Full-length Dhh and N-terminal Shh act as competitive antagonists to regulate angiogenesis and vascular permeability

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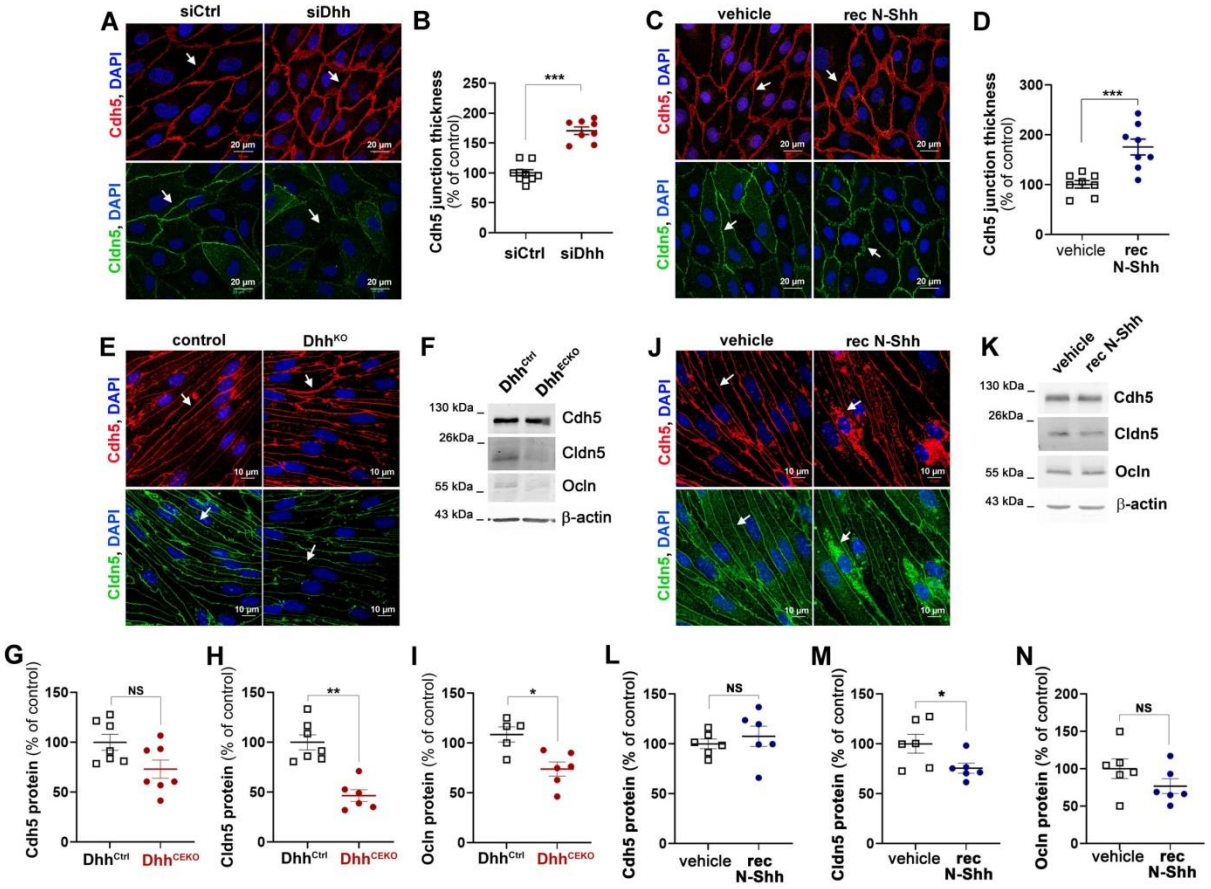
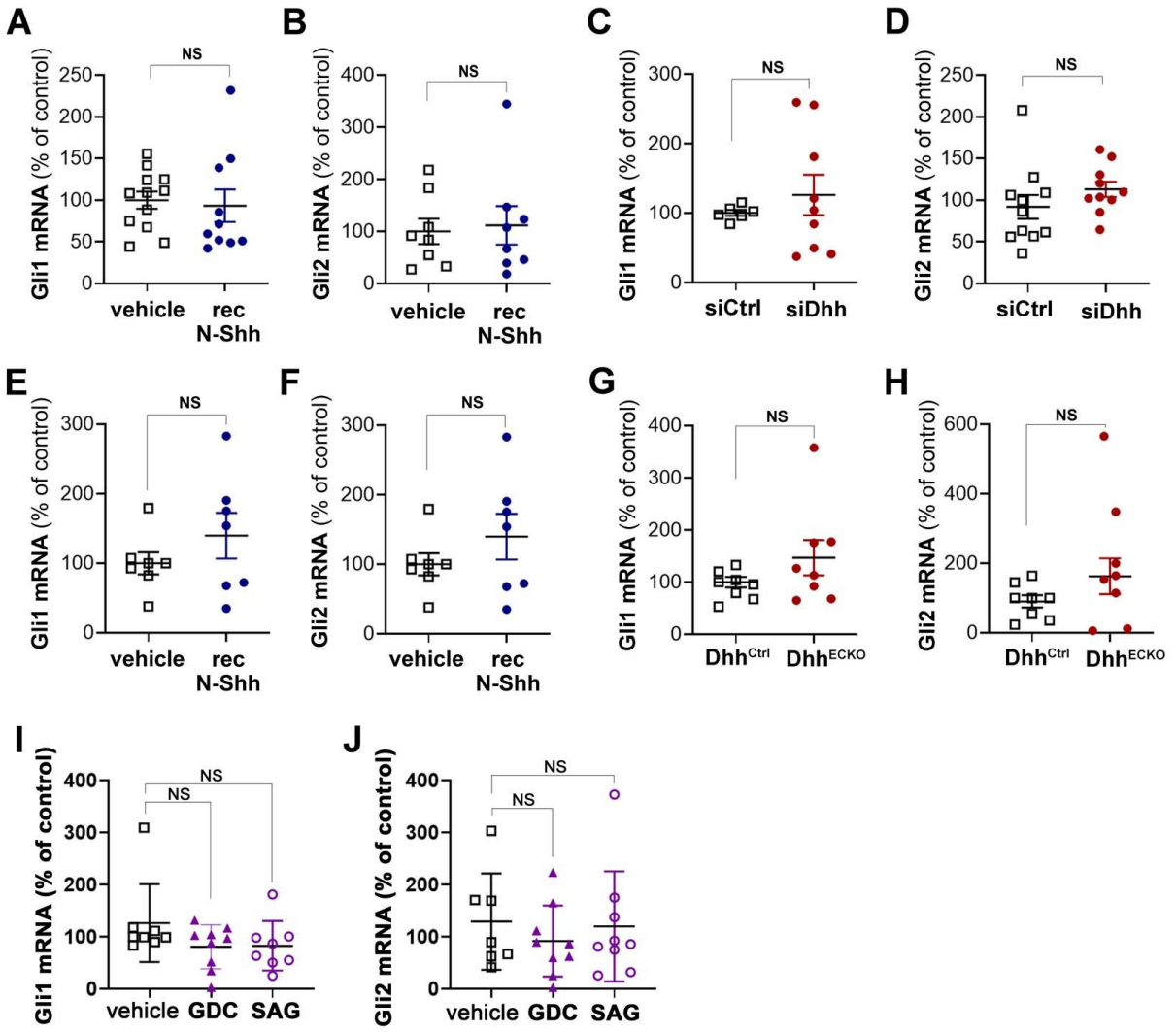
Supplementary data

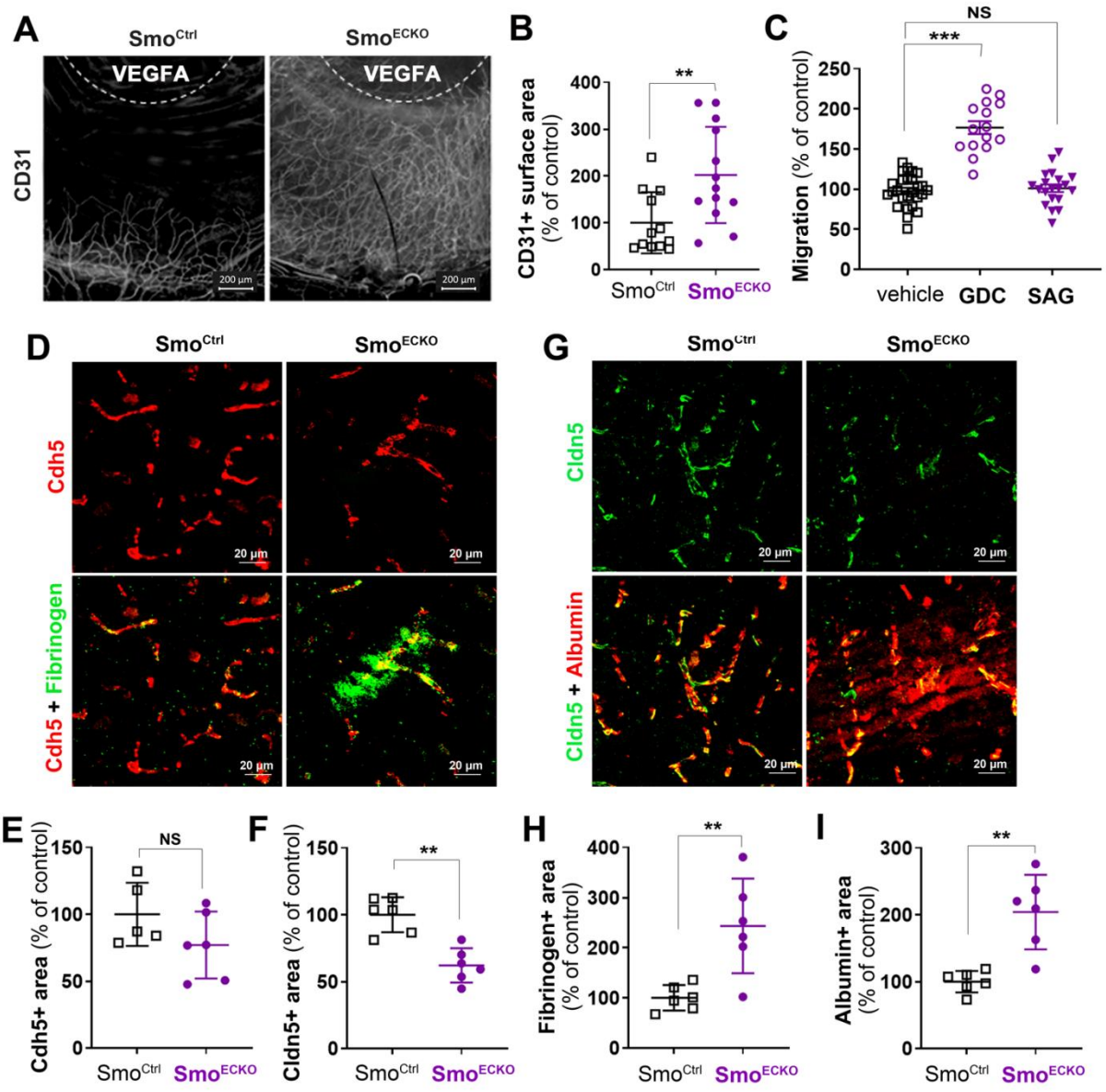
Supplementary Figures and Supplementary Figure legends



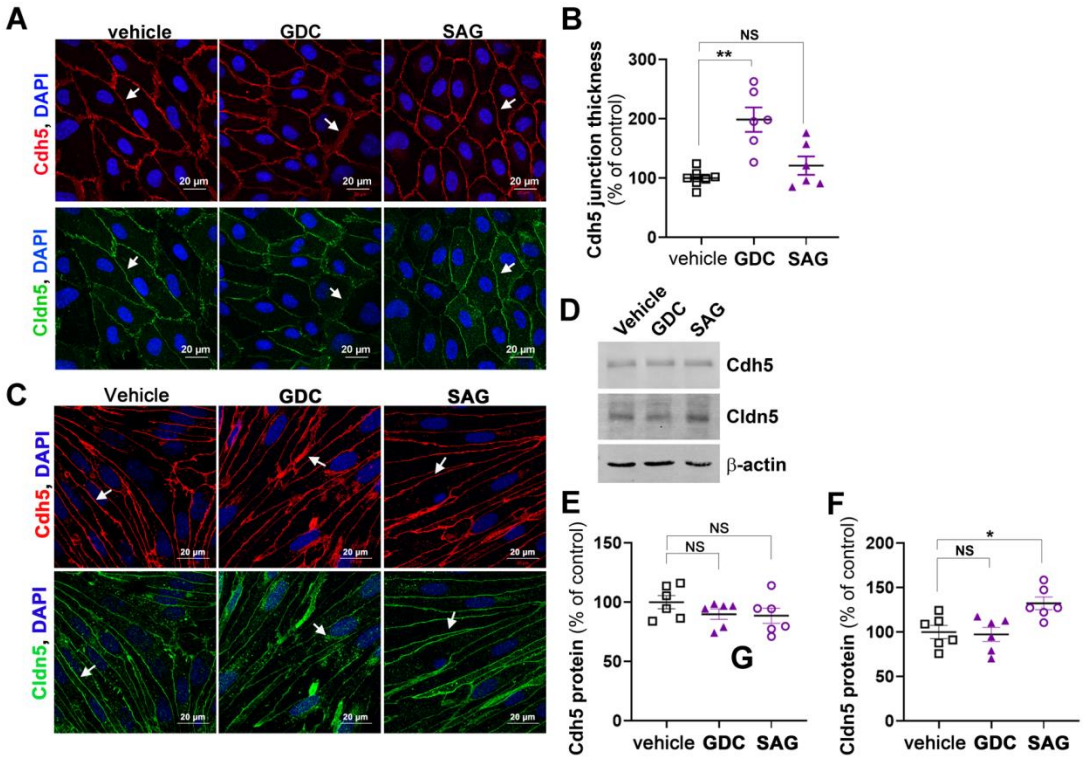
Supplementary Figure 1: ***EC-derived Dhh improves intercellular junction integrity while recombinant N-Shh disrupts it.*** (**A-B**) HUVECs were transfected with Dhh or control siRNAs. (**A**) Cdh5 (in red) and Cldn5 (in green) localization was evaluated by immunofluorescent staining of a confluent cell monolayer and (**B**) quantified as the mean junction thickness using Image J software. The experiment was repeated 8 times. (**C-D**) HUVECs were treated or not with 1 µg/mL recombinant N-Shh (rec N-Shh) for 30 minutes. (**C**) Cdh5 (in red) and Cldn5 (in green) localization was evaluated by immunofluorescent staining of a confluent cell monolayer and (**D**) quantified as the mean junction thickness using Image J software. The experiment was repeated 6 times. (**E-I**) mouse brain ECs were isolated from Cdh5-Cre^{ERT2}; Dhh$^{Flox/Flox}$ (Dhh^{ECKO}) and Dhh$^{Flox/Flox}$ (Dhh^{Ctrl}) mice that had been previously administered with tamoxifen. (**E**) Cdh5 (in red) and Cldn5 (in green) localization was evaluated by immunofluorescent staining of a confluent cell monolayer. (**F**) Cdh5, Cldn5 and Ocln protein levels were evaluated by western blot analyses and (**G-I**) quantified using Image J software (n=7 mice per group). (**J**) Mouse brain ECs were isolated from WT mice, and then treated or not with 1 µg/mL rec N-Shh for 30 minutes. Cdh5 (in red) and Cldn5 (in green) localization was evaluated by immunofluorescent staining of a confluent cell monolayer. (**K-N**) mouse brain ECs were isolated from WT mice, and then treated or not with 1 µg/mL rec N-Shh for 24 hours. (**K**) Cdh5, Cldn5 and Ocln protein levels were evaluated by western blot analyses and (**L-N**) quantified using Image J software (n=6 mice per group). *: p≤0.05; **: p≤0.01; ***: p≤0.001; NS: not significant. Mann-Whitney test.



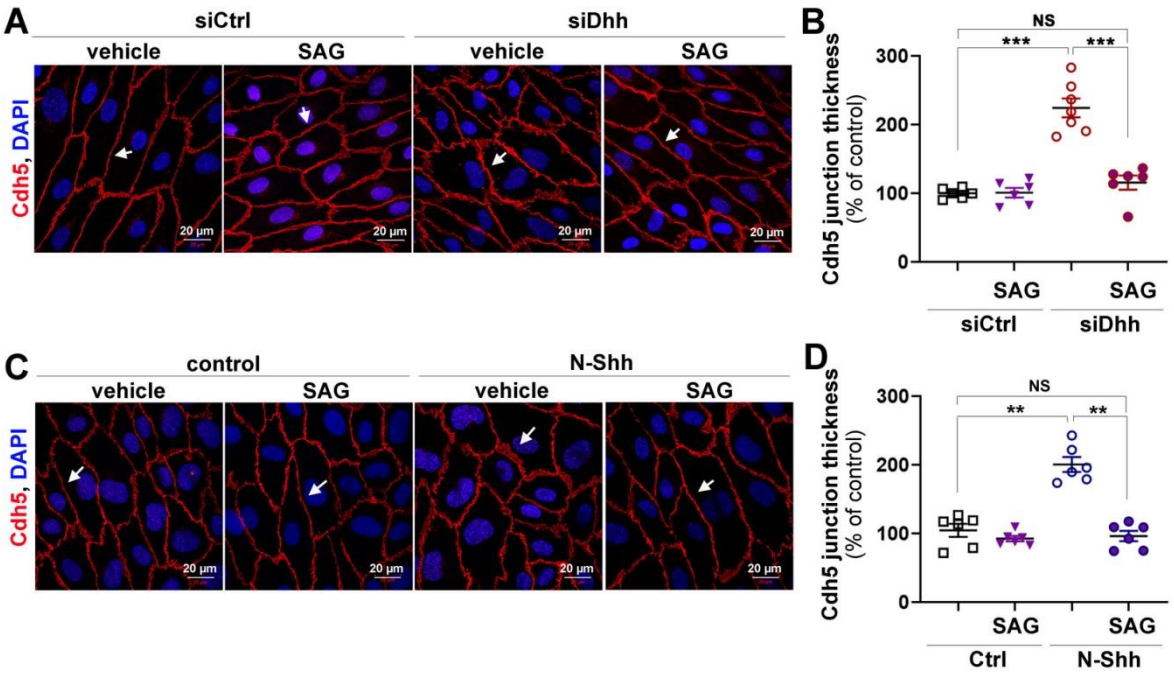
Supplementary Figure 2: ***Hh ligands do not modulate Gli expression in ECs*****.** (**A-B**) HUVECs were treated or not with 1 µg/mL recombinant N-Shh (recN-Shh) for 24 hours. Gli1 (**A**) and Gli2 (**B**) mRNA expression was quantified via RT-qPCR. The experiment was repeated 3 times, each experiment included triplicates. (**C-D**) HUVECs were transfected with Dhh or control siRNAs. Gli1 (**C**) and Gli2 (**D**) mRNA expression was quantified via RT-qPCR. The experiment was repeated 3 times, each experiment included triplicates. (**E-F**) Mouse brain ECs were isolated from WT mice, and then treated or not with 1 µg/mL rec N-Shh for 24 hours. Gli1 (**E**) and Gli2 (**F**) mRNA expression was quantified via RT-qPCR (n=7 mice in each group) (**G-H**) mouse brain ECs were isolated from Cdh5-Cre^{ERT2} Dhh$^{Flox/Flox}$ (Dhh^{ECKO}) and Dhh$^{Flox/Flox}$ (Dhh^{Ctrl}) mice that had been previously administered with tamoxifen). Gli1 (**G**) and Gli2 (**H**) mRNA expression was quantified via RT-qPCR (n=8 mice in each group) (**I-J**) HUVECs were treated with either 30 nmol/L GDC-0449, 100 nmol/L SAG or control vehicle for 24 hours. Gli1 (**I**) and Gli2 (**J**) mRNA expression was quantified via RT-qPCR. The experiment was repeated 3 times, each experiment included triplicates. NS: not significant. Mann-Whitney test.



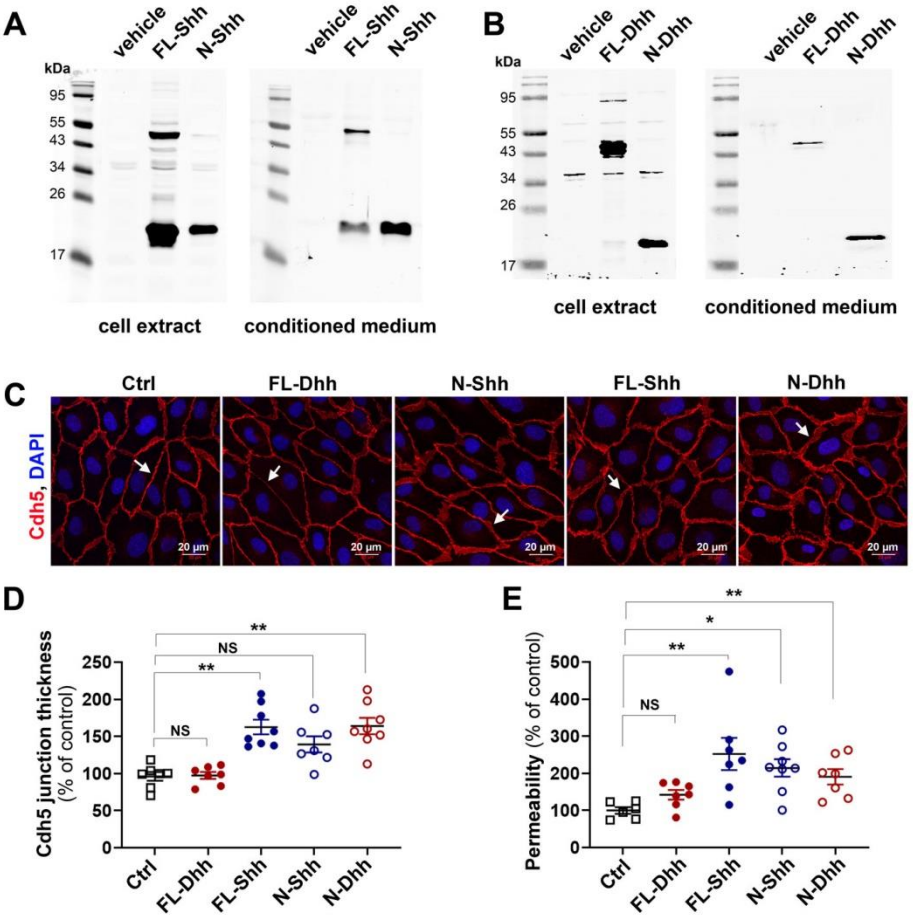
Supplementary Figure 3: ***Inhibition of Hh signaling in ECs promotes angiogenesis and destabilizes the BBB***. (**A-B)** VEGFA containing pellets were implanted in the corneas of Cdh5-Cre^{ERT2} Smo$^{Flox/Flox}$ (Smo^{ECKO}) and Smo$^{Flox/Flox}$ (Smo^{Ctrl}) mice 2 weeks after they were administered with tamoxifen. Whole mount corneas were immunostained with anti-CD31 antibodies to identify blood vessels. (**A**) Representative pictures are shown. (**B**) Angiogenesis was quantified as the percentage of CD31+ surface area (n=13 and 12 corneas respectively). **(C)** HUVECs were treated either with 30 nmol/L GDC-0449, 100 nmol/L SAG or control vehicle. Cell migration was assessed in a chemotaxis chamber. The experiment was repeated 4 times, each experiment included n=4 wells/conditions. **(D-I)** Smo^{ECKO} and Smo^{Ctrl} mice were sacrificed 2 weeks after they were administered with tamoxifen (n= 6 mice in each group). (**D**) Brain sagittal sections were immunostained with either anti-Cdh5 (in red), and anti-Fibrinogen (in green) antibodies (**E**) or anti-Cldn5 (in green) and anti-Albumin (in red) antibodies. Representative confocal images are shown. (**F**) Cdh5 expression was quantified as the Cdh5+ surface area. (**G**) Cldn5 expression was quantified as the Cldn5+ surface area. (**H**) Fibrinogen extravasation was quantified as the fibrinogen+ surface area. (**I**) Albumin extravasation was quantified as the albumin+ surface area. **: p≤0.01; ***: p≤0.001; NS: Not significant. Mann-Whitney test.



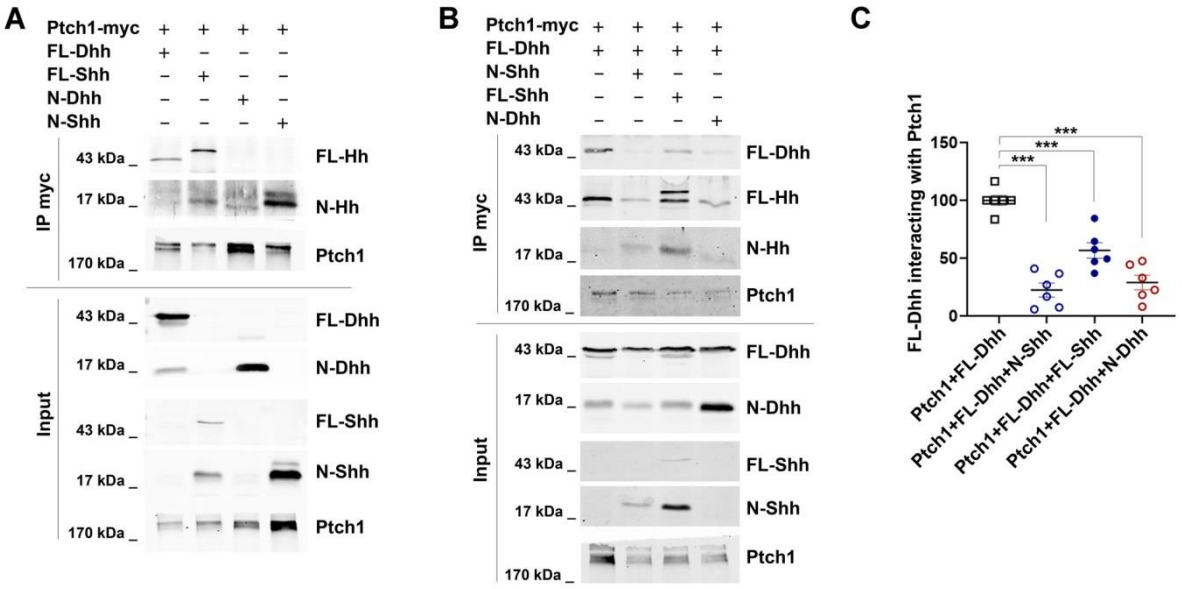
Supplementary Figure 4: ***Inhibition of Hh signaling in ECs destabilizes intercellular junctions sand promotes EC migration.*** (**A-B**) HUVECs were treated either with 30 nmol/L GDC-0449, 100 nmol/L SAG or control vehicle. (**A**) Cdh5 (in red) and Cldn5 (in green) localization was evaluated by immunofluorescent staining of a confluent cell monolayer and (**B**) quantified as the mean junction thickness using Image J software. The experiment was repeated 6 times. (**C-F**) Mouse brain ECs were isolated from C57BL/6 WT mice and then treated either with 30 nmol/L GDC-0449, 100 nmol/L SAG or control vehicle for 24 hours. (**C**) Cdh5 (in red) and Cldn5 (in green) localization was evaluated by immunofluorescent staining of a confluent cell monolayer. (**D**) Cdh5 and Cldn5 protein levels were evaluated by western blot analyses and (**E-F**) quantified using Image J software (n=6 in each group). *: p≤0.05; **: p≤0.01; NS: not significant. Kruskal-Wallis test followed by Dunn's multiple comparison test.



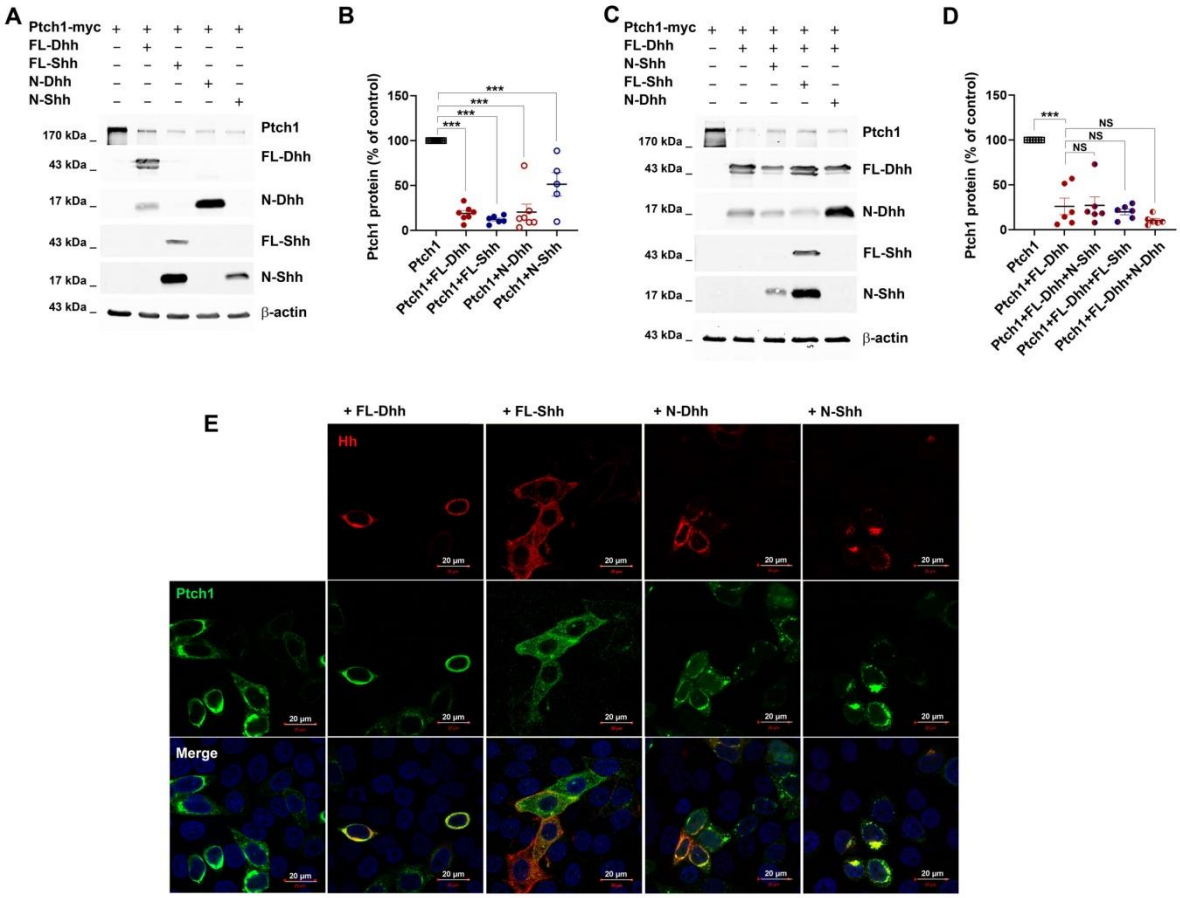
Supplementary Figure 5: ***FL-Dhh activates Hh signaling in ECs while N-Shh inhibits it.*** (**A-B**) HUVECs were transfected with Dhh or control siRNAs and then treated or not with 100 nmol/L SAG for 16 hours. (**A**) Cdh5 (in red) localization was evaluated by immunofluorescent staining of a confluent cell monolayer and (**B**) quantified as the mean junction thickness using Image J software. The experiment was repeated 6 times. (**C-D**) HUVECs were treated or not with 1 µg/mL rec N-Shh for 16 hours in the presence or not of 100 nmol/L SAG (**C**) Cdh5 (in red) localization was evaluated by immunofluorescent staining of a confluent cell monolayer and (**D**) quantified as the mean junction thickness using Image J software. The experiment was repeated 6 times. **: p≤0.01; ***: p≤0.001; NS: not significant. Kruskal-Wallis test followed by Dunn's multiple comparison test.



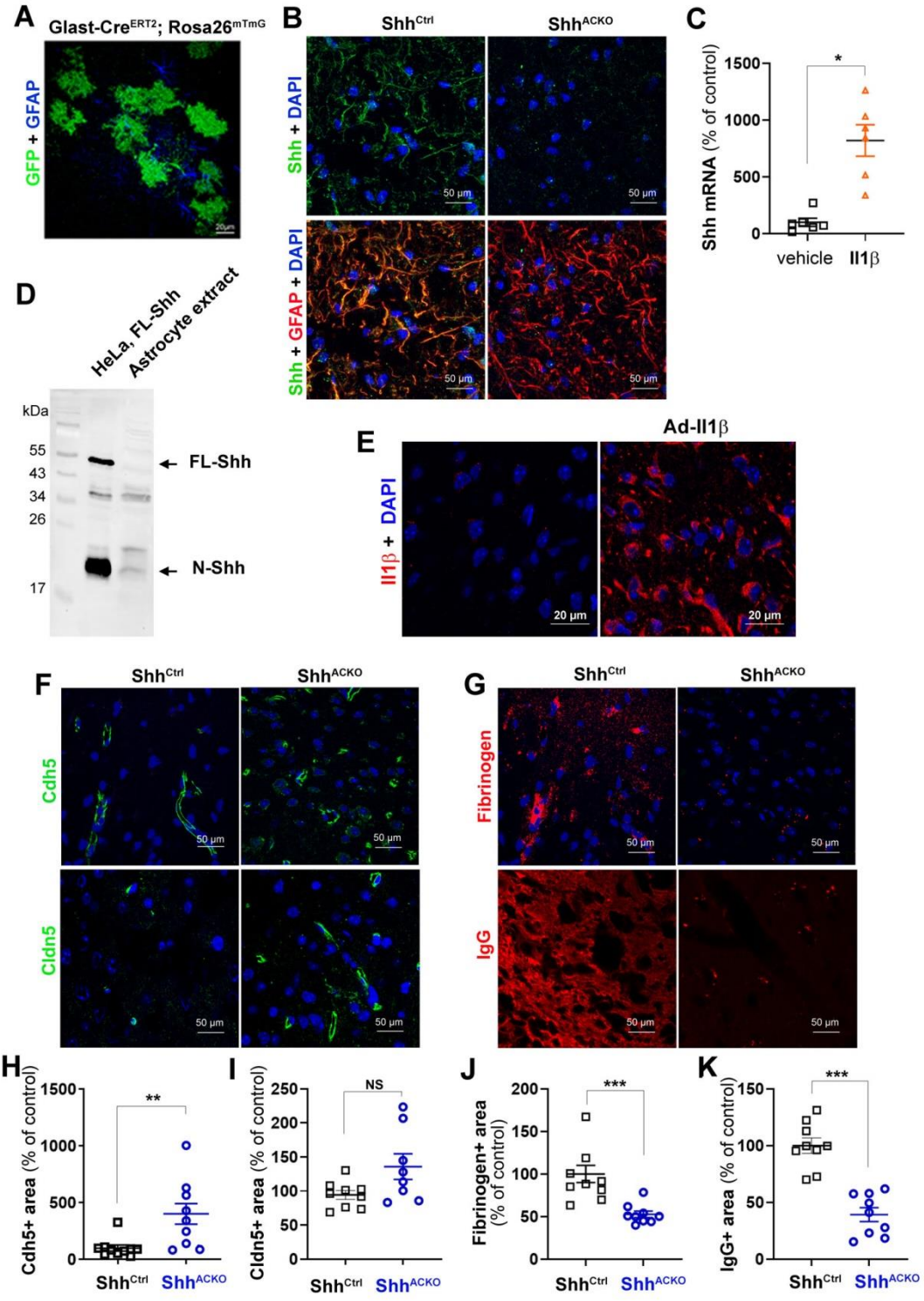
Supplementary Figure 6: (**A**) HeLa cells were transfected either with FL-Shh or N-Shh encoding plasmids. Expression and secretion of Shh was assessed by western blot analysis. (**B**) HeLa cells were transfected either with FL-Dhh or N-Dhh encoding plasmids. Expression and secretion of Dhh was assessed by western blot analysis. (**C-E**) HUVECs were treated with conditioned medium from HeLa containing or not FL-Dhh, N-Shh, FL-Shh or N-Dhh. (**C**) Cdh5 localization was evaluated by immunofluorescent staining (in red) of a confluent cell monolayer and (**D**) quantified as the mean junction thickness using Image J software. The experiment was repeated 6 times. (**E**) Endothelial monolayer permeability to 70 kDa FITC-Dextran was assessed using Transwells. The experiment was repeated 3 times, each experiment included duplicates. *: p≤0.05; **: p≤0.01; NS: not significant. Kruskal-Wallis test followed by Dunn's multiple comparison test.



Supplementary Figure 7: (**A**) HeLa cells were co-transfected Ptch1-myc together with FL-Dhh, FL-Shh, N-Dhh or N-Shh encoding plasmids. Hh ligands interaction with Ptch1 was assessed by co-immunoprecipitation assay. (**B**) HeLa were co-transfected Ptch1-myc and FL-Dhh encoding plasmids together with or without N-Shh, FL-Shh or N-Dhh encoding plasmids. Hh ligands interaction with Ptch1 was assessed by co-immunoprecipitation assay and (**C**) quantified using Image J software. The experiment was repeated 6 times. ***: p≤0.001. Kruskal-Wallis test followed by Dunn's multiple comparison test.

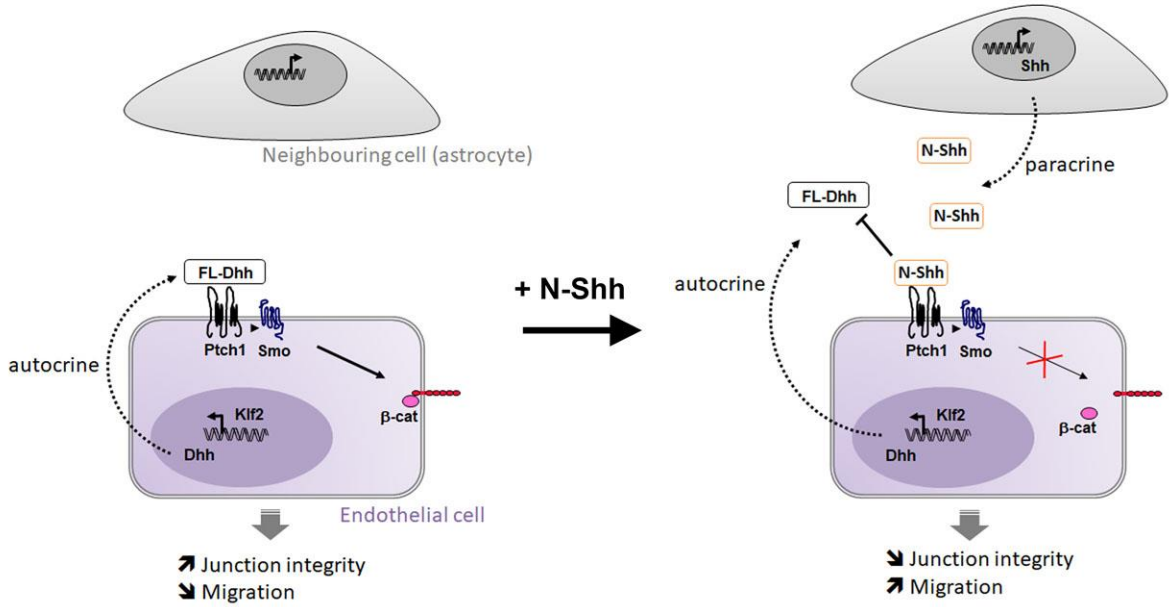


Supplementary Figure 8: (**A**) HeLa cells were co-transfected with Ptch1-myc encoding plasmids together with plasmids expressing or not FL-Dhh, FL-Shh, N-Dhh or N-Shh. Ptch1, Dhh, and Shh protein level was assessed by western blot analysis. (**B**) Ptch1 protein level was quantified using Image J software. The experiment was performed 6 times. (**C**) HeLa cells were co-transfected Ptch1-myc and FL-Dhh encoding plasmids together with or without N-Shh, FL-Shh or N-Dhh encoding plasmids. Ptch1, Dhh, and Shh protein level was assessed by western blot analysis. (**D**) Ptch1 protein level was quantified using Image J software. The experiment was performed 6 times. (**E**) HeLa cells were co-transfected with Ptch1-myc encoding plasmids together with plasmids expressing or not FL-Dhh, FL-Shh, N-Dhh or rec N-Shh. Ptch1 (in green), Dhh (in red), and Shh (in red) protein localization was assessed by immune-fluorescent staining. ***: p≤0.05; NS: not significant. Kruskal-Wallis test followed by Dunn's multiple comparison test.



Supplementary Figure 9: (**A**) Spinal cord sections from Glast-Cre^{ERT2}; Rosa26$^{mT/mG}$ were co-immunostained with anti-GFP (in green) and anti-GFAP (in blue) antibodies. The co-localization of GFAP and GFP staining validate the specific activity of the Cre recombinase within the astrocytes in the Glast-Cre^{ERT2} mouse model. (**B**) EAE was induced in both Glast-Cre^{ERT2}; Shh$^{Flox/Flox}$ (Shh^{ACKO}) and Shh$^{Flox/Flox}$ (Shh^{ctrl}) mice. Mice were sacrificed 32 days later. Spinal cord sections were co-immunostained with anti-Shh (in green) and anti-GFAP (in red) antibodies. (**C**) Cultured normal

human astrocytes were treated with or not with 10 ng/mL Il1β for 24 hours. Shh mRNA expression was quantified by RT-qPCR. The experiment was repeated 6 times. (**D**) Shh protein expression in cell extract from both HeLa transfected with FL-Shh encoding plasmids and Il1β-treated astrocytes was analysed by western blot. (**E-K**) Both Glast-Cre^{ERT2}; Shh$^{Flox/Flox}$ (Shh^{ACKO}) and Shh$^{Flox/Flox}$ (Shh^{ctrl}) mice were administered in the cerebral cortex with adenoviruses encoding Il1β. Mice were sacrificed 7 days later. (**E**) Brain coronal sections were immunostained with anti-Il1β (in red) antibodies. (**F**) Brain coronal sections were immunostained with anti-Cdh5 (in green) or anti-Cldn5 (in green) antibodies. Representative confocal images are shown. (**G**) Cdh5 and (**H**) Cldn5 expression was quantified as the Cdh5+ (n=9 mice in each group) and Cldn5+ (n=8 and 9 mice respectively) surface area respectively. (**I**) Brain coronal sections were immunostained with anti-Fibrinogen (in red) and anti-IgG (in red) antibodies. Representative confocal images are shown. (**J**) Fibrinogen and (**K**) IgG extravasation was quantified as the fibrinogen+ surface area (n=9 mice in each group) and Albumin+ surface area respectively (n=9 mice in each group). *: p≤0.05; **: p≤0.01; ***: p≤0.001; NS: not significant. Mann-Whitney test.



Supplemental Figure 10: Schema recapitulating the findings. In healthy conditions, FL-Dhh which is produced by endothelial cell themselves promotes endothelium integrity and quiescence. N-Shh which is either produced in a neighbouring cell in pathological conditions (e.g. astrocytes in the setting of brain inflammation) or administered ectopically prevents FL-Dhh binding to Ptch1 which leads to destabilization of endothelial intercellular junctions and an increased EC migration.

Supplemental table

hActb	**F**	5′-GGAGGAGCTGGAAGCAGCC-3′
	R	5′-GCTGTGCTACGTCGCCCTG-3′
hDhh	F	5′-AACAGCTTACTTCCGGCTCC-3′
	R	5′-CGACTCTTGTGGGCTCTGTT-3′
hDisp1	F	5′-TGCAGAAGCACTGGATCGTT-3′
	R	5′-CTGTGACAAGCTCAGCTCCA-3′
hGli1	F	5′-TTCCTACCAGAGTCCCAAGT-3′
	R	5′-CCCTATGTGAAGCCCTATTT-3′
hGli2	F	5′-CAGATCCACATGTACGAACAG-3′
	R	5′-CCATGATGGCATCGAAGTC-3′
hShh	F	5′-CAGTTTATCCCCAATGTGGC-3′
	R	5′-GCCAAAGCGTTCAACTTGTC-3′
mActb	F	5′-CCTGAACCCTAAGGCCAACC-3′
	R	5′-TAGCCCTCGTAGATGGGCAC-3′
mGli1	F	5′-GAAGGAATTCGTGTGCCATT-3′
	R	5′-GCAACCTTCTTGCTCACACA-3′
mGli2	F	5′-TGGTTGAGCGGAAGGTTGAA-3′
	R	5′-ACAGGTTTGGGATACGCACA-3′
mDhh	F	5′-CTTGGACATCACCACGTCTG-3′
	R	5′-ATGTAGTTCCCTCAGCCCCT-3′
mIhh	F	5′-TGTCAAGTCTGAGCATTCGG-3′
	R	5′-GTCTCGATGACCTGGAAAGC-3′
mShh	F	5′-GTCGAGCAGTGGACATCACC-3′
	R	5′-GAAACAGCCGCCGGATTTGG-3′

F forward, R reverse
Actb was used as the household gene

Supplementary Table I: List of primers used for reverse transcription (RT) quantitative polymer chain reaction (qPCR)

Supplementary methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured in endothelial basal medium-2 (EBM-2) supplemented with EGM™-2 BulletKit™ (Lonza). Cell from passage 3 to passage 6 were used. Each experiment was performed on at least 2 independent cell batches. HeLa ATCC®CCL-2™ cells were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% fetal bovine serum. Normal human astrocytes (Lonza) were cultured in astocytic basal medium (ABM) supplemented with AGM bulletKit™ (Lonza). Cell from passage 2 to passage 5 were used.

Recombinant proteins and chemicals

Recombinant N-Shh was from Shenedoah, the Hhat inhibitor RU SKI 43 was from Sigma Aldrich, the Smoothened agonist SAG was from Sigma Aldrich, while GDC-0449 was from Selleckchem.

siRNA/transfection

HUVECs were transfected with human Dhh siRNA: rArCrUrCrCrUrUrArArArGgArGrGrArCrUrArUrUrUrArGCC, human Disp1 siRNA: rGrGrArUrCrUrArArCrArArGrUrUrArCrArUrUrGrUrArUAG or universal scrambled negative control siRNA duplex (Origen) using JetPRIME™ transfection reagent (Polyplus Transfection), according to the manufacturer's instructions.

Plasmids/Transfection

pIRES-NDhh [47], pIRES-NShh [16] and pcDNA3-FL-Dhh [43] were described previously. Human FL-Dhh sequence was obtained after EcoRI digestion of pBS hSHH (Addgene ID13996) and then cloned at the EcoRI site of pCDNA3.1 myc His to generate the pcDNA3-FL-Shh plasmid. The myc-tagged human Ptch1, Ptch1-1B-myc was kindly given by R. Toftgard [48] and the human full length Dhh was previously described [43]. HeLa cells were transfected using JetPRIME™ transfection reagent (Polyplus Transfection), according to the manufacturer's instructions.

Quantitative RT-PCR

RNA was isolated using Tri Reagent® (Molecular Research Center Inc) as instructed by the manufacturer, from $3x10^5$ cells or from tissue that had been snap-frozen in liquid nitrogen and homogenized. For quantitative RT-PCR analyses, total RNA was reverse transcribed with M-MLV reverse transcriptase (Promega) and amplification was performed on a DNA Engine Opticon®2 (MJ Research Inc) using B-R SYBER® Green SuperMix (Quanta Biosciences). Primer sequences are reported in Supplementary table 1.

The relative expression of each mRNA was calculated by the comparative threshold cycle method and normalized to Actb mRNA expression.

Migration assay

Cell migration was evaluated with a chemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD, USA). Briefly, a polycarbonate filter (8 µm pore size) (GE Infrastructure, Fairfield, CN, USA) was coated with a solution containing 0.2% gelatin (Sigma-Aldrich Co.) and inserted between the chambers. $5×10^4$ cells per well were seeded in the upper chamber, and the lower chamber was filled with EBM-2 containing 0.5% FBS. Cells were incubated for 8 hours at 37°C then viewed under 20× magnification,

and the number of cells that had migrated to the lower chamber were counted in 3 HPFs per well; migration was reported as the mean number of migrated cells per HPF. Each condition was assayed in triplicate and each experiment was performed at least three times.

In vitro permeability assay

100 000 cells were seeded in Transwell® inserts. The day after, 0.5 mg/mL 70 kDa FITC-dextran (Sigma) was added to the upper chamber. FITC fluorescence in the lower chamber was measured one hour later.

Immunoprecipitation/western blot analysis

Prior to western blot analysis, Ptch1 was immunoprecipitated with anti myc-tag antibodies (Millipore, Cat# 05-724). Expression of Dhh, Shh, Ptch1 and Smo were evaluated by SDS PAGE using mouse anti-Dhh antibodies (Santa Cruz Biotechnology, Inc, Cat# sc-271168), mouse anti-Shh antibodies (Santa Cruz Biotechnology, Inc, Cat#sc-365112), rabbit-anti Hh antibodies (Santa Cruz Biotechnology, Inc, Cat# sc-9024), rabbit anti-Ptch1 antibodies (Abcam, Cat#ab53715).

Expression of junction protein Cdh5, Ocln and Cldn5 was evaluated by SDS PAGE using goat anti-mouse Cdh5 antibodies (R&D systems, cat#AF1002), mouse anti-Ocln (Invitrogen, cat#33-1500) and mouse anti-Cldn5 antibodies (Invitrogen, cat#33-1500.

Protein loading quantity was controlled using rabbit monoclonal anti-β-actin antibodies (Cell Signaling Technology, Cat#4970). Secondary antibodies were from Invitrogen, Cat#A-21039, A-21084, A-21036). The signal was then revealed by using an Odyssey Infrared imager (LI-COR).

Proximity ligation assay

Proximity ligation assay was performed using the Duolink® In Situ Orange Starter Kit Mouse/Rabbit (Sigma) according to the manufacturer instructions. Images were taken at the 63x magnification under a confocal microscope. 8-12 transfected cells per conditions were imaged for quantification.