SUPPLEMENTAL MATERIAL

Detailed Methods

Animals. Notch3$^{-/-}$ mice in a C57BL/6 background were a generous gift from Dr. Tom Gridley.[1] Notch3$^{-/-}$, Notch3$^{+/-}$ and wild type littermates were obtained by crossing Notch3$^{+/-}$ and Notch3$^{+/-}$ mice. All experimental procedures on mice were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Committee for the Use of Animals in Research.

Cell culture. Human aortic smooth muscle cells from Lonza were grown in DMEM supplemented with 10% FBS, 100I.U./ml penicillin-streptomycin. Cells between passages 6 and 8 were used for experiments. Mouse aortic smooth muscle cells were isolated from aortas of Notch3$^{+/-}$ and Notch3$^{-/-}$ mice. Cells between passages 3 and 8 were used for experiments. All cultures were maintained in humidified 5% CO_2 at 37 °C. Lentiviral and adenoviral transduction was performed as described previously.[2, 3] Briefly, recombinant lentiviruses were produced by transient transfection of TN-293 cells (Clontech). Subconfluent TN-293 cells were transiently co-transfected with pCDF1-MCS2-EF1-copGFP-NICD3 or the control plasmid without insert, and the lentiviral packaging plasmids pFIV34N and pVSV-G overnight. 48 hours after transfection, viral supernatant was harvested and used for infection. The human HIF1α cDNA was cloned into the pAdTrack shuttle vector in front of the CMV promoter using KpnI and EcoRI restriction sites. The adenovirus plasmids containing GFP alone (AdGFP) and GFP together with HIF1α (AdHIF1α) were transfected into HEK293 cells, and the viral particles were amplified and purified. For infection, human aortic smooth muscle cells were seeded in a 12-well plate at a density of $2x10^4$ cells per well 24 hours prior to viral infection. To each well, 0.5ml of lentivirus suspension diluted in 0.5ml DMEM with 10% FBS was added. Polybrene was supplemented at a final concentration of 6μg/ml. 24 hours later, cells were transferred to fresh DMEM containing 10% FBS. 72 hours after infection, cells were infected with adenoviral particles, which were diluted in 400μl Opti-MEM (Invitrogen). 2 hours after incubation, 600μl normal growth media was supplemented. The following day, the virus-containing media was replaced with fresh media. Efficiency of transduction was monitored by GFP expression.

Immunostaining on whole-mount retinas. The immunostaining in retinas was performed as previously described.[3] Briefly, eyes were isolated from Notch3$^{-/-}$ and Notch3$^{+/-}$ mice at indicated time points and fixed in 4% paraformaldehyde for 30 minutes. The cornea, sclera, lens, vitreous, and hyaloid vessels were removed to isolate retinas and radial incisions were made at equal intervals along the retinal edge. Subsequently retinas were placed in cold methanol for 20 minutes, blocked and permeabilized in PBS containing 5% donkey serum and 0.3% Triton-X-100 for 1 hour. Primary antibody, Notch3 (1:100) (Santa Cruz), sm α-actin (1:1000) (Sigma), NG2 (1:200) (Millipore),

Ang-2 (1:200) (Zymed), Collagen IV (1:200) (Millipore), or phospho-histone H3 (1:200) , was costained with 10μg/ml TRITC labeled-isolectin B4 (*Griffonia simplicifolia*) (Invitrogen) for 2 hours at 37°C. Incubations with fluorescently tagged secondary antibodies were performed at 4°C overnight, including Alexa-Fluor 488 donkey anti-mouse (1:250), Alexa-Fluor 594 goat anti-rabbit and Alexa-Fluor 488 goat anti-rabbit (Invitrogen). Retinas were flat mounted in Vectashield (Vector Laboratories). Confocal images were captured and vessels were quantified with NIH ImageJ software by a blind observer.

Cell death detection ELISA. For the detection of apoptosis, a cell death detection ELISA^{PLUS} kit (Roche) was used to quantify DNA fragmentation according to the manufacturer's instructions. Briefly, isolated retinas were homogenized in 100μl lysis buffer, incubated for 30 minutes at room temperature, and centrifuged for 10 minutes at 2000rpm. 20μl of the supernatant of each retina were assayed. For cells, aortic smooth muscle cells from Notch3$^{+/-}$ and Notch3$^{-/-}$ mice were plated at a density of 5x10^4 cell in a 12-well plate, starved in DMEM with 0.25% FBS for 72 hours. An equal number of cells were lysed in 100μl lysis buffer and were utilized for cell death detection.

Aortic ring assay. Aortic sprouting assays were performed as previously described with modification.[4] Briefly, aortas from Notch3$^{+/-}$ and Notch3$^{-/-}$ littermates at P11 were dissected, cut into 1-mm-thick rings, and incubated overnight in complete EBM-2 media (Lonza) containing 10% FBS before being placed into a rat tail collagen gel (1.5mg/ml). 48 hours after embedding, rings were stained with 10μg/ml TRITC labeled-isolectin B4 and NG2, and imaged by confocal microscopy. Vessels were quantified with ImageJ software.

Oxygen-induced retinopathy (OIR). OIR was induced as previously described, with minor modifications.[5] In brief, at postnatal day (P)7 pups, along with nursing mothers, were placed in 70% oxygen. At P12, they were returned to 21% oxygen for 5 days. From P12 to P17, the animals were anesthetized, their retinas were collected and iso-lectinB4 immunostaining on whole-mount retinas were performed. Avascular area was quantified as percentage of whole retinal area. Neovascularization was quantified using ImageJ software by calculating the area of iso-lectinB4 staining of neovascular tufts as reported.[6]

RNA isolation and quantitative reverse transcriptase-polymerase chain reaction (qPCR). Total RNA was extracted from mouse retinas using RNAqueous-4PCR kit (Invitrogen) according to the manufacturer's instruction, and reverse transcribed with M-MLV reverse transcriptase (Invitrogen) to generate cDNA. Quantitative PCR was performed using a StepOne PCR system (Applied Biosystems) with Power SYBR Green. The fold difference in various transcripts was calculated by the ΔΔCT method using 18S as the internal control. After PCR, a melting curve was constructed in the range of 60°C to 95°C to evaluate the specificity of the amplification products. Primer sequences for mouse transcripts were as follows: 18S For-5'-GTT GGT TTT CGG AAC TGA GGC-3'; 18S Rev-5'-GTC GGC ATC GTT TAT GGT CG-3'; Notch3 For-5'-TTG TCT GGA TGG AAG CCC ATG T-3'; Notch3 Rev-5'-ACT GAA CTC TGG CAA ACG CCT-3'; Angiopoietin-2 For-5'-ACA CCG AGA AGA TGG CAG TGT-3'; Angiopoietin-2 Rev-

5’-CTC CCG AAG CCC TCT TTG TA-3’; Angiopoietin-1 For-5’-GGG CTG GAA GGA GTA TAA AAT GG-3’; Angiopoietin-1 Rev-5’-GAA CTC GTT CCC AAG CCA ATA T-3’; Tie-2 For-5’-CAA TCA GGC CTG GAA ATA CAT TG-3’; Tie-2 Rev-5’-TCC GCG GCT CCA AGT AGT T-3’. For human Angiopoietin-2 For-5’-AAC AGG AGG CTG GTG GTT TG-3’; Rev-5’-TGT GGA TAG TAC ATT CCG TTC AAG TT-3’. Hrt3 For-5’-CGC AGA GGG ATC ATA GAG AAA CG-3’; Hrt3 Rev-5’-GCC AGG GCT CGG GCA TCA AAG AA-3’; Hes1 For-5’-CCC CAG CCA GTG TCA ACA C-3’; Hes1 Rev-5’-TGT GCT CAG AGG CCG TCT T-3’; Hrt2 For-5’-CAC ATC AGA GTC AAC CCC ATG T -3’; Hrt2 Rev-5’-GCC ATG AGC AGA AGG CAC TT-3’.

Statistical analysis. Data analyses were performed using PrismGraph and comparisons between data sets were made using Student’s t test. Differences were considered significant if $P < 0.05$, and data are presented as mean ± standard error of the mean (SEM). Data shown are representative of at least three independent experiments.

Supplemental References

1. Krebs LT, Xue Y, Norton CR, Sundberg JP, Beatus P, Lendahl U, Joutel A, Gridley T. Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. *Genesis.* 2003;37:139-143.

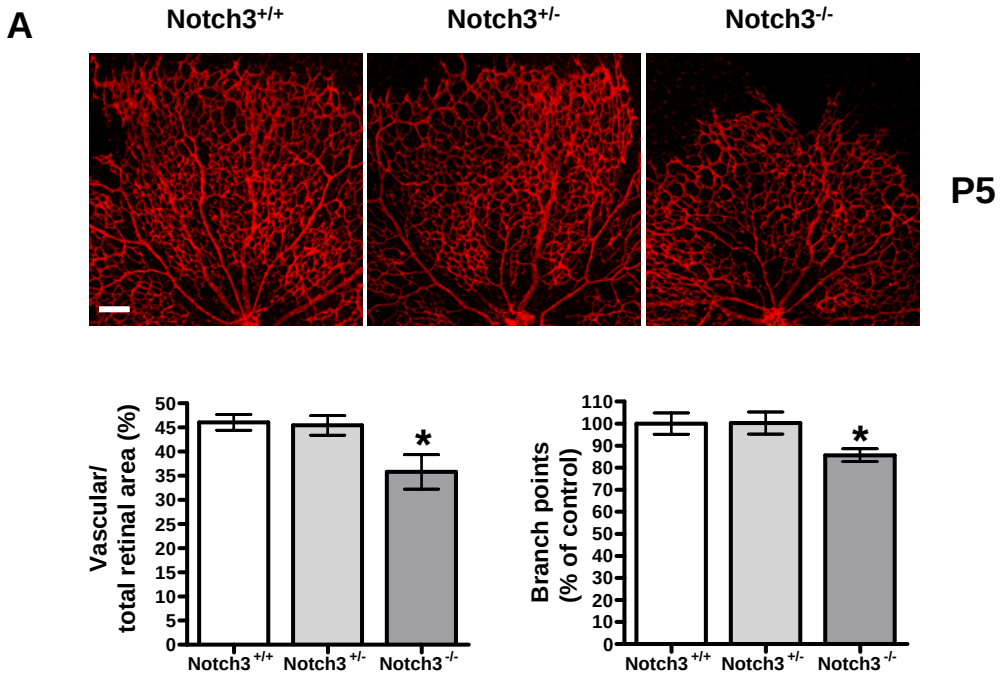
2. Corley KM, Taylor CJ, Lilly B. Hypoxia-inducible factor 1alpha modulates adhesion, migration, and FAK phosphorylation in vascular smooth muscle cells. *J Cell Biochem.* 2005;96:971-985.

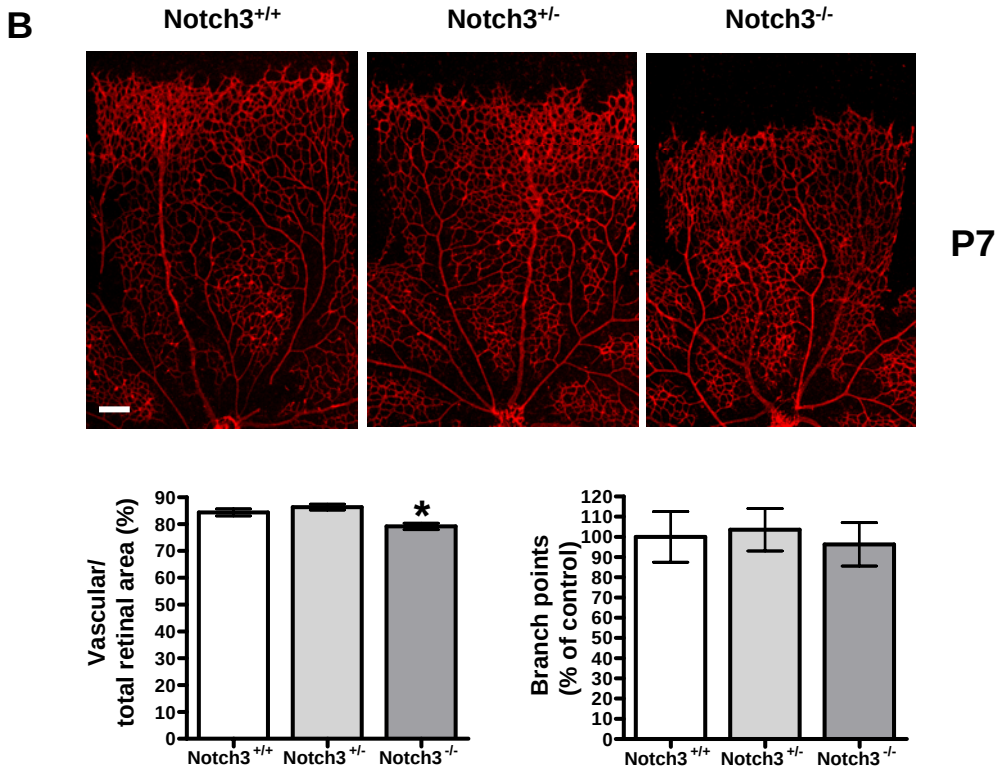
3. Liu H, Kennard S, Lilly B. NOTCH3 expression is induced in mural cells through an autoregulatory loop that requires endothelial-expressed JAGGED1. *Circ Res.* 2009;104:466-475.

4. Masson VV, Devy L, Grignet-Debrus C, Bernt S, Bajou K, Blacher S, Roland G, Chang Y, Fong T, Carmeliet P, Foidart JM, Noel A. Mouse Aortic Ring Assay: A New Approach of the Molecular Genetics of Angiogenesis. *Biol Proced Online.* 2002;4:24-31.

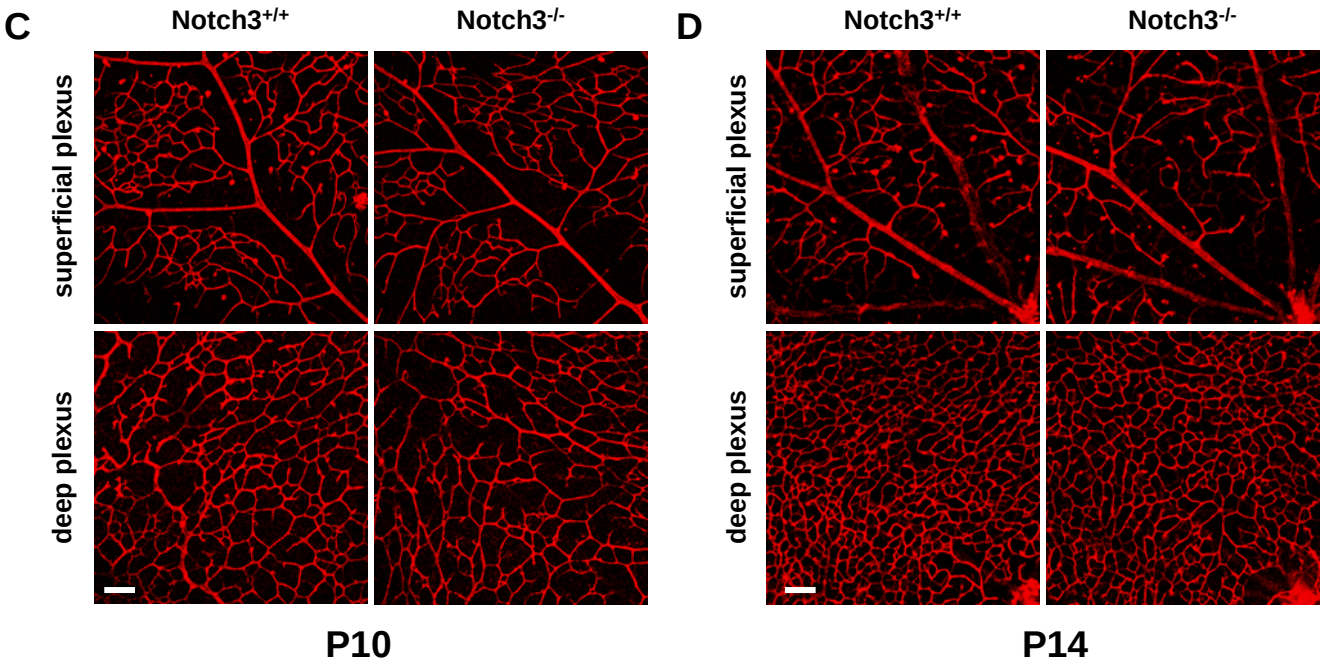
5. Smith LE, Wesolowski E, McLellan A, Kostyk SK, D'Amato R, Sullivan R, D'Amore PA. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci.* 1994;35:101-111.

6. Stahl A, Connor KM, Sapieha P, Willett KL, Krah NM, Dennison RJ, Chen J, Guerin KI, Smith LE. Computer-aided quantification of retinal neovascularization. *Angiogenesis.* 2009;12:297-301.

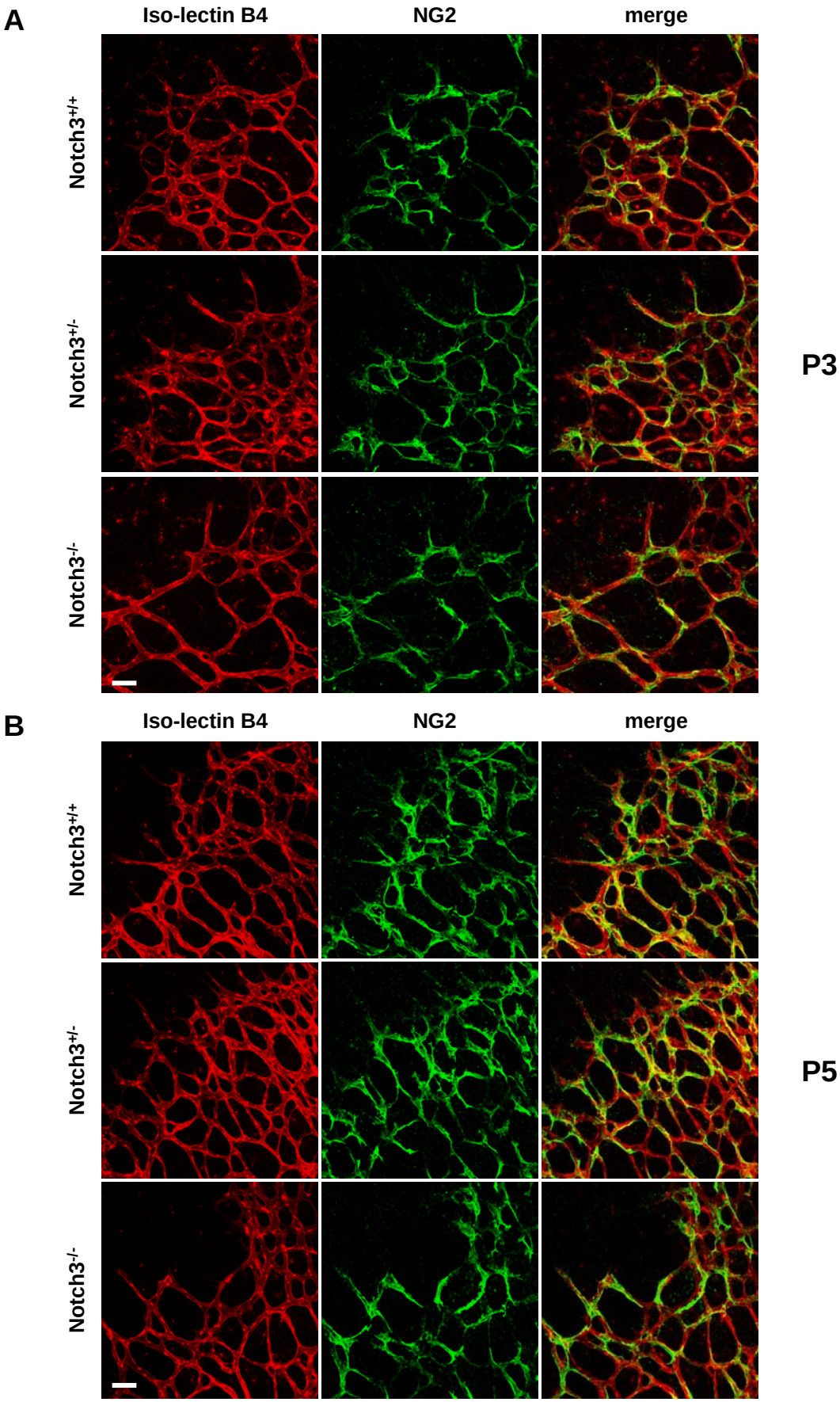




Online Figure I

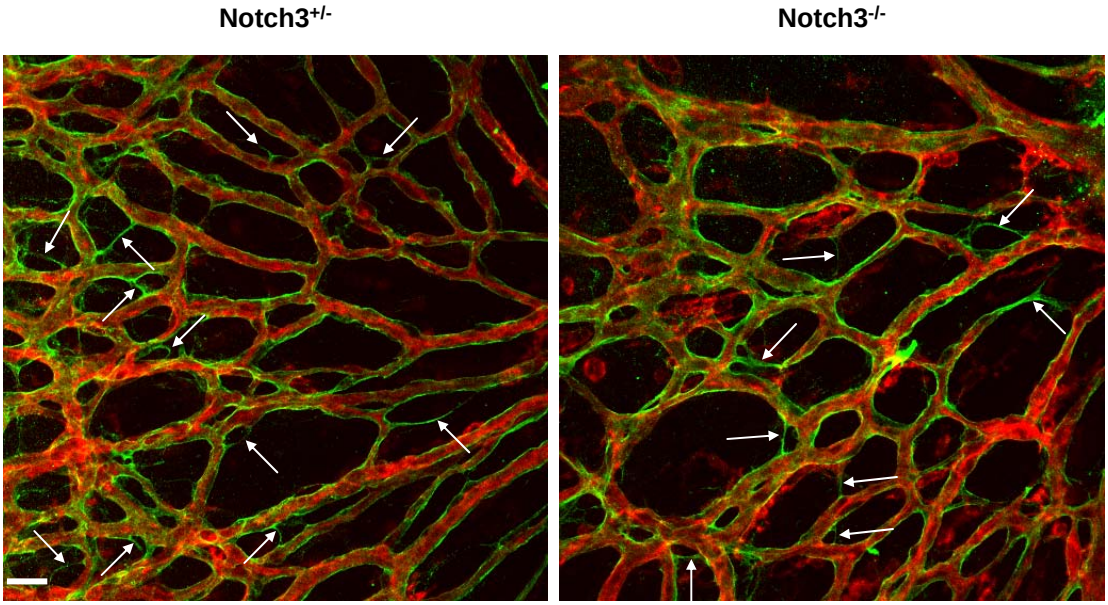


Online Figure I. Notch3 deletion compromises retinal angiogenesis *in vivo*. (A, B) Retinal vasculature from Notch3$^{+/+}$, Notch3$^{+/-}$ and Notch3$^{-/-}$ littermates were stained with iso-lectin B4 at P5 and P7. Graphs show quantification of vascularized area and branch points in retinas. * $P < 0.05$ compared to heterozygous and wild type. Images were taken at 50X by confocal microscopy. Bar, 25μm. (C, D) Superficial and deep vascular plexus from Notch3$^{+/+}$ and Notch3$^{-/-}$ littermates were stained with iso-lectin B4 at P10 and P14. Images were taken at 100X by confocal microscopy. Bar, 100μm.

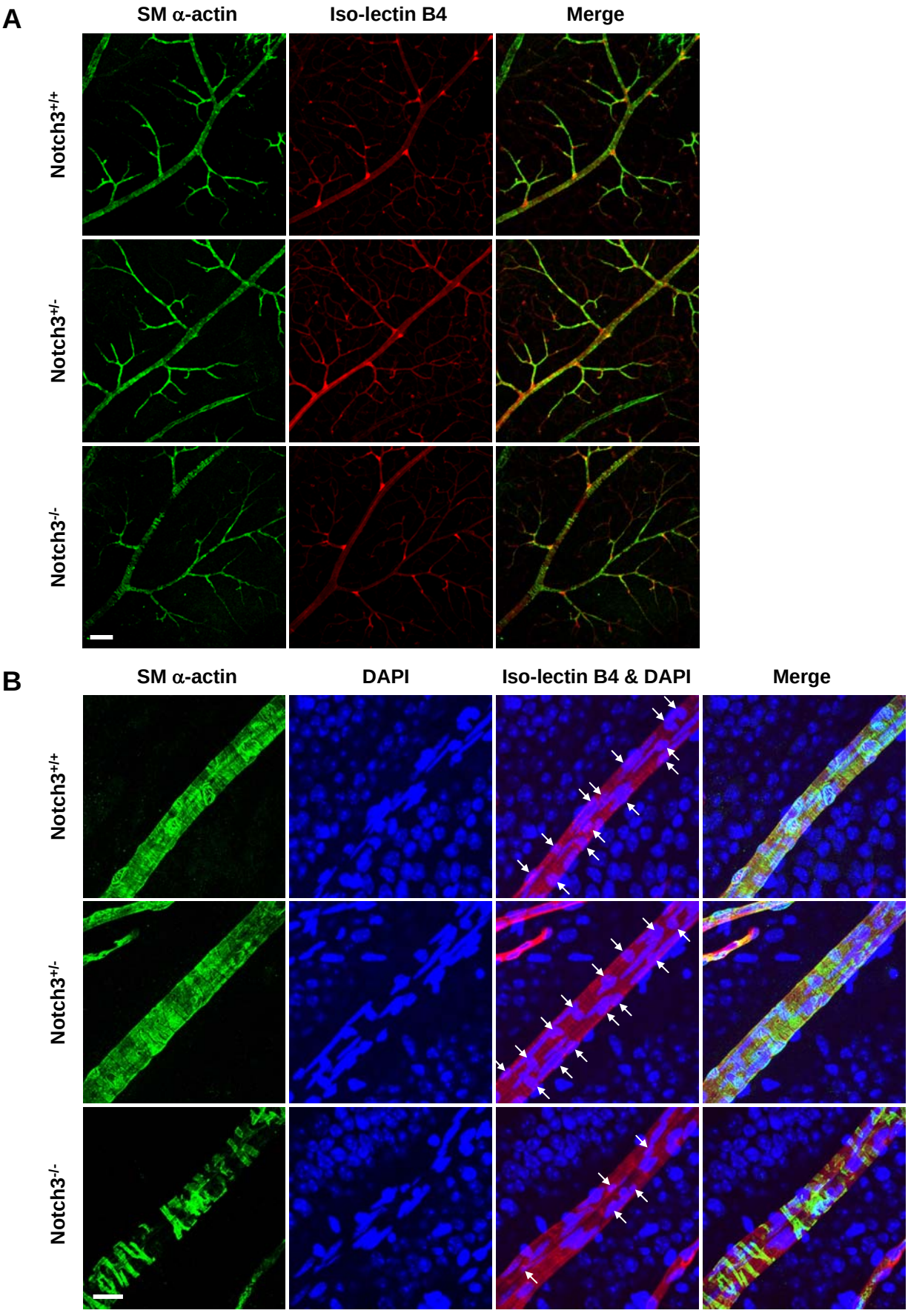


Online Figure II

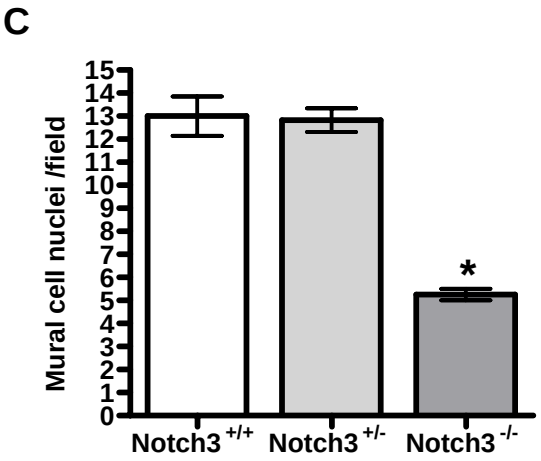
Online Figure II. Mural cells in the angiogenic front of the retina. Retinas isolated from Notch3$^{+/+}$, Notch3$^{+/-}$ and Notch3$^{-/-}$ at P3 (A) and P5 (B) were stained with iso-lectin B4 (red) and mural cell marker NG2 (green). Confocal images were taken at 200X magnification. Bar, 50μm.



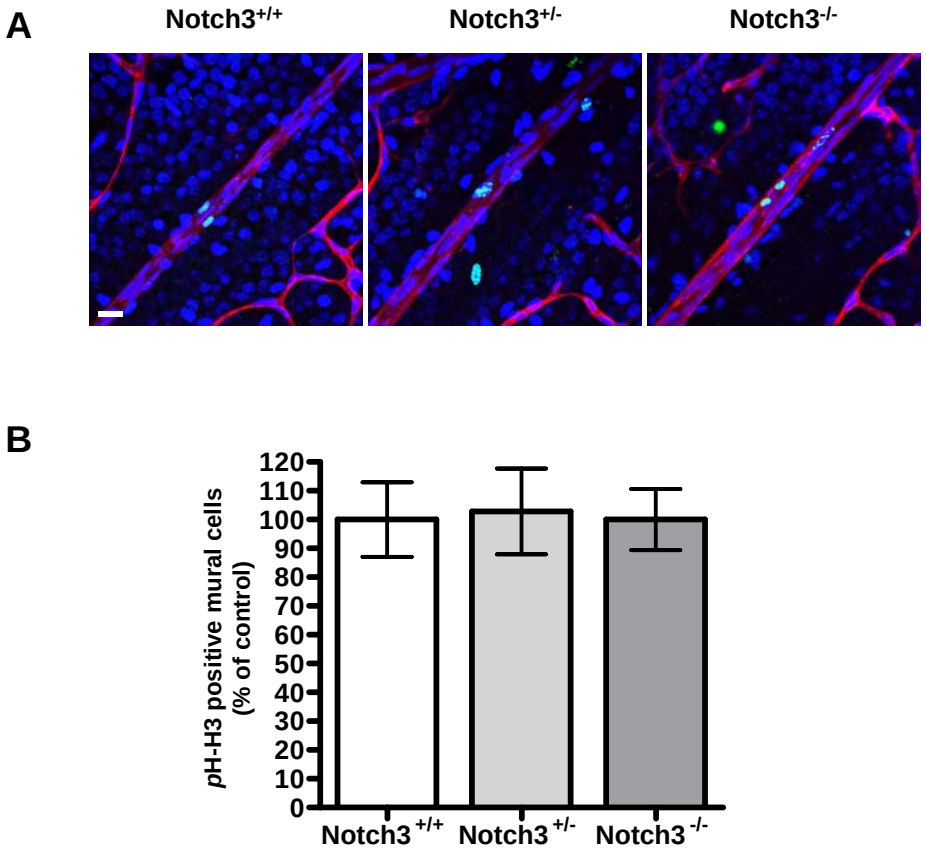
Online Figure III. Notch3 deletion does not increase vessel regression. Retinas were isolated from Notch3$^{+/-}$ and Notch3$^{-/-}$ littermates at P3, and stained with iso-lectin B4 (red) and anti-collagen IV (green). Confocal images were taken at 250X magnification. Bar, 25μm. Arrows indicate the empty sleeves (collagen IV+ iso-lectin B4−) resulting from vessel regression.



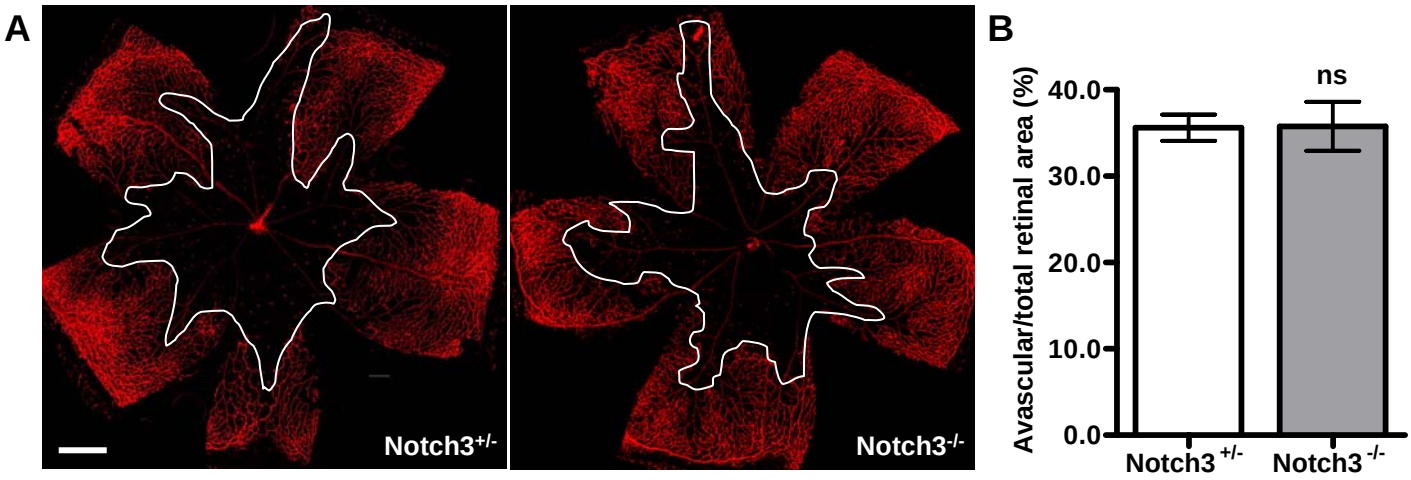
Online Figure IV



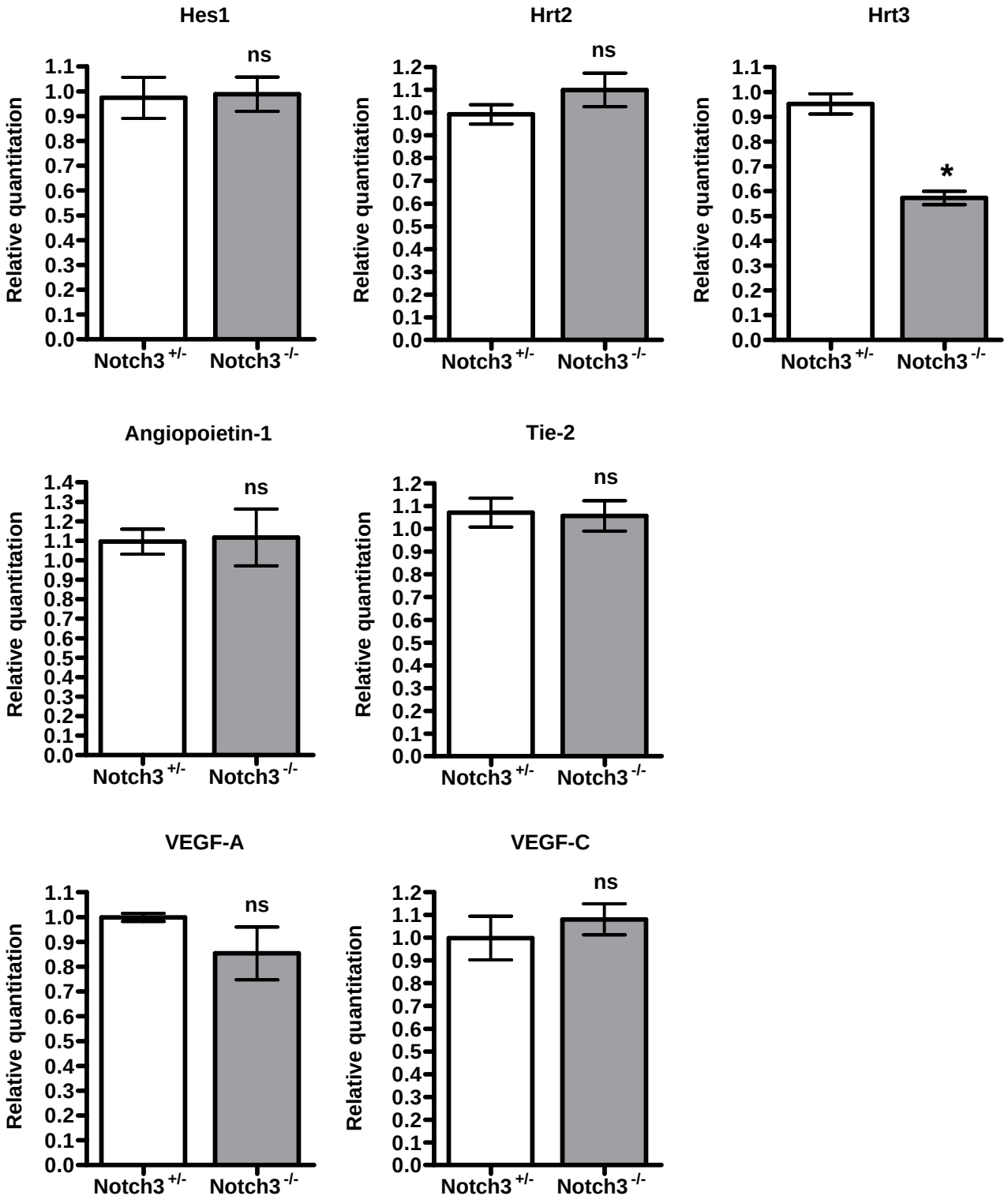
Online Figure IV. Notch3 deletion decreases the coverage of mural cells in retinal vasculature. Retinas were isolated from Notch3$^{+/+}$, Notch3$^{+/-}$ and Notch3$^{-/-}$ littermates at P43, and stained with mural cell marker sm α-actin (green) and iso-lectin B4 (red) (A). Confocal images were taken at 100X magnification. Bar, 100μm. Retinas were stained with sm α-actin (green), DAPI (blue, for nuclei) as well as iso-lectin B4 (red) (B). Arrows indicate the nuclei of mural cells. Confocal images were taken at 630X magnification. Bar, 20μm. (C) Quantification of mural cell nuclei in retinal arteries of Notch3$^{+/+}$, Notch3$^{+/-}$ and Notch3$^{-/-}$ at P43. * $P < 0.05$ compared to relevant controls.



Online Figure V. Notch3 deletion does not affect proliferation of mural cells in retinal arteries. (A) Retinas were isolated from Notch3$^{+/+}$, Notch3$^{+/-}$ and Notch3$^{-/-}$ littermates at P10, and stained with iso-lectin B4 (red), anti-phospho-histone H3 (*p*H-H3, green, for proliferation) and DAPI (blue, for nuclei). Confocal images were taken at 400X magnification. Bar, 20μm. (B) Quantification of proliferating mural cells in retinal arteries.

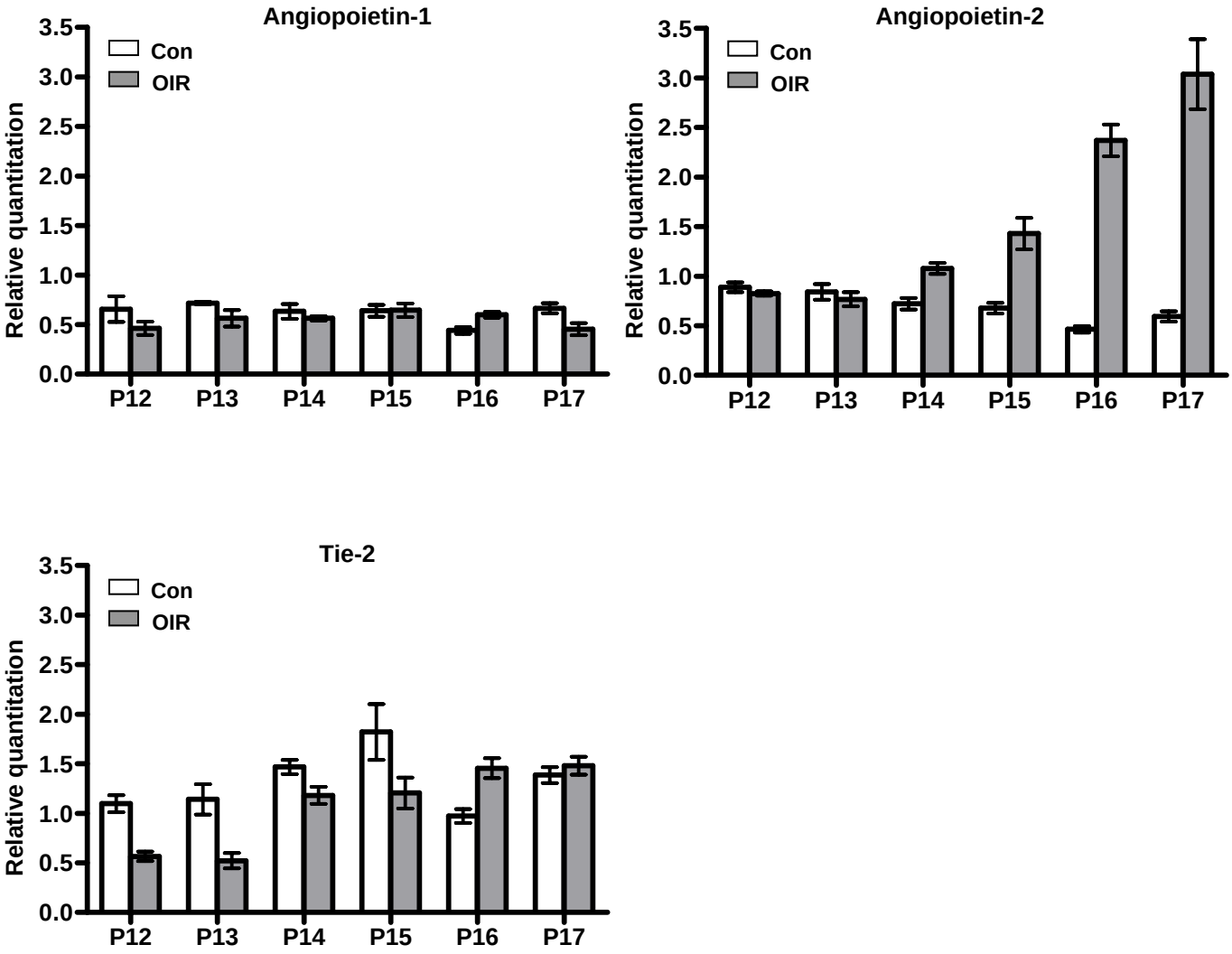


Online Figure VI. Notch3 deletion does not affect vessel obliteration at P12 in the OIR model. Retinas from Notch3$^{+/-}$ and Notch3$^{-/-}$ littermates were examined at P12 by microscopy after oxygen-induced retinopathy. (A) Representative retinal vasculature from Notch3$^{+/-}$ (left) and Notch3$^{-/-}$ (right) littermates at P12. White lines outline the area of vaso-obliteration. Bar, 500μm. (B) Graph represents vaso-obliteration in Notch3$^{+/-}$ and Notch3$^{-/-}$ mice at P12 (n=6 per group); ns, not significant.

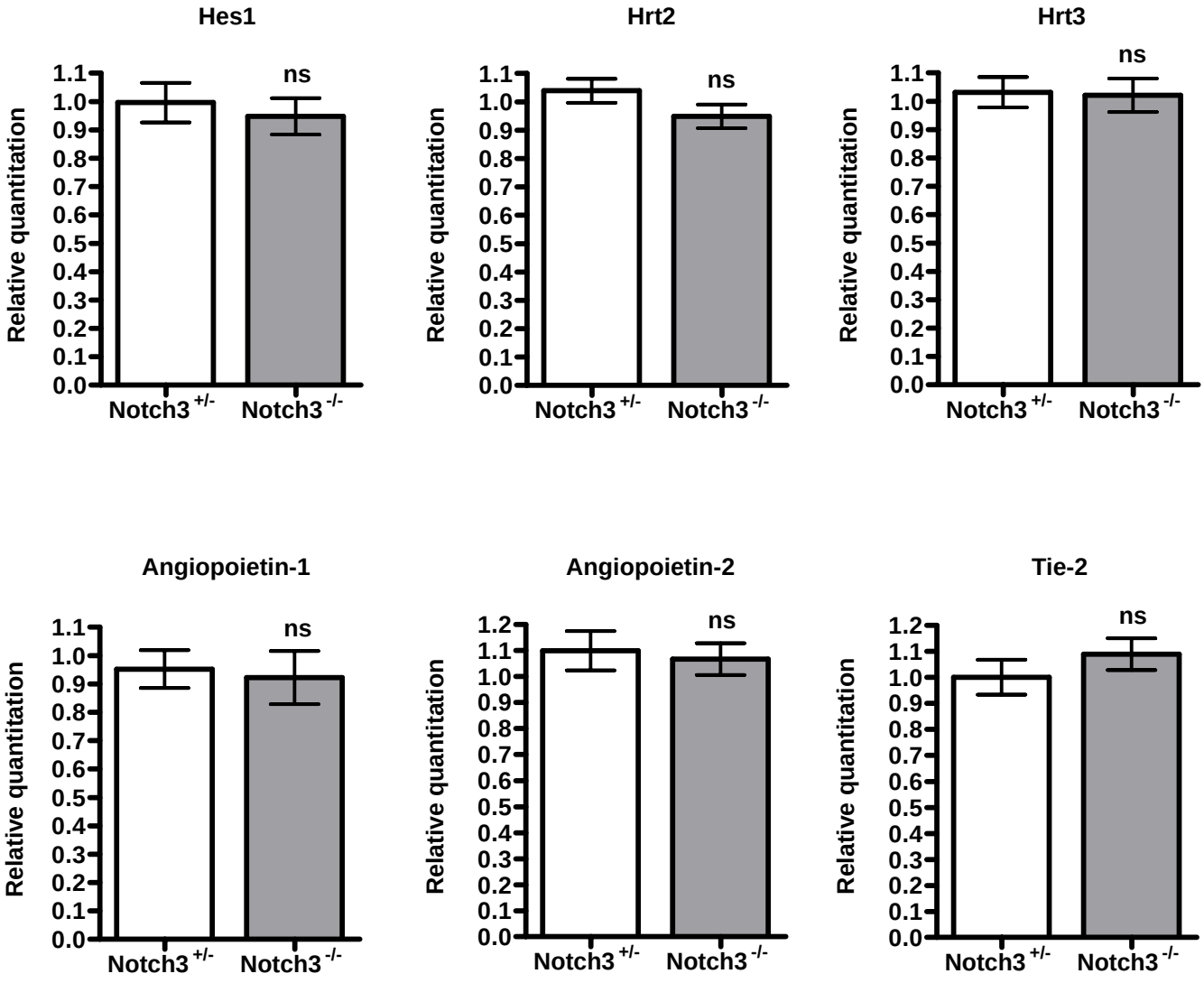


Online Figure VII. Gene expression comparison in P17 retinas of Notch3$^{+/-}$ and Notch3$^{-/-}$ mice subjected to OIR. Quantitative PCR analysis of Hes1, Hrt2, Hrt3, Angiopoietin-1, Tie-2, VEGF-A and VEGF-C mRNA expression in the retinas of Notch3$^{+/-}$ and Notch3$^{-/-}$ mice at P17 after oxygen-induced retinopathy (n=3); ns, not significant; * $P < 0.05$ compared to relevant control.

Online Figure VII



Online Figure VIII. Gene expression in the course of OIR and normal conditions. Quantitative PCR analysis of Angiopoietin-1, Angiopoietin-2 and Tie-2 mRNA expression in the retinas of wild type mice at indicated time points (P12-P17) in OIR model and normal condition (Con) (n=3).



Online Figure IX. Gene expression comparison in P17 retinas of Notch3$^{+/-}$ and Notch3$^{-/-}$ mice under normal conditions. Quantitative PCR analysis of Hes1, Hrt2, Hrt3, Angiopoietin-1, Angiopoietin-2 and Tie-2 in P17 retinas of Notch3$^{+/-}$ and Notch3$^{-/-}$ mice under normal conditionS (n=3); ns, not significant.

Online Figure IX