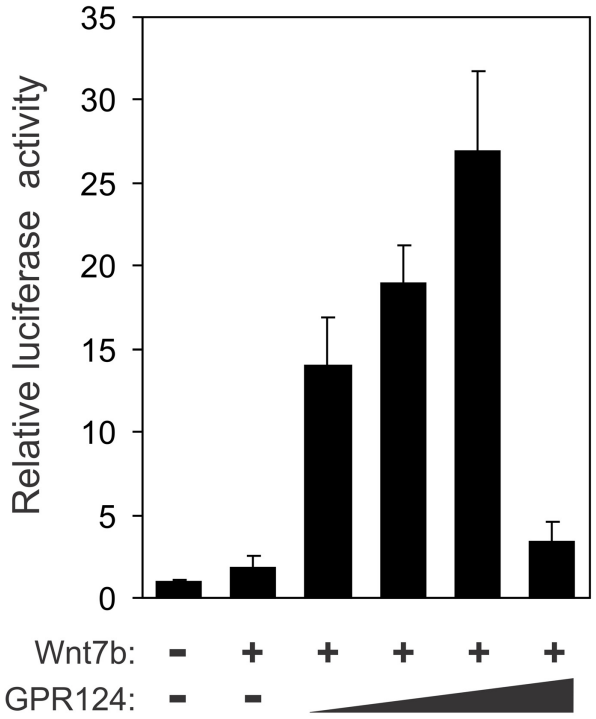

Figure S1. Luciferase Activity in HEK293 Reporter Cells Increases in Response to Co-Expression of WNT7B and GPR124, Related to Figure 2.

In this experiment a WNT7B expression vector was transfected into 293 reporter cells that also contained a stably integrated doxycycline-inducible GPR124 vector. The wedge indicates increasing GPR124 levels achieved by adding increasing amounts of doxycycline to the culture medium.

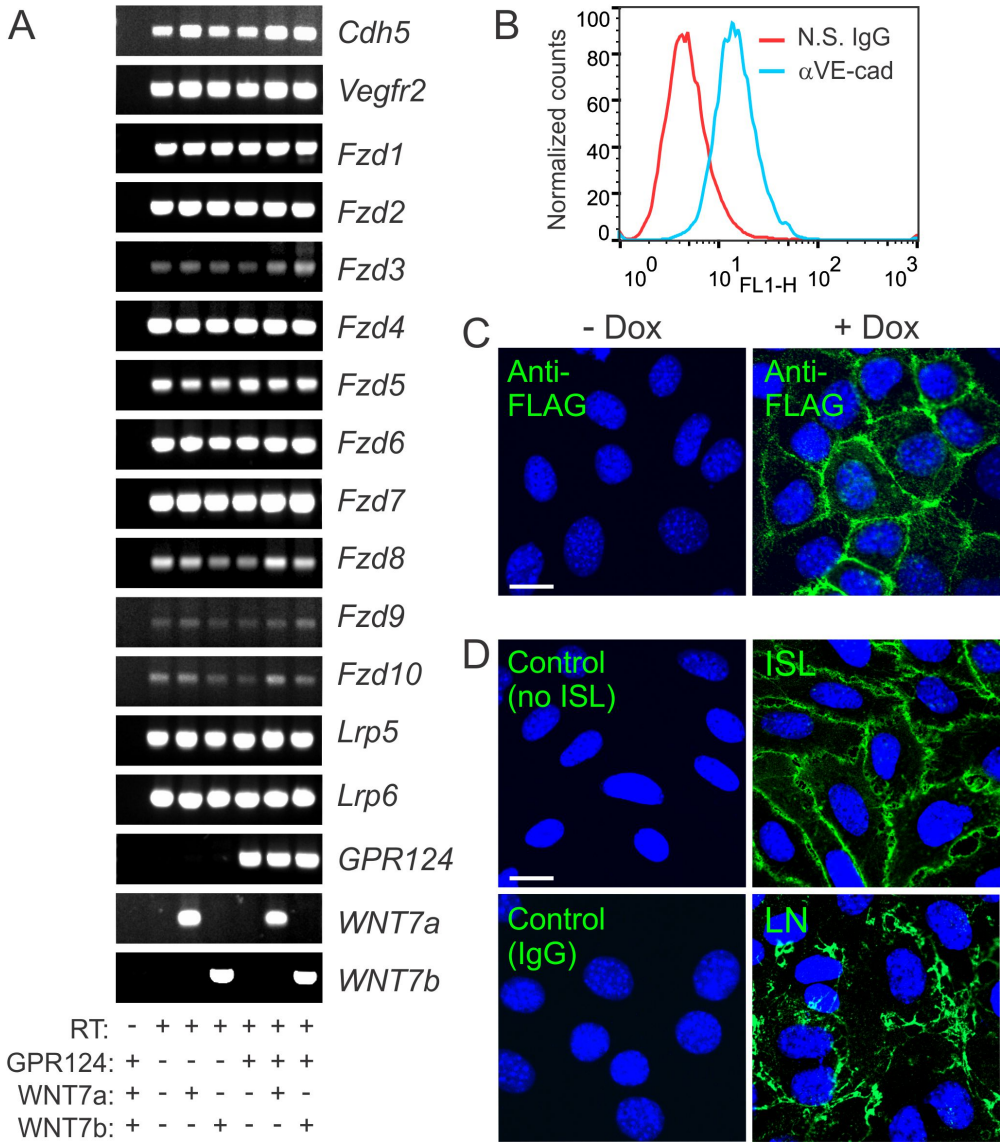

Figure S2. Characterization of BKO-124i Reporter Cells, Related to Figure 2.

A) RT-PCR was used to demonstrate the expression of the endothelial markers VE-cadherin (cadherin 5; *Cdh5*) and *Vegfr2* in BKO-124i cells. Transcripts for each of the frizzleds (*Fzd1* to *Fzd10*) and *Lrp5* and *Lrp6* could also be detected in BKO-124i cells. Expression of *WNT7A*, *WNT7B* and *GPR124* was also verified in the *WNT7A*- or *WNT7B*-transfected or doxycycline-induced (GPR124) cells. In the first lane reverse

transcriptase (RT) was omitted from the cDNA synthesis reaction in order to verify that the products observed were amplified from cDNA and not contaminating genomic DNA.
B) Flow cytometry revealed the expression of VE-cadherin protein on the surface of BKO-124i cells. Cells were stained with a goat anti-mouse VE-cadherin antibodies (αVE-cad) or non-specific goat IgG (N.S. IgG) as a negative control.
C) Immunofluorescence staining of non-permeabilized BKO-124i cells using anti-FLAG antibodies (green) revealed expression of FLAG-GPR124 at the cell surface 24h post treatment with doxycycline (+ Dox).
D) Staining of non-permeabilized BKO-124i cells revealed strong reactivity with isolectin B4 (ISL) and anti-laminin (LN) antibodies.
Nuclei in C and D were counterstained with DAPI (blue).

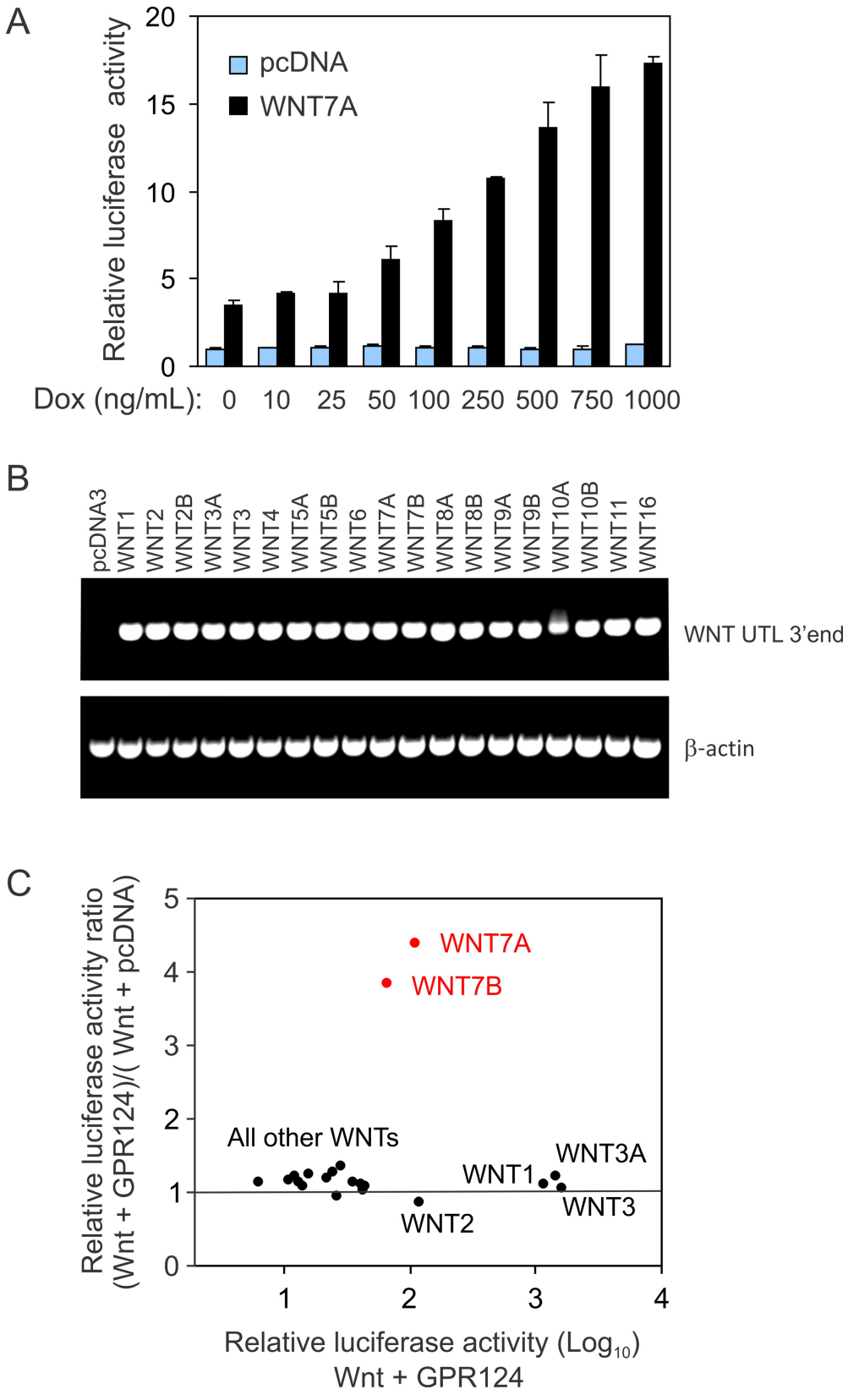
A
Relative luciferase activity
20
15
10
5
0
pcDNA
WNT7A
Dox (ng/mL): 0 10 25 50 100 250 500 750 1000
B
pcDNA3
WNT1
WNT2
WNT2B
WNT3A
WNT3
WNT4
WNT5A
WNT5B
WNT6
WNT7A
WNT7B
WNT8A
WNT8B
WNT9A
WNT9B
WNT10A
WNT10B
WNT11
WNT16
WNT UTL 3'end
β-actin
C
Relative luciferase activity ratio
(Wnt + GPR124)/(Wnt + pcDNA)
5
4
3
2
1
0
WNT7A
WNT7B
All other WNTs
WNT3A
WNT1
WNT3
WNT2
1
2
3
4
Relative luciferase activity (Log10)
Wnt + GPR124

Figure S3. WNT7 Enhances β-catenin Activity in BKO-124i Reporter Cells, Related to Figure 2.

A) WNT7A induced reporter activity in BKO-124i cells increased dose-dependently in response to increasing levels of doxycycline (Dox) in the culture medium. The pcDNA control, which was transfected with pcDNA3.1 empty vector instead of WNT7A, revealed no effect of doxycycline treatment or GPR124 expression on luciferase activity.

B) RT-PCR analysis was used to monitor expression of each of the 19 *WNTs* following transfection into BKO-124i cells. The PCR primers used were designed to amplify a region of the cDNA that was common to all 19 WNTs and derived from the 3' untranslated region (UTR) present in the vector backbone. Cells transfected with empty vector (pcDNA3.1) served as a negative control.

C) A dot plot was used to depict the GPR124-specific enhancement of WNT7A and WNT7B stimulated luciferase activity in BKO-124i cells. The same data were used to generate Figure 2F of the main text except here GPR124 independent WNT activity is also displayed on the x-axis.

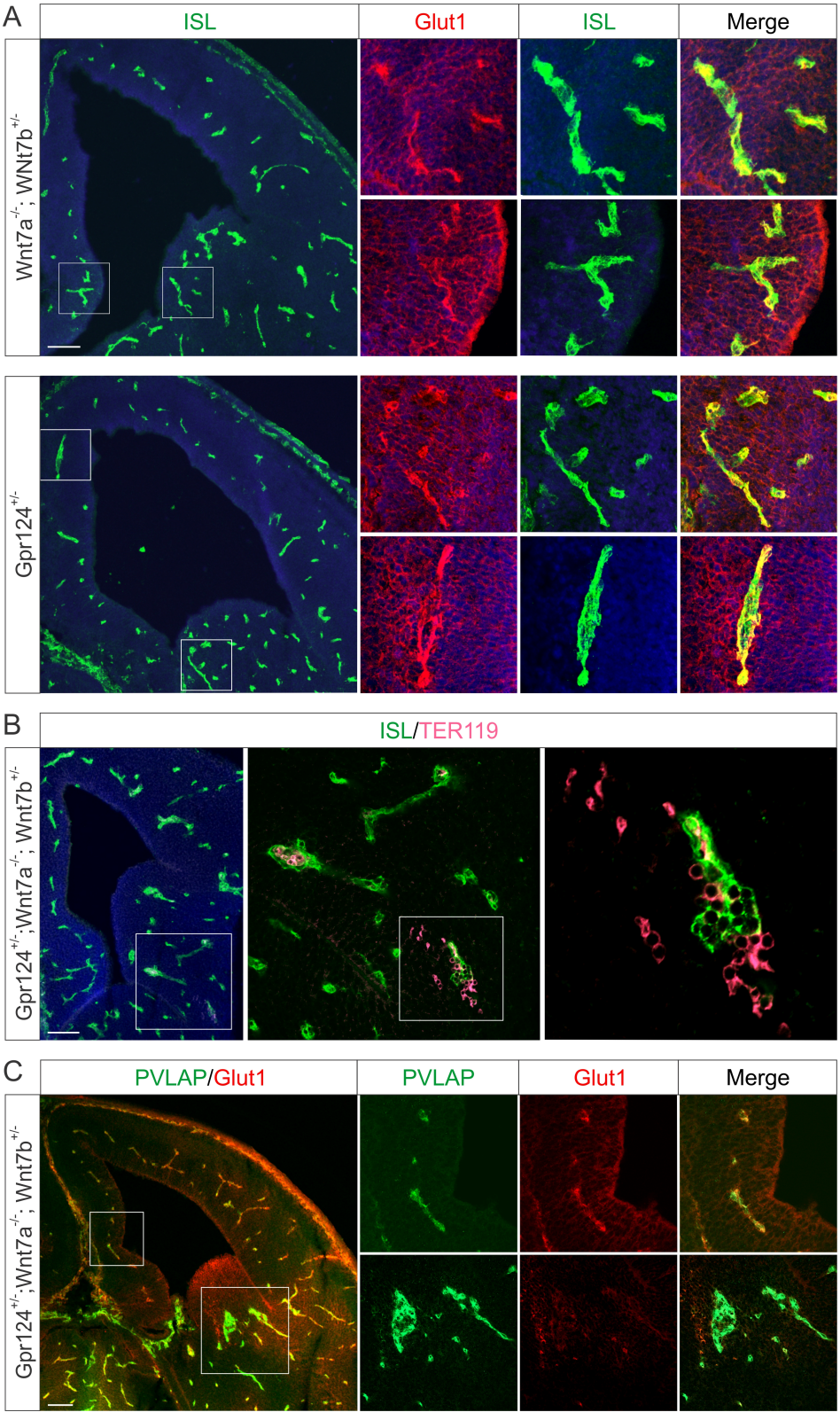
A
ISL
Glut1
ISL
Merge
Wnt7a-/-; WNt7b+/-
Gpr124+/-
B
ISL/TER119
Gpr124+/-;Wnt7a-/-; Wnt7b+/-
C
PVLAP/Glut1
PVLAP
Glut1
Merge
Gpr124+/-;Wnt7a-/-; Wnt7b+/-

Figure S4. Compound Mutant Mice Display Loss of BBB Integrity, Related to Figure 4.

A) Unlike in compound mutants (Figure 4 of main text), no unusual glomeruloids were observed in the telencephalon of *Gpr124*$^{+/-}$ mice or *Wnt7a*$^{-/-}$; *Wnt7b*$^{+/-}$ mice. Glut1 was expressed at similar levels in vessels throughout the forebrain. Bar: 100μm.

B) Immunofluorescent staining for the erythroid cell marker TER119 revealed occasional leakage of red blood cells from glomeruloid structures in the forebrains of compound (Gpr124$^{+/-}$; Wnt7a$^{-/-}$; Wnt7b$^{+/-}$) mutants. Leakage was not observed in the corresponding regions of *Gpr124*$^{+/-}$ or *Wnt7a*$^{-/-}$; *Wnt7b*$^{+/-}$ mice (S.S and B.S.C, unpublished data).

C) The unusual glomeruloids found in the ganglionic eminence of *Gpr124*$^{+/-}$; *Wnt7a*$^{-/-}$; *Wnt7b*$^{+/-}$ forebrains expressed higher levels of PVLAP and lower levels of Glut1 (bottom inset) compared to vessels in other regions of the brain (top inset).

Supplemental Experimental Procedures

Animals

Gpr124 mutant mice were originally generated in our laboratory (Cullen et al., 2011) and are available from The Jackson laboratory (JAX 016881). The BAT-gal (Maretto et al., 2003) (JAX 005317), *Wnt7a* (Parr and McMahon, 1995) (JAX 004715) and floxed *Wnt7b* (Rajagopal et al., 2008) (JAX 008467) strains were obtained from The Jackson laboratory, and the immortomouse (Jat et al., 1991) (strain CBA;B10-Tg(H2Kb-tsA58)6Kio/Crl) was obtained from Charles River. The Wnt7b null allele was generated by crossing the JAX 008467 strain (floxed *Wnt7b* allele) with a transgenic β-actin-cre deleter strain.

Generation of reporter cells

HEK293 reporter cells were generated by infecting HEK293 cells with Cignal Lenti particles containing a TCF/LEF luciferase vector (CLS-018L; Qiagen). Stably infected cells were cloned by limiting dilution and individual clones screened for luciferase activity in response to transfection with a constitutively active β-catenin plasmid. Once a stable reporter clone was identified, a tetracycline inducible GPR124 vector [3xFlagT5i/pcDNA4-TO (Cullen et al., 2011)] was introduced into the cell line and a GPR124 doxycycline inducible subclone identified. To generate BKO reporter cells, $Gpr124^{+/-}$ mice were crossed with the immortomouse that contains a temperature sensitive the SV40 TAg transgene (Jat et al., 1991). $Gpr124^{+/-}$; $tsA58TAg^{+}$ mice were then crossed with $Gpr124^{+/-}$ mice to obtain the desired $Gpr124^{-/-}$; $tsA58TAg^{+}$ embryos.

E13.5 embryonic brains were micro-dissected and endothelial cells isolated using biotinylated isolectin-B4 conjugated to streptavidin magnetic beads as previously described (Cullen et al., 2011). The immortalized cells were cloned and analyzed for endothelial markers by QPCR. Clones that expressed endothelial markers (Vegfr2+, VE-cadherin+ and isolectin+) were infected with the Cignal Lenti TCF/LEF luciferase vector and several stable subclones that responded to β-catenin pathway activation identified. One of the clones (c36) that showed consistent activation in response to β-catenin stimulation, called BKO cells, was used for the studies reported here. An additional reporter line that contained a doxycycline inducible GPR124 vector, called BKO-124i, was generated by sequentially introducing a Tet-On transactivator (pCMV-Tet3G) and then a tetracycline inducible 3xFLAG-tagged GPR124 vector (3xFlagT5i/pTRE3G) into BKO cells (see plasmids for vector details).

Luciferase assays

For luciferase assays 15,000 reporter cells per well were plated onto 96 well white tissue culture plates (Falcon) at 33^{o}C and various plasmids were transfected in-well 24h later with Lipofectamine 2000 (Invitrogen). As an internal control a Renilla luciferase plasmid was co-transfected along with each plasmid. After transfection cells were cultured at 37ºC to silence the temperature sensitive SV40 TAg transgene. To induce GPR124 expression, BKO-124i cells were treated with doxycycline 3 h after transfection. Luminescence was measured 48 hrs post-transfection using the Dual-Glo luciferase Assay System (Promega). For co-culture experiments HEK293 cells were transfected at ~70% confluency using Lipofectamine LTX (Invitrogen). 24hrs later, HEK293 cells were

rinsed, trypsinized, counted, and 15,000 cells were plated on top of reporter cells. Luminescence was measured 24 hours later.

RT-PCR and real-time quantitative PCR

Total RNA was isolated using the RNAeasy isolation kit (Qiagen) and samples were treated with DNAse to remove contaminating DNA. After re-isolating the RNA to remove DNAse, RNA was converted into cDNA using the Superscript III reverse transcriptase kit (Invitrogen). 30 cycles of PCR was used for each of the genes shown in Figure S2. Real-time QPCR was performed on an MX3000 system (Stratagene) as previously described (Cullen et al., 2011). The primers used, which are available from the authors upon request, were designed to span large introns to avoid potential amplification of contaminating DNA.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described (Cullen et al., 2009) using E13.5 embryos that had been fixed for 30 min in PBS containing 2% paraformaldehyde, frozen in OCT, and cryosectioned. Sections were treated with blocking solution [1% blocking reagent, cat. no. 11096176001; Roche) in 100 mM Tris (pH 7.5), 150 mM NaCl] for 20 min before IF staining. For immunocytochemistry BKO cells were plated onto poly-D-lysine coated chamber slides (BD Biosciences) and transfected 24 hours later. 48h later, cells were rinsed, fixed with ice-cold 4% paraformaldehyde for 15 min, and stained. Nuclei were counterstained with DAPI (Invitrogen). Fluorescence images were captured using a Zeiss LSM510 confocal

microscope. The following lectins or antibodies were used: biotin or FITC-labeled isolectin-B4 (Sigma), rat anti-PLVAP (clone Meca32; BD Pharmingen), rabbit anti-Glut1 (Millipore), rabbit anti-laminin (Sigma), mouse anti-FLAG (cloneM2; Sigma), and mouse anti-myc (clone 9E10; Sigma). Secondary antibodies were acquired from Invitrogen, Jackson Immunoresearch, Rockland Immunochemicals, and Vector Laboratories.

Flow cytometry

Cells were labeled with non-specific goat IgG (SouthernBiotech) or goat anti-mouse VE-cadherin (R&D) and analyzed on a FACScalibur flow cytometer (Becton Dickinson) and graphed using FlowJo v10 software.

β-galactosidase staining

20 μm frozen sections of brain or spinal cord were cut onto Superfrost slides (Fisher), air dried 2 min. and immersed in PBS containing 2mM $MgCl_2$. Slides were equilibrated for 15 min. in X-gal buffer [PBS containing 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 2mM MgCl, 0.25% Triton X-100] then stained at 37^0C under incubation Chamber Gaskets (Grace Bio-Labs) with 0.5mg/mL X-gal in the same buffer. Once blue staining was evident vessels were then stained with biotin-labeled isolectin-B4 (Sigma) followed by AP-labeled anti-biotin (Dako), and visualized using Fast Red (Sigma). The quantification of β-gal+ vessels was performed on all vessels that penetrated the neural epithelium in the lateral ventricular walls and ganglionic eminence and on all vessels infiltrating the spinal cord. The vessels of the PNVP were excluded from this analysis.

Plasmids

To generate the 3xFlagGPR124-RGE/pcDNA3.1 vector encoding the RGE mutation, a PCR-based based approach was used to incorporate the missense mutation 1071C→G (nucleotide number originates from start of untagged sequence) into 3xFlagGPR124/pcDNA3.1 (Cullen et al., 2011). The doxycycline inducible GPR124 vector used to generate BKO-124i cells, called 3xFlagT5i/pTRE3G, was generated by subcloning the 3×FLAG-tagged GPR124 gene from 3×FlagT5i/pcDNA4-TO (Cullen et al., 2011) into pTRE3G (Clontech). The vector 3×FlagT5i-4aaDel/pcDNA4, which lacks the last 4 amino acids of GPR124, was derived from 3×FlagT5i/pcDNA4-TO using a PCR-based strategy. Mouse *Gpr124* was PCR amplified from cDNA and cloned into pCMVsport2 (Invitrogen) to generate Gpr124/pCMVsport2. The Dlg1-HA/pcDNA3 vector was made by subcloning the full-length murine *Dlg1* cDNA from IMAGE clone 6410247 into pcDNA3 and then using PCR based tag insertion to introduce a 3X-HA tag (YPYDVPDYAYPYDVPDYAYPYDVPDYA) immediately before to the stop codon. Human WNT7A/pcDNA3 and WNT7B/pcDNA3 were generated by subcloning the cDNA from IMAGE clones 3949579 and 5457045 respectively, into pcDNA3.1 (Invitrogen) and using PCR to optimize the Kozak sequence. Using PCR insertion, a myc-tag was incorporated into the C-terminus of WNT7A/pcDNA3 and WNT7B/pcDNA3 to generate WNT7A-myc/pcDNA3 and WNT7B-myc/pcDNA3. Single point mutations of WNT7A-myc/pcDNA3 were generated using a QuickChange site directed mutagenesis kit (Stratagene). The following mutants were made 214G→A (E72K), 325A→G (A109T), 610G→A (G204S), 664C→T (R222W), 874C→T (R292C), 911G→T (C304F). The following GPR124-myc/pcDNA3 deletions were generated

using gBlocks (IDT) and a Gibson Assembly cloning kit (NEB): ΔLRR (aa75-245), ΔIG (aa75-341), ΔHorm (aa75-698), ΔGPS (aa75-751). The GPR125/pcDNA3 expression vector was made by subcloning a full length human GPR125 EST cDNA (IMAGE clone ID:5551209) into pcDNA3.1. The vector encoding the GPR124/125LRR fusion protein, called GPR124-myc-125LRR/pcDNA3, was generated by digesting GPR124-myc/pcDNA3 with SgrAI and HindIII, purifying the vector backbone, and filling the excised region with custom gBlocks. The final vector contains aa1-77 of GPR124, aa82-236 of GPR125, aa234-1331 of GPR124 followed by a C-terminal myc tag. The 19 human WNTs, which were cloned into pcDNA3.2/V5-DEST, were obtained from the Open Source WNT project. Each of these WNTs is untagged and contains a common signal peptide, a stop codon, and a common untranslated 3' sequence. All vectors used in this study were sequence verified.

Western blotting

24hrs post-transfection HEK293 or BKO cells were rinsed and lysed in 1xPBS buffer containing an additional 163 mM NaCl (300mM NaCl total), 1% Triton X-100, and protease inhibitor cocktail (Roche) and clarified by centrifugation. Protein extracts were separated by SDS-PAGE, transferred to a PDVF membrane (Millipore), and probed with mouse anti-Wnt7a/b (Santa Cruz), rabbit anti-c-myc (GenScript), mouse HRP-labeled anti-β-actin (Santa Cruz), HRP-labeled anti-FLAG antibodies (Sigma) or HRP-labeled anti-HA (Roche). All secondary antibodies were from Jackson ImmunoResearch. Pierce ECL2 Western blotting substrate (Thermo) was used for detection.

Immunoprecipitation

24h post transfection HEK293 cells were rinsed with PBS and lysed in PBS containing 1% triton X-100, 300mM NaCl, and protease inhibitor cocktail (Roche). Lysates were clarified by centrifugation then incubated with Protein G sepharose beads (GE Life Sciences) armed with 2μg of anti-DYKDDDDK (FLAG) antibody (BioLegend). Beads were washed with the lysis buffer, and bound proteins were eluted in SDS Laemmli sample buffer containing 0.2 M DTT and analyzed by Western blotting.

Mass spectrometry

hCMEC/GPR124i cells treated with 0.5mg/mL doxycycline for 24 hours, untreated hCMEC/GPR124i, or parent hCMEC were lysed in TBS (50mM Tris-HCl pH 7.6, 75mM NaCl), 1% Triton X-100 containing protease inhibitor cocktail (Roche) and clarified by centrifugation. FLAG-tagged proteins were immunoprecipitated using anti-FLAG antibodies (M2 clone, Sigma), separated by SDS-PAGE and stained with SimpleBlue (Invitrogen). Proteins were extracted from the gel matix by trypsin digestion, and peptides analyzed by mass spectrometry as previously described (Zofall et al., 2009).

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