Methods

Mice. *Eng*$^{flox/flox}$ mice[1] were crossed with *Cdh5(PAC)-CreER*^{T2} mice[2] to generate endothelial specific inducible *Eng* knockout. These mice were in turn crossed with transgenic fluorescent reporter mouse lines B6.Cg-*Gt(ROSA)26Sor*$^{tm3(CAG-EYFP)Hze}$/J (Stock Number 007903, The Jackson Laboratory) or *R26R-H2B-mCherry* (Acc. No. [CDB0203K], http://www.cdb.riken.jp/arg/reporter_mice.html)[3] allowing for *in vivo* lineage tracing and ex vivo time-lapse imaging. For induced gene knockout in neonatal mice, tamoxifen was administrated by postnatal intraperitoneal injections. *Eng* full knockout mice (*Eng*$^{lacZ/lacZ}$) were generated in collaboration with Regeneron by targeted insertion of the *lacZ* gene into exon 2 of *Eng*. TgENG^{LoxP} mice[4] were crossed with *Cdh5(PAC)-CreER*^{T2} mice to generate endothelial specific inducible ENG overexpression. The ENG overexpression mice were then crossed with *Eng* $^{flox/flox}$ to generate *Eng*$^{i\Delta EC+iOE}$ mice. Both males and females are included. Animal housing and procedures were in accordance with Swedish legislation and approved by the local animal ethics committees.

Tamoxifen administration. 10 or 50 µg of tamoxifen was administrated by intraperitoneal (IP) injection at postnatal day (P) 1 or 4 to induce nearly complete gene knockout in neonatal mice. For mosaic *Eng* knockout, 3-10 µg of tamoxifen was administrated by IP injection at the indicated time point. For mosaic ENG overexpression in *Eng*^{iECOE} or *Eng*$^{i\Delta EC+iOE}$ mice, 100 µg of tamoxifen was administrated by IP injection at P3.

***In vivo* imaging and quantification of EC migration.** Mice received tamoxifen (0.2-1mg) one week prior cornea suture implantation. The cornea suture method was performed as previously described [5] . In brief, mice were anesthetized and 3 sutures of Nylon surgical silk (11-0, AROSurgical) were implanted per cornea (considered day 0). At days 5-7, mice were anaesthetized and the newly formed vasculature of the corneas was imaged utilising a Leica SP8 microscope (Leica microsystems) with 25x/1.0 objective. Z-stacks of epifluorescence images were acquired every 24 hours. Time-lapse bright field images were acquired to record blood flow direction. Displacement of ECs between two imaging time points, were defined by the distance to a selected reference point (adjacent vessel branches, or cells without relative change of distance to their neighbouring cells within 24 hours) using LAS AF built-in modules.

***In vivo* VEGFR2 or AKT inhibition.** The mice (*Eng* $^{flox/flox}$; Cdh5(PAC)-CreER^{T2}) were injected with tamoxifen (50µg) at P3 followed by IP injection of SU5416 (20mg/kg/day, Sigma) or Wortmannin (0.3mg/kg/day, Sigma) from P4-P6. The same volume of DMSO was injected to corresponding genotypes, serving as controls. Mice were sacrificed at P7 and retinas processed for immunofluorescent analysis.

Analysis of tip cell competence within retinal or brain vasculature of P7 mice. Retinas or brains with 15-90% recombination were used for the quantification. Contributions of recombined (YFP+) or non-recombined (YFP-) cells to tip-cell position were calculated as number of YFP+ tip cells/(YFP+/CD31+ area) and number of YFP- tip cells/(YFP-/CD31+ area) followed by calculation of the ratio of the two for each sample. Tip cells in five different regions covering both cerebral cortex and striatum were counted and summed for each brain.

Analysis of distribution of recombined cells in the retina. Mosaic recombination was induced at P1 by tamoxifen. Retinas were taken at P7 and stained for CD31, YFP and human ENG and flat mounted to slides by cutting into 4 leaflets. Images of whole retinas were taken by z-stack tile scanning with confocal microscope (Leica SP8). For each leaflet, general recombination ratio for *Eng* knockout was measured as YFP+ area/CD31+area in Volocity; recombination ratio for *Eng* overexpression was measured as human ENG+ area/CD31+ area. Arteries and veins were identified using several well-established criteria: Arteries have smaller diameter, fewer branches and higher smooth muscle cell coverage than veins. Also, arteries and veins appear in an alternating pattern in the retina. Areas within approximately 50 μm around the main arteries and veins are defined as A and V regions respectively. Areas beyond these regions until capillary are defined as Vessels near A or Vessels near V regions. Regional recombination ratio was also measured in the same way as general recombination. The ratio of regional/total recombination was used to assess the distribution of recombined cells.

Analysis of anatomical localisation of malformation initiation. Mosaic recombination was induced at P4 by tamoxifen. Retinas were taken at P7 and stained for CD31 and αSMA and mounted for imaging. Images of whole retinas were acquired by z-stack tile scanning with confocal microscope. A site of malformation was scored positive if the diameter of the vessel exceeded its respective feeding (arterioles, capillaries) or draining (venules, capillaries) vessel.

Hypoxia detection in mouse retina. Hypoxia in the retina was assessed by IP injection of 60mg/kg of pimonidazole (Hypoxyprobe) to P7 mice 90 min prior sacrifice. Eyes were fixed in 4% paraformaldehyde (PFA) for 2 hours at room temperature. Retinas were isolated and immunostained for CD31 and pimonidazole adducts.

Aortic ring assay. Thoracic aortas were taken from mice at P7 and cut into 1 mm rings after removal of the connective tissues. The rings were embedded between two layers of rat tail collagen, type I (Life technologies) and cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and VEGFA (Peprotech, 30 ng/ml). For growth factor treatment, medium was changed to DMEM with 2% BSA and with or without the inclusion of VEGFA (30 ng/ml) after 4 days or initial cultivation. Samples were fixed in 4% PFA and analysed by microscopy after immunostaining with antibodies.

Live imaging and cell tracking in angiogenic sprouts of aortic rings. Aortic rings were cultured as described above in 24-well glass bottom plates (Mattek) and imaged from day four using a Leica SP8 laser confocal microscope system (Leica Microsystems; equipped with a motorized stage and a CO_2 incubator) maintained at 37°C and 5% CO2 with a humidifier. Twenty-nine Z slices were acquired at each position every 20 minutes. Medium was changed every day until termination. Cellular migration was analysed using MTrackJ in ImageJ (NIH) and Imaris software (Bitplane). Single cell tracks were selected manually and data were collected for quantitative analysis of sprout elongation, cellular migration speed and directionality. Distances of the nuclei at the tip position from the first to the last time points were measured as elongation of sprouts. Migration speed of one cell was obtained by averaging the transient speed at each time point (measured by the software). The relative number of time-points a cell migrated towards the sprouting direction is defined as anterograde migration and

analogous for retrograde migration. A cell that migrated less than 2 µm between two time points was defined "still". 17-38 cells were measured for each genotype or treatment. Statistics were analysed using Prism 5.0 software (GraphPad).

Cell culture. HDMECs (PromoCell) were cultured in endothelial cell growth medium MV2 with supplied supplements (PromoCell). The cell lines were authenticated by the vendor (Promocell) for cell morphology and cell-type specific markers using flow cytometric analyses. The cell lines were not authenticated thereafter. The cell lines were tested for mycoplasma contamination by the vendor (Promocell). The cell lines were not tested for mycoplasma contamination thereafter. No cell lines used in this study are found in the database of commonly misidentified cell lines (ICLAC and NCBI Biosample). For VEGFA stimulation, sub-confluent cells were starved in MV2 with 0.2% FBS without supplements for 5 hours. The same medium with VEGFA (50 ng/ml) was then added to the cells and incubated at 37**°**C. Cells were lysed at indicated time points for total or membrane protein isolation or fixed immediately in 4% PFA for antibody staining.

Isolation of mouse lung ECs. Isolation of ECs from mouse lung was done by using anti-CD31-antibody (BD Pharmingen)-conjugated dynabeads (Life Technologies) as previously described[6] . Cells were cultured in MV2 medium (PromoCell) with 4-OH-tamoxifen (5µM, Sigma) for 5 days before splitting for experiments.

Flow-mediated behavioural analysis. HDMECs or mouse lung ECs were seed fully confluent in a flow chamber (CellDirector 2D, Gradientech) and cultured in incubator for 3 hours before experiment. The chamber was then connected to a syringe pump and placed on microscope (equipped with cell culture system) for imaging. Pre-warmed endothelial cell growth medium MV2 was pumped through the flow chamber with a flow rate of 150µl/min (7.5 dyne/cm^2). For VEGFR2 or AKT inhibition SU5416 (5µM) or Wortmannin (100nM) was added to the medium. Bright field images were taken every 5 minutes for 5 hours. Cellular migration was analysed using MTrackJ in ImageJ.

RNA interference. Lentivirus was used to introduce specific short hairpin RNAs (shRNA) into HDMECs. The lentiviral vector pLKO.1-TRC was a gift from David Root (Addgene plasmid #10878). The oligonucleotide sequences used to target human *ENG* were selected from the MISSION shRNA library (TRCN0000083142, Sigma). The control shRNA sequence lacks targets (5'-CCTAAGGTTAAGTCGCCCTCG-3'), (Addgene, plasmid #1864). Lentiviruses were generated using the packaging cell line 293FT. Supernatants containing viral particles were collected at 48 and 72 hour after transfection. Viral supernatants were diluted 1:5 and supplemented with polybrene (8 µg/mL) for infection of HDMECs. The virus-containing medium was replaced with fresh medium 24 hours after infection. The cells were selected by puromycin (1µg/ml) for 7days and then split for experiments.

Immunofluorescence. Mice were sacrificed and brains and eyes immediately removed and fixed in 4% PFA. Brain coronal sections (200 µm thickness) were generated using a vibratome. Retinas were dissected and processed for whole mount antibody staining. Samples were incubated in phosphate buffered saline (PBS) with 0.5% triton X-100 and 1% BSA for 3 hours at room temperature and then with primary antibodies in the same solution overnight at 4°C with agitation. After

3 washes at room temperature, samples were incubated with secondary antibodies overnight at 4°C, washed 3 times and mounted to slides for imaging.

For staining of cell lines, cells were cultured on coverslips and fixed in 4% PFA after treatment. After fixation, the cells were washed 3 times in PBS and permeabilized in PBS with 0.1% tritionX-100 for 5 minutes, followed by block in 1% BSA for 30 minutes and incubation with primary antibodies overnight at 4°C. Cells were washed 3 times in 0.1% tritonX-100 and incubated with secondary antibodies for 1 hour at room temperature. They were then washed 3 times and mounted to slides for imaging. Specimens were imaged by LSM 700 (Carl Zeiss AG) or Leica SP8 (Leica Microsystems) laser confocal microscopes. Image processing and analysis was done using Volocity (PerkinElmer) and ImageJ software.

Antibodies: mouse anti α-smooth muscle actin (ASMA) (Clone: 1A4)(C6198, Sigma, 1:100); goat anti-mouse CD31 (AF3628, R&D Systems, 1:500); rat anti-Endoglin (Clone: MJ7/18)(14-1051, eBioscience, 1:400); chicken anti-GFP (ab13970, Abcam, 1:1000); rabbit anti-ERG (Clone: EPR3864)(ab92513, Abcam, 1:500); rabbit anti-VEGFR2 (Clone: 55B11)(2479, Cell Signaling, 1:200); goat anti-VEGFR2 (AF357, R&D Systems, 1:200); mouse anti-human Endoglin (Clone: SN6)(14-1057-82, eBiosciences, 1:200); goat anti human Endoglin (AF1097, R&D systems, 1:500); alexa555-mouse anti-GM130 (Clone: 35/GM130)(560066, BD, 1:200); mouse anti-EEA1 (Clone: mAbcam18175)(ab18175, Abcam, 1:500); rabbit anti-Rab5 (Clone: C8B1)(3547S, Cell signaling, 1:200); rabbit anti-Rab7 (Clone: D95F2)(9367S, Cell signaling, 1:200); goat anti-Calnexin (sc6465, Santa Cruz, 1:200). Secondary antibodies conjugated with Alexa Fluor dyes were obtained from Life Technologies or Jackson ImmunoResearch Laboratories.

Proliferation. Proliferation in the developing brain and retina was assessed by IP injection of 5-ethynyl-2′-deoxyuridine (EdU, 100 μg/mouse) at postnatal day 7. Mice were sacrificed 2 hours after injection and brains and retinas were removed and fixed in 4% PFA. EdU staining on brain coronal vibratome sections (200 μm thickness) or retinas was done according to the instruction of Click-iT EdU imaging kit (Life Technologies) apart from 3 hours incubation in the reaction mix at room temperature. The samples were co-stained for ERG for EC identification as described above. Images were taken by confocal microscope and analysed using Volocity software. Percentages of proliferative ECs among non-recombined ECs are calculated as: mCherry-, ERG+, EdU+ cells/mCherry-, ERG+ cells; percentages of proliferative ECs among recombined ECs are calculated as: mCherry+, ERG+, EdU+ cells/mCherry+, ERG+ cells.

Quantitative PCR. RNA was extracted and purified using RNeasy Plus kit (Qiagen). RNA concentrations were measured by Nanodrop spectrophotometer (ThermoFisher Scientific) and adjusted to equal, followed by reverse transcription using SuperScript III (Life Technology). Real-time quantitative PCR were performed on ABI 7300 real-time PCR machine using SYBR Green master mix (Kapa Biosystem). Housekeeping gene ribosomal protein L19 (*Rpl19*) was used as internal control. The comparative Ct method was used to calculate fold differences.
Primers for mouse genes: *Cxcr4*, forward: 5′-TCAGTGGCTGACCTCCTCTT-3′, reverse: 5′-TTTCAGCCAGCAGTTTCCTT-3′; *Dll4*, forward: 5′-CAGAGACTTCGCCAGGAAAC-3′, reverse: 5′-TCATTTTGCTCGTCTGTTCG-3′; *Eng*, forward: 5′-CTTCCAAGGACAGCCAAGAG-3′, reverse: 5′-TTCTGGCAAGCACAAGAATG-3′; *Hey1*, forward: 5′-GAGACCATCGAGGTGGAAAA-3′, reverse: 5′-CTTCTCGATGATGCCTCTCC-3′; *Id1*, forward: 5′-GGTACTTGGTCTGTCGGAGC-3′, reverse: 5′-TCATGTCGTAGAGCAGGACG-3′; *Rpl19*, forward: 5′- GGTGACCTGGATGAGAAGGA-3′, reverse: 5′-

TTCAGCTTGTGGATGTGCTC-3´; *Vegfa*, forward: 5´- AGCACAGCAGATGTGAATGC-3´, reverse: 5´-AATGCTTTCTCCGCTCTGAA-3´; *Efnb2*, forward: 5'- AGGAATCACGGTCCAACAAG-3', reverse: 5'-GTCTCCTGCGGTACTTGAGC-3'.
Primers for human genes: *ENG*, forward: 5'- CACTAGCCAGGTCTCGAAGG-3', reverse: 5'-CTGAGGACCAGAAGCACCTC-3'; *JAG1*, forward: 5'- AGGCCGTTGCTGACTTAG-3'; reverse: 5'-GCAGAAGTGGGAGCTCAA-3'; *KLF2*, forward: 5'- CCTCCCAAACTGTGACTGGT -3', reverse: 5'-ACTCGTCAAGGAGGATCGTG-3'; *HEY1*, forward: 5'- CGAGGTGGAGAAGGAGAGTG-3', reverse: 5'-GCGCGTCAAAGTAACCTTTC-3'.

X-gal staining. Fresh brain sections or retinas fixed in 4% PFA for 1h at 4°C were washed in rinse buffer (0.1 M Phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40) twice, followed by incubation in staining solution (0.1 M Phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mM potassiumferricyanide, 5 mM potassiumferrocyanide, 1 mg/ml X-gal) for 1-2 hours at 37°C and then washed in rinse buffer twice, 20 minutes each time at room temperature. Samples were then post fixed in 4% PFA for 10 min and continue with immunostaining with antibodies.

Sorting (FACS) of brain ECs from mice harbouring conditional fluorescent reporters. P7 pups with conditional EC-specific fluorescent reporters were anesthetized and perfused with 2 ml Hank's Balanced Salt Solution (HBSS). Pups were then decapitated and brains were transferred to tubes with cold PBS (without Ca or Mg) with 2mM EDTA and 1% BSA and then cut into small pieces with scalpel. Materials were transferred into a 15 ml tube containing 10 ml pre-warmed trypsin (0.05%), rotated end-over-end at 37°C for 12 minutes. Cell suspension was pipetted up and down for several times and passed through a 40 µm cell strainer into a 50 ml tube on ice. Cells were spun down at 400g at 4°C and re-suspended in cold PBS (without Ca or Mg) with 2mM EDTA and 1% BSA to a concentration of 1-40×10^6 cells/ml. GFP+, mCherry- and GFP+, mCherry+ cells were sorted simultaneously by FACS (BD FACSARIA III) into RNAlater (Sigma) in different tubes. RNA was extracted using RNeasy micro kit (Qiagen).

Surface biotinylation. HDMECs (PromoCell), transduced with either control shRNA or Endoglin targeting shRNA, were starved for 4 h in Endothelial Cell Basal Medium MV2 with 0.2% FBS. Following the starvation, the transduced HDMECs were stimulated for 10, 30 or 60 min with 50 ng/ml VEGFA (Peprotech). For the biotinylation of surface proteins after the stimulation, the cells were washed twice with ice cold PBS and incubated with 0.5 mg/ml Sulfo-NHS-biotin (EZ-Link, Thermo Scientific) for 30 min at 4°C. To block the remaining reactive biotin, cells were washed twice with ice cold PBS containing 100 mM Glycine. Then cells were lysed in lysis-buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1% Igepal CA-630, 1% Triton X-100 (Sigma)) supplemented with protease and phosphatase inhibitor cocktails (Roche), Complete and PhosSTOP (Roche), respectively, for 60 min at 4°C. Insoluble cell debris were pelleted by centrifugation for 15 min at 4°C with 14000 x g. Input protein samples were taken from the supernatant, mixed with β-Mercaptoethanol-containing Laemmli-buffer, boiled and frozen for later use at -20°C. Biotinylated proteins were precipitated by adding the supernatant to Streptavidin-sepharose beads (GE Healthcare) and their incubation for 16 h at 4°C, end-over-end. The Streptavidin-sepharose beads were then washed with ice cold TBS and proteins were denatured and released from the beads by boiling in β-Mercaptoethanol-containing Laemmli buffer.

Immunoprecipitation. The transduced HDMECs were starved for 4 h in Endothelial Cell Basal Medium MV2 with 0.2% FBS. Following the starvation, the cells were stimulated for 5, 10, 15 or 30 minutes with 50 ng/ml VEGFA and lysed on ice in lysis buffer (50 mMTris-Hcl pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40) supplemented with protease and phosphatase inhibitor cocktails, Complete and PhosSTOP. Lysates were incubated with VEGFR2 antibody (55B11, Cell Signalling) coupled to Protein G beads, overnight at 4°C. Beads were washed 5 times with lysis buffer and proteins were denatured and released from the beads by boiling in β-Mercaptoethanol-containing Laemmli buffer.

Western blot. The protein samples were separated on a SDS-PAGE (4-12% gradient gel) (Life Technologies), transferred to nitrocellulose membranes (Life Technologies), and incubated sequentially with primary and appropriate horseradish peroxidase (HRP) conjugated secondary antibodies. Signals were detected using the ECLprime reagent kit (GE Healthcare). Pictures were obtained by the imaging system FluroChem Q (AlphaInnotec) and analysed using ImageJ software. The following antibodies were used: mouse anti-Endoglin (Clone: SN6)(14-1057-82, eBioscience);rabbit anti-VEGFR2 (Clone: 55B11)(2479, Cell Signaling); mouse anti-phospho Tyrosine (Clone: 4G10) (16-103, Millipore); mouse anti-phospho Tyrosine (Clone: PY99) (sc-7020, Santa Cruz Biotechnology) rabbit anti-Endoglin (ab135528, AbCam); rabbit anti-phospho ERK-1/2 (D.13.14.4E) (T202/Y204) (4370, Cell Signaling); mouse anti-total ERK-1/2 (Clone: L34F12) (4696, Cell Signalling) goat anti-Calnexin (sc-6465, Santa Cruz Biotechnology); rabbit anti-p*Akt (Ser473)(Clone: D9E)(4060, Cell Signaling); rabbit anti-panAKT (Clone: C67E7)(4691, Cell Signaling); mouse anti pan-AKT (Clone: 40D4) (2920, Cell Signaling), sheep anti-mouse IgG HRP (RPN4201, GE Healthcare); goat anti-rabbit IgG HRP (RPN4301, GE Healthcare). For detection of the complete biotinylated protein fraction, membranes were probed with Streptavidin-HRP (GE Healthcare). Primary antibodies were used at a dilution of 1:1000, secondary antibodies were used at a dilution of 1:7500. Dilution for Streptavidin-HRP is 1:2500.

Statistics and Reproducibility. Statistical analysis was performed using GraphPad Prism software. Statistical significance in figures 1c, 1d, 2c, 2e, 2g, 2i, 2k, 2m, 3c-h, 4d, 4e, 4g, 5b, 5d, 5f, 5h, 5j, 5l, 6b, 6d-i, 7i, 8b, 8c and Supplementary Fig. 6d-f, 8a, 8b was determined by unpaired two-tailed Student's t-test. Chi-square test was used in 6l. Two-way ANOVA was used to compare between groups in time course experiments (figures 7b, 7d, 7f-g, 7j). Variances were similar between the groups compared. Differences were considered significant with a $P < 0.05$. For animal experiments, no statistical methods were used for predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiment and outcome assessment.

Data availability. Source data for Fig. 1d, 2g, 2m, 4d, 6f-i, 7b, 7d, 7f, 7g, 7i, 7j have been provided as Supplementary Table 1. All other data supporting the findings of this study are available from the corresponding author on request.

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