

# The $\beta$ 8 Integrin cytoplasmic domain activates extracellular matrix adhesion to promote brain neurovascular development

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## Abstract

In the developing mammalian brain neuroepithelial cells interact with blood vessels to regulate angiogenesis, blood-brain barrier (BBB) maturation, and other key neurovascular functions. Genetic studies in mice have shown that sprouting neurovascular development is controlled, in part, by *Itgb8* which encodes the neuroepithelial cell-expressed integrin  $\beta$ 8 subunit. However, these studies have involved complete loss-of-function *Itgb8* mutations, and have not discerned the relative roles for the  $\beta$ 8 integrin extracellular matrix (ECM) binding region versus the intracellular signaling tail. Here, Cre/lox strategies have been employed to selectively delete the cytoplasmic domain of *Itgb8* without perturbing its transmembrane and extracellular domains.

We report that the  $\beta 8$  integrin cytoplasmic domain is essential for inside-out modulation of adhesion, including activation of latent-TGF $\beta$ s in the ECM. Quantitative sequencing of the brain endothelial cell transcriptome identifies TGF $\beta$ -regulated genes with putative links to blood vessel morphogenesis, including several genes linked to Wnt/ $\beta$ -catenin signaling. These results reveal that the  $\beta 8$  integrin cytoplasmic domain is essential for the regulation of TGF $\beta$ -dependent gene expression in endothelial cells and suggest that cross-talk between TGF $\beta$ s and Wnt pathways is critical for neurovascular development.

## Introduction

Members of the  $\alpha v$  family of integrins, comprised of  $\alpha v\beta 1$ ,  $\alpha v\beta 3$ ,  $\alpha v\beta 5$ ,  $\alpha v\beta 6$ , and  $\alpha v\beta 8$ , bind with high specificity to RGD peptide motifs present in many ECM proteins (McCarty, 2020). Embryos null for the  $\alpha v$  integrin gene (*Itgav*) or  $\beta 8$  integrin gene (*Itgb8*), and thus lacking the  $\alpha v\beta 8$  integrin heterodimer, develop nearly identical phenotypes that include aberrant brain angiogenesis and intracerebral hemorrhage (Bader et al., 1998; McCarty et al., 2002; Zhu et al., 2002) that initiate in the ganglionic eminences of the midbrain. Conditional deletion of *Itgav* or *Itgb8* using Nestin-Cre (Tronche et al., 1999) leads to similar angiogenic pathologies as those that develop in *itgav*<sup>-/-</sup> and *Itgb8*<sup>-/-</sup> embryos (Arnold et al., 2012; Arnold et al., 2019; Arnold et al., 2014; Hirota et al., 2015; Hirota et al., 2011; Lee et al., 2015; Mobley and McCarty, 2011; Mobley et al., 2009). The major ligands for  $\alpha v\beta 8$  integrin in the brain are latent-TGF $\beta$ s, which are embedded in the ECM as inactive latent complexes (Worthington et al., 2011). Integrin-induced conformational changes in the latent-TGF $\beta$  complex leads to cytokine release, engagement with TGF $\beta$  receptors, and activation of canonical signaling events involving Smad transcription factors (Dong et al., 2014; Shi et al., 2011). Genetically targeting TGF $\beta$  receptor genes in endothelial cells, and particularly *Tgfr2* which encodes the type 2 TGF $\beta$  receptor,

leads to CNS vascular pathologies that are strikingly similar to those that develop in mice lacking *Itgav* or *Itgb8* in neuroepithelial cells (Allinson et al., 2012; Nguyen et al., 2011).

Integrin engagement with the ECM is regulated largely by inside-out activation, which involves a conformational transition from a low affinity inactive state to a high affinity ECM binding state. Inside-out activation is controlled by interactions between the  $\beta$  subunit cytoplasmic tail, intracellular signaling effectors, and the cytoskeleton (Kadry and Calderwood, 2020). Structural studies of  $\alpha v \beta 8$  integrin, however, reveal that it exists mainly in an atypical adhesion-competent conformation termed 'extended-closed', rather than in a mixture of 'bent' (inactive) or 'extended' (active) conformations detected in other integrins (Wang et al., 2017). The extended-closed  $\alpha v \beta 8$  integrin structure suggests that a transition to a fully extended conformation might depend on activating components that interact with the transmembrane and/or cytoplasmic domain. Interestingly, the cytoplasmic tail of  $\beta 8$  integrin is divergent from other integrins (McCarty et al., 2005a) and lacks conserved sequences that recruit signaling effectors that function in inside-out activation (Chen et al., 2006; Jannuzi et al., 2004; Mathew et al., 2012; Menezes et al., 2014).

The unique properties of the  $\beta 8$  integrin cytoplasmic domain raise many questions about its roles in regulating intracellular signaling versus ECM adhesion. For example, the cytoplasmic domain could serve to promote a transition from the extended-closed conformation to a fully active conformation with higher affinity ECM binding. Alternatively, the  $\beta 8$  integrin extracellular domain may be fixed in the extended-closed conformation, with the cytoplasmic tail having no roles in inside-out activation. Indeed, cell-based assays (Mu et al., 2002) and cryo-EM studies of  $\alpha v \beta 8$  integrin (Campbell et al., 2020; Cormier et al., 2018) reveal that engagement with latent-TGF $\beta$  and activation of TGF $\beta$  signaling occurs independently of the cytoplasmic tail. Thus far, all *Itgb8* gene targeting approaches have generated complete loss-of-function mutants that do not

distinguish roles for the cytoplasmic domain from the extracellular ECM binding domain in regulating neurovascular development and physiology.

Here, Cre/lox strategies in mice have been used to selectively delete the Itgb8 cytoplasmic domain while keeping the transmembrane and extracellular domains functionally intact. Mutant mice develop brain vascular pathologies that are linked to impaired latent-TGF $\beta$  activation and signaling in vascular endothelial cells. We also detect unique cell signaling defects in the cytoplasmic domain-deficient mice that are distinct from complete loss-of-function (Itgb8<sup>-/-</sup>) mutants. Whole transcriptome sequencing reveals that integrin activation of TGF $\beta$  signaling in brain vascular endothelial cells leads to changes in angiogenic gene expression, including alterations in several genes with links to canonical Wnt signaling. Collectively, these data establish a key function for the  $\beta$ 8 integrin cytoplasmic tail in both extracellular adhesion and intracellular signaling in brain neurovascular units. These results also suggest that inhibition of the cytoplasmic domain may be an effective therapy for blocking ECM adhesion, and particularly activation of the TGF $\beta$  pathway.

## Results

Analysis of the murine  $\beta$ 8 integrin protein sequence revealed that amino acids 682-702 constitute the transmembrane domain, with the final 65 amino acids (702-767) comprising the cytoplasmic domain. Amino acids 673-727 are encoded by Itgb8 exon 13 and amino acids 728-767 are encoded by exon 14. Thus, by following a standard Cre/lox knockout approach, the deletion of exons 13 and 14 would ablate both the  $\beta$ 8 integrin transmembrane domain and the cytoplasmic domain. We assumed that perturbing the transmembrane domain would likely be problematic, since in other integrins this domain is involved in cell surface expression and heterodimerization with the  $\alpha$ v subunit (Yang et al., 2009). Alternatively, inserting loxP sites flanking exon 14 alone would not result in the removal of the entire cytoplasmic domain.

Therefore, we chose a strategy involving insertion of a premature STOP codon in exon 13 of the *Itgb8* gene to prevent expression of the downstream cytoplasmic sequences (Figure 1A). To avoid potential dominant negative effects of the knock-in, we also inserted a cDNA mini-gene comprised of exon 13/14 coding sequences and a polyA tail flanked by loxP sites upstream of exon 13 containing the engineered STOP codon. Cre-mediated deletion of the mini-gene enables transcriptional readthrough to the premature STOP in exon 13, leading to a truncated  $\beta 8$  integrin (ConKO/ $\beta 8\Delta$ cyto). Correct *Itgb8* targeting was confirmed by Southern blotting and PCR-based analysis of purified genomic DNA (Figure 1B, C). Deletion of the ConKI cassette was also confirmed using neurospheres cultured from control and ConKI/+ mice and infected with adenovirus-Cre (Figure 1D). We also bred a Nestin-Cre transgenic strain (Tronche et al., 1999) to ConKI/+ mice to selectively delete the  $\beta 8$  integrin cytoplasmic domain in neuroepithelial cells of the developing brain. We confirmed that Nestin-Cre is active in neuroepithelial cells of the embryonic brain as early as E11, as revealed with the Rosa26-loxSTOPlox-YFP reporter (Supplemental Figure 1). As shown in Figure 1D, genomic PCR analysis of neurospheres isolated from E14 control and Nestin-Cre;ConKO/+ embryonic brain validated Cre-mediated deletion of the mini-gene and resulting  $\beta 8$  integrin protein truncation. This result is similar to the deletion of the floxed mini-gene using cultured neurospheres infected with adenovirus-Cre (Figure 1E). A schematic summary of the  $\beta 8$  integrin transmembrane and cytoplasmic sequences encoded by exons 13 and 14 as well as the expression of truncated  $\beta 8$  integrin protein resulting from Cre-mediated removal of the mini-gene is highlighted in Figure 1F. Sanger sequencing was performed using cDNA prepared from Nestin-Cre control and Nestin-Cre;ConKO/+ E14 neurospheres, confirming Cre-mediated deletion of the floxed mini-gene and premature termination of the *Itgb8* cytoplasmic domain coding sequence (Supplemental Figure 2).

Next, we generated a rabbit polyclonal antibody directed against the extracellular domain of  $\beta 8$  integrin. This antibody as well as additional anti- $\alpha v$  and anti- $\beta 8$  antibodies directed against cytoplasmic sequences were utilized to test expression of  $\alpha v\beta 8$  integrin before and after Cre-mediated recombination (Figure 2A). Immunoblot analysis of whole brain lysates revealed a ~93 kDa truncated protein expressed by the ConKO allele as well as a ~100 kDa full-length protein expressed by the ConKI or wild type allele (Figure 2B). Truncated  $\beta 8$  integrin protein was not detected in samples that lacked the Nestin-Cre transgene. A second  $\beta 8$  integrin antibody developed against the cytoplasmic tail revealed expression of only the full-length  $\beta 8$  integrin protein (Figure 2B). Immunoblotting for  $\alpha v$  integrin protein did not reveal differences in expression after truncation of  $\beta 8$  integrin. We also analyzed  $\alpha v$  and  $\beta 8$  integrin expression in neurospheres isolated from E14 Nestin-Cre control and Nestin-Cre;ConKO/+ embryos. Immunoblot analysis revealed diminished levels of full-length  $\beta 8$  integrin protein in Nestin-Cre;ConKO/+ samples owing to mutation of one *Itgb8* allele (Supplemental Figure 3). We also cultured neurospheres from the brains of adult ConKI/+ mice and infected them with control adenovirus control or adenovirus-Cre to induce  $\beta 8$  integrin protein truncation. Diminished levels of full-length  $\beta 8$  integrin protein were detected following adenovirus-Cre infection (Supplemental Figure 3). Neuroepithelial cells give rise to astrocyte progenitors in the post-natal brain (Toutouchian and McCarty, 2017; Verkhatsky and Nedergaard, 2018); therefore, we next examined whether cultured astrocyte progenitors from control and Nestin-Cre;ConKO/+ mice could bind to different ECM substrates using in vitro adhesion assays. As shown in Figure 2C, in comparison to control cells, Nestin-Cre;ConKO/+ cells displayed defective adhesion to ECM proteins including collagen IV and fibronectin. Analysis of  $\beta 8^{-/-}$  cells revealed a similar deficiency in adhesion to ECM proteins (Supplemental Figure 3). Collectively, these data reveal that selective loss of the  $\beta 8$  integrin cytoplasmic domain leads to defects in cell-ECM adhesion.

Next, neurovascular development was analyzed *in vivo* using F1 progeny resulting from Nestin-Cre/+ males mated with ConKI/ConKI or ConKI/+ females. Nestin-Cre;ConKO/+ mutant mice were born in expected Mendelian ratios and did not display obvious developmental abnormalities (26 mutants of 115 total mice). Like controls, Nestin-Cre;ConKO/+ mutants did not develop obvious brain vascular pathologies such as intracerebral hemorrhage or hydrocephalus (Figure 3A, B) that was grossly evident in  $\beta 8^{-/-}$  mutant embryos (Figure 3C). The lack of severe brain vascular pathologies was not due to poor Nestin-Cre transgene expression or enzymatic activities since conditional deletion of the  $\beta 8^{\text{flox/flox}}$  allele, and thus leading to CNS-specific *Itgb8* loss-of-function, resulted in grossly obvious intracerebral hemorrhage (Supplemental Figure 4). Microscopic analyses of E14 brains, and particularly midbrain regions including the ganglionic eminences in Nestin-Cre;ConKO/+ mutants revealed defective blood vessel patterning (Figure 3D-F). Control brain sections showed close juxtaposition between neuroepithelial cells and radially migrating angiogenic blood vessels (Figure 3D). In contrast, cerebral blood vessels in Nestin-Cre;ConKO/+ mutants did not closely juxtapose the neuroepithelium as revealed by double immunofluorescence imaging with anti-Nestin and anti-CD31 antibodies (Figure 3G). In addition, brain endothelial cells in the Nestin-Cre;ConKO/+ mutants did not sprout radially along neuroepithelial cell processes and displayed abnormal morphologies as determined by several quantitative parameters (Figure 3H-K). The *in vivo* adhesion and sprouting abnormalities support the *in vitro* defects in cell-ECM binding (Figure 2C). Lastly, while total numbers of microglia were not significant in control versus Nestin-Cre;ConKO/+ mutant mice, there were increased numbers of perivascular microglial cells in mutants as revealed by double labeling with anti-Iba1 and anti-CD31 antibodies (Figure 3L-O). In particular, significantly higher numbers of Iba1<sup>+</sup> microglial cells were detected near distended blood vessels in ganglionic eminences of the midbrain (Figure 3P). Analysis of E18 embryos revealed lack of macroscopic intracerebral hemorrhage in Nestin-Cre;ConKO/+ mice as

compared to controls, whereas hemorrhage was obvious in  $\beta 8^{-/-}$  embryos (Supplemental Figure 4). However, in E18 Nestin-Cre;ConKO/+ mutants there were continued defects in the juxtaposition between vascular endothelial cells and neuroepithelial cells (Supplemental Figure 5). Cerebral blood vessels in E18 Nestin-Cre;ConKO/+ mutant mice were associated with increased numbers of microglia as revealed by labeling with anti-Iba1 antibodies (Supplemental Figure 5). Similar to controls, vascular endothelial cells in Nestin-Cre;ConKO/+ and  $\beta 8^{-/-}$  mutant brains were associated with pericytes as revealed by double immunofluorescence with anti-CD31 and anti-NG2 antibodies (Supplemental Figure 5).

Integrin-dependent TGF $\beta$  activation and signaling via Smad transcription factors was next analyzed in control and mutant E14 brains. As shown in Figure 4A-G, reduced levels of Smad3 phosphorylation were detected in blood vessels within Nestin-Cre;ConKO/+ mutant brains as revealed by immunofluorescence staining of fixed sections with anti-CD31 and a phospho-specific Smad3 antibody. There was also significantly less phosphorylated Smad3 in brain endothelial cells of  $\beta 8^{-/-}$  embryonic brain sections (Figure 4H-N). We also detected reduced levels of phospho-Smad3 in vascular endothelial cells in Nestin-Cre; $\beta 8^{\text{flox/flox}}$  conditional knockout brains (Supplemental Figure 6). Collectively, these data reveal that the vascular pathologies in the Nestin-Cre;ConKO/+ mutant brains are most likely related to defects in TGF $\beta$  signaling in vascular endothelium.

$\beta 8$  integrin-dependent activation of latent-TGF $\beta$ s was also quantified using in vitro cell-based assays. Purified LAP-TGF $\beta 1$  was added to wild-type or Nestin-Cre;ConKO/+ astrocyte progenitors and conditioned media was subsequently transferred to HEK-293T reporter cells expressing a Smad binding element inducible secreted alkaline phosphatase reporter construct (SBE-iSEAP) (Figure 5A). As shown in Figure 5B, conditioned medium from Nestin-Cre control cells that had been pre-treated with exogenous LAP-TGF $\beta 1$  induced robust SBE-iSEAP activities in a time-dependent manner. Conversely, conditioned medium from Nestin-



Cre;ConKO/+ astrocyte progenitors treated with exogenous LAP-TGF $\beta$ 1 induced lower levels of SBE-iSEAP activities (Figure 5B). Similarly, reduced latent-TGF $\beta$ 1 activation and signaling was detected in  $\beta$ 8<sup>-/-</sup> cells using the SBE-iSEAP reporter assay (Figure 5C).

To identify signaling pathways that are linked to the  $\beta$ 8 integrin cytoplasmic tail we utilized the antibody-based reverse-phase protein array (RPPA) platform (Nishizuka and Mills, 2016). Astrocyte progenitor cultures were prepared from P0 wild type mice (adhesion, transmembrane and signaling domains intact),  $\beta$ 8<sup>-/-</sup> mice (adhesion, transmembrane, and signaling domains deleted), and Nestin-Cre;ConKO mice (signaling domain deleted) and detergent-soluble lysates were tested using RPPA (Figure 6A). These cultures are mostly comprised of Nestin<sup>+</sup>/GFAP<sup>+</sup> astrocyte progenitors, but also contain some vascular endothelial cells and microglia (Tchaicha et al., 2010). Several signaling proteins involved in growth, polarity, and/or migration were differentially expressed or phosphorylated in cultured astrocyte progenitors lacking the  $\beta$ 8 integrin cytoplasmic domain (Figure 6B). Interestingly, the levels of these proteins were inversely correlative in  $\beta$ 8<sup>-/-</sup> samples versus Nestin-Cre;ConKO/+ samples (Figure 6C). For example, in comparison to controls the ECM protein Collagen VI showed elevated expression in Nestin-Cre;ConKO/+ lysates and reduced expression in  $\beta$ 8<sup>-/-</sup> lysates. These data suggest that complete loss of adhesion and signaling domains ( $\beta$ 8<sup>-/-</sup>) versus selective loss of only the signaling domain (Nestin-Cre;ConKO/+) have differential impacts on protein expression and/or stability. Next, we tested expression of Collagen VI and the GTP exchange factor Vav1 identified by RPPA by immunoblotting detergent-soluble lysates from control and mutant cells. As shown in Figure 6D, in Nestin-Cre;ConKO/+ astrocyte progenitor lysates we detected elevated expression of Collagen VI as well as reduced expression of Vav1. Quantitation of these immunoblot data show integrin-dependent changes in expression that are consistent with the RPPA results (Figure 6E). Lastly, in comparison to Nestin-Cre control littermates immunohistochemical staining of P0 brain sections revealed increased expression of

Collagen VI protein throughout the cerebral cortex of Nestin-Cre;ConKO/+ mutants

(Supplemental Figure 7).

While Nestin-Cre;ConKO/+ mutant mice displayed microscopic angiogenesis defects during embryonic and neonatal development, all mutant animals analyzed thus far (n=11) have survived to adulthood (>1 year) without the onset of age-related neurological defects. Nestin-Cre;ConKO/+ adult mice showed reduced body weights in comparison to age-matched controls (Figure 7A); however, there were no neurodegenerative phenotypes or obvious motor deficits as assessed with a rotarod (Figure 7B). The normal post-natal survival as well as the lack of obvious neurological deficits in Nestin-Cre;ConKO/+ mice are unlike the diminished survival of  $\beta 8^{-/-}$  or Nestin-Cre; $\beta 8^{\text{flox/flox}}$  mice, which die by 6 months due to spinocerebellar neurodegeneration (McCarty et al., 2005b; Mobley and McCarty, 2011; Mobley et al., 2009). Microscopic analysis of brain sections from adult Nestin-Cre;ConKO/+ mutant mice did not reveal persistent defects in blood vessel morphologies, and differences in astrocytes and microglia were not apparent (Figure 7C-H). In contrast, adult  $\beta 8^{-/-}$  and Nestin-Cre; $\beta 8^{\text{flox/flox}}$  mutant brains (P60) showed increased numbers of astrocytes and microglial cells (Supplemental Figure 8). Interestingly, analysis of brains from neonatal (P10) Nestin-Cre;ConKO/+ mutants did not reveal microscopic hemorrhage, although we detected increased numbers of astrocytes in the cortex and striatum as revealed by immunohistochemistry for GFAP (Supplemental Figure 9). A prior report has shown that intracerebral hemorrhage in  $\beta 8^{-/-}$  and Nestin-Cre; $\beta 8^{\text{flox/flox}}$  mice leads to microglial activation and subsequent motor dysfunction (Arnold et al., 2019). However, we did not detect obvious differences in Iba1<sup>+</sup> microglial cell numbers in Nestin-Cre;ConKO/+ at P10, although  $\beta 8^{-/-}$  mice of a similar age displayed microhemorrhage and increased numbers of perivascular glial cells (Supplemental Figure 9).

Collectively, our results reveal that during development the  $\beta 8$  integrin cytoplasmic domain promotes activation of ECM-bound latent-TGF $\beta$ s and subsequent TGF $\beta$  receptor

signaling in endothelial cells (Figures 2-5). These data are consistent with previous reports showing that deletion of the *Tgfr2* gene, which encodes the type 2 TGF $\beta$  receptor, in vascular endothelial cells using a tamoxifen-inducible endothelial cell-specific Cre transgenic model, *Cdh5-CreERT2*, leads to similar angiogenesis pathologies (Allinson et al., 2012). We also detect abnormal angiogenesis and microscopic hemorrhage when the *Tgfr2* gene is acutely ablated in vascular endothelial cells using the PDGFB-CreERT2 model (Figure 8A-F). The *Tgfr2*-regulated genes and pathways in the brain vascular endothelium that control developmental angiogenesis remain largely unknown; therefore, we performed quantitative RNA sequencing (RNAseq) to identify differentially expressed genes in *Tgfr2*<sup>-/-</sup> brain endothelial cells. To facilitate sorting of endothelial cells we also intercrossed *Tgfr2*<sup>flox/flox</sup> mice with Cre-inducible ROSA26-loxSTOPlox-EYFP (R26-EYFP) reporter mice (Mao et al., 2001). Neonatal mice were injected with tamoxifen once per day for three consecutive days, and vascular endothelial cells were isolated from freshly dissected P7 brains of based on YFP and CD31 expression (Figure 8G). As shown in Figure 8H, I and Supplemental Figure 10, several genes showed differential expression in *Tgfr2*<sup>+/-</sup> control versus *Tgfr2*<sup>-/-</sup> cells. Five genes (*Mfsd2a*, *ApoD*, *Htra3*, *Spock2* and *Wfdc1*) identified to be down-regulated in *Tgfr2*<sup>-/-</sup> brain endothelial cells were independently validated by qRT-PCR (Figure 8J). Analysis of the brainrnaseq.org database confirmed enriched expression of these five genes in brain vascular endothelial cells isolated from P7 mice (Supplemental Figure 11). Stimulation of the bEnd.3 brain endothelial cell line with TGF $\beta$ 1 led to significantly increased expression of four of the five genes analyzed (Figure 8K). We have previously shown TGF $\beta$ 1-dependent regulation of *Mfsd2a* expression in brain endothelial cells (Tiway et al., 2018). Interestingly, other reports have shown that expression of *ApoD*, *Htra3*, *Spock2* and *Wfdc1* (Jensen et al., 2019) as well as *Mfsd2a* (Wang et al., 2020) is controlled by canonical Wnt signaling. Therefore, we treated brain endothelial cells with Wnt7a and analyzed expression of these five genes. As shown in Figure 8L, we confirmed Wnt-

dependent increase in expression of four of the five genes, with levels of Htra3 not impacted by Wnt7a treatment.

Collectively, these data reveal that the  $\beta 8$  integrin cytoplasmic domain is necessary for neuroepithelial cell adhesion to ECM protein ligands, and particularly latent-TGF $\beta$ s, in the brain microenvironment. Our results support a model in which engagement of the  $\beta 8$  integrin cytoplasmic sequences with the cytoskeleton and/or intracellular effector proteins is essential for the propagation of the extended-closed conformation and possibly the generation of novel ECM adhesion-competent conformations in  $\alpha v\beta 8$  integrin (Figure 9). These results also indicate that functionally blocking the  $\beta 8$  integrin cytoplasmic domain may be an effective strategy for perturbing ECM adhesion, including inhibition of the TGF $\beta$  pathway.

## Discussion

Most integrin  $\beta$  subunits, including  $\beta 1A$ ,  $\beta 2$ ,  $\beta 3A$ ,  $\beta 5$ , and  $\beta 6$ , utilize NPXY motifs in their cytoplasmic domains to recruit talins and/or kindlins, which induce dramatic inside-out structural changes in the extracellular region that convert the inactive conformation to ECM binding-competent conformations (Ginsberg, 2014). In contrast, the  $\beta 8$  integrin cytoplasmic tail lacks NPXY motifs and its isolated extracellular region is detected mainly in an extended-closed conformation that can bind effectively to ECM ligands (Dong et al., 2014; Wang et al., 2017). It will be important to further elucidate the cytoplasmic domain-dependent mechanisms that propagate the extended-closed state or convert it to a more active conformation, including the relative contributions of intracellular proteins that interact with the  $\beta 8$  integrin cytoplasmic tail. For example, the F-actin binding protein Band 4.1B (McCarty et al., 2005a) could control cytoskeleton-dependent alterations in  $\alpha v\beta 8$  integrin ECM adhesion. Alternatively, PTP-PEST could dynamically promote extracellular adhesion via reversible control of integrin phosphorylation (Lee et al., 2015) or RhoGDI1-mediated Rac1/Cdc42 activation (Reyes et al.,

2013). The  $\beta 8$  integrin cytoplasmic tail might also mediate interactions with endothelial cell-expressed Neuropilin 1 (Nrp1), which is linked to TGF $\beta$  receptor signaling and is a counter-receptor for  $\alpha v\beta 8$  integrin (Hirota et al., 2015). Altered interactions between  $\alpha v\beta 8$  integrin and Nrp1 might result in impaired neuroepithelial-endothelial cell adhesion leading to aberrant activation of latent TGF $\beta$  and/or TGF $\beta$  receptor signaling in endothelial cells. Nrp1 is also expressed at lower levels in the developing brain neuroepithelium, and the cytoplasmic domain of Nrp1 binds to PDZ domain-containing proteins (Lanahan et al., 2013). Interestingly, Spinophilin, which binds to the  $\beta 8$  integrin cytoplasmic tail (Cheerathodi et al., 2016), contains a PDZ domain that may mediate associations between Nrp1 and  $\alpha v\beta 8$  integrin.

The RPPA experiments using lysates from astrocyte progenitor cultures reveal differential expression of Collagen VI, Vav1 and other factors, indicating that  $\alpha v\beta 8$  integrin controls the expression and/or stability of these proteins. In the adult mouse brain, however, Vav1 and Col6a1 mRNAs are enriched in microglia and fibroblasts, respectively (Vanlandewijck et al., 2018). It is possible that these cells are admixed in the astrocyte cultures, with  $\alpha v\beta 8$  integrin promoting intercellular adhesion and communication between astrocytes and other stromal cells. Indeed, perivascular microglia play important roles in regulating brain angiogenesis (Paredes et al., 2018). Alterations in  $\alpha v\beta 8$  integrin functions might affect the morphology and activation status of nearby microglia, possibly involving leucine rich repeat containing protein 33 (LRRC3). LRRC33, an extracellular protein secreted into the microenvironment by microglia is essential for latent-TGF $\beta$  activation possibly through cooperative binding with  $\alpha v\beta 8$  integrin in perivascular astrocytes (Qin et al., 2018).

The quantitative RNA sequencing results and cell signaling data reveal novel cross-talk mechanisms between TGF $\beta$ s and Wnts in vascular endothelial cells. Prior reports have shown that  $\beta$ -catenin plays a crucial role in vascular development, particularly in the brain (Liebner et al., 2008). Selective ablation of Wnt7a/Wnt7b genes in neuroepithelial cells, or Ctnnb1/ $\beta$ -catenin

in endothelial cells (Daneman et al., 2009; Stenman et al., 2008), leads to brain angiogenesis pathologies that are similar to those that develop in *Itgav*, *Itgb8* or *Tgfb2* mutant mice (McCarty, 2009). *Norrin*, which has structural homology to TGF $\beta$ s but signals via Wnt receptors, regulates developmental angiogenesis in the brain and retina (Wang et al., 2018). Inhibition of Wnt/ $\beta$ -catenin signaling in brain endothelial cells during development revealed significant down-regulation of several ECM genes with links to the TGF $\beta$  pathway (Jensen et al., 2019). A more recent report has shown cross-talk between TGF $\beta$ s and Wnts in vascular endothelial cells during retinal angiogenesis and formation of the blood-retina barrier (Zarkada et al., 2021). The proteins GPR124 and Reck are also linked to Wnt signaling and it will be important to determine if this complex is linked to  $\alpha$ v $\beta$ 8 integrin adhesion and signaling pathways (Vallon et al., 2018). In cancer, cross-talk between TGF $\beta$ /Smad signaling and Wnt/ $\beta$ -catenin signaling occurs at multiple levels to regulate gene expression (Attisano and Labbe, 2004). The Smad3 C-terminal region interacts with  $\beta$ -catenin to form a Smad4/Smad3/ $\beta$ -catenin complex following engagement of TGF $\beta$ 1 with the TGF $\beta$  receptor complex (Tian and Phillips, 2002; Zhang et al., 2010). Our data identify specific genes that are regulated via cross-talk between TGF $\beta$ s and Wnts during neurovascular development. It will be interesting to further dissect these interactions in development and also determine if these events are altered in vascular-related neurological disorders.

## **Materials and Methods**

### **Experimental mice**

All animal protocols were reviewed and approved prior to use by the Institutional Animal Care and Use Committee (IACUC) and The UT MD Anderson Subcommittee on Animal Studies, both AAALAC accredited institutions. Details for generating Nestin-Cre; $\beta$ 8<sup>flox/flox</sup> conditional knockout and  $\beta$ 8<sup>-/-</sup> whole body knockout have been reported previously (Mobley et al., 2009; Proctor et al.,

2005). A conditional knock-in model was generated by insertion of a premature STOP codon within one of the exons coding for cytoplasmic domain of  $\beta 8$  integrin and a floxed cDNA (mini-gene) upstream. Such conditional knock-in females (ConKi/+) were bred with Nestin-Cre/+ males to achieve Cre/lox removal of two coding exon sequences along with an intervening intron coding for  $\beta 8$  integrin cytoplasmic tail, thus generating conditional knock-out mice (Nestin-Cre/+; $\beta 8$ -ConKi/+) that harbored a truncated form of  $\beta 8$  integrin in neuroepithelial cells. The Nestin-Cre transgenic mice (Tronche et al., 1999) were purchased from Jackson Laboratories (stock 003771). ROSA26-loxSTOPlox-EYFP mice were also purchased from Jackson Laboratories (stock 006148). The PDGFB-CreERT2 (Claxton et al., 2008) and Tgfb $\beta$ 2flox/flox (Nguyen et al., 2011) models have been described previously. Mouse genotypes were confirmed by genomic DNA isolation from ear snips and standard PCR protocols. Embryo staging involved timed mating, with the morning of the plug date defined as E0. For mouse behavioral analyses, a Rotarod device (Ugo Basile) was used to measure motor coordination and strength. The times at which mice fell from the rod were recorded. Mice were allowed to stay on the rod for a maximum of 360 seconds (6 minutes). Six separate trials were performed over a 3-day period (three trials per day).

### **Tissue sectioning, immunofluorescence and immunohistochemistry**

Embryonic and neonatal mouse brains were harvested and fixed in cold 4% PFA (15714, Electron Microscopy Sciences) in 1X PBS (21-040-CV, Corning and SH30256.01, Hyclone) for 15 hrs. at 4°C. Fixed brains were washed with 1X PBS and embedded in 4.5% low melting point agarose (16520, Invitrogen, Life Technologies) for embryonic brains or 3% agarose (GR140, Denville Scientific Inc.) for post-natal brains. Embedded brains were sectioned under ice-cold conditions using a vibratome (VT 1000S, Leica) to collect 100  $\mu$  m sagittal or coronal brain slices. Tissue sections were blocked using 0.25% Triton X-100 (Fisher BioReagents) in 1X PBS,

containing 10% species-specific serum (Jackson ImmunoResearch Laboratories, Inc.) matching the secondary antibody host. Commercially available primary and secondary antibodies (Supplemental Table 1) were used for immunostaining. Brain tissue sections were mounted using Vectashield antifade mounting medium (H-1200, Vector laboratories Inc.). For all immunohistochemistry, heat induced antigen retrieval was performed using 1X sodium citrate buffer, pH 6.0 (C9999, Millipore Sigma) or 1 mM EDTA, pH 8.0 (unless otherwise indicated as per manufacturer's instructions). Permeabilization of brain tissue sections (formalin-fixed paraffin embedded), was performed using 0.1% Triton X-100 in 1X PBS, followed by blocking with 10% species-specific serum (Jackson ImmunoResearch Laboratories, Inc.) matching the secondary antibody host (Jackson ImmunoResearch Laboratories, Inc.) for both IHC-P and -F, and developed using ImmPACT DAB substrate, peroxidase (HRP, SK-4150, Vector Laboratories) for IHC-P. Primary and secondary antibodies used are included in Suppl. Table 1. Bright field microscopy was performed using Carl Zeiss Axio Imager.Z1 and Olympus BX43 microscopes.

### **Image acquisition, analysis and quantitation**

Immunofluorescence images were acquired using Olympus FLUOVIEW FV3000 confocal laser scanning microscope. Multi-dimensional acquisition was carried out using Z-stacks with 2.5  $\mu\text{m}$  slicing intervals at a scan rate of 4us/pixel with a resolution of at least 1024 x 1024 pixels per slice and digitally compiled in FV31S-SW (ver. 2.4.1.198). Image acquisition parameters including laser power, exposure time, voltages and gain were assigned uniformly across experimental and control samples for each imaging channel. All images were analyzed using Fiji and Image J software packages (Schindelin et al., 2012; Schneider et al., 2012) and serial z-stack images (three 2.5  $\mu\text{m}$  slices per image) were used for all quantification. Multiple areas of different brain regions were imaged across experimental and control mice groups (n=3). Fluorescence intensity of pSmad3<sup>+</sup> nuclei and CD31<sup>+</sup> blood vessel signals were measured from



corresponding channels using standard “analyze color histogram” module. Blood vessel adjacent regions were analyzed for quantitating Nestin<sup>+</sup> neuroepithelial cell contacts with CD31<sup>+</sup> blood vessels, and pSmad3<sup>+</sup>/CD31<sup>+</sup> co-localization. Digitally magnified microscopic images were used to manually draw around blood vessels using “freehand tool” and fluorescence signal co-localization was quantitated using “co-localization” algorithm in Fiji. Mander’s correlation coefficients of signal co-localization were measured with or without background-corrected threshold values. A similar approach was adopted to quantify pSmad3<sup>+</sup> nuclei by using digitally magnified microscopic images and manually drawing around CD31<sup>+</sup> vascular areas. Similar threshold parameters were used for all images from both control and mutant brains and pSmad3<sup>+</sup> nuclei were counted using “analyze particles” algorithm in Fiji (Kumari and Rana, 2015). Numbers of pSmad3<sup>+</sup> nuclei were normalized to the total CD31<sup>+</sup> vascular area.

All images were scaled ( $\mu\text{m}$ ) as per the objective lens used for acquisition to measure cellular parameters. Acquired image stacks were projected for maximum intensity to include all signals, but auto-threshold module was then used to include only cellular signals for quantitation and exclude non-specific background or noise. CD31<sup>+</sup> blood vessel parameters (counts, area coverage, mean fluorescence signal intensity) were quantified using “vessel analysis” module in Fiji (Simms et al., 2017). Numbers of perivascular microglial cells quantitated by measuring Iba1<sup>+</sup> cell parameters in both low magnified microscopic images to include larger brain regions and digitally magnified images to include areas adjacent to CD31<sup>+</sup> blood vessels. Cell counts, area coverage, mean fluorescence signal intensity, major and minor axes for shape descriptors, and cell circularity were analyzed using “analyze particles” algorithm (Kumari and Rana, 2015). Iba1<sup>+</sup> cells were counted above the set threshold of  $50 \mu\text{m}^2$  to avoid any non-specific background or noise. Integrated density parameter measures total cellular fluorescence intensity per cell area. A cell with higher area coverage and/or mean signal intensity gives increased integrated density values. Maximum and minimum distances between two points on

the cell periphery are defined by the major and minor axes (McWhorter et al., 2013). A completely circular cell will have identical major and minor axes values whereas the difference between the major and minor axes will change in accordance with cell shape alterations. For example, the difference between the major and minor axes will be larger when a cell becomes less circular and attains more ameboid or elongated morphology. Shape factor determines such differences between major and minor cellular axes to validate dynamic transitions in cell morphology.

### **Mouse astrocyte progenitors and brain endothelial cell line cultures**

Neonatal brains (P1-P3) were harvested and cerebral cortices were carefully collected after removal of meninges and other structures including the hippocampus. Cortical sheets were minced and washed in sterile 1 g/L (low glucose) Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and sodium pyruvate (10-014-CV, Corning) by centrifugation at 1,000 rpm for 5 mins at RT. Tissue pellets were re-suspended and gently agitated in fresh and sterile DMEM containing 150 U/mL of collagenase (Type I, LS004196, Worthington Biochemical Corp.) and 40 µg/mL of DNase I (D7291, Sigma-Aldrich), for 30 mins at 37°C. After centrifugation at 1,000 rpm for 5 mins. at RT, the pellet was re-suspended in sterile astrocytic growth medium [1 g/L DMEM containing 10% bovine calf serum (SH30072.03, Hyclone) and 1 U/mL Penicillin-Streptomycin (MT30004CI, Corning)], and triturated. The suspension was filtered through 40 µm nylon cell strainer (Falcon) and 10 mL of suspension was added to T-75 tissue culture flasks (Corning) pre-coated with laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (L2020, Sigma), used at 1:300 dilution in sterile 1X PBS. Primary astrocyte progenitor cultures were maintained (37°C, 5% CO<sub>2</sub>) by replacing with fresh and sterile astrocytic growth medium every 3-4 days until they were confluent. Mouse brain endothelial cells (bEnd.3; ATCC) were maintained in Dulbecco's modified Eagle's medium (30-2002, ATCC) containing 10% fetal

bovine serum (F0926, Sigma) and 1% antibiotic-antimycotic solution (30-004-CI, Corning). Cells were serum starved overnight at confluence and treated with either 1 ng/mL recombinant human TGF $\beta$ 1 protein (7754-BH, R&D Systems) for 12 hours or 50 ng/mL human Wnt7a protein (GTX53868-pro, GeneTex) for 24 hours.

### **TGF $\beta$ -Smad reporter assays**

HEK293T cell line stably transfected with human TGF $\beta$ RI, Smad3 and Smad4 genes, was purchased from InvivoGen (Cat. code hkb-tgfb). This HEK-Blue<sup>TM</sup> TGF- $\beta$  SEAP reporter cell line detects bioactive TGF- $\beta$  through activation of downstream TGF- $\beta$ -Smad signaling pathway and production of detectable secreted alkaline phosphatase protein (SEAP). Cells were grown and passaged (according to manufacturer's instructions) in sterile 4.5 g/L (high glucose) DMEM (10-017-CV, Corning) containing 10% heat-inactivated fetal bovine serum (F4135, Sigma-Aldrich), 1 U/mL Penicillin-Streptomycin and 100  $\mu$ g/ml Normocin<sup>TM</sup> (InvivoGen), and maintained through selection (according to manufacturer's instructions) by the same growth medium but containing 30  $\mu$ g/ml Blastocidin<sup>TM</sup> (ant-bl, InvivoGen), 200  $\mu$ g/ml Hygromycin B gold<sup>TM</sup> (ant-hg, InvivoGen) and 100  $\mu$ g/ml Zeocin<sup>TM</sup> (ant-zn, InvivoGen). Cells were passaged (Trypsin-free) every 3-4 days or when they were ~75% confluent. Primary astrocyte progenitors cultured from neonatal mice brains were used to measure their ability to activate latent TGF- $\beta$  1, thus generating bioactive TGF- $\beta$ . Confluent astrocytic cultures were passaged by 0.25% Trypsin, 0.1% EDTA in HBSS [without calcium, magnesium and sodium bicarbonate] (MT 25053CI, Corning) and cells collected by centrifugation (1000 rpm, 5 mins. at RT) were re-suspended in fresh sterile astrocyte growth medium (heat-inactivated 10% BCS used for assay) and counted (Vi-cell Analyzer, Beckman Coulter).  $1 \times 10^5$  viable astrocytes were seeded per well of 12-well tissue culture plate (Corning), pre-coated with laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (L2020, Sigma) used at 1:300 dilution in sterile 1X PBS,

and containing 1 mL of growth medium per well. Cells were allowed to grow (37°C, 5% CO<sub>2</sub>) for 3-4 days. Growth medium was replaced with 1 g/L DMEM containing 10% BCS (heat-inactivated), 1 U/mL Penicillin-Streptomycin, and conditioned with 1 ng/mL latent-TGF- $\beta$  1 (rh-Latent-TGF- $\beta$  1, 299-LT/CF, R7D Systems), 1 ng/mL active TGF- $\beta$  (7754-BH-005/CF, R&D Systems), and no TGF- $\beta$  (control). All TGF- $\beta$  treatments were done in triplicates and cells were conditioned for 15 hrs. at 37°C and 5% CO<sub>2</sub>. Concurrently, antibiotics-selected Hek-Blue TGF- $\beta$  cells were passaged at 75% confluence in T-75 tissue culture flasks using methods described above. Passaged cells were grown for 15 hrs. (37°C, 5% CO<sub>2</sub>) on 48-well tissue culture plate (Corning) with 2x10<sup>5</sup> viable cells/mL/well. After 15 hrs. of incubation, growth medium was replaced by conditioned medium from astrocytic cultures (described above) and HEK-blue-TGF- $\beta$  cells were further incubated at 37°C, 5% CO<sub>2</sub>. 20  $\mu$  L of cell supernatant was collected from each well independently at 24, 48 and 72 hrs. and added to one well of a 96-well plate. 180  $\mu$ L of Quanti-blue™ assay reagent (rep-qbs, InvivoGen) was added per well containing cell supernatant and incubated at 37°C for 15-60 mins. Finally, O.D. (absorbance) was measured at 655 nm (Synergy HTX multi-mode reader, BioTek).

### **Adenovirus infection of cells**

Control or ConKI/+ neurosphere were grown in serum-free media and infected either with Ad5 adenovirus expressing Cre (UIOWA cat#Ad5CMVCre) or control adenovirus (UIOWA cat#Ad5CMVempty). After initial titering of virus, an MOI of 100 was used to infect cells for 48 hours for genomic PCR analysis or 4-7 days for immunoblot analysis.

## **ECM adhesion assays**

Primary cell cultures from  $\beta 8^{-/-}$ , *Nestin-Cre/+*;  $\beta 8$ -*ConKi/+* and paired wild type (control) P0/P1 mice brains were passaged once they were confluent in T75 tissue culture flasks [pre-coated with laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (L2020, Sigma) used at 1:300 dilution in sterile 1X PBS] containing astrocyte growth media. Standard cell culture technique involving 0.25% Trypsin, 0.1% EDTA in HBSS [without calcium, magnesium and sodium bicarbonate] (MT 25053CI, Corning) was used to detach cells followed by trypsin neutralization (#0113, ScienCell Research Laboratories), centrifugation (1000 rpm, 5 mins., RT) and re-suspension of cell pellets in 2 mM EDTA (Invitrogen™ 15575020, Fisher Scientific) containing 1X PBS (RT). Re-suspended cells were washed again by centrifugation (1000 rpm, 5 mins., RT) and re-suspended in 4 mL of assay buffer (ECM540, Millipore Sigma). Viable cell count was obtained (Vi-cell Analyzer, Beckman Coulter) and  $2 \times 10^4$  viable cells were added per well of the 96-well strip (ECM540, Millipore Sigma). The assay was carried out using the ECM cell adhesion array kit (ECM540, Millipore Sigma) following manufacturer's instructions. Final absorbance readings were taken at 650 nm (Synergy HTX multi-mode reader, BioTek).

## **Immunoblotting and immunoprecipitation**

Confluent astrocyte progenitor cultures were lysed using radioimmunoprecipitation assay buffer (RIPA: 50mM Tris-HCl pH 8.0, 150 mM NaCl, 1% sodium deoxycholate, 1% triton X-100, 0.1% SDS, 1% NP-40 and 1 mM EDTA) containing protease and phosphatase inhibitors (A32955, A32957, Thermo Scientific) to obtain soluble protein fractions. Total protein concentration in lysates was determined using bicinchoninic acid assay (Pierce™ BCA, 2327, Thermo Scientific). For western blot analysis, SDS-PAGE was performed with 7.5%, 10% or 12% polyacrylamide gels, under reducing or non-reducing conditions, transferred to nitrocellulose

membranes (Bio-Rad) and blocked using Intercept<sup>®</sup> TBS blocking buffer (927-60001, LI-COR) or 3% milk in 1X PBS containing 0.2% Tween-20. For immunoprecipitation, cell surface proteins of confluent astrocyte cultures were biotinylated (EZ-link<sup>™</sup> Sulfo-NHS-Biotin, 21217, Thermo Scientific) followed by quenching with 1X TBS (Tris-buffered saline, T60075-4000, RPI). Cells were lysed using the same protocol described above to obtain total soluble protein lysates and protein concentration was determined using BCA method. 100-200  $\mu$ g of protein lysate was incubated overnight with 2.5-5  $\mu$ g of primary antibody (Suppl. Table 1) at 4°C. 50-100  $\mu$ L of Protein A agarose (11134515001, Roche), washed with cold 1X PBS by centrifugation and finally re-suspended in RIPA buffer] was added per tube containing protein- primary antibody conjugates and incubated at 4°C for 1 hr. The primary antibody-agarose conjugate was immunoprecipitated by centrifugation (15,000 rpm, 10 mins., 4°C) and washed with RIPA buffer containing protease and phosphatase inhibitors. Finally, the pellet was re-suspended in non-reducing sample buffer and western blot analysis was performed using the same protocol described above. All primary and secondary antibodies used are included in (Supplemental Table 1). Immunoblots were scanned using Odyssey CLx infrared imaging system with Image Studio (LI-COR).

### **Reverse-phase protein arrays**

Adherent cells were washed twice with ice-cold PBS, lysed in RIPA or RPPA lysis buffer containing 1% Triton X-100, 50 mM Hepes pH 7.4, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyruvate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10% glycerol, and a cocktail of protease and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany) for 20–30 min with frequent mixing on ice, centrifuged at 14,000 RPM at 4 °C for 15 min to isolate the detergent-soluble protein supernatant. The protein concentration was determined using the BCA assay (or Bradford) and the optimal protein concentration of lysates for RPPA was 1.2 mg/ml. Lysates

were denatured in 4X sample buffer (35% glycerol, 8% SDS, 0.25 M Tris-HCl, pH 6.8; without  $\beta$ -mercaptoethanol) for 5 min at 95°C. Lysates were stored at -80°C and subsequently analyzed at RPPA core facility at MD Anderson Cancer Center. Samples were serially diluted and probed with 466 antibodies and arrayed on nitrocellulose-coated slides. Relative protein levels were normalized for loading and compared to the standard curve. Normalized data points were transformed to a linear value used for analysis.

### **Isolation of primary mouse brain endothelial cells for transcriptomic profiling**

PDGFBB-CreERT2f/+;Tgfr2f/+;R26-YFP/+ control or PDGFBB-CreERT2;Tgfr2f/f;R26-YFP/+ mutant were intragastrically injected with 50 mg tamoxifen (50 ml, 1mg/ml) from P1 to P3. At P7 mice were euthanized by CO<sub>2</sub> in accordance with IACUC regulations. Mouse brains were dissected out under sterile conditions and all subsequent work was done within a tissue culture hood. The brains were manually dissociated and CD31 positive selected by use of FACS sorting of CD31-APC and YFP positive cells. Control (n=3) and mutant (n=5) cell samples were subsequently transcriptionally profiled RNAseq analysis. After RNA quality was validated, eight samples of a RIN of  $\geq 7$  were loaded onto HiSeq400 instrument. An average of ~40 million paired-end reads were generated for each of the eight samples. Sequenced reads fastq files were mapped to mouse reference genome GRCm3 (mm10).using Tophat v2.0.13 (Kim et al., 2013). Raw reads count were calculated using HTseq 0.12.4 (Anders et al., 2015). Differentially expressed genes between control and Tgfr2 mutant cells were obtained with edgeR package (Robinson et al., 2010). Differentially expressed genes are those having logFC > 2 and FDR < 0.01.

## **Cultured brain endothelial cell RNA extraction and quantitative real time PCR**

Total RNA was extracted from both treated and untreated bEnd.3 cells using the RNeasy mini kit (74106, Qiagen) and quantified using NanoDrop 2000c (Thermo Scientific) spectrophotometer. cDNA was synthesized using ready-to-go you-prime first-strand beads (27926401, Cytiva) and random hexamers (N8080127, Invitrogen). Quantitative real time PCR was performed using iTaq™ Universal SYBR® Green Supermix (1725121, Bio-Rad) on a 7500 Fast Real-Time PCR system (Applied Biosystems). The qRT-PCR primers are listed in Supplemental Table 2. The relative gene expression was quantified using the  $\Delta\Delta Ct$  method and fold changes of targeted genes were normalized by Gapdh mRNA. Expression level of targeted genes in the untreated bEnd.3 cells and/or wild type mouse brain endothelial cells was taken as 1 and the other values are expressed as fold change over basal expression. Each sample was tested in triplicate. The RNAseq files have been deposited online and are freely accessible at <https://data.mendeley.com/datasets/3g8x5cmx6z/2>.

## **Statistical analyses**

Quantitation of confocal images were performed using Image J (NIH, USA) (Rueden et al., 2017; Schindelin et al., 2012). Graph Pad prism 8.0 was used to plot mean values (n=3 or greater  $\pm$  SEM, unless otherwise indicated) compare between experimental and control groups and to determine statistical differences by unpaired Student's t-test and two-way ANOVA (Tukey post-hoc analysis) at 95% confidence intervals ( $\alpha$  value 0.05). Statistical p-values less than \*0.05, \*\*0.01, \*\*\*0.001 and \*\*\*\*0.0001 were considered significant.



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## Contributions

A.D., J.E.M., Z.C., and S.S. performed experiments and analyzed data.

A.D. and J.H.M. collated data and wrote the manuscript.

## Conflict of Interest Statement

The authors declare no potential conflicts of interest

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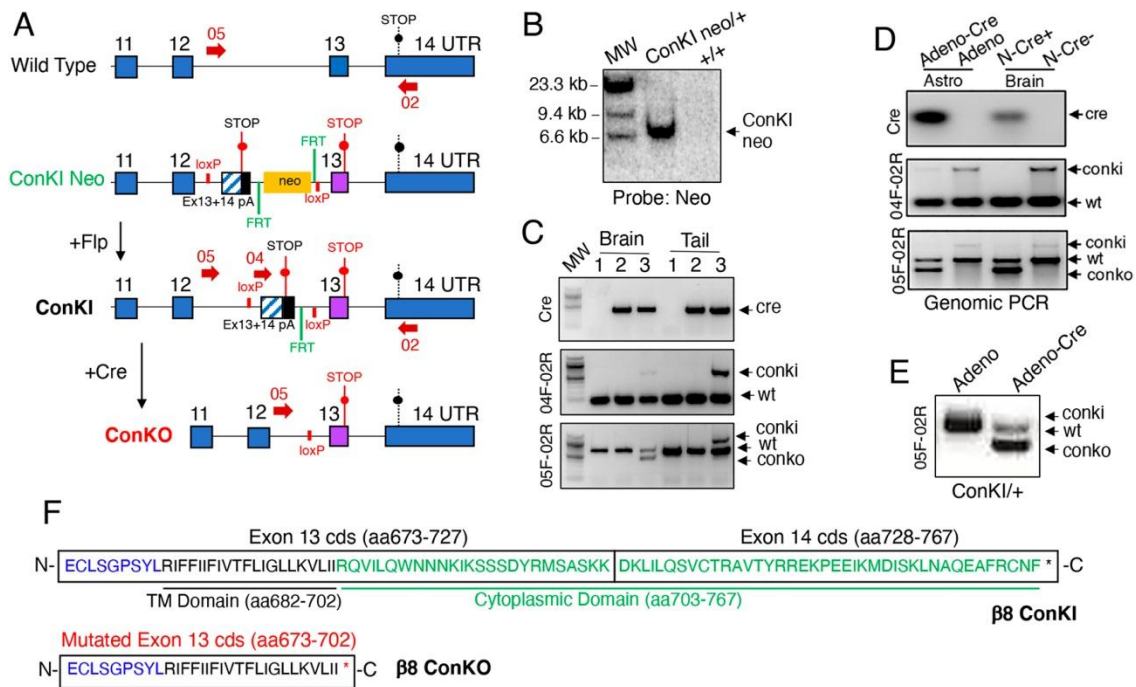
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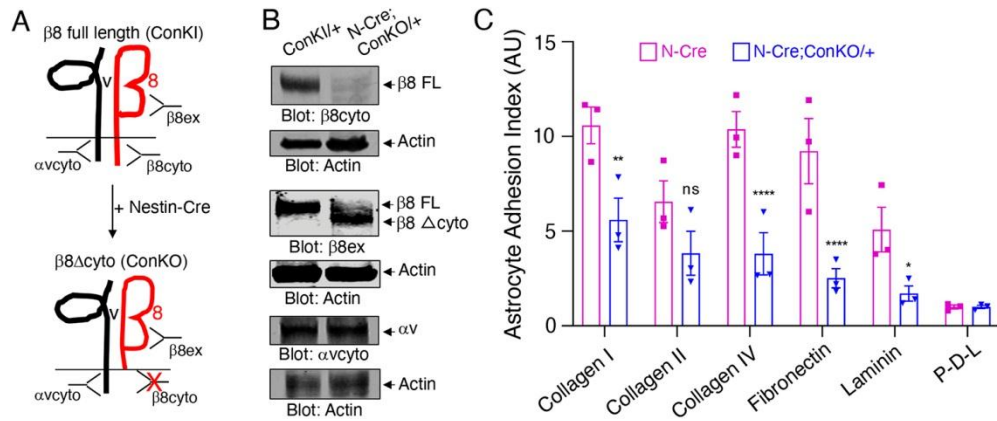
## Figures



**Figure 1. Development of a Cre/lox mouse model to selectively delete the Itgb8 cytoplasmic domain. (A);** General strategy for targeting the murine Itgb8 gene to inhibit expression of the cytoplasmic domain. The 3' region of the wild type Itgb8 gene is shown at top with the endogenous STOP codon in exon 14. A premature STOP codon was knocked into exon 13. A floxed “mini-gene” comprised of a cDNA containing exon 13 and the coding sequence of exon 14 as well as a 3' poly-A (pA) sequence was inserted upstream of the knock-in exon to avoid potential dominant-negative effects of the engineered STOP (ConKI-Neo). The Neo cassette was subsequently removed using Flp recombinase to generate the conditional knock-in allele (ConKI). Cre-mediated deletion of the floxed mini-gene sequence results in premature termination of translation (via the engineered STOP in exon 13) and generation of the conditional knock-out allele (ConKO), which encodes a truncated protein that lacks the cytoplasmic domain. **(B);** Southern blot of genomic DNA isolated from wild type control of ConKI

Neo/+ heterozygote mice, digested with BamHI, and hybridized with a probe corresponding to the Neo cassette. **(C)**; Genomic PCR analysis using DNA isolated from E14 brain and tail tissue samples confirms correct targeting and Cre-mediated recombination at the Itgb8 ConKI locus. Sample identities from brain and tail tissues in (C) are as follows: lane 1, Nestin-Cre-;+/+ (Cre-negative, wild type); lane 2, Nestin-Cre+;+/+ (Cre-positive, wild type); lane 3, Nestin-Cre+;ConKI/+ (Cre-positive, mutant). Forward and reverse DNA primer pairs used for PCR (05-02 and 04-02) are shown in the schematic (arrows in A). Note that Cre-mediated recombination occurs in the brain samples, but not in the tail samples. **(D)**; Cortical astrocytes (Astro) cultured from mouse pups were infected with control adenovirus (Adeno) or Adeno-Cre and recombination at the engineered Itgb8 ConKI allele was analyzed with PCR. Alternatively, dissected brain tissue from E18 ConKI/+ control (N-Cre-) or Nestin-Cre;ConKO/+ mice (N-Cre+) were analyzed by genomic PCR. Primer pairs used for genomic PCR analyses are shown by red arrows in Figure 1A. **(E)**; Cre-mediated recombination excises the mini-gene (Ex13/14), as confirmed by RT-PCR using RNA isolated from ConKI/+ brain neurospheres infected with control Adeno or Adeno-Cre. **(F)**; Schematic diagram showing the amino acid sequences for the  $\beta 8$  integrin transmembrane domain (black) and cytoplasmic domain (green) and corresponding exon 13 and 14 coding sequences (cds) of the wild type Itgb8 allele or the engineered Itgb8 ConKI allele containing the mini-gene insertion ( $\beta 8$  ConKI, upper panel). The black asterisk indicates the endogenous STOP codon. Cre-mediated deletion of the loxP-flanked mini-gene allows transcriptional read through to a STOP codon engineered in exon 13, leading to termination of the Itgb8 coding sequence and truncation of the  $\beta 8$  integrin cytoplasmic domain (ConKO, lower panel). The red asterisk indicates the inserted premature STOP codon shown in panel A.



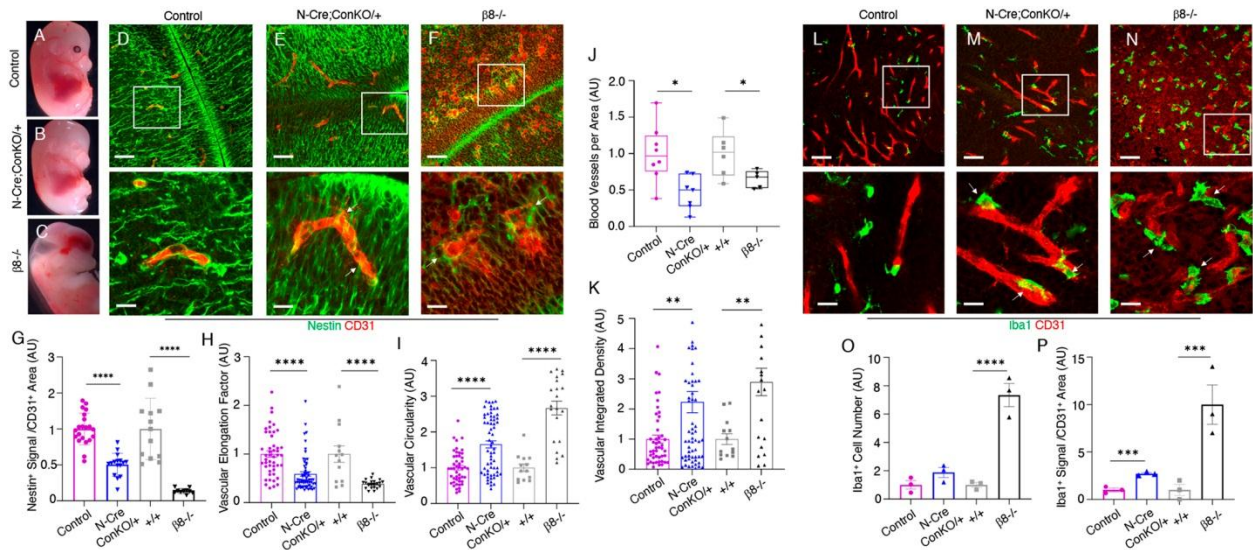


**Figure 2. The  $\beta 8$  integrin cytoplasmic domain promotes adhesion to the ECM. (A);**

Summary of antibodies directed against different regions (extracellular or cytoplasmic) of  $\alpha v$  or  $\beta 8$  integrin proteins. Note that the  $\beta 8$ cyto antibody cannot recognize the truncated  $\beta 8$  integrin protein (ConKO) owing to deletion of the cytoplasmic domain, whereas the  $\beta 8$ ex antibody can recognize both the full-length (FL) and truncated  $\beta 8$  proteins. **(B);** Brain lysates from control (ConKI/+) or Nestin-Cre;ConKO/+ neonatal mice (P0) were analyzed by immunoblotting with antibodies directed against the  $\beta 8$  integrin extracellular domain ( $\beta 8$ ex), the  $\beta 8$  integrin cytoplasmic domain ( $\beta 8$ cyto), and the  $\alpha v$  integrin cytoplasmic domain ( $\alpha v$ cyto). **(C);**

Quantification of  $\beta 8$  integrin cytoplasmic domain-dependent adhesion to various ECM proteins. Astrocyte progenitors from Nestin-Cre control or Nestin-Cre;ConKO/+ brains were added to wells coated with the indicated ECM proteins (x-axis) and cell adhesion was quantified after 16 hours. Note that loss of the  $\beta 8$  integrin cytoplasmic tail leads to defects in ECM adhesion.

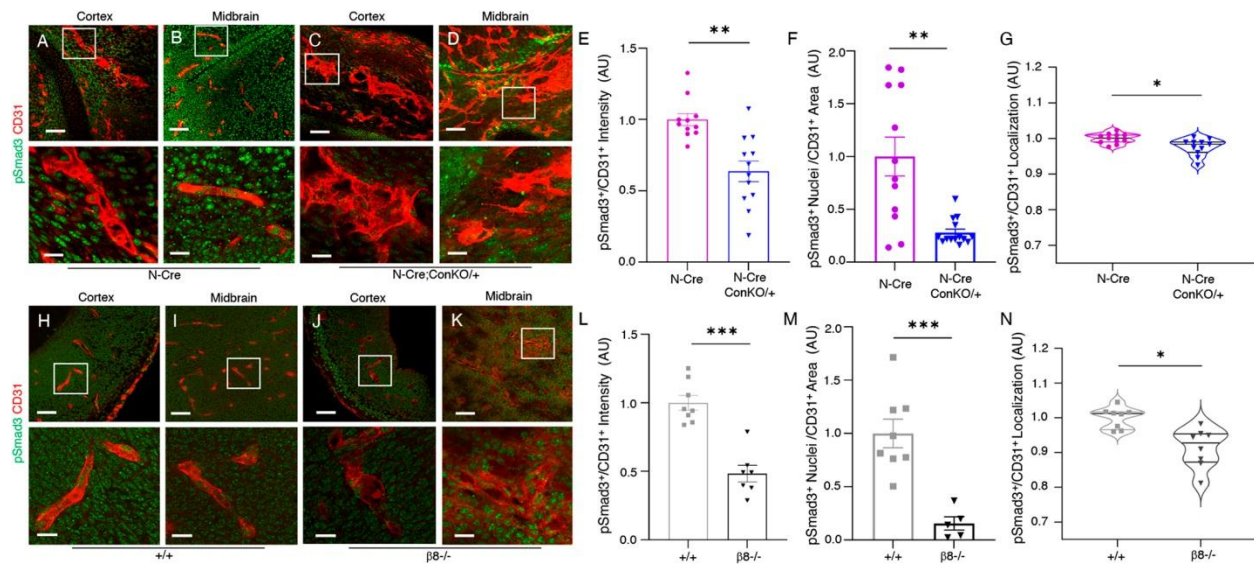
Differences between groups were analyzed using two-way ANOVA and Tukey post-hoc analysis (n=3, mean  $\pm$  SEM, \*p<0.05, \*\*p<0.01, \*\*\*\*p<0.0001).



**Figure 3. Deletion of the  $\beta 8$  integrin cytoplasmic domain leads to defective cell adhesion and aberrant blood vessel morphogenesis during brain development. (A-C);**

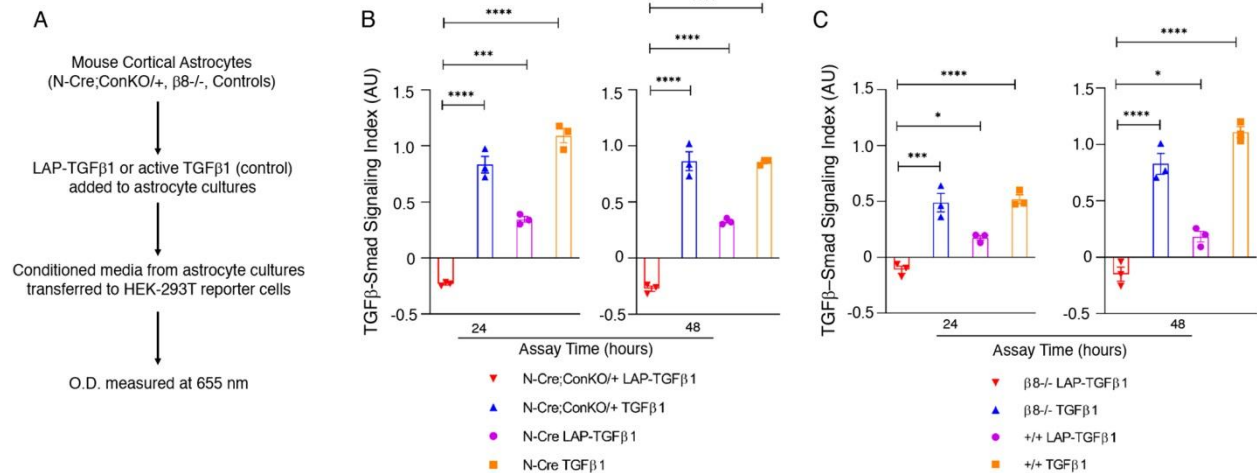
Representative images of E14 Nestin-Cre control (A), Nestin-Cre;ConKO/+ (B), and  $\beta 8^{-/-}$  (C) embryos. Note that unlike  $\beta 8^{-/-}$  embryonic brains, which are hemorrhagic and hydrocephalic, there is a lack of grossly obvious hemorrhage in Nestin-Cre;ConKO/+ embryos. **(D-F)**; Sagittal sections through the ganglionic eminence regions of E14 Nestin-Cre control (D), Nestin-Cre;ConKO/+ (E), or  $\beta 8^{-/-}$  embryos (F) were analyzed by double immunofluorescence using anti-CD31 antibody (red) to detect vascular endothelial cells combined with anti-Nestin (green) antibody to detect neuroepithelial cells. Note that in comparison to blood vessels in control mice, microscopic analysis of blood vessels in the Nestin-Cre;ConKO/+ and  $\beta 8^{-/-}$  brains reveal abnormal, distended morphologies as well as disrupted contacts with perivascular neuroepithelial cells. The lower panels are higher magnified images of boxed areas in upper panels. Scale bars in the upper and lower panels are 20  $\mu\text{m}$  and 5  $\mu\text{m}$ , respectively. **(G)**; Quantitation of neuroepithelial-endothelial cell juxtapposition in the ganglionic eminences of E14 Nestin-Cre control versus Nestin-Cre;ConKO/+ mutant embryos as well as wild type control versus  $\beta 8^{-/-}$  mutant embryos. **(H, I)**; Quantitation of blood vessel morphologies in the ganglionic

eminences of E14 Nestin-Cre control versus Nestin-Cre;ConKO/+ mutant embryos and wild type control versus  $\beta 8^{-/-}$  mutant embryos. Note the significant decrease in vascular elongation along the neuroepithelium (H) and a corresponding increase in circularity indicative of shape defects (I).  $\beta 8^{-/-}$  mutant mice brains revealed similar alterations in cerebral blood vessel morphology. **(J)**; Quantitation of blood vessels per area in the ganglionic eminences of control and mutant mice. Note the decrease in the number of CD31<sup>+</sup> blood vessels per area in Nestin-Cre;ConKO/+ and  $\beta 8^{-/-}$  mutant embryos as compared to the controls. **(K)**; Quantitation of blood vessel shapes within the ganglionic eminences of control, Nestin-Cre;ConKO/+ and  $\beta 8^{-/-}$  mutant embryos. In comparison to controls, CD31<sup>+</sup> blood vessels in mutant brains were more dilated. Aberrantly shaped blood vessels covered larger areas and thus showed enhanced anti-CD31 fluorescence intensity, as characterized by higher integrated density values. **(L-N)**; Sagittal sections through midbrain regions of E14 Nestin-Cre control (L), Nestin-Cre;ConKO/+ (M), or  $\beta 8^{-/-}$  embryos (N) were analyzed by double immunofluorescence using anti-CD31 (red) antibody to detect vascular endothelial cells combined with anti-Iba1 (green) antibody to detect microglial cells. Note that in comparison to blood vessels in control mice, the blood vessels in the Nestin-Cre;ConKO/+ and  $\beta 8^{-/-}$  brains have abnormal morphologies and show increased juxtaposition with microglia. The lower panels are higher magnified images of boxed areas in upper panels. Scale bars in the upper and lower panels are 20  $\mu\text{m}$  and 5  $\mu\text{m}$ , respectively. **(O, P)**; Quantitation of perivascular microglial cells in the ganglionic eminences of E14 control and mutant embryos. In comparison to control mice, higher number of Iba1<sup>+</sup> cells were identified near blood vessels in Nestin-Cre;ConKO/+ mutant mice brains.  $\beta 8^{-/-}$  mutant brain sections contained higher numbers of total microglia and perivascular microglia. Differences between groups were analyzed using Student's t-test (n=3, mean  $\pm$  SEM, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001).



**Figure 4. Deletion of  $\beta 8$  integrin cytoplasmic domain in brain neuroepithelial cells leads to reduced TGF $\beta$ -Smad signaling in vascular endothelial cells. (A-D);** Analysis of Smad3 phosphorylation in brains from E14 Nestin-Cre control (A, B) and Nestin-Cre;ConKO/+ embryos (C, D). Shown are sagittal sections through the forebrains/cerebral cortices (A, C) and the ganglionic eminence regions of the midbrain (B, D). Note the abnormal CD31<sup>+</sup> blood vessel morphologies in Nestin-Cre;ConKO/+ brains with reduced levels of pSmad3. The lower panels are higher magnification images of boxed areas in upper panels. Scale bars in the upper and lower panels are 20  $\mu$ m and 5  $\mu$ m, respectively. **(E-G);** Quantification of pSmad3 levels in E14 Nestin-Cre control and Nestin-Cre;ConKO/+ midbrain regions as plotted in a bar graph measuring fluorescence intensity of pSmad3 relative to CD31 (E), numbers of pSmad3<sup>+</sup> nuclei normalized to total CD31<sup>+</sup> area (F), or by violin plots showing pSmad3<sup>+</sup>/CD31<sup>+</sup> co-localization (G). Note the reduced levels of pSmad3 in Nestin-Cre;ConKO/+ brain samples. **(H-K);** Analysis of Smad3 phosphorylation in brains from E14 wild type control (H, I) and  $\beta 8^{-/-}$  embryos (J, K). Shown are sagittal sections through the developing cerebral cortices (H, J) and the ganglionic eminence regions of the midbrain (I, K). Note the abnormal CD31<sup>+</sup> blood vessel morphologies in  $\beta 8^{-/-}$  brains with defects in levels of pSmad3. The lower panels are higher magnification images

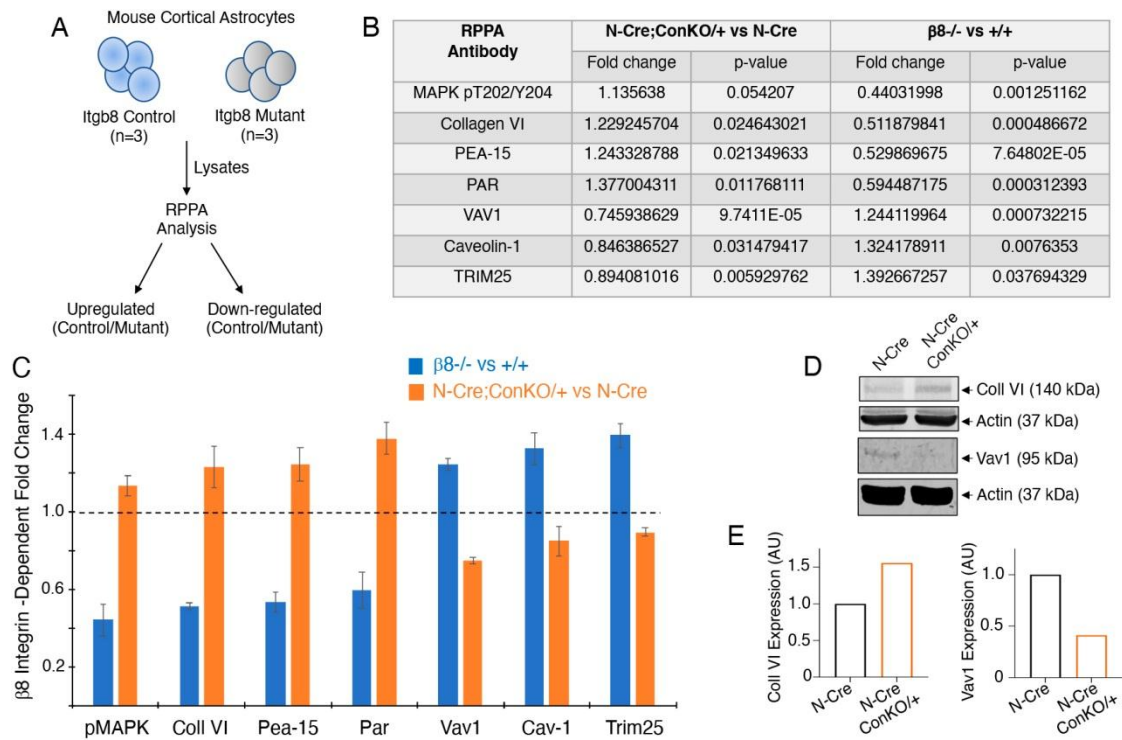
of boxed areas in upper panels. Scale bars in the upper and lower panels are 20  $\mu\text{m}$  and 5  $\mu\text{m}$ , respectively. **(L-N)**; Quantification of pSmad3 levels in E14 wild type control and  $\beta 8^{-/-}$  midbrain regions as plotted in a bar graph measuring fluorescence intensity of pSmad3 relative to CD31 (L), numbers of pSmad3<sup>+</sup> nuclei normalized to total CD31<sup>+</sup> area (M), or by violin plots showing pSmad3<sup>+</sup>/CD31<sup>+</sup> co-localization (N). Note the reduced levels of pSmad3 in  $\beta 8^{-/-}$  brain samples. Differences between groups were determined using Student's t-test (n=3, mean +/- SEM, \*\*p<0.01, \*p<0.001).



**Figure 5. The  $\beta 8$  integrin cytoplasmic domain promotes latent-TGF $\beta$  activation in vitro.**

**(A)**; Experimental strategy for quantifying  $\beta 8$  integrin-dependent latent-TGF $\beta$  activation and signaling in vitro using control and mutant astrocytes and HEK-293 cells expressing SBE-iSEAP reporter constructs. **(B, C)**; Quantification of  $\beta 8$  integrin-mediated activation of latent-TGF $\beta 1$  using Nestin-Cre control and Nestin-Cre;ConKO/+ astrocytes (B) as well as wild type and  $\beta 8^{-/-}$  astrocytes (C) in cell-based SBE-iSEAP reporter assays. Astrocyte conditioned media (+/- exogenous LAP-TGF $\beta 1$  or bioactive TGF $\beta 1$ ) was transferred to HEK-SBE Blue cells followed by quantification of reporter activities at 24 and 48 hours. Note that both Nestin-Cre;ConKO/+ and  $\beta 8^{-/-}$  astrocytes show reduced latent-TGF $\beta$  activation and signaling at 24 and 48 hours in comparison to control astrocytes.



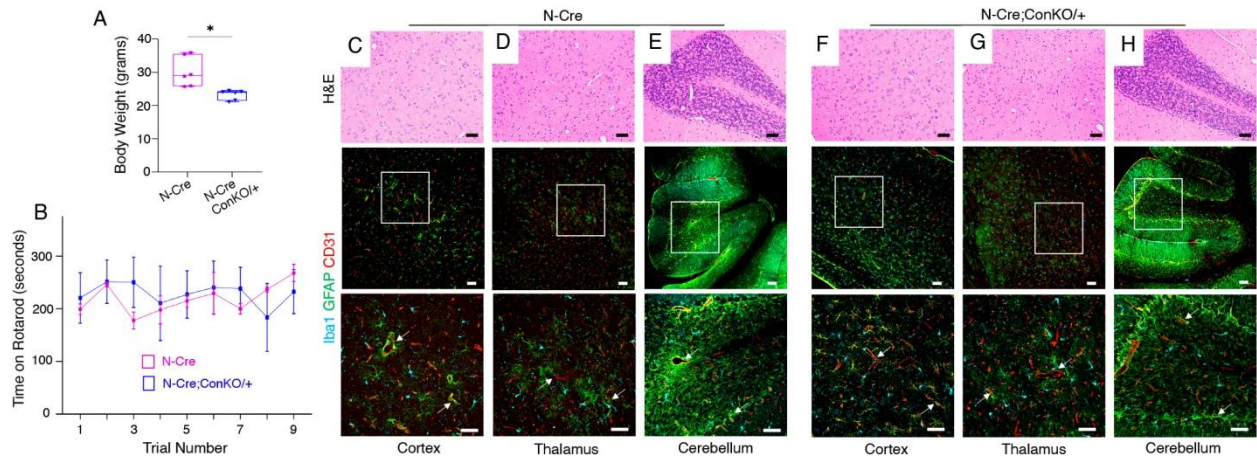


**Figure 6. The  $\beta 8$  integrin cytoplasmic domain regulates signal transduction in astrocytes.**

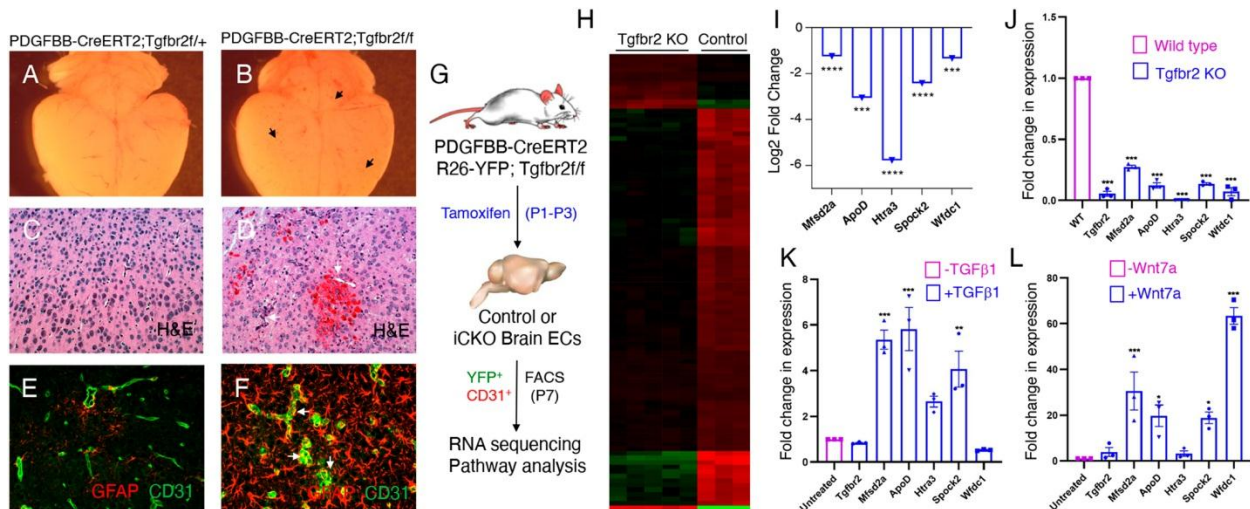
**(A)**; Strategy to identify  $\beta 8$  integrin-dependent changes in protein expression or phosphorylation in mouse astrocytes using reverse phase protein array (RPPA) platforms. **(B)**; Table summarizing the seven most differentially expressed and/or phosphorylated proteins (with relevant p-values) in mutant versus control astrocytes identified by RPPA. **(C)**; Bar graph summarizing select proteins that show statistically significant differences in expression and/or phosphorylation in control versus  $\beta 8^{-/-}$  astrocytes or Nestin-Cre control versus Nestin-Cre;ConKO/+ astrocytes. Shown are the top seven proteins displaying differential expression and/or phosphorylation in astrocyte progenitors cultured from Nestin-Cre;ConKO/+ versus  $\beta 8^{-/-}$  pups. **(D)**; Detergent-soluble lysates from Nestin-Cre control and Nestin-Cre;ConKO/+ mutant astrocytes were immunoblotted using anti-Collagen VI and anti-

Vav1 antibodies. Note the integrin-dependent changes in expression which correlates with the expression differences identified in the RPPA screen (B, C). **(E)**; Densitometry-based quantification of Collagen VI (left graph) and Vav1 (right graph) protein levels based on immunoblot data in (D).





**Figure 7. Absence of brain vascular pathologies and neurodegenerative phenotypes in adult Nestin-Cre;ConKO/+ mice. (A);** Body weights were recorded for seven month old male Nestin-Cre control and Nestin-Cre;ConKO/+ mice, p-value < \*0.05. **(B);** Rotarod analyses of seven month old male Nestin-Cre control (n=3) and Nestin-Cre;ConKO/+ mutant mice (n=3). The time (y-axis) the mice remained on the rod rotating at an increasing speed versus the trial number (x-axis) is plotted. Note the lack of statistically significant differences in time spent on the rod for mutant versus control mice. **(C-H);** Sagittal brain sections from seven month old Nestin-Cre control (C-E) or Nestin-Cre;ConKO/+ mutant mice (F-H) through the cerebral cortex (C, F), thalamus (D, G), and cerebellum (E, H) were stained with H&E (upper panels) or fluorescently labeled with anti-Iba1 (cyan), anti-GFAP (green), and anti-CD31 (red) antibodies (lower panels) to reveal microglia, astrocytes and vascular endothelial cells, respectively. Note the absence of obvious neurovascular pathologies in Nestin-Cre;ConKO/+ mutant brain sections (F-H). White arrows in the fluorescent panels indicate blood vessels with closely juxtaposed astrocytes and/or microglial cells. The lower panels are higher magnification images of the boxed areas within the middle panels. Scale bars are 50  $\mu$ m.

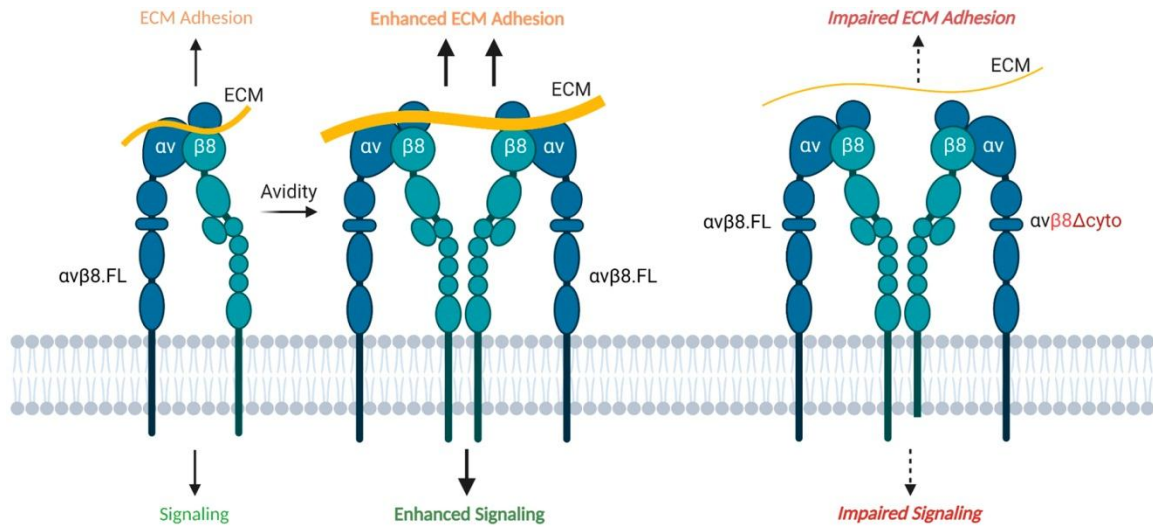


**Figure 8.  $\beta 8$  integrin-activated TGF $\beta$  signaling regulates gene expression in brain**

**endothelial cells. (A-F);** Images of brains and fixed sections from tamoxifen-injected *Pdgfb-*CreERT2** control (A) or *Pdgfb-*CreERT2*; *Tgfr2f/f** conditional knockout mice (B) stained with H&E (C, D) or anti-CD31 and anti-GFAP antibodies (E, F). Note that *Pdgfb-*CreERT2*; *Tgfr2f/f** mutant mice develop focal brain hemorrhages and angiogenesis defects (arrows in B, D) as well as higher numbers of perivascular astrocytes (arrows in F). Mice received three consecutive intragastric tamoxifen injections between P1 and P3 and were analyzed at P7. **(G);** Strategy to identify TGF $\beta$ -regulated genes involved in cerebral angiogenesis and neurovascular development. Bulk transcriptome sequencing was performed on YFP<sup>+</sup>/CD31<sup>+</sup> brain endothelial cells (ECs) isolated from P7 PDGFB-*CreERT2*; R26-YFP/+ control or PDGFB-*CreERT2*; *Tgfr2f/f*; R26-YFP/+ inducible knockout (iCKO) mice. **(H);** Heat map listing the top differentially expressed genes in control *Pdgfb-*CreERT2*; *Tgfr2f/f*; R26-YFP/+* (n=3) and mutant *Pdgfb-*CreERT2*; *Tgfr2f/f*; R26-YFP/+* (n=5) brain endothelial cells. **(I);** Decreased relative expression of five select genes (*Mfsd2a*, *Apod*, *Htra3*, *Spock2*, *Wfdc1*) as measured by quantitative mRNA sequencing from isolated control and *Tgfr2*<sup>-/-</sup> brain endothelial cells.

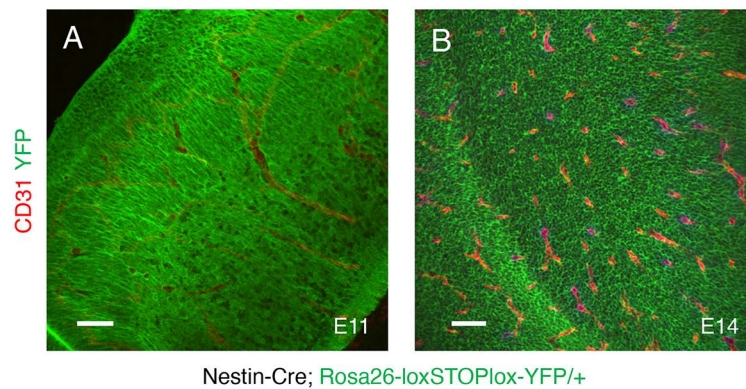
\*\*\*p<0.001, \*\*\*\*p<0.0001. **(J);** Quantitative RT-PCR analysis validates differential expression in

control and *Tgfr2*<sup>-/-</sup> brain endothelial cell samples for five genes identified from transcriptome sequencing. **(K)**; Brain endothelial cells (bEnd.3) were treated with TGFβ1 for 12 hours and expression of *Tgfr2*, *Mfsd2a*, *ApoD*, *Htra3*, *Spock2* and *Wfdc1* was quantified by RT-PCR. **(L)**; Brain endothelial cells were treated with Wnt7a for 24 hours and expression levels of *Tgfr2*, *Mfsd2a*, *ApoD*, *Htra3*, *Spock2* and *Wfdc1* mRNAs were quantified by RT-PCR. Statistical differences of means were determined by two-way ANOVA (n=3± SEM); p-values < \*0.05, \*\*0.01, \*\*\*0.001



**Figure 9. Model for the  $\beta 8$  integrin cytoplasmic tail in control of ECM adhesion and intracellular signaling.** Activated full-length  $\alpha v \beta 8$  integrin promotes ECM engagement and clustering, leading to enhanced avidity to protein ligands and robust intracellular signaling. Genetic deletion of the  $\beta 8$  cytoplasmic domain impairs intracellular signaling and cytoskeletal linkages, depending on the presence of full-length  $\beta 8$  integrin.  $\alpha v \beta 8 \Delta \text{cyto}$  is shown in a clustered complex with full-length  $\alpha v \beta 8$  integrin (ConKO/+). This figure was created with [awb://BioRender.com](http://awb://BioRender.com).

Supplemental Figure 1



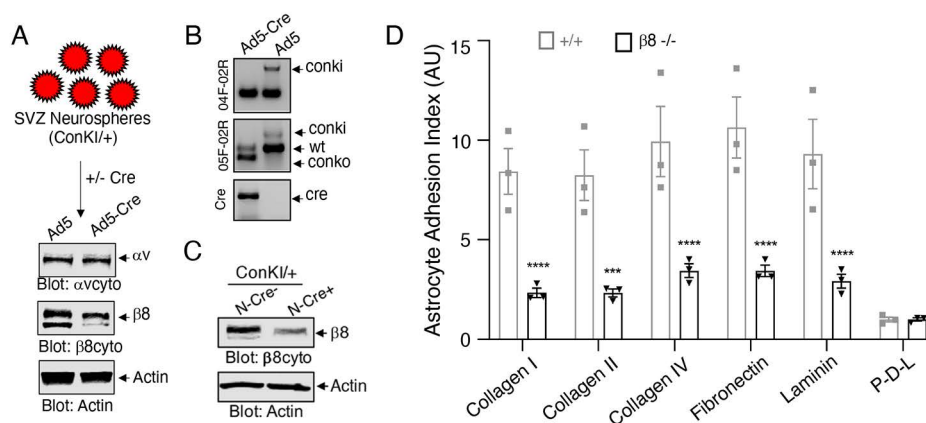
**Fig. S1. Analysis of Nestin-Cre-mediated recombination in embryonic brain neuroepithelial cells. (A, B);** The Nestin-Cre transgene is expressed in embryonic neuroepithelial cells, but is not expressed in brain blood vessels at E11 (A) and E14 (B) as revealed by the crossing with the Rosa26-loxSTOPllox-YFP reporter model.



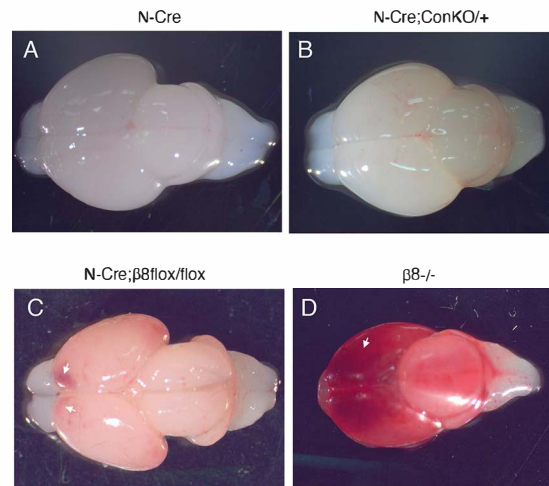
**Fig. S2. DNA sequencing confirms cytoplasmic domain deletion in cultured mouse Nestin-Cre;ConKO/+ cells. (A);** Cre-mediated recombination of the engineered Itgb8 locus removes the mini-gene (Ex13/14), resulting in a premature STOP codon that truncates the  $\beta 8$  integrin protein after the transmembrane domain. **(B, C);** Sanger sequencing of cDNA from E14 Nestin-Cre control and Nestin-Cre;ConKO/+ neurospheres confirmed selective ablation of the  $\beta 8$  integrin cytoplasmic tail coding sequence in Nestin-Cre;ConKO/+ neural progenitor cells. DNA sequences of the ConKI control (B) and ConKO mutant (C) Itgb8 allele confirms Cre-mediated deletion of the Ex13/14 mini-gene and expression of the truncated  $\beta 8$  integrin protein lacking the cytoplasmic signaling domain. The transmembrane sequence is indicated by the red line, and the cytoplasmic domain is indicated by the blue shaded region. The endogenous STOP codon in (B) and the engineered STOP codon in (C) are indicated by red asterisks.



## Supplemental Figure 3

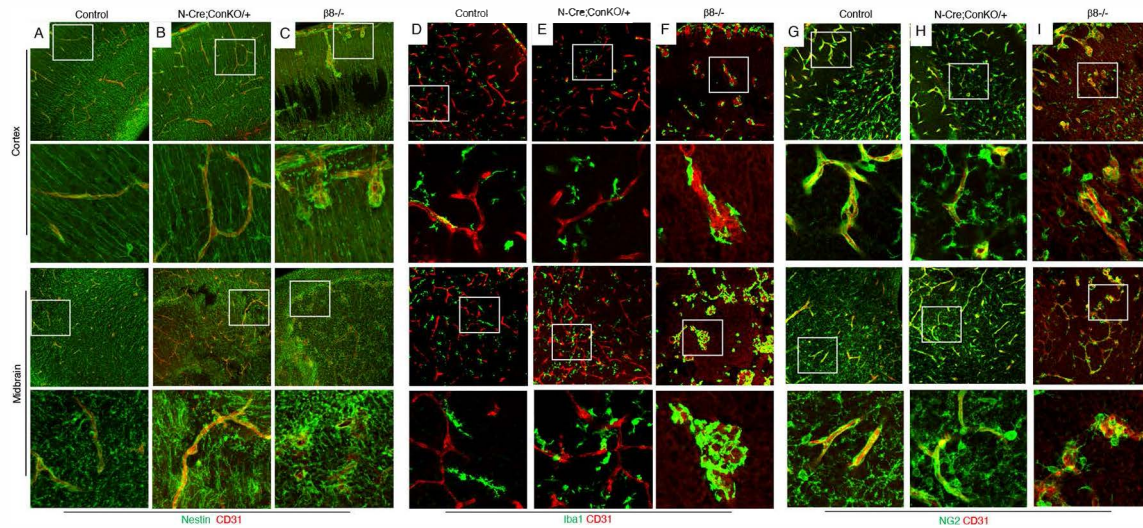


**Fig. S3. In vitro validation of Cre-mediated *Itgb8* mini-gene deletion and quantitation of b8 integrin ECM adhesion. (A, B);** Neurospheres cultured from the subventricular zone of the lateral ventricles of P60 ConKI/+ mice were infected with empty adenovirus (Ad5 control) or adenovirus-Cre (Ad5-Cre). Detergent-soluble lysates were analyzed by immunoblotting with anti- $\alpha$ vcyto or anti-b8cyto integrin antibodies (see Figure 2A). Note the reduction in b8 integrin protein due to truncation of the cytoplasmic domain. Alternatively, Cre-mediated deletion of the floxed mini-gene in the engineered *Itgb8* locus was confirmed by genomic PCR (B). **(C);** Neurospheres from E14 control (ConKI/+) or Nestin-Cre;ConKO/+ P0 mice were analyzed by immunoblotting. Note the reduce expression of full-length b8 integrin protein in the neurosphere samples from Nestin-Cre;ConKO/+ embryos. **(D);** Wild type control or  $\beta$ 8<sup>-/-</sup> astrocytes were added to tissue culture wells coated with the indicated ECM proteins and cell adhesion was quantified after 2 hours. Note that  $\beta$ 8<sup>-/-</sup> astrocytes show severe ECM adhesion defects similar to the defects with N-Cre;ConKO/+ astrocytes (Figure 2C). Differences between groups were analyzed using two-way ANOVA and Tukey post-hoc analysis (n=3, mean  $\pm$  SEM, \*\*\*p<0.001, \*\*\*\*p<0.0001).

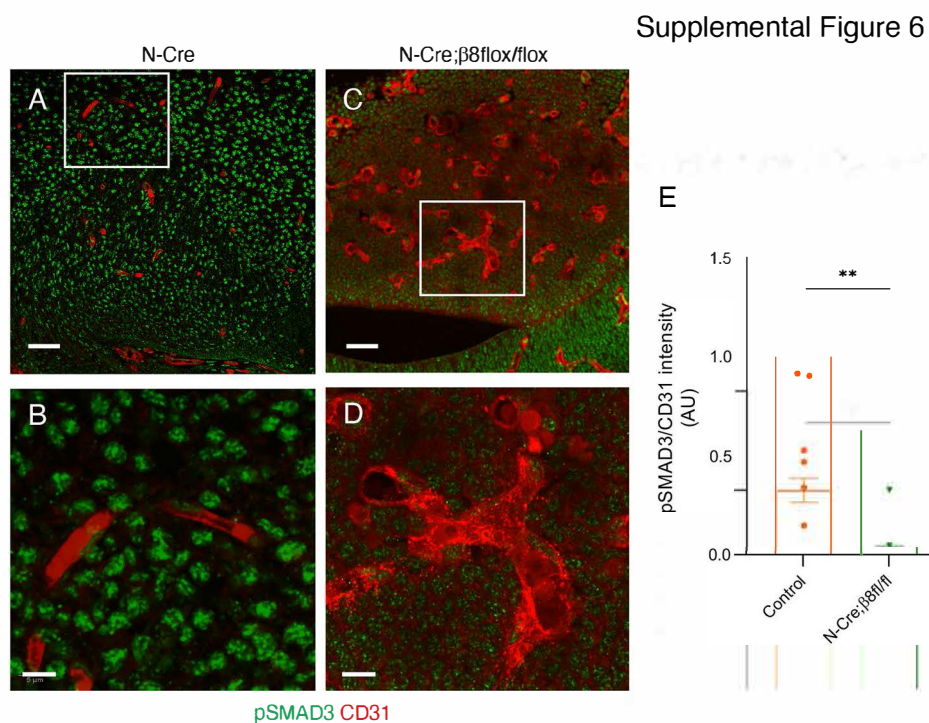


**Fig. S4. Lack of intracerebral hemorrhage in Nestin-Cre;ConKO embryos. (A-D);** Images of isolated brains from an E18 control (A), Nestin-Cre;ConKO/+ (B), Nestin-Cre;β8flox/flox (C) and β8<sup>-/-</sup> embryo (D). Note the absence of grossly obvious hemorrhage in the Nestin-Cre;ConKO/+ brains that is less severe than the hemorrhage in the Nestin-Cre;β8flox/flox and β8<sup>-/-</sup> mutants.



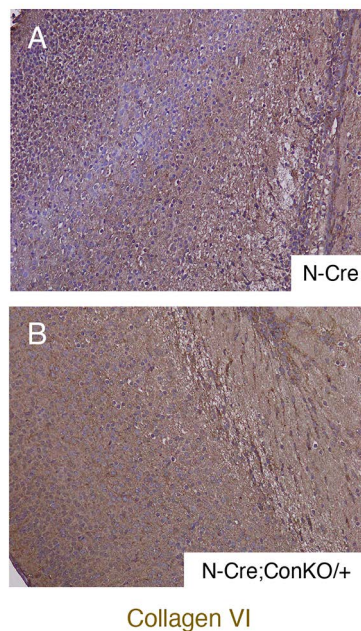


**Fig. S5. Neurovascular pathologies in the E18 Nestin-Cre;ConKO/+ brain. (A-C);** Sagittal sections through forebrain/neocortical regions or midbrain regions of E18 Nestin-Cre control (A), Nestin-Cre;ConKO/+ (B), or  $\beta 8^{-/-}$  embryos (C) were analyzed by double immunofluorescence using anti-CD31 antibodies to detect vascular endothelial cells combined with anti-Nestin to detect neuroepithelial cells. **(D-F);** Sagittal sections through the forebrains/neocortical regions of E18 Nestin-Cre control (D), Nestin-Cre;ConKO/+ (E), or  $\beta 8^{-/-}$  embryos (F) were analyzed by double immunofluorescence using anti-CD31 antibodies to detect vascular endothelial cells combined with anti-Iba1 to detect microglial cells. **(G-I);** Sagittal sections through the forebrains/neocortical regions of E18 Nestin-Cre control (G), Nestin-Cre;ConKO/+ (H), or  $\beta 8^{-/-}$  embryos (I) were analyzed by double immunofluorescence using anti-CD31 antibodies to detect vascular endothelial cells combined with anti-NG2 to detect vascular pericytes.



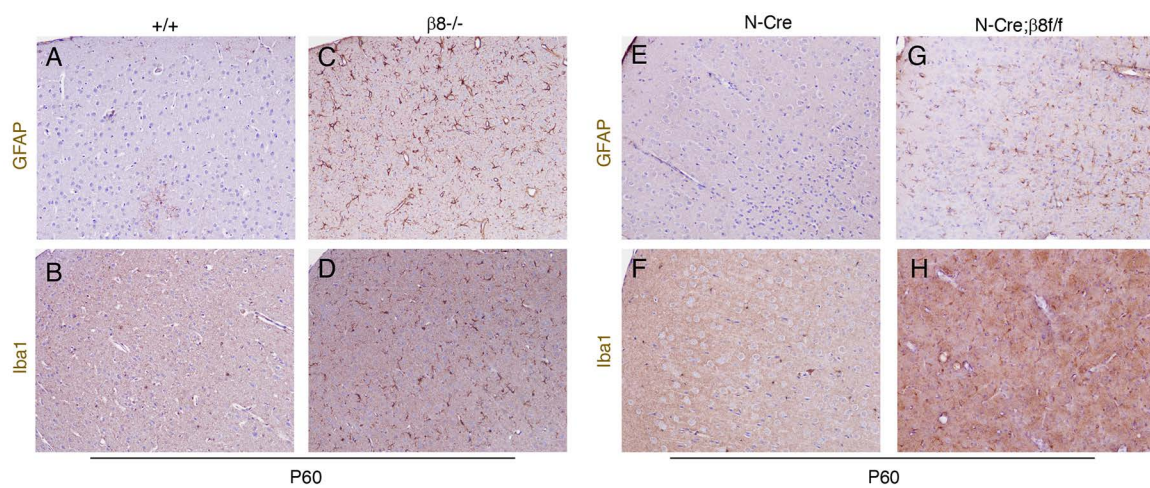
**Fig. S6. Conditional knockout of b8 integrin in brain neuroepithelial cells leads to reduced canonical TGFb receptor signaling in vascular endothelial cells. (A-D);** Analysis of Smad3 phosphorylation in sagittal brain sections through telencephalic regions of E14 N-Cre control (A, B) and N-Cre;b8flox/flox embryos (C, D). Note the abnormal CD31+ blood vessel morphologies in N-Cre;b8flox/flox brains, with reduced levels of pSmad3. The lower panels are higher magnification images of the boxed areas in the upper panels. (E): Quantification of pSmad3 levels in E14 Nestin-Cre control and Nestin-Cre;b8flox/flox mutant midbrain regions as determined by the bar graph measuring fluorescence intensity of pSmad3 relative to CD31

Supplemental Figure 7



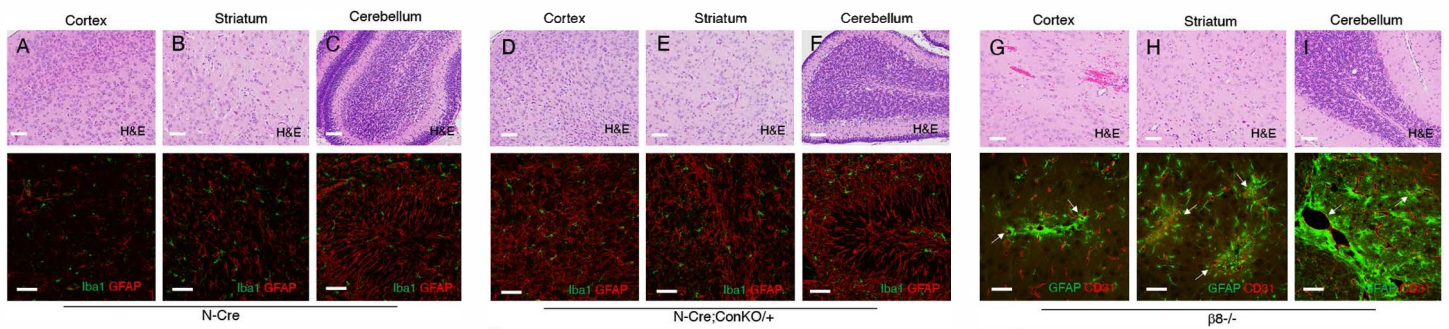
**Fig. S7. Analysis of collagen VI protein expression in Nestin-Cre control and Nestin-Cre;ConKO/+ P0 brain sections. (A, B);** Formalin fixed and paraffin embedded control (A) and mutant (B) sagittal brain sections were labeled with an anti-Collagen VI antibody. Note the increased levels of Collagen VI expression in the Nestin-Cre;ConKO/+ cortex, versus the control cortex.

Supplemental Figure 8

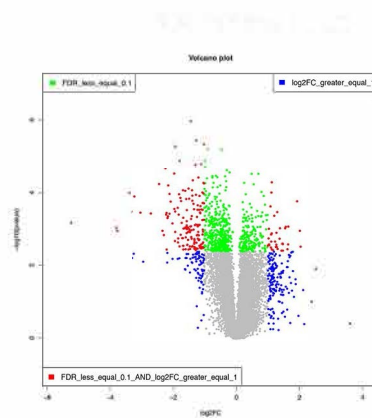


**Fig. S8. Brain vascular pathologies in  $\beta 8^{-/-}$  and Nestin-Cre; $\beta 8^{flox/flox}$  adult mice. (A-D);** Coronal brain sections through the cerebral cortices of wild type control (A, B) or  $\beta 8^{-/-}$  (C, D) P60 mice were immunohistochemically labeled with anti-GFAP (A, C) and anti-Iba1 (B, D) antibodies to visualize astrocytes and microglia, respectively. **(E-H);** Coronal brain sections through the cerebral cortices of Nestin-Cre control (E, F) or Nestin-Cre; $\beta 8^{flox/flox}$  (G,H) P60 mice were immunohistochemically labeled with anti-GFAP (E, G) and anti-Iba1 (F, H) antibodies to visualize astrocytes and microglia, respectively. In comparison to control brain sections, note the obvious perivascular Iba1+ microgliosis and GFAP+ astroglialosis in mutant brain sections.

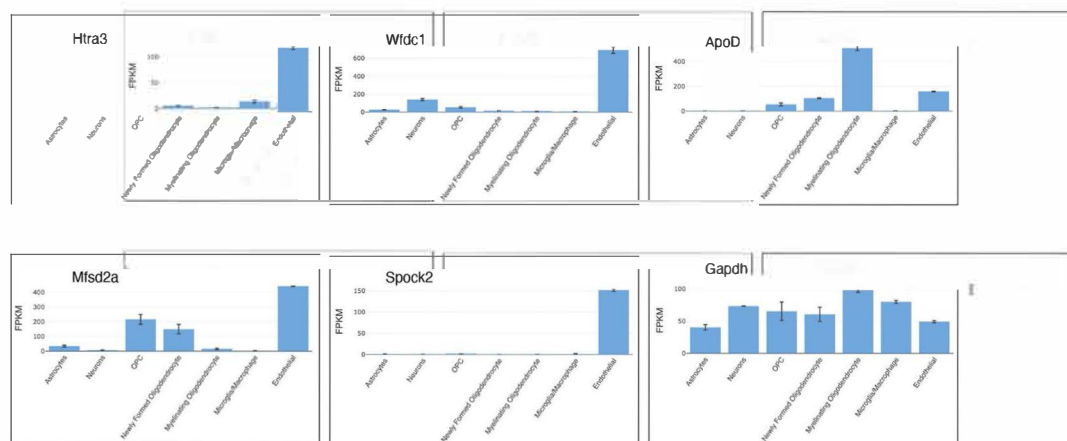




**Fig. S9. Microscopic analysis of brains from Nestin-Cre control and Nestin-Cre;ConKO/+ neonatal mice at P10. (A-F);** Sagittal brain sections from P10 Nestin-Cre control (A-C) or Nestin-Cre;ConKO/+ mutant mice (D-F) through the cerebral cortex (A, D), striatum (B, E), and cerebellum (C, F) were fixed, paraffin embedded and stained with H&E (upper panels). Alternatively, P10 brains were sectioned with a vibratome and fluorescently labeled with anti-Iba-1 (green) and anti-GFAP (red) antibodies to reveal microglia and astrocytes, respectively. Note the astrogliosis in Nestin-Cre;ConKO/+ mutant brain sections, whereas there is an obvious lack in microgliosis. (D-F). **(G-I);** Sagittal brain sections from P16  $\beta 8^{-/-}$  mutant mice through the cerebral cortex (G), striatum (H), and cerebellum (I) were fixed, paraffin embedded and stained with H&E (upper panels). Alternatively, paraffin embedded brain sections were fluorescently labeled with anti-GFAP (green) and anti-CD31 (red) antibodies to reveal astrocytes and endothelial cells, respectively. Note the microscopic hemorrhage in the cortex (G) as well as robust astrogliosis throughout the  $\beta 8^{-/-}$  brain.



**Fig. S10. Volcano plot showing mRNAs in brain endothelial cells that are upregulated and downregulated after *Tgfr2* gene ablation.** Quantitative RNA sequencing comparisons reveal differentially expressed genes in PDGFBB-CreERT2f/+ or PDGFBB-CreERT2;*Tgfr2*f/f brain endothelial cells isolated from P7 mice as revealed by a color-coded volcano plot. Red indicates higher mRNA expression levels and blue indicates lower expression. Differentially expressed genes were identified using the EdgeR package with adjusted p-value cutoffs  $<0.05$  and  $\log_2$  fold changes  $> 2$ .



**Fig. S11. Validation of brain endothelial cell expression of Tgfr2-regulated mRNAs identified by quantitative RNA sequencing.** Analysis of the brain RNA-seq database (brainrnaseq.org) confirms that the various genes down-regulated after ablation of Tgfr2 with PDGFBB-CreERT2 at P7 are expressed mainly in brain endothelial cells. In these experiments brain endothelial cells were isolated from P7 Tie2-GFP mice. Gapdh, which is a more broadly expressed gene, is shown as a relative control.



**Table S1. Commercial primary antibodies**

Primary Antibody	Host(s)	Dilution/ Concentration	Application	Catalog #	Manufacturer
Nestin	Chicken mAb	1:100	IF	CH23001	Neuromics
Iba1	Rabbit pAb	1:250	IF	019-19741	Fujifilm WPC
Iba1	Rabbit pAb	1:250	IHC-P	013-27691	Fujifilm WPC
pSMAD3 (S423+S425)	Rabbit mAb	1:200	IF	ab-52903	Abcam
NG2	Rabbit pAb	1:200	IF	AB5320	Millipore Sigma
CD31	Rat mAb	1:200	IF	553370	BD Biosciences
CD31	Goat pAb	1:250	IHC-F	AF3628	R&D Systems
GFAP	Chicken pAb	1:4000	IHC-F, IF	NBP1-05198	Novus
GFAP	Rabbit pAb	1:500	IHC-F, IF	Z0334	Dako
GFP	Chicken	1:2000	IHC-F, IF	GFP-1020	Aves
GFP	Rabbit	1:1000	IHC-F, IF	ab290	Abcam
Isolectin GS-IB <sub>4</sub> -Alexa Fluor™ 488	G. <i>simplicifolia</i>	1:500	IF	I21411	ThermoFisher
Laminin	Rabbit pAb	1:100	IHC-P	L9393	Sigma Aldrich
Vav1	Rabbit pAb	1:1000	WB	2502	Cell Signaling Technology
Collagen VI	Rabbit pAb	1:1000	WB	17023-1-AP	Proteintech
β8-cyto	Rabbit pAb	1:3000	WB, IP	Custom-made	Covance
αv-cyto	Rabbit pAb	1:3000	WB, IP	Custom-made	Covance
β8-ex	Rabbit pAb	1:1000-1:3000	WB, IP	Custom-made	Covance
β-actin	Mouse mAb	1:3000	WB	A5441	Sigma Aldrich
α-actinin	Mouse mAb	1:3000	WB	AB18061	Abcam
β-actin	Rabbit pAb	1:1000-1:3000	WB	BS-0061R-TR	Bioss
IgG (control)	Rabbit, rat, chicken, goat	1-5 µg/mL (titled based on application)	WB, IF, IHC-P, IHC-F	AB-105-C, 6-001-A, AB-101-C, AB-108-C	R&D Systems

**Table S1. Commercial secondary antibodies:**

<b>Secondary Antibody</b>	<b>Host</b>	<b>Dilution</b>	<b>Catalog #</b>	<b>Manufacturer</b>
IRDye® anti-Rabbit 800CW	Goat	1:15,000	926-32211	LI-COR
IRDye® anti-Mouse 680CW	Goat	1:15,000	926-68070	LI-COR
IRDye® anti-Rabbit 800CW	Donkey	1:15,000	926-32213	LI-COR
IRDye® anti-Mouse 680CW	Donkey	1:15,000	926-68072	LI-COR
IRDye® Streptavidin 800CW	na	1:5,000	926-32230	LI-COR
Alexa Flour® 488 anti-Chicken	Donkey	1:500	703-545-155	Jackson ImmunoResearch
Alexa Flour® 488 anti-Rabbit	Donkey	1:500	711-545-152	Jackson ImmunoResearch
Alexa Flour® 594 anti-Rat	Donkey	1:500	712-585-153	Jackson ImmunoResearch
Alexa Flour® 594 anti-Goat	Donkey	1:500	705-585-147	Jackson ImmunoResearch

**Table S2.** Primers used for quantitative real time PCR

<b>Gene</b>	<b>Primer Sequence (5'-3')</b>
<i>mouse</i> Gapdh	<i>Forward:</i> GAATGGGAAGCTTGTCATCAACGG
	<i>Reverse:</i> GTAGACTCCACGACATACTCAGCAC
<i>mouse</i> Tgfbr2	<i>Forward:</i> TTAACATGATGTCATGGCCAGCG
	<i>Reverse:</i> AGACTTCATGCGGCTTCTCACAGA
<i>mouse</i> Mfsd2a	<i>Forward:</i> CCGGTCCAGGTGAAGAAGGAAC
	<i>Reverse:</i> GCTCGGCCACAAAAAGGATA
<i>mouse</i> Apod	<i>Forward:</i> GAAGCCAAACAGAGCAACG
	<i>Reverse:</i> TGTTTCTGGAGGGAGATAAGGA
<i>mouse</i> Htra3	<i>Forward:</i> CTCGGGCTTCATCATGTCAGA
	<i>Reverse:</i> AATCGTGGCAATGTCCGACTT
<i>mouse</i> Spock2	<i>Forward:</i> ACTGTGATGACATCGTGGGTT
	<i>Reverse:</i> TCTTCTGGCCTGTCTTTCTGG
<i>mouse</i> Wfdc1	<i>Forward:</i> TGTCCTCAGGCTATGAGTG
	<i>Reverse:</i> AAGTGCCTCTGTTGTCCCTTC