Essential regulation of CNS angiogenesis by the orphan G protein-coupled receptor GPR124

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Methods

**Generation of mice.** GPR124 KO mice were generated by targeting the first coding exon of GPR124 and replacing it with a lacZ cassette. For the conditional GPR124 KO allele, the first coding exon was flanked by loxP sites. Successfully targeted 129Sv ES cells were injected into C57/Bl6 blastocysts to generate chimeras. Additional details are available upon request. For the Tie2-GPR124 transgenic mice, full-length GPR124 CDS was cloned into the long enhancer-containing Tie2 expression construct (T. Sato) (1). TOP-Gal Wnt signaling reporter mice were obtained from R. Nusse (Stanford University), Tie2-Cre mice from JAX. PDGFB-Cre mice from M. Fruttiger (UC London), Tie2-Cre/floxed β-catenin embryos from R. Daneman (Stanford University).

**Immunofluorescence analysis.** Embryos or adult tissues were fixed in 4% PFA for one hour, cryoprotected in 30% sucrose and embedded in OCT. Frozen sections were stained according to standard protocols. Polyclonal GPR124 antibody was raised by immunizing rabbits with N-terminal, extracellular domain (ECD) of GPR124 (AA 1-665) fused to human IgG2α Fc portion purified from transfected 293T cell supernatant. Production bleeds were affinity purified using GPR124 ECD-Fc fusion protein, followed by two rounds of negative selection against human Fc protein. Antibodies: hamster α-CD31, rabbit α-1,3-Glucose-1 (Millipore), rat α-PDGFRβ, rabbit α-VEGFR2 (eBioscience), goat α-Nrp1 (R&D Systems), goat α- EphB4 (R&D Systems), rabbit α-lacZ (ICN), rabbit α-phospho-histone H3, rabbit α-cleaved caspase-3 (Cell Signaling Technology), rabbit α-Id1 (CalBioreagents), rabbit α-giantin (Covance). Where applicable, fluorescein conjugated isolectin B4 (Vectorlabs) or Alexa 488 labeled phalloidin (Invitrogen) was added to the secondary antibody mix to mark endothelial cells and the actin cytoskeleton, respectively.

**Whole-mount β-galactosidase staining.** Embryos were fixed in 4% PFA for 20 minutes, rinsed with PBS several times and incubate in β-galactosidase staining solution (5mM KferroCN, 5mM KferriCN, 2mM MgCl2, 500 µg/ml X-gal in PBS) at 37°C for 16 hours.

**Histology.** Histological analysis was performed according to standard protocols. For PCNA staining of paraffin-embedded tissues biotinylated mouse α-PCNA (Invitrogen) was used.

**Vascular casting.** Mice were anesthetized by isoflurane inhalation, perfused with saline containing heparin (1 unit/ml) until the liver became pale, followed by 10 ml of 4% buffered paraformadehyde through an 18 gauge needle inserted in the left ventricle of the heart. Microfil was prepared by mixing 5 ml of MV-Diluent, 4 ml of Compound and 5% of MV Curing agent as per manufacturer's instructions (MV-130, Flow Tech, Inc.) and perfused through left ventricle, 10 ml for each mouse using a 18 gauge needle. Brains were collected, fixed in 4% buffered paraformadehyde at 4°C overnight, dehydrated and cleared by immersion in methyl salicylate for 24 hours. Vascular structures were observed and imaged under bright field using a Leica dissection microscope.
EM analysis. Embryonic brains were fixed in 2% gluaraldehyde/2% formaldehyde for 2 hours, washed with phosphate buffer, post-fixed with 1% osmium tetroxide for one hour, and incubated in 0.5% uranyal acetate over night at 4°C. Samples were dehydrated, washed with propylene oxide twice and embedded with Polybad/812. Samples were sectioned with a diamond knife at 70 nm, stained with 2% uranyal acetate and 1% lead citrate and examined on CM 12 Phillips electron microscope.

FACS analysis. Embryonic tissues were digested in 0.5mg/ml Liberase I (Roche)/ 0.06 U DNaseI (Worthington) in PBS containing Mg²⁺, Ca²⁺ for 20-30 minutes with frequent tituration to generate a single cell suspension. Cells were stained with hamster anti-CD31 (Millipore), rat anti-PDGFRβ (eBioscience) and rabbit anti-GPR124 and analyzed using s LSRII FACS analyzer.

Isolation of embryonic forebrain and hindbrain endothelial cells. Single cell suspensions from embryonic brain tissue were generated as described above. Endothelial cells were isolated using magnetic bead isolation (Miltenyi Biotec) with biotinylated rat α-CD31 antibody (BD Biosciences) as per manufacturer’s instructions.

Generation of GPR124 over-expressing/GPR124 knockdown bEND3 cells. GPR124 knockdown was achieved by infection of bEND3 cells with shRNA containing lentivirus targeting GPR124 (Sigma) per manufacturer’s instructions. Four different shRNA designs were used, with resultant knockdown efficiencies of greater than 95% for all four. For GPR124 overexpression, full-length GPR124 expressing adenovirus Ad GPR124 was generated according to standard protocols using a high-capacity adenovirus vector. For migration assays, bEND3 cells were infected at a multiplicity of infection (MOI) of 100. For the dominant negative (DN) Cdc42 experiments, bEND3 cells were co-infected with Ad GPR124, Ad tTA (both at MOI of 100) and 125 µl of crude Ad TRE-Cdc42DN (N17) (kind gift of Daniel Kalman) or Ad GFP control virus.

Endothelial cell migration assays. For Boyden chamber assays, the BD Biosciences endothelial migration system was used according to manufacturer’s instructions.

For microfluidic assays (2), microfluidic devices were fabricated using standard soft lithography and micromolding techniques at the Stanford Microfluidics Foundry clean room. Briefly, fabrication of master molds was performed by patterning two layers of negative SU8 photoresist (MicroChem, Newil typeon, MA) on a silicon wafer. The devices were manufactured by pouring liquid polydimethyl siloxane (PDMS) (Sylgard 184 Silicone elastomer kit) into the silicon mold and baking in the oven for ~1 hr. Inlets and outlets to the cell culture chamber and reagent channels were punched using sharpened needles and the devices were bonded irreversibly to glass slides by treating both the surfaces of glass and PDMS with plasma cleaner. The cell culture chamber was adsorbed with fibronectin overnight (10 µg/ml) and rinsed three times with buffer.

Cells (~500,000 cells/mL) suspended in starvation medium were injected into the cell culture chamber and allowed to adhere for ~3 h. The inlet and outlet of the cell culture chamber were plugged, and tubings (Upchurch Scientific, Oak Harbor, WA) were
inserted into the inlets of the reagent channels and connected to 100 ul syringes (Hamilton, Reno, NV) mounted on a syringe pump (World Precision Instruments, Sarasota, FL). By supplying conditioned medium to the source reagent channel and starvation medium to the sink reagent channel (flow rate = 10 nl/min), a stable linear-gradient of conditioning molecules is formed across the cell culture chamber. Previous experiments utilizing this device and fluorescently-labeled molecules demonstrated that the gradient is formed within 50 min and then remains stable for the duration of the 750-min experiment (1).

For tracking the migrating cells, a time-lapse videomicroscopy system was used (Zeiss Axiocvert 200 microscope, Carl Zeiss AB, Stockholm, Sweden), consisting of an inverted microscope, a motorized X/Y stage and an incubator equipped with systems for temperature and CO₂ control, set to 37 °C and 5% CO₂ in air. Cells were imaged every 30 minutes for 750 min using a phase contrast 10X objective and AxioVision time-lapse software (Zeiss). For each condition, three independent trials were performed with ~30 migratory cell tracks recorded per trial. Cell-tracking analysis was performed using ImageJ software with MtrackJ plug-in. Cell translocation was determined by tracking the center of each cell nucleus over time; migratory cells were defined as those whose nucleus moved more than 50 µm from its initial position during the 750-min experiment. The average center of mass for all cell tracks is marked with a yellow cross.

Chemotactic index of a cell was defined to be the ratio of the net distance a cell migrates in the direction of the gradient (i.e., across the width of the device perpendicular to the source and sink channels) to the total length of the cell track (i.e., total distance traveled). Therefore, random migration yields a chemotactic index of zero while migration in a straight line directly up the gradient yields a chemotactic index of one. Chemotactic indices are shown on a scatter plot overlaid with a box plot; center line marks the median, the upper box marks the upper quartile, and the lower box marks the lower quartile. Due to the non-Gaussian distribution of the individual chemotactic indices, Kolmogorov-Smirnov (KS) tests were used to compare the probable statistical significance between cell populations using sampled chemotactic indices for each experimental condition. P-values are reported in Supplemental Information. Directional histograms of migrating cells were generated using Chemotaxis and Migration Tool freeware (Ibidi, Munich, Germany), which generates a smoothed histogram for the number of cells migrating within a specific angular trajectory (angular bins of 10 degrees).

For generation of embryonic cortical cell-conditioned medium (CM), E12.5 to E13.5 C57/Bl6 forebrain or hindbrain were isolated and digested to generate a single cell suspension (see above). Cells were grown until confluency in complete medium, which was then switched to DMEM/0.1%BSA. CM was harvested 48 hours later. Protein concentration in the forebrain and hindbrain CM was 10mg/ml. Where applicable, recombinant soluble VEGFR1 ectodomain (sVEGFR1/sFlt1) (400 ng/ml) (3) was added to the CorCM.

**Sprout formation assay.** Endothelial sprout formation was studied in a slightly modified version of the microfluidic device used for chemotaxis assays. These devices consisted of
two culture chambers connected by micro-capillaries (width ~30 µm, height ~25 µm). Endothelial cells were injected into one of the culture chambers and allowed to form a confluent 2D monolayer. An extracellular matrix (1.9 mg/ml collagen I blended with 50 µg/ml fibronectin) without cells was injected into the second culture chamber, and the entire device was perfused with a stable gradient of conditioned forebrain or hindbrain media. Sprout formation and/or cell migration through the micro-capillaries was observed by time-lapse phase contrast microscopy over three days. Sprout length was determined using ImageJ (NIH freeware) for at least three independent devices at each condition. Sprout directionality was defined as the ratio of the sprout length elongated towards the gradient divided by the total sprout length.

Sprouts within collagen gels were fixed by injecting 4% paraformaldehyde into the source and sink channels and incubating at 4°C for ~1 hr. The samples were washed at least four times by injecting PBS into the source and sink channels. The cells were blocked with 10% normal goat serum in PBST (0.3% Triton X-100 in PBS solution) for ~3 hrs. For confocal microscopy, demonstration of lumen formation, the actin cytoskeleton was stained overnight at 4°C using Alexa Fluor 555-conjugated phalloidin (Invitrogen). Samples were washed with PBS at least four times and then incubated with PBS at 4°C for ~12 hrs prior to imaging. A laser scanning confocal inverted fluorescence microscope (Zeiss) was used to acquire images. Sprout data are reported as averages ± standard deviation. For each condition reported, at least n=3-5 independent experiments were performed. One-tailed, non-paired, Student T-test was used to determine the statistical significance of differences between pairs of conditions.

**Scratch assay.** bEnd3 cells +/- GPR124 expression were grown to confluency in chamber slides. After reaching confluency, bEnd3 cells were serum starved for 4 hours (DMEM/10%FCS). Using a pipet tip a scratch was applied medially across the confluent cell surface area generating an acellular zone. Cells were stimulated with complete medium (DMEM/10% FCS) for 24 hours. Cells were then fixed in 4% PFA and analyzed for polarization and cytoskeletal organization by giantin and phalloidin staining, respectively.

**Endothelial cell proliferation assay.** Cell titer 96AQ cell proliferation assay (Promega) was used according to the manufacturer’s instructions.

**Quantitative PCR.** The Quantitect system (Qiagen) was used for qPCR analysis. Assays were performed on a BioRad iCycler.

**Northern blot analysis.** Northern blot analysis using a 3’ UTR probe was performed according to standard protocols.

**Southern blot analysis.** Southern blot analysis was performed according to standard protocols. The Southern blot probe was designed to sit just outside of the 3’ homology arm.
Western blot analysis. Western blot analysis was performed according to standard protocols. 50 µg of E10.5 whole cell extract were loaded. GPR124 protein was detected using a polyclonal rabbit antibody raised against a peptide corresponding to the C-terminus of GPR124 (AA 1255-1276).

Supporting Figure Legends

Fig. S1. Embryonic expression of GPR124. Domain structure of GPR124/TEM5. GPR124/TEM5 encodes a 1331 amino-acid open reading frame with a 760 amino acid N-terminal extracellular domain (A). Endothelial expression of GPR124 in embryonic CNS, GPR124 immunofluorescence (IF) colocalizes with the endothelial marker CD31 in vasculature of telencephalon (B) to (D), midbrain (E) to (G) and neural tube (H) to (J). Fluorescence-activated cell sorting (FACS) analysis of primary cells isolated from E12.5 organs demonstrated that GPR124 was expressed in the endothelium (CD31+) of many embryonic organs, ranging from ~85% in forebrain and midbrain to 15-43% in liver, heart and kidney (K). Similarly, FACS analysis revealed that GPR124 expression was widely prevalent amongst embryonic pericytes (PDGFRβ+) with highest expression in brain and kidney amongst tissues examined (L). GPS, GPCR proteolysis site; HormR, hormone binding domain; Ig, immunoglobulin domain; LRR, leucine-rich repeat; LRRCT, C-terminal leucine-rich repeat; PDZB, PDZ binding domain; RGD, Arginine-Glycine-Aspartic acid motif; 7TM, seven-transmembrane region.

Fig. S2. Embryonic expression of GPR124 in non-CNS organs. Microvascular expression of GPR124 is detected in E12.5 liver (A-C), heart (D-F) and lung (G-I). Co-immunofluorescence staining with CD31 is shown. Note also the GPR124-positive endothelium in the dorsal aorta and the abundant non-vascular GPR124 expression in the epithelia of lung and oesophagus and the mesenchyme surrounding the dorsal aorta. da, dorsal aorta, lu, lung, oe, oesophagus.

Fig. S3. Adult CNS expression of GPR124. GPR124 is uniformly expressed in the microvasculature of the adult mouse brain cortex. Co-localization with endothelial marker CD31 is shown (A-C). Confocal microscopy analysis demonstrated GPR124 expression in CD31+ endothelial cells (E, G, arrow) and PDGFRβ+ pericytes (F, asterisk). Control staining for CD31 and PDGFRβ (D), single stain for GPR124 (G) and triple stain (H) are included. Similar images of adult mouse brain retina are shown (I-K).

Fig. S4. Adult expression of GPR124 in non-CNS organs. In the kidney glomerulus (A), the endocrine pancreas (B) and during corpus luteum angiogenesis (C and D), GPR124 co-localizes with PDGFRβ+ pericytes (purple color in A and B = red/GPR124 + blue/PDGFRβ, yellow color in D = green/GPR124 + red/PDGFRβ). No GPR124 co-localization is seen with CD31+ endothelial cells in A-C (lack of yellow indicates lack of costaining of green/GPR124 + red/CD31).

Fig. S5. Targeting of the GPR124 locus. Targeting strategy. A lacZ expression cassette followed by a neomycin selection marker was inserted in the 5’ UTR of the GPR124 gene. EcoRV sites and 3’ probe location used for Southern blot genotyping of ES cells are indicated (A). Southern blot analysis to identify correctly targeted ES cell clones. Insertion of the lacZ/neo cassette yields a 4.5 kb recombinant EcoRV fragment in addition to the 9kb wild type fragment (B). Analysis of embryonic GPR124 expression using the inserted lacZ reporter gene. Whole-mount lacZ staining of E10.5 GPR124+/− embryos revealed vascular GPR124 expression in the dorsal aorta, the intersomitic
vessels, the cardiac outflow tract, the developing CNS and the head mesenchyme (C). Histological sections of lacZ stained embryos demonstrated vascular GPR124 expression in the dorsal aorta (D) and the neural tube (E). CNS, central nervous system; DA, dorsal aorta; HM, head mesenchyme; ISV, intersomitic vessels; OT, outflow tract.

**Fig. S6. Validation of the GPR124 lacZ knock-in allele.** Insertion of the lacZ cassette into the 5’UTR of the GPR124 gene resulted in a null allele. Absence of GPR124 mRNA in GPR124<sup>−/−</sup> embryos is confirmed by quantitative RT-PCR (A) and Northern blot analysis (B). Absence of GPR124 protein is demonstrated by Western blot using a rabbit polyclonal antisera raised against a peptide corresponding to the C-terminus of GPR124 (C) and immunofluorescence staining with rabbit polyclonal antibody recognizing the GPR124 extracellular domain (ECD). Single staining for GPR124 (D and E) and co-IF staining for CD31 and GPR124 (F and G) are shown.

**Fig. S7. Embryonic lethality from CNS hemorrhaging in GPR124<sup>−/−</sup> embryos.** Table of genotypes from GPR124<sup>+/−</sup> intercrosses. Deletion of GPR124 results in embryonic lethality starting at E15.5 (A). Forebrain hemorrhage in GPR124<sup>−/−</sup> embryos at E12.5 (arrow) becomes progressively more pronounced as development proceeds and results in embryonic lethality starting at E15.5. Transient neural tube hemorrhage is also present at E12.5 (*). At E17.5 omphalocele is also detectable (B).

**Fig. S8. CNS hemorrhaging in E11.5 GPR124<sup>−/−</sup> embryos.** Hemorrhaging in telencephalon (A and B, arrows) and neural tube (A to D, asterisks) is first observed in E11.5 GPR124<sup>−/−</sup> embryos. Histological analysis displays prominent hemorrhaging in the forebrain ganglionic eminences (E, F, arrows) and the ventral neural tube (G, H, arrows).

**Fig. S9. Vascular patterning controls in GPR124<sup>−/−</sup> embryos.** IF staining for CD31 (green) demonstrated vascular patterning defects in the ventral half of the neural tube of E11.5 GPR124<sup>−/−</sup> embryos, while overall trunk vascular patterning in the GPR124<sup>−/−</sup> embryos was indistinguishable from wild type controls (A, B, transverse section) except for the ventral neural tube (C, D). Vascular patterning in the hindbrain was unaffected in GPR124<sup>−/−</sup> embryos (E, F, transverse section, E14.5). V, ventricle. White boxes in (A, B) indicate the region enlarged in (C, D).

**Fig. S10. Normal vascular patterning in non-CNS organs in GPR124<sup>−/−</sup> embryos.** Microvascular beds in embryonic heart (A, B), lung (C, D), liver (E, F) and intestine (G, H) of E12.5 GPR124<sup>−/−</sup> embryos as analyzed by CD31 IF staining are indistinguishable from wild type littermate controls.

**Fig. S11. CNS endothelial proliferation, apoptosis and VEGF receptor expression are unperturbed in GPR124<sup>−/−</sup> embryos.** Endothelial cell proliferation and apoptosis, as analyzed by IF staining for phospho-histone H3 (A, B) and cleaved caspase 3 (C, D), respectively, are unchanged in the E12.5 GPR124<sup>−/−</sup> telencephalon compared to wild type controls. Pial-ventricular direction is indicated. VEGF co-receptor Nrp1 (E and F) and VEGFR2 (G and H) are expressed normally in E12.5 GPR124<sup>−/−</sup> forebrain endothelial cells. PNVP=perineural venous plexus.
**Fig. S12. Tie2-Cre-mediated GPR124 deletion.** Vascular deletion of GPR124 using a floxed GPR124 allele crossed to a Tie2-Cre driver recapitulated the global GPR124 deletion phenotype. Forebrain hemorrhaging (A and B) and the formation of perineural vascular plexus-associated glomeruloid vascular malformations (C and D) are detected in E14.5 GPR124<sup>flox/−</sup>; Tie2-Cre embryos.

**Fig. S13. GPR124 expression in Tie2-GPR124 transgenic mice.** Analysis of GPR124 expression by co-IF staining with CD31. Endogenous adult brain endothelial cell expression of GPR124 is superimposed by transgenic Tie2-GPR124 expression (A-D), while heart (E-H) and liver (I-J), as examples of tissues where GPR124 is not normally expressed in the adult vasculature, exhibit marked ectopic vascular GPR124 expression in the Tie2-GPR124 adult mice.

**Fig. S14. Vascular patterning in Tie2-GPR124 transgenic mice.** IF staining for CD31 revealed hypervascularization in the cortex of Tie2-GPR124 transgenic animals (A and B), while vascular patterns in heart (C and D), kidney (E and F), spleen (G and H) and liver (I and J) are unaltered.

**Fig. S15. CNS vascular malformations in Tie2-GPR124 transgenic mice.** Frequency of CNS vascular malformations in Tie2-GPR124 transgenic mice (A). Hyperplastic vessels in Tie2-GPR124 brains are invested with PDGFRβ<sup>+</sup> pericytes (B and C). Brain calcifications are frequently associated with vascular malformation in the Tie2-GPR124 transgenic brains (E).

**Fig. S16. Shear-minimized microfluidic migration chamber and modulation of GPR124 expression in bEND3 cells.** Schematic representation of the microfluidic migration chamber used in Fig. 4. A stable, shear-minimized 63% to 37% gradient of conditioned medium is created across the cell culture chamber. (A). FACS histogram of GPR124 expression. Wild type expression levels of GPR124 in control adenovirus- (Ad Fc, expressing an antibody IgG2a Fc fragment) infected bEND3 cells (blue) can be augmented by increasing multiplicities of infection with Ad GPR124 (encoding full-length mouse GPR124 cDNA) (orange and green). Conversely, GPR124 levels can be suppressed by infection with lentivirus expressing a short-hairpin inhibitor RNA (shRNA) targeting GPR124 (red). Values in parentheses indicate multiplicities of infection. FACS staining with the anti-GPR124 ectodomain antisera from Fig. S1 is shown. (B). Four different GPR124 shRNA designs with GPR124 knockdown efficiencies of greater than 95% were analyzed by FACS as in (A), above. (C). Lack of GPR124 expression has no effect on bEND3 cell proliferation. MTS cell proliferation assay was performed in 50% and 100% embryonic forebrain cortical cell conditioned medium (CorCM) (D).

**Fig. S17. P-values for microfluidic bEND3 cell migration assays.** Table of P-values for the microfluidic bEND3 cell migration assays.
**Fig. S18. Dual chamber microfluidic sprout formation assay.** Endothelial cells are cultured in the left chamber at initiation of assay. Microcapillaries (box) allow migration into the right chamber filled with a collagen/fibronectin matrix. Chemoattractant gradient is present on the right side. After 3 days sprout formation and turning in the direction of the chemoattractant gradient can be observed and quantitated.

**Fig. S19. GPR124−/− endothelial cells assume tip cell positions in forebrain vascular sprouts in chimeric embryos.** GPR124−/− (i.e. GPR124lacZ/lacZ) ES cell lines were established from blastocysts derived from heterozygous GPR124 intercrosses and microinjected into wild-type blastocysts to derive chimeric embryos. Completely GPR124−/−ES cell derived embryos recapitulate the CNS hemorrhaging phenotype (A, control embryo in B). GPR124−/−ES cell-derived CNS endothelial cell were identified by IF staining for β-galactosidase (C) and by co-localization with the endothelial marker isolecitin B4 (IB4) (D). A GPR124−/− CNS endothelial cell assuming the tip cell position in a forebrain vascular sprout in a GPR124−/−/wild type chimera is marked by the arrow (E), indicating that GPR124 ko does not ablate the assumption of a tip cell identity.

**Fig. S20. GPR124 epistasis analyses.** The GPR124 CNS vascularization knockout phenotype is strikingly similar to those recently reported in the Wnt7A/B and endothelial β-catenin KO studies, with identical location and appearance of CNS vascular malformations and the endothelial down regulation of the BBB marker Glut-1. Thus, we sought to determine the epistatic relationship between Wnt7/b-catenin signaling and GPR124 in CNS endothelial cells. β-catenin signaling activity, as analyzed using the TOP-Gal reporter strain, was clearly detectable in the endothelial cells of GPR124 KO (GPR124flo/flo; CMV-Cre; TOP-Gal) CNS vascular malformations (A, B). Conversely, GPR124 expression in CNS endothelial cells was found to be unaltered in β-catenin mutants (β-cateninflo/flo; Tie2-Cre) when compared to heterozygous controls (C, D). Similarly, expression of the transcription factor Id1 with the Id1/Id3 double KO phenotype characterized by CNS vascular malformations reminiscent of the GPR124 KO phenotype was unchanged in GPR124−/− CNS endothelial cells (E, F).
Fig. S1. Embryonic expression of GPR124 in endothelium and pericytes.
Fig. S2. Embryonic expression of GPR124 in non-CNS organs

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Fig. S3. Adult CNS expression of GPR124
Fig. S4. Adult expression of GPR124 in non-CNS organs

- **A**: Kidney glomerulus
- **B**: Endocrine pancreas
- **C**: Corpus luteum
**Fig. S5. Targeting of the GPR124 locus**

A diagram illustrating the targeting of the GPR124 locus using a recombinant targeting vector. The diagram includes restriction enzyme sites (EcoRV) and genomic regions such as 5'UTR, CDS, SDKlacZpA, and PGKneopA. An arrow indicates the site of integration into the genome, resulting in a recombinant locus.

**B**

An ES cell Southern blot showing bands at 9 kb and 4.5 kb for different genotypes (+/-, +/-, +/+).

**C**

Imagery of whole-mount embryo showing CNS, HM, OT, DA, ISV, and the dorsal aorta (DA).

**D**

Imagery of dorsal aorta with histological staining.

**E**

Imagery of neural tube with histological staining.
Fig. S6. Validation of the GPR124\(^{\text{lacZ}}\) knock-in allele

A

qRT-PCR E12.5

GPR124 RNA relative to β-actin

B

GPR124 Northern blot E12.5

C

Western blot
anti-GPR124 C-terminal antibody

D

E10.5 embryo

-5.5kb

293T cells

GPR124 transfection

E

F

G

IF E11.5 neural tube; anti-GPR124 ECD antibody

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Fig. S7. Embryonic lethality from CNS hemorrhaging in GPR124<sup>−/−</sup> embryos

### Table

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### Legend

- **GPR124<sup>++</sup>**
- **GPR124<sup>−/−</sup>**

**E12.5**

**E14.5**

**E15.5**

**E17.5**
Fig. S8. CNS hemorrhaging in E11.5 GPR124−/− embryos

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E11.5
Fig. S9. Vascular patterning controls in GPR124⁻/⁻ embryos
Fig. S10. Normal vascular patterning in non-CNS organs in GPR124^{+/−}-embryos

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The table above shows the normal vascular patterning in various non-CNS organs in GPR124^{+/−}-embryos. The images illustrate the vascular structures in the heart, lung, liver, and intestine, comparing wild-type (GPR124^{+/+}) and mutant (GPR124^{−/−}) conditions.
Fig. S11. CNS endothelial cell proliferation, apoptosis and VEGF receptor expression are unperturbed in GPR124−/− embryos.
Fig. S12. Tie2-Cre-mediated GPR124 deletion

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<thead>
<tr>
<th></th>
<th>GPR124_{flox/-}</th>
<th>GPR124_{flox/-}, Tie2-Cre</th>
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<tbody>
<tr>
<td>E14.5</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>CD31</td>
<td>C</td>
<td>D</td>
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PNVP

E14.5
Fig. S13. GPR124 expression in Tie2-GPR124 transgenic mice

<table>
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<tr>
<th></th>
<th>brain</th>
<th>heart</th>
<th>liver</th>
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<tbody>
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<td>A</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>E</td>
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<tr>
<td>F</td>
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<td>I</td>
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CD31/GPR124
Fig. S14: Vascular patterning in Tie2-GPR124 transgenic mice

<table>
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<tr>
<th>brain</th>
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<th>kidney</th>
<th>spleen</th>
<th>liver</th>
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<td>E</td>
<td>G</td>
<td>I</td>
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<tr>
<td>B</td>
<td>D</td>
<td>F</td>
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<td>J</td>
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CD31
Fig. S15. CNS vascular malformations in Tie2-GPR124 transgenic mice

<table>
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<tr>
<th>Age</th>
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<tbody>
<tr>
<td>4 weeks</td>
<td>1/2</td>
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<tr>
<td>3 months</td>
<td>1/3</td>
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<tr>
<td>6 months</td>
<td>3/5</td>
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<tr>
<td>12 months</td>
<td>7/10</td>
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A

B

C

D

E

wild type

Tie2-GPR124

CD31/PDGFRβ

H&E
Fig. S16. Shear-free microfluidic migration chamber and modulation of GPR124 expression in bEND3 cells

A. Diagram of the microfluidic migration chamber with source and sink channels.

B. Flow cytometry analysis of GPR124 expression in different conditions.

C. Western blotting analysis showing GPR124 expression levels.

D. Bar graph showing relative bEND3 cell proliferation under different conditions.
Fig. S17. P-values for microfluidic bEND3 cell migration assay

<table>
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<tr>
<th>KS test P values</th>
<th>GPR124+ Forebrain Gradient</th>
<th>GPR124+ Forebrain Uniform</th>
<th>GPR124 shRNA Forebrain Gradient</th>
<th>GPR124 shRNA Forebrain Uniform</th>
<th>GPR124+/Gradient sFlt1</th>
<th>GPR124+/Gradient 293T CM</th>
<th>GPR124+/Gradient Hindbrain CM</th>
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<tbody>
<tr>
<td>GPR124+ Forebrain Gradient</td>
<td>N/A</td>
<td>0.001</td>
<td>0.001</td>
<td>0.74</td>
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<tr>
<td>GPR124+ Forebrain Uniform</td>
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<td>0.87</td>
<td>0.77</td>
<td>0.001</td>
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<td>0.001</td>
<td>0.43</td>
<td>0.22</td>
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<tr>
<td>GPR124 shRNA Forebrain Uniform</td>
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<td>0.001</td>
<td>0.79</td>
<td>0.15</td>
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<tr>
<td>GPR124+/Gradient sFlt1</td>
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<td>0.001</td>
<td>0.001</td>
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<tr>
<td>GPR124+/Gradient 293T CM</td>
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<td>GPR124+/Gradient Hindbrain CM</td>
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<td>N/A</td>
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</table>
At initiation of assay, endothelial cells are cultured on the left side chamber. Microcapillaries (box) allow cell migration into the right side chamber filled with a collagen/fibronectin matrix and a soluble gradient of conditioned medium.

After 3 days, collective cell migration and sprout formation within the matrix is observed using phase contrast, inverted fluorescence, and confocal microscopy.
Fig. S19. GPR124<sup>-/-</sup> endothelial cells assume tip cell positions in forebrain vascular sprouts in chimeric embryos.
Fig. S20. GPR124 epistasis analyses

The GPR124 CNS vascularization knockout phenotype is strikingly similar to those recently reported in the Wnt7A/B and endothelial β-catenin KO studies, with identical location and appearance of CNS vascular malformations and the endothelial down regulation of the BBB marker Glut-1. Thus, we sought to determine the epistatic relationship between Wnt7/β-catenin signaling and GPR124 in CNS endothelial cells. β-catenin signaling activity, as analyzed using the TOP-Gal reporter strain (anti-β-gal), was clearly detectable in the endothelial cells of GPR124 KO (GPR124^{fl/fl}; CMV-Cre; TOP-Gal) CNS vascular malformations, indicating that Wnt signaling is not altered by GPR124 deficiency (A, B). Conversely, GPR124 expression in CNS endothelial cells was found to be unaltered in β-catenin mutants (β-catenin^{fl/fl}; Tie2-Cre) when compared to heterozygous controls (C, D). Similarly, expression of the transcription factor Id1 with the Id1/Id3 double KO phenotype characterized by CNS vascular malformations reminiscent of the GPR124 KO phenotype was unchanged in GPR124/- CNS endothelial cells (E, F). These studies do not support an epistatic relationship between GPR124 and Wnt signaling or Id1. Compound heterozygote analysis for GPR124 and integrin β8 has also been negative.