (Life Technologies, C10340) according to the manufacturer’s instructions.
**Image acquisition and quantitative analysis.** Whole-brain pictures and overview images from entire, unstained coronal slices were taken using a dissection microscope (Leica MZ205C) coupled to a digital camera (QImaging MicroPublisher 5.0 RTV) with Velocity 6.3 (Perkin Elmer) software.

Confocal images were acquired with a Zeiss LSM780 or LSM880 confocal laser scanning microscope equipped with the following objective lenses: 10x Plan APO NA 0.45, 20x Plan APO NA 0.80, water immersion 40x LD C APOchromat NA 1.0, 63x Plan APO NA 1.4 oil, and 100x Plan APO NA 1.4 oil. Confocal laser scanning microscope was used, equipped with the following objective lenses: 10x HC PL APO NA 0.40, 40x HC PL APO CS2 NA 0.75, water immersion 40x HC PL APO W motCORR CS2 NA 1.10, and glycerol immersion 63x HC PL APO Glyc Corr CS2 NA 1.30. Image acquisition was done with ZEN Software 2.3 SPI FI 14.0 (Carl Zeiss) or Leica Application Suite X 2.0.11.4392 (Leica). Images analysis and processing (brightness and contrast correction, background subtraction) was performed using Velocity 6.3 (Perkin Elmer), Photoshop CS6 13.0 (Adobe), Illustrator CS6 16.00.0 (Adobe), and Fiji-IJ 2.0-rc-64. All confocal images are represented as maximum intensity projections unless stated otherwise.

For quantification of vascular area, the blood vessel markers CD31 or GLUT1 were used. Based on their expression pattern, objects were defined with the Find Objects tool from the Measurements package in Volocity and their areas (μm²) measured. Vascular density was calculated by dividing the total perimeter of vessels identified with the luminal marker ICAM2 into the corresponding area calculated as already described. Vessel diameter of arteries and veins was measured manually in Fiji from CD31 stained vessels. Arteries and veins were identified based on their morphology and the distinctive coverage of SMC (asSMC-). Capillary diameter was measured using a specific plugin in ImageJ. Details of number of vessels measured per animal and condition can be found in the Source Data File.

Quantification of sprouts was performed in GLUT1 stained sections. Visual identification of sprouts was based in the identification of vascular projections with obvious filopodia leading their growth into the avascular space. The number of sprouts was normalized to the vascular area present in each image quantified.

Red blood cell (RBC) leakage was measured manually by defining the extravascular areas in the brain parenchyma where RBCs (identified by staining with GLUT1) were obvious and normalizing it to the whole brain region imaged. Percentage (%) values represent the proportion of the brain area in which extracellular RBCs are found.

For quantification of EC numbers combination of a vascular marker together with the EC-specific nuclear marker ERG was used for unambiguous identification. EC density reflects the number of ECs found in 100 μm² of vascular area. Proliferating ECs (EdU™ and ERG(E)) were visually identified, manually counted and their number normalized to the total amount of ECs in 100 μm² of vascular area.

Analysis of apoptosis in brain ECs was based on the identification of apoptotic ECs either by terminal deoxynucleotidyl transferase dUTP nick end labelling using the Click-iT TUNEL Alexa Fluor 647 Imaging Assay (Thermo Fischer Scientific, C10247) or by immunostaining against cleaved Caspase-3. The number of apoptotic ECs per 100 μm² of vascular area (measured as already described) was plotted.

Mural cell coverage was calculated by dividing the surface area positive for either pericyte (PDGFRβ) or SMC (asSMCA) markers into the surface area stained for vascular cells (e.g., CD31 or GLUT1). Likewise, astrocyte endfoot coverage was calculated by dividing the surface area positive for Aqp4 immunostaining into the total vascular area. Recombination efficiency in mural cells was quantified by establishing a percentage relation between the area covered by recombined (GFP+) pericytes with regards to the total perivascular PDGFRβ area.

GEAP and Iba1+ regions were identified with the Find Objects tool from the Measurements package in Velocity and their areas (μm²) measured. Values are reported as such without additional normalization. The size of the fields of view used for quantitation was equal for control and mutant mice.

For quantification of phSMAD3, phSMAD1/5, and KLF4 immunoreactivity signal intensity, the vascular area was identified and segmented based on blood vessel-specific staining (i.e., ICAM2) and antibody (i.e., EdU) signal intensity. The signal intensity in the vascular area after cropping out the DAPI+ nuclei (where the specific staining is expected) was measured. Active Tβh1 staining quantitation was based in measurement of the signal intensity within the vascular area normalized to the non-vascular regions of the image. All signal intensity plots correspond to the value obtained after subtracting the background-specific mean intensity from the standardized mean intensity measurement for antibody and antibody of interest. Detailed information regarding the number of biological replicates (animals or individual experiments), and the number of samples measured for each animal (or technical replicates in each experiment) are detailed in the Source Data File. Settings for scanner confocal detection and laser excitation were always kept identical between samples whenever comparisons between mutant mice and their respective controls were done.

**Blood flow assessment using two-photon in vivo microscopy.** P10 pups were anaesthetized by intraperitoneal injection of xylazine (Bayer, Rompun 2%; 3.3 mg kg⁻¹) and ketamine (Zoetics, Ketavet 100 mg ml⁻¹; 33.3 mg kg⁻¹) dissolved in saline and kept thereafter in a heating pad at 37 °C. Once the stage of surgical anaesthesia was achieved, a single midline incision on the skin of the head was performed and a custom-made titanium ring was glued on top of the intact skull. Next, 25 μL of Texas Red-Dextran (70,000 MW, Neutral, Thermofisher Scientific, D1830) were administered by intracardiac injection and the animal was anaesthetized by intraperitoneal injection of ketamin (100 mg ml⁻¹). Whole-brain images were taken using a TriM Scope II multi photon system (LaVision BioTec) fitted with a Chameleon Ultra II Ti Sapphire laser (Coherent) a Chameleon Compact optimal parametric oscillator (OPO, Coherent) and a water dipping 16x CFI-75 NA 0.8 objective (Nikon) with a working distance of 2.0 mm. Images were acquired and processed with ImSpector Pro 5.1.347 (LaVision BioTec).

In vivo imaging of blood flow dynamics at cellular resolution in brain blood vessels and calculation of blood flow velocities was based on centreline scans. To ensure flow velocities remained constant, selected vessels were measured twice, at the beginning and end of an experiment. Overview images were captured at the beginning of each imaging session.

**Transmission electron microscopy.** For electron microscopy, 10 ml of PBS and 40 ml of 2% PFA and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) were perfused through the left ventricle of an anaesthetized mouse. The brain was removed and further perfused through the left ventricle of an anaesthetized mouse with 10 ml of PBS and 40 ml of 2% PFA and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). In brief, P10 pups were anaesthetized and perfused with 10 ml of PBS. Brain hemispheres without olfactory bulb and cerebellum were collected in a weighted tube. After addition of 300 μl of double distilled water, the samples were homogenized with a tissue grinder (Pellet Pestle Cordless Motor, Kimble Chase) and centrifuged at 20,000 g, 30 min, 4 °C.
200 μL of tissue homogenate were collected, mixed with 800 μL of Drabkin’s reagent (Sigma, D5941) and incubated for 15 min at RT. Absorbance at 550 nm was measured (BioPhotometer plus, Eppendorf) and compared to a standard curve of known haemoglobin concentration.

**BBB analysis and identification of AV malformations.** For BBB analysis, adult animals were injected i.p. with 2% Evans Blue (960 Da, 4 ml kg\(^{-1}\) of body weight, Sigma, E2129) and 10% fluorescein sodium salt (376 Da, 0.6 ml kg\(^{-1}\) of body weight, Sigma, F6377) in normal saline. The stain was allowed to circulate for 24 h. Afterwards, the animals were transcardially perfused with 20 ml of PBS and 15 ml of ice-cold 4% PFA. Brains were collected and postfixed in 4% PFA ON at 4 °C. Vibratome sections were obtained and imaged by confocal microscopy as already described. Note that sodium fluorescein is used as a reporter of barrier leakage to small molecules (smaller than 0.4 kDa), while Evans Blue reflects albumin (66.5 kDa) extravasation.
For identification of arteriovenous (AV) malformations, P10 pups were anesthetized and euthanized by transcardial perfusion of PBS through the left ventricle. Immediately after and without removing the needle used for perfusion, 100 μL of 1% Evans Blue in normal saline were injected following by 300 μL of lactate (Naloxon). Efficient labelling was monitored by visual inspection of main superficial arteries in the sternum and neck region. Brains were collected, fixed in 4% PFA ON AT 4°C, washed in PBS, and imaged in a dissection microscope.

**FACS of brain mural and endothelial cells.** Brain cortices from P5, P7, or P10 pups were collected in ice-cold dissection media (DMEM Sigma, D6564) supplemented with penicillin/streptomycin (PAA, P11-010) and 25 mM HEPES (Hepes, H3357) and transferred to digestion media: 37°C pre-warmed dissection media supplemented with 100 μg/ml 1 Listerase DH (Roche, 5401054) and 50 μg/ml 1 DNAseI (DNAse, Sigma, DN25). Samples were minced and digested for 15 min at 37°C with occasional pipetting to disaggregate tissue pieces. FACS buffer (PBS with 2% FCS) was added and the tissue homogenate was mixed with 1.7 volumes of 22% albumin fraction (RBA, Carl Roth, 80762). After centrifugation (1000g, 12 min at RT) the supernatant was removed and the cell pellet resuspended in 5 ml of FACS buffer, filtered through a 40 μm nylon mesh (Corning, 352240) and centrifuged (300g, 5 min at RT). The cell pellet, consisting mostly of single cells was resuspended in a suitable volume of FACS buffer together with the following antibodies: rat anti-TER-119-Pacific Blue (1:100, BioLegend, 116232), rat anti-CD45-Pacific Blue (1:200, BioLegend, 103126), rat anti-CD31-FITC (1:100, Invitrogen, RM5201), rat anti-CD13-PE (HE, 1:100, BD Pharmingen, 357847), rat anti-CD140a-PE/CY7 (1:100, Fscience, 25-1401), and rat anti-CD140b-APC (APC) (1:300, eBioscience, 17-1401-82). For cell sorting of astrocytes and microglia, which perichondelial expression of CD31, the CD31-APC antibody was replaced by rat anti-CD31-BV711 (1:100, BioLegend, 740680). After 30 min incubation on ice, cells were washed with FACS buffer and resuspended in FACS buffer with DAPI (1 mg ml⁻¹) to exclude dead cells. Cells were sorted directly into a RT-Plus buffer (Qiagen, 1053393) supplemented with 1% β-mercaptoethanol (Sigma, M6250) using a FACS Aria IIu (BD Biosciences) with a 70 FACS Diva software (BD Bioscience, Version 6.0).

Gating strategies to discriminate cell populations based on their immunoboldelling were defined according to reference experiments using single-stained samples and FMO (fluorescence minus one) controls.

**RNA extraction and RT-qPCR.** Total RNA from freshly sorted or cultured pericytes and ECs was isolated using the RNeasy Plus Micro Kit (Qiagen, 74034) following the manufacturer’s instructions. Whole RNA was reverse transcribed and converted to complementary DNA (cDNA) using the iScript cDNA synthesis kit (BioRad, 170-8890). The following FAM-conjugated TaqMan gene expression probes (all from Thermo Fisher Scientific) were used: Dl1 (Mm00441691_m1), Dl2 (Mm00449533_m1), Myc (Mm00487256_m1), Anpep (Mm00476227_m1), Rgs5 (Mm00654112_m1), Cg5ped1 (Mm00507257_m1), Notch3 (Mm01345646_m1), Acta2 (Mm00724512_s1), Tgfb1 (Mm00416661_g1), Hey1 (Mm00488856_m1), Bmp2 (Mm01340178_m1), Bmp4 (Mm00432087_m1), Nrp1 (Mm00435379_m1), Cg5ped1 (Mm01192932_g1), Tgfb1 (Mm00335418_m1), Krill (Mm01316552_m1), Ccm2 (Mm00452581_m1), Pdzd10 (Mm00772342_s1), Klf2 (Mm00492799_g1), Klf4 (Mm00551604_m1), Rbpj (Mm01217627_g1), Id3 (Mm00775963_g1), Id3 (Mm00492257_m1), Tgfb1 (Mm01336705_m1), Pnt1 (Mm01265744_m1), Tgf3 (Mm00436960_m1), Ifg8 (Mm01249558_m1), Adamts8 (Mm01497220_m1), and Cxcl2 (Mm00414224_m1). VlC-conjugated Arib (435230), TaqMan probe was used to normalize gene expression after house-keeping gene selection using a TaqMan Array Mouse Endogenous Control Plate (Thermo Fisher Scientific, 426697).
off was set as 100 for the cell population of interest. For comparison of lists of genes, the Compare two lists online tool (https://projects.tcg.aic.nationalcancerinstitute.nih.gov/hi/) was used.

**Primary brain pericyte isolation and culture.** Brains from 6–8 weeks old mice homozygous for Rbpjfl/fl mice, either TAT-Cre or Cre-expressing lentivirus-infected Rbpjfl/fl mice (gift from Steven Dowdy; TAT-Cre: protein was produced in Rosetta 2(DE3)pLysS Escherichia coli cells (Novagen, 71403) and purified on Ni-NTA Agarose (Qiagen, 30210) and EndoTrap HD1/1 (Hyglos, 800305). Lentivirus were produced in HEK293T cells co-transfected with the packaging plasmids pSPAX2 (Addgene, 12260), PMD2.G (Addgene, 12269) and pHelper Ultra-Blasticidin or pHelper-Ultra-Blasticidin using Fugene6 transfection reagent (Promega, E2691). pHelper (Addgene, 24129) was a gift from Malcolm Moore; the CDNAS encoding Cre and Blasticidin resistance were subcloned into the BamHI, NheI, and SalI restriction sites.

TAT-Cre, or an equal amount of BSA (negative control), were diluted in MEM media ( Gibco, 31095-029), filtered in 0.22 μm low binding protein filter units (Milw-GV, SLGV/0135L) and added pericytes seeded 24 h earlier. 15 h later cells were washed once in PBS and pericyte medium added. Gene recombination analysis or experimental procedures were done 48 h after removal of TAT-Cre.

**Collagen gel contraction assay.** Assessment of in vitro contractility of brain pericytes after Rbpjfl/fl mice deletion was performed with gel contraction assay22. In brief, recombined or control pericytes were suspended at 1 × 105 cells ml⁻¹ in 1.0 mg ml⁻¹ type 1 collagen dissolved in 0.8× DMEM supplemented with 8% FBS with pH adjusted by addition of 0.1 M NaOH. The cell–matrix mixture was added drop-wise in wells of a 24-well plate and was allowed to solidify for 20 min at 37 °C. Pericyte-derived cells were washed once with PBS and left in culture for 48 h before evaluation of gene recombination or experimental analysis.

**Chromatin immunoprecipitation (ChIP), library, and sequencing.** ChIP was performed essentially as previously described23. The following antibodies were used: H3 (Abcam, ab1791), H3K4me1 (Abcam, ab8955), H3K4me3 (Diagenode, pAb-003-050), and RBP (Cell Signaling, #5313). Libraries were prepared using the Diagenode MicroPlex Library Preparation kit v2 (Diagenode) following the manufacturer’s instructions with few modifications. Libraries were purified with Agencourt AMPure XP Beads (Beckman Coulter, #A68811), quantified, analyzed on a Tapestation device (Agilent), and pooled. Finally, sequencing was performed by Centro de Análisis Genómico (CNAG-CRG). Spain.

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**Statistical analysis.** All data are presented as mean ± standard error of the mean (s.e.m.). No statistical methods were used to predetermine sample size; instead sample number was defined according to previous experience and reproducibility of the results across several independent experiments. For all experiments the number of animals (n) analyzed per group was derived from at least two different litters. No animals were excluded from the analysis and no randomization or blocking was used. Unless otherwise indicated, results are based on three or more independent experiments to guarantee reproducibility of the findings. Sample distribution was assessed with the D Agostino & Pearson omnibus test and a two-tailed Student’s t-test was used to determine statistical significance when comparing two independent groups with normal distribution and no significant difference in their standard deviation (SD); alternatively, Welch’s t-test was used for non-parametric distributed data with unequal variances. When comparing two independent groups

**Distal middle cerebral artery occlusion (dMCAO).** The dMCAO procedure were performed as previously described22 in 20-week-old (adult) male mice. Surgical procedures started 1 week after tamoxifen administration. The animals were anesthetized by intraperitoneal injection of ketamine (80 mg kg⁻¹) and xylazine (16 mg kg⁻¹). The skin incision was performed to gain access to the temporal muscle, which was partially removed using electrocoagulation forceps with the high-frequency generator set to 5–7 W. After removing the muscle flap, the distal middle cerebral artery (MCA) was identified and the skull above the MCA branch was thinned using a microdrill. After removing the bone, the MCA was coagulated proximally and distally to the vessel bifurcation using bipolar forceps with the energy of the high-frequency generator set to 3–5 W. After ensuring that the blood flow is permanently interrupted, the temporal muscle was fixed at the muscle flap and the craniectomy was closed with 1.5–2.0 suture. In the next day, cells were washed once in PBS and pericyte medium added. Gene recombination analysis or experimental procedures were done 48 h after removal of TAT-Cre.

**Magnetic resonance imaging (MRI).** Animals were subjected to MRI on days 1, 2, 5, and 7 after dMCAO operation. MRI was performed using a 7 Tesla research scanner (Pharmascan 70/16 US, Bruker Biospin) with a 16 cm horizontal bore magnet and a 9 cm (inner diameter) shielded gradient with a H-resonance frequency of 300 MHz and a maximum gradient strength of 300 μT m⁻¹. For imaging a 1-H RF quadrature-volume resonator with an inner diameter of 20 mm was used. Data acquisition and image processing were carried out with Paravision 6.0 software (Bruker). During the examination mice were anesthetized with isoflurane and then placed in the warm cage in 70% N₂O and 30% O₂ via a vaporizer and placed on a heated circulating water blanket to ensure constant body temperature of 37 °C. Brains were imaged with a T2-weighted 2D turbo spin-echo sequence with imaging parameters TR per TE = 2000–20000 ms, 30 ms, 8 and 4 averages. In each case, 32 axial slices with a slice thickness of 0.5 mm, a field of view of 256 × 256, and a matrix size of 256 × 256 were obtained. The whole object map total volume was automatically calculated. The space-occupying effect due to brain oedema, expressed as the volume increase of the affected hemisphere (%HSE), and the oedema-corrected lesion volume (LVc) were calculated according to standardized protocols24.
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