Supporting Information

Daneman *et al.* 10.1073/pnas.0805165106

SI Materials and Methods

Immunohistochemistry. Embryos were isolated and fixed for 1 h in 4% paraformaldehyde. Fixed tissue was submerged in 30% sucrose and then embedded in 2:1 30% sucrose:OCT mixture. Ten micrometer tissue cryosections were then generated and stained as follows. Sections were incubated in a blocking/permeabilization solution containing 50% goat serum and 0.2% Triton X-100 for 30 min at room temperature, followed by incubation in primary antibody solution overnight at 4 °C. Appropriate secondary antibodies, conjugated to alexa fluorophores, were then incubated for 2 h at room temperature before mounting in vectashield with DAPI (Vector H 1200). When applicable, fluorescein conjugated-BSL (Vector, FL-1101 diluted 1:500) was added to the secondary antibody mix, and these samples were then postfixed for 10 min with 4% paraformaldehyde to stabilize BSL. All sections were analyzed with a Nikon Eclipse E800 microscope and images were taken with a Diagnostics Instruments SPOT camera and analyzed by SPOT software. For TOP-GAL transgenic mice, tissue sections of E12.5 were double-labeled with a rabbit anti-lacZ antibody (ICN) diluted 1:5000 and BSL-FITC (Vector, FL-1101) diluted 1:500. For conditional β-catenin mutants and littermate controls, tissue sections were stained with a rat anti-CD31 antibody (BD PharMingen, 553370), rabbit anti-NG2 antibody (chemicon, AB5320), rabbit anti-glut-1 antibody (chemicon AB1340) a rabbit antiserum against Pard6 (a generous gift from Anthony Pawson, Toronto, Canada) that stained blood and BSL-FITC all diluted 1:500. Adenoviral injected mice were stained with BSL-FITC and with the rat anti-CD31 antibody.

Cell Purification and Cell Culture. For GeneChip experiments on acutely purified brain, liver, and lung endothelial cells, cell suspensions were prepared from cerebral cortex, liver, or lung of Tie2GFP adult mice based on procedures previously described (1, 2). For brain samples, the cerebral cortex was dissected away from the forebrain, and the meninges were peeled off with fine forceps. For liver samples, peripheral regions of each lobe were utilized to avoid the hepatic portal vein ensuring the tissue vasculature consisted primarily of sinusoidal capillaries. Whole lung lobes were utilized. Each tissue was diced with a scalpel, and enzymatically dissociated with papain (40 units/mL Worthington-3126) solution containing L-cystein (0.4 mg/mL, Sigma C 7477) and DNase (125 units/mL, Worthington LS002007) for 1.5 hours, prior to mechanical trituration in a solution containing ovomucoid (2 mg/ml, Roche 109878), DNase (125 units/mL) and BSA 91 mg/mL, Sigma A8806), to yield a cell suspension, which was recovered by centrifugation. Cell suspensions were resuspended in FACS buffer (DPBS, 0.02%BSA with propidium iodide), and endothelial cells were FACS purified based on GFP fluorescence utilizing a FACS Vantage SE sorter (Becton Dickinson) and CellQuest software. For each tissue, background GFP fluorescence was determined by FACS analysis of cell suspensions from wild-type FvB mice (Charles River), and dead cells were eliminated by high propidium iodide fluorescence. Forward scatter and side scatter analyses were also used as gates to limit the sorting to single live cells. In each case, 2 rounds of sorting were performed for maximal purity, based on reanalysis.

For cell culture experiments, endothelial cells were purified from the cerebral cortex of adult mice through modification of methods described elsewhere (2, 3). Those modifications include: using sequential panning steps with rat anti-CD45 (serotec, MCA1031GA) coated dishes to deplete microglia, followed by selection of endothelial cells on a rat anti-CD31 (BD PharMingen, 553370) coated dish. The endothelial cells were recovered by trypsinization, and plated on CIV (BD, 72441) coated coverslips and grown in a neurobasal based medium (Invitrogen 21103), containing SATO (100 μg/ml transferrin Sigma T1147; 100 μg/ml BSA Sigma A4161; 60 ng/ml progesterone Sigma P8782; 16 μg/ml putrescine Sigma P5780, 40 ng/ml sodium selenite Sigma S5261), insulin (5 μg/ml, Sigma I-6634), pyruvate (1 mM Invitrogen 11360–070), penicillin (100 U/ml Invitrogen 15140–122), streptomycin (100 U/ml Invitrogen 15140–122), glutamine (2 mM Invitrogen 25030), N-acetyl-L-cysteine (5 μg/ml Sigma A8199), T3 (40 ng/ml Sigma T6397), forskolin (4.2 μg/ml Sigma F6886), bFGF (50 ng/ml, peprotech), and 0.5% serum. The cells were grown for 2 weeks, and 0.25 μg/ml of recombinant Wnt 7a (R&D systems) or vehicle control was added 15 h before mRNA isolation.

GeneChip Experiments. Purification of RNA, generation of biotinylated cRNA, subsequent hybridization to Affymetrix Mouse Genome 430 2.0 Arrays and raw image analysis with Affymetrix GCOS 1.3 software was performed as previously described (2). The Significance Analysis of Microarrays (SAM) method was used to determine genes that were significantly different between cell populations.

1. Huettner JE, Baughman RW (1986) Primary culture of identified neurons from the visual cortex of postnatal rats. *J Neurosci* 6:3044–60.
2. Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA, Thompson WJ, Barres BA (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: A new resource for understanding brain development and function. *J Neurosci* 28:264–278.
3. Mi H, Haeberle H, Barres BA (2001) Induction of astrocyte differentiation by endothelial cells. *J Neurosci* 21:1538–1547.

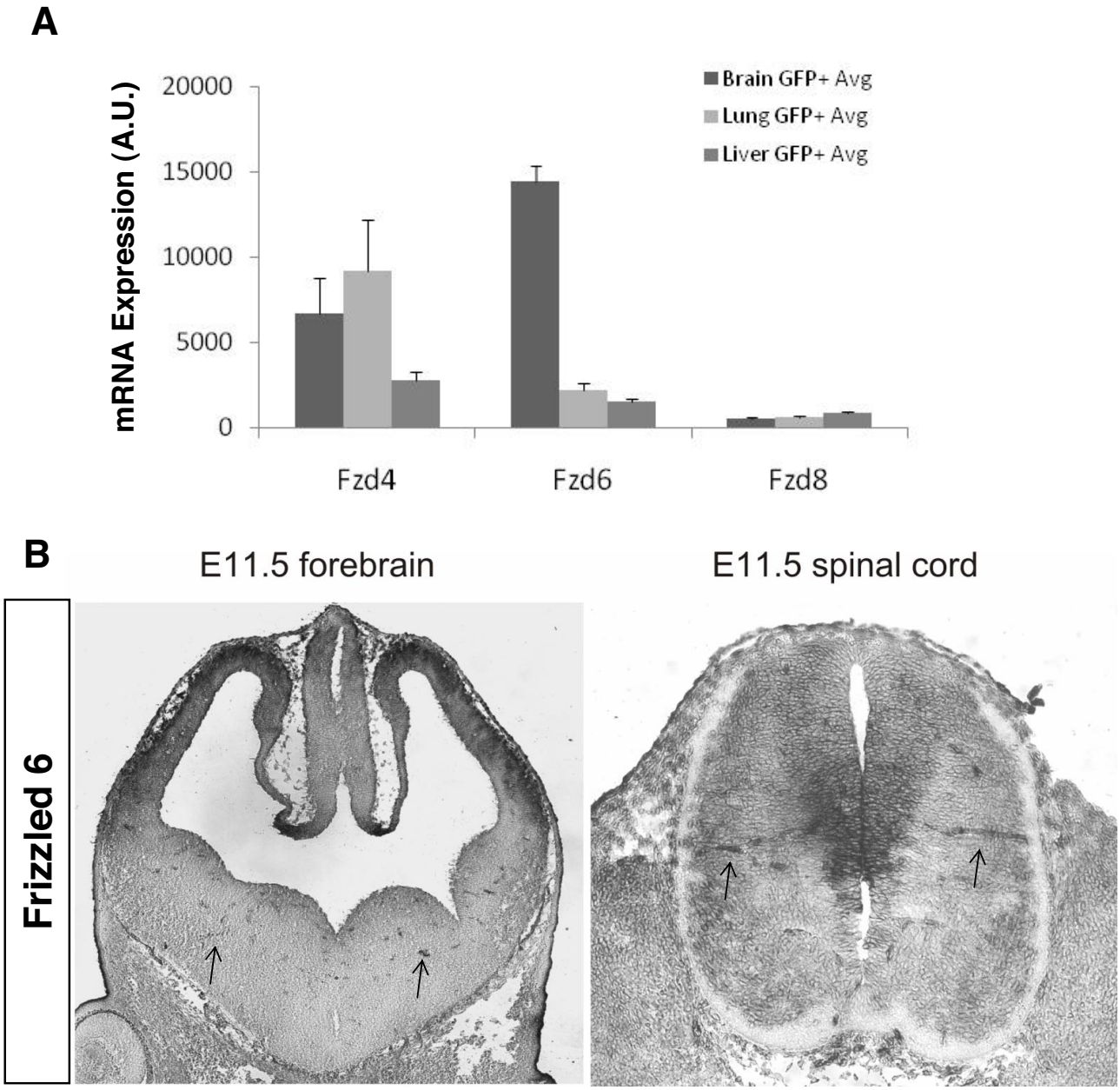

Fig. S1. Expression of Frizzleds in CNS endothelial cells. (*A*) GeneChip analysis of Frizzled receptors in purified endothelial cells. FACS analysis was used to purify endothelial cells from the brain, liver, and lung of Tie2GFP mice, and gene expression was analyzed using Affymetrix microarray analysis. Expression of Fzd4, Fzd6, and Fzd8 was observed in CNS endothelial cells, with Fzd6 enriched in CNS endothelial cell compared with the liver and lung samples. (*B*) In situ hybridizations demonstrating Fzd6 expression in CNS endothelial cells in the E12.5 mouse forebrain and spinal cord. Fzd6 is expressed in the vascular cells (black arrows) in the neural tissue.

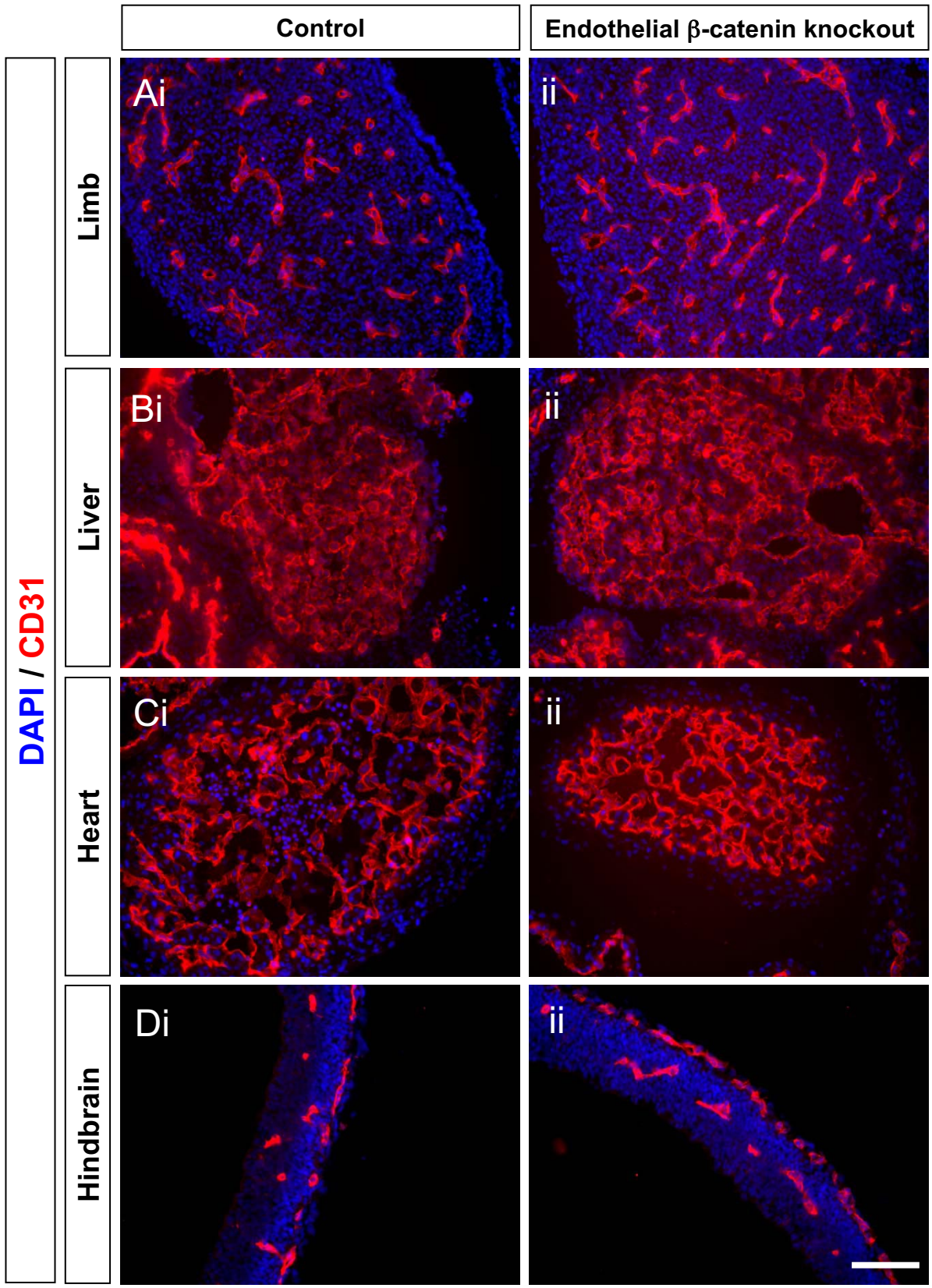

Fig. S2. Normal vasculature in non-neural tissue and hindbrain of endothelial-specific β-catenin mutants. (*A-D*) Tissue sections of the developing limbs (*A*), liver (*B*), heart (*C*), and hindbrain (*D*) of E11.5 (*ii*) endothelial-specific β-catenin mutants and (*i*) litter-mate controls were stained with the nuclear marker DAPI (blue); the vascular marker CD31 (red) demonstrating that β-catenin is not required for blood vessel formation in these tissues. (Scale bar, 100 μm.)

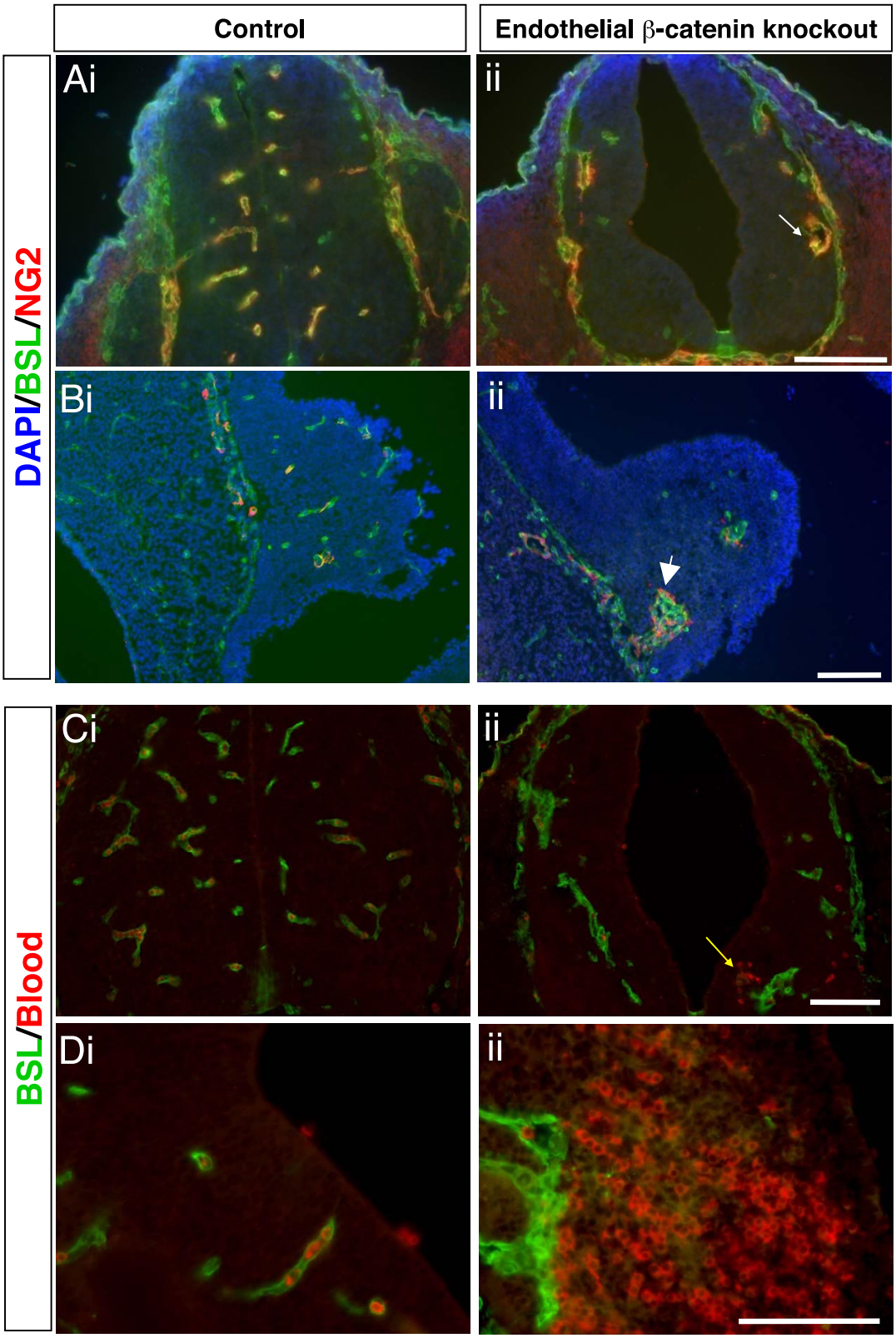

Fig. S3. Cellular analysis of vascular malformations in endothelial specific β-catenin mutants. (A) Cross sections of the developing neural tubes, and (*B*) sagittal sections of the forebrain of E11.5 (*ii*) endothelial-specific β-catenin mutants and (*i*) litter-mate controls were stained with the nuclear marker DAPI (blue), the vascular marker BSL (green), and an antibody against the pericyte marker NG2 (red). Vascular malformations in the endothelial-specific β-catenin mutants consisted of aggregates of endothelial cells surrounded by pericytes. The aggregates could be found to form disorganized balls of cells with no discernable lumen (white arrow head in *Bii*) or layers of cells surrounding a lumen carrying blood (white arrow in *Aii*). (Scale bar, 100 μm.) (*C-D*) Cross sections of developing neural tubes of E11.5 (*ii*) endothelial specific β-catenin mutants and (*i*) litter-mate controls were stained with the vascular marker BSL (green) and anti-serum directed against PARD6 which stains blood cells (red). Vascular malformations in the β-catenin mutants were associated with small leakage of blood cells (yellow arrow in *Cii*) and large hemorrhages (*Dii*). (Scale bar, 100 μm.)

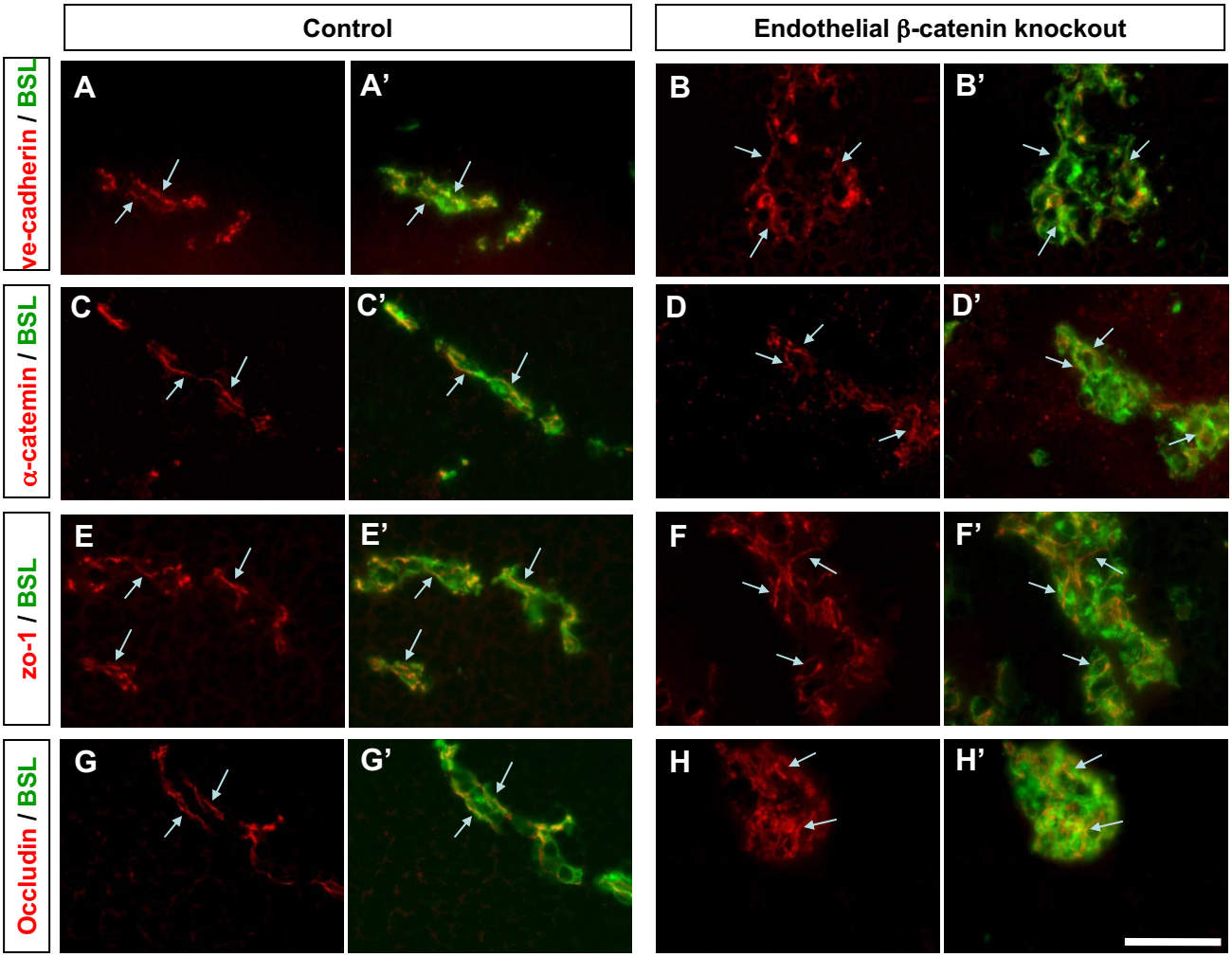

Fig. S4. Expression of adherens junctions and tight junctions in endothelial-specific β-catenin mutants. Sagittal sections of the spinal cord of E11.5 endothelial-specific β-catenin mutants (*B*, *D*, *F*, and *H*) and litter-mate controls (*A*, *C*, *E*, and *G*) were stained with antibodies directed against ve-cadherin (*A* and *B*), α-catenin (*C* and *D*), zo-1 (*E* and *F*), and occludin (*G* and *H*) and double labeled with the vascular marker BSL (green in merge images *A'*-*H'*). Each adherens junction (ve-cadherin and α-catenin) and tight junction component (zo-1 and occludin) was observed at cellular junctions in both normal vessels in the control animals and in the vascular malformations in the endothelial-specific β-catenin mutants. White arrows point to cell junctions. (Scale bar, 50 μm.)

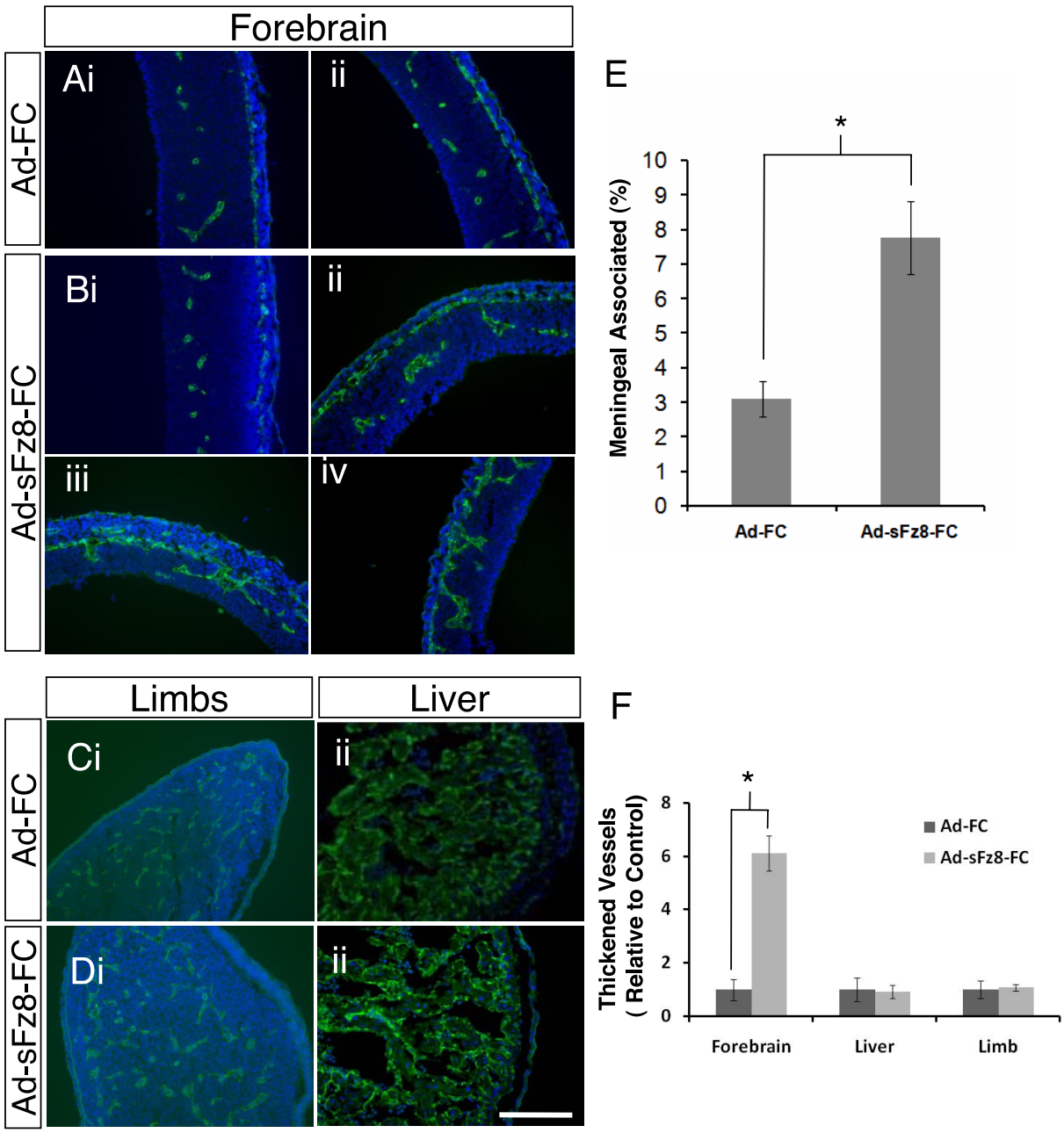

Fig. S5. Inhibition of Wnt signaling leads to CNS-specific vascular defects. (*A-D*) Pregnant female C57bl6 mice were administered IV an adenovirus encoding a (*B*) soluble Frizzled 8 receptor-FC fusion (Ad-sFz8-FC) or (*A*) a control FC (Ad-FC) at 9 days of gestation. Cross sections of E12.5 forebrain were stained with the nuclear stain DAPI (blue) and the vascular stain BSL (green). Normal tissue capillary beds were observed throughout the forebrain in embryos treated with Ad-FC (*A i* and *ii*), whereas CNS vascular defects were observed in the forebrains of embryos treated with Ad-sFz8-FC (*B*). The tissue vasculature of Ad-sFz8-FC treated animals ranged from normal (*Bi*), to small aggregates of endothelial cells (*Bii*), to large malformations that were stuck to the meningeal surface (*B iii* and *iv*). Apparently normal vasculature was found in non-neural tissues including the limbs (*Ci* and *Di*) and the liver (*Cii* and *Dii*) in animals treated with both viruses. (Scale bar, 100 μm.) (*E*) The vasculature of embryos treated with Ad-FC and Ad-sFz8-FC were quantified for percent forebrain vascular length associated with the meningeal surface. Ad-sFz8-FC treated embryos exhibited blood vessels with an increase in association with the meningeal surface. Error bars represent standard error of the mean. *$<$0.002 as analyzed by a two tailed standard T-test not assuming normal variance. (*F*) The vasculature of embryos treated with Ad-FC and Ad-sFz8-FC were quantified for percent vascular length in forebrain, liver, and limbs with abnormally thick vessels identified as vascular segments thicker than 3 cell bodies across. The values were graphed relative to the average of the Ad-FC. Ad-sFz8-FC treated embryos exhibited thicker blood vessels in the forebrain but not the liver or limbs. Error bars represent standard error of the mean. *$<$0.002 as analyzed by a two tailed standard T-test not assuming normal variance.