SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Reagents and Antibodies

Human fibrinogen (F8630, Sigma), Slit2 (5444-SL, R&D systems), VEGF-A165 (293-VE, R&D systems), FGF-2 (233-FB, R&D systems). Antibodies: anti-Robo1 (MAB7118, R&D systems), anti-NCK1 (#2319, Cell Signaling), anti-NCK1 (ab168940, Abcam), anti-NCK2 (ab109239, Abcam), anti-GM130 (610823, BD), anti-NG2 (AB5320, Millipore), anti-Collagen IV (AB769, Millipore), anti-ERG1/2/3 (SC353, Santa Cruz), anti-phospho-histone 3 (PH3, 06-570, Millipore), anti-α-smooth muscle actin CY3 (CY3-SMA, C6198, Sigma), anti-DLL4 (AF1389, R&D systems), anti-GFP (G10362, Life Technologies), anti cleaved caspase-3 (#9661S, Cell Signaling), anti-CD144 (555289, BD Biosciences), anti-Podocalyxin (AF1556, R&D systems), anti-Endomucin (HM1108**,** Hycult Biotech), CY3-phalloidine (P5282, Sigma), Dapi (D1306, Life Technologies), anti-CDC42 (ab155940, Abcam), anti-pVEGFR2 1175 (#2478, Cell Signaling), anti-pVEGFR2 1214 (#2477, Cell Signaling), anti-pVEGFR2 (#9698, Cell Signaling), p44/42 MAP kinase (phospho-ERK, #9106, Cell Signaling), anti-p44/42 MAP kinase (total ERK, #9102, Cell Signaling), anti-phospho-tyrosine (p-Y, #8954, Cell Signaling). Appropriate secondary antibodies were conjugated to horseradish peroxidase (Vector Laboratories) or fluorescently labeled (Life Technologies). IsolectinB4 was purchased from Life Technologies. DAPT (565770, Calbiochem) was dissolved in 10% ethanol and 90% oil (10mg/ml) for mice injection. Bmp9 and 10 blocking antibodies were from Genentech.

Murine endothelial cell isolation

MLECs were isolated as described with modifications[1]. Briefly, cells were isolated from collagenase-digested lung tissue using rat-anti-mouse CD31 mAb-coated Dyna-beads followed by enrichment using ICAM-2 mAb. Primary MLECs were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml endothelial cell mitogen (Biomedical Technologies, Inc.), and 10 U/ml heparin. Cells were used under passage three.

Embryo Whole-mount immunostaining

Embryos were harvested and fixed in 4% paraformaldehyde (PFA) in PBS for 2 hours at room temperature, washed with PBS and incubated for 1 hour in blocking solution (1% Triton-X100, 3% Bovine Serum Albumin (BSA, Sigma-Aldrich) and 3% heat-inactivated Bovine Serum (Fisher)) at room temperature. The embryo were incubated with the primary antibody overnight (1:100 Endomucin (Hycult Biotech) in blocking solution. Samples were washed and incubated in secondary antibody (1:200 Goat anti-Rat AlexaFluor 488 (Invitrogen) in blocking solution) overnight. Immunofluorescent images were taken using Leica DM 6000B microscope connected to a Leica TCS SP5 system and Leica LAS AF imaging software.

Oxygen Induced Retinopathy

OIR was performed as described[2, 3]. The mother and P7 pups were placed in 80% O2 until P12. Next, the pups were injected with 400µg of tamoxifen at P12 and P13 and placed with a nursing mother for adoption. Eyes were collected at P17, retinas were stained with IB4 and avascular area and the tufts were quantified [3].

Wound Healing Assay and Analysis

Wound healing studies were done by the microsurgery core at Yale Cardiovascular Research Center. Wounds were created with a sterile 6-mm biopsy punch in the back skin (Miltex Inc, PA) without injuring the underlying muscle. Wound regions were photographed using a Leica M125 microscope with an HC80 HD camera (Leica, Germany) on days 0, 1, 3, 5, 7, 9, 11, 13 and 15. Wound area was calculated using NIH ImageJ software. Wound sizes were expressed as percentage of the wound area on day 0.

In vitro sprouting assay

In vitro sprouting assay were done as previously described [4]. Briefly, HUVECs were spread between two fibrin layers. Wi-38 fibroblasts were then plated on top of the fibrin layers in EBM-2 supplemented with 2% FBS and growth factors. After 4–6 days, the cells were labeled with 4 mg/ml Calcein (Life Technologies) for 1 hr, and imaged.

Immunohistochemistry

The eyes of P5 pups were prefixed in 4% PFA for 20 minutes at room temperature (RT). The retinas were dissected out and blocked during 30 min at RT in blocking buffer TNBT (0.1 M Tris-HCl, 150 mM NaCl 1% Blocking Reagent (PerkinElmer), 0.5% Triton X-100). The retinas were incubated with antibodies in TNBT overnight. After washing with Pblec (1 mM MgCl2, 1 mM CaCl2, 0.1 mM MnCl2, 1% Triton X-100 in PBS), the retinas were incubated with IsolectinB4 and the corresponding secondary antibody in Pblec for 2 hr at RT. Then the retinas were mounted in fluorescent mounting medium (DAKO, Carpinteria, CA, USA). High resolution pictures of retinas were acquired using a Leica SP5 confocal microscope with a Leica spectral detection system (Leica 15 SP detector) and the Leica application suite advanced fluorescence (LAS-AF) software. Quantification of retinal vascular development was done using the Biologic CMM Analyser Software[5, 6].

Cell culture and siRNA transfection

HUVECs were obtained from Lonza and cultured in EGM2-Bulletkit (Lonza). HUVECs were starved overnight in EBM-2 supplemented with 2% FBS and treated with Slit2 or VEGF-A during the time indicated. For the rescue experiments, QS11 (10μM) is added from the starvation to the end of the experiments.

siRNAs (FlexiTube siRNA) were purchased from Dharmacon, ROBO1 (SMARTpool: ON-TARGETplus ROBO1 siRNA L-011381-00-0005), ROBO2 (SMARTpool: ON-TARGETplus ROBO2 siRNA L-023273-01-0005), NCK1 (SMARTpool: ON-TARGETplus NCK1 siRNA L-006354-00-0005), NCK2 (SMARTpool: ON-TARGETplus NCK2 siRNA L-019547-00-0005), RAC1 (SMARTpool: ON-TARGETplus Rac1 L-003560-00-0005), RAC2 (SMARTpool: ON-TARGETplus RAC2 siRNA L-007741-00-0005) and the negative control (ON-TARGETplus Non-targeting Pool D-001810-10-05). HUVECs were transfected with 25pmol siRNA per well in 6-well with 2.5μl RNAiMax (Invitrogen) according to the manufacturer's instructions and used for experiments 48hr after transfection.

Immunoprecipitation

Cell lysates were prepared in 50 mM Tris-HCl at pH 7.4, 50 mM NaCl, 0.5% Triton X-100, phosphatase and protease inhibitors, centrifuged at 16,000g for 20 min, and incubated for over-night at 4 °C with relevant antibodies. Then, the antibodies were coupled to protein A/G magnetic beads (88802, Thermo Scientific), washed in lysis buffer and resuspended in 2× Laemmli's sample buffer.

GST pulldown assay

Top10 E. coli cells harboring pGEX-PAK-CRIB were induced with IPTG (0.3 mM). After 3–4 h at 37 °C, cells were centrifuged at 2,800g for 5 min, cells were lysed in 20 mM Tris-HCl at pH 7.2, 150 mM NaCl, 1% Triton X-100, 10mM MgCl2, 1mM PMSF, lysozyme (1 µg.ml−1), DTT (10mM), 10µg DNase I and protease inhibitors. The GST fusion proteins were purified with glutathione-Sepharose 4B beads and washed with binding/wash buffer (50 mM Tris-Cl at pH 7.2, 150 mM NaCl, 10mM DTT, 1% Triton X-100 and protease inhibitors). The beads were incubated with the cell lysate for 45min at 4°C, washed in binding/wash buffer and resuspended in 2× Laemmli's sample buffer. Bound CDC42 was detected by Western blotting using anti-CDC42.

Proliferation assay

We performed the proliferation analysis using Click-iT EdU Alexa Fluor 647 Imaging kit (Life Technologies). P3 or P7 pups were injected with 300µg of EdU (5mg/ml) and killed 4h later. EdU staining was done according the manufacturer's protocol.

The proliferation assays were done as previously described[7]. The xCELLigence RTCA DP analyzer was used to measure proliferation of wildtype, *ROBO1/2* and *NCK1/2* knockout HUVEC cells (10,000 cells/well) in response to VEGF- A (100ng/ml). The plate was monitored every 15 minutes for 48 hours.

Apoptosis Analysis

The *in vitro* apoptosis analysis is performed using cleaved caspase-3 staining of

confluent HUVEC monolayers. 24 h after siRNA transfection, the confluent cells were starved during 16h (EBM2) and cultured with EBM2 supplemented with 50 ng/ml VEGF-A during 24 h.

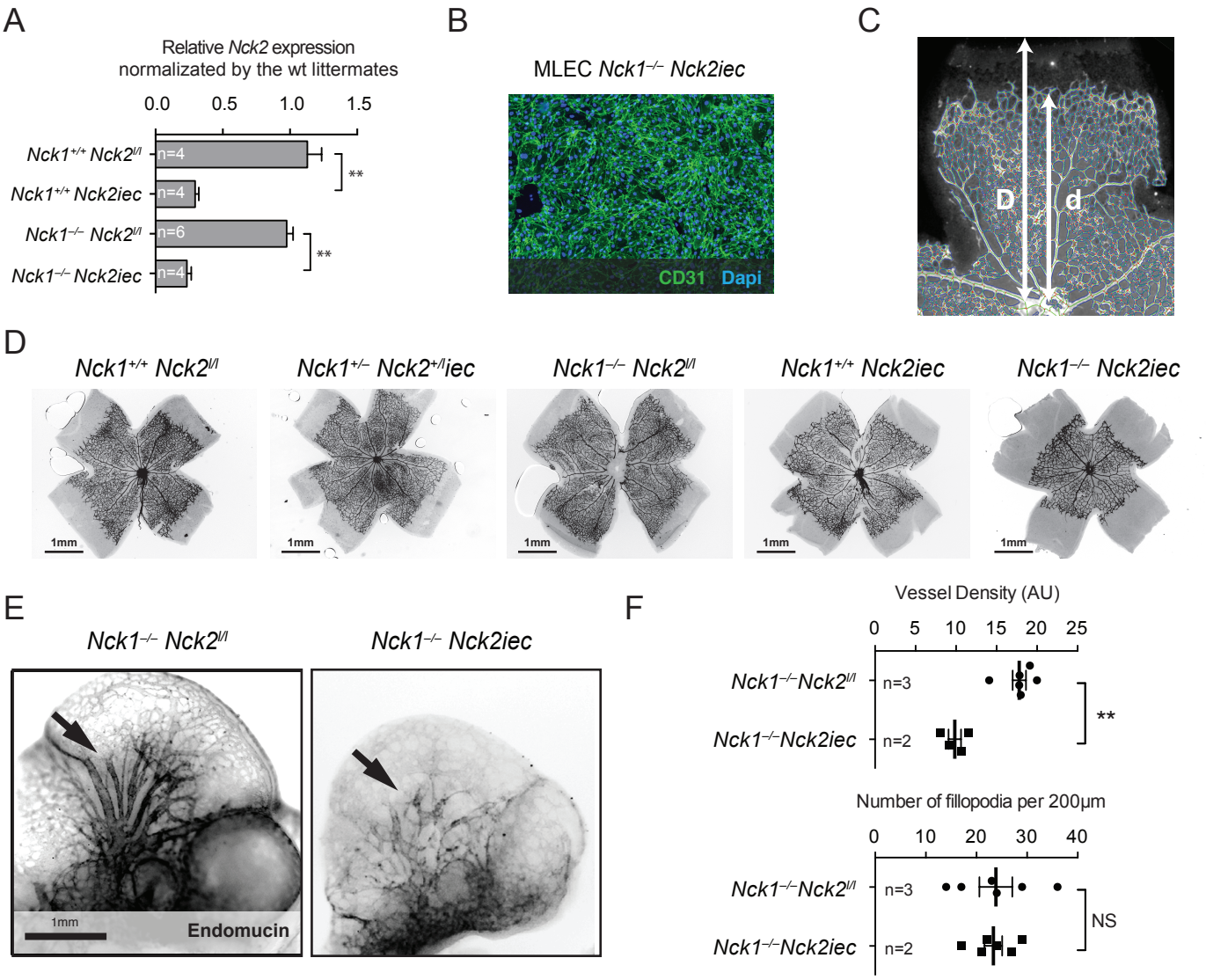
Western blotting

Cells were lysed in lysis buffer including phosphatase and protease inhibitors (Thermo Scientific, 78420, 1862209). Equal amounts of proteins were separated on 4–15% Criterion precast gel (#567-1084, Bio-rad) and transferred on nitrocellulose membrane (Biorad). Western blots were developed with chemiluminescence HRP substrate (Millipore, WBKLS0500) on a Luminescent image analyser, ImageQuant LAS 4000 mini (Ge Healthcare).

Quantitative real-time PCR

RNAs from HUVEC or from MLECs were purified using RNeasy-kit (Qiagen). 1µg RNA was reverse transcribed using SuperScript III (Invitrogen) and quantitative PCR were assayed using the corresponding primers (Qiagen): HsNCK1 (QT00077945), HsNCK2 (QT00245441), HsKDR (QT00069818) HsNRP1 (QT00023009), HsDLL4 (QT00081004), HsANGPT2 (QT00100947), HsID1 (QT00230650), HsESM1 (QT00022491), MmNck2 (QT0014324). The expression levels were normalized to ACTIN for HUVECs.

Dubrac et al. 2015 supp Figure 1



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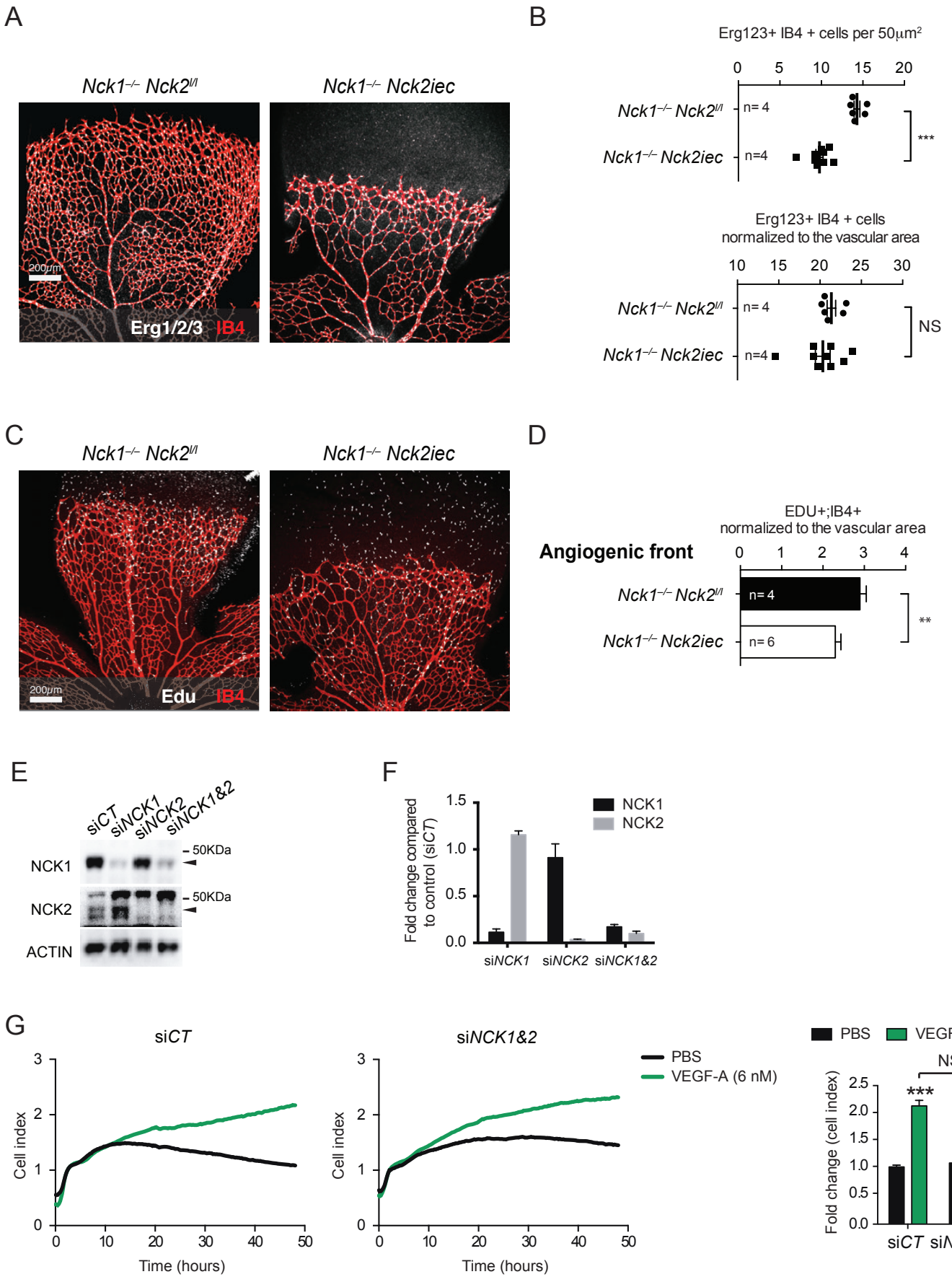

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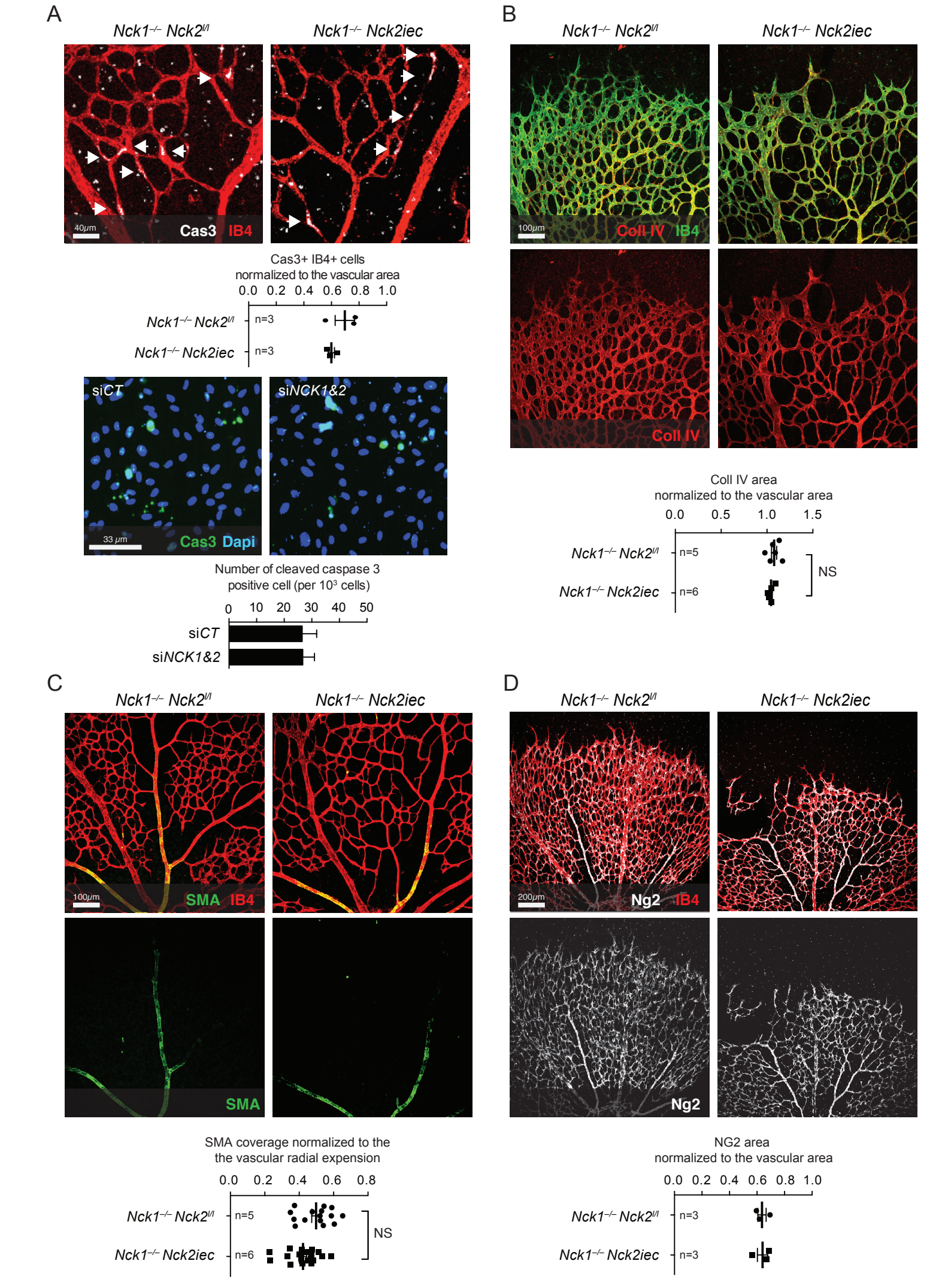

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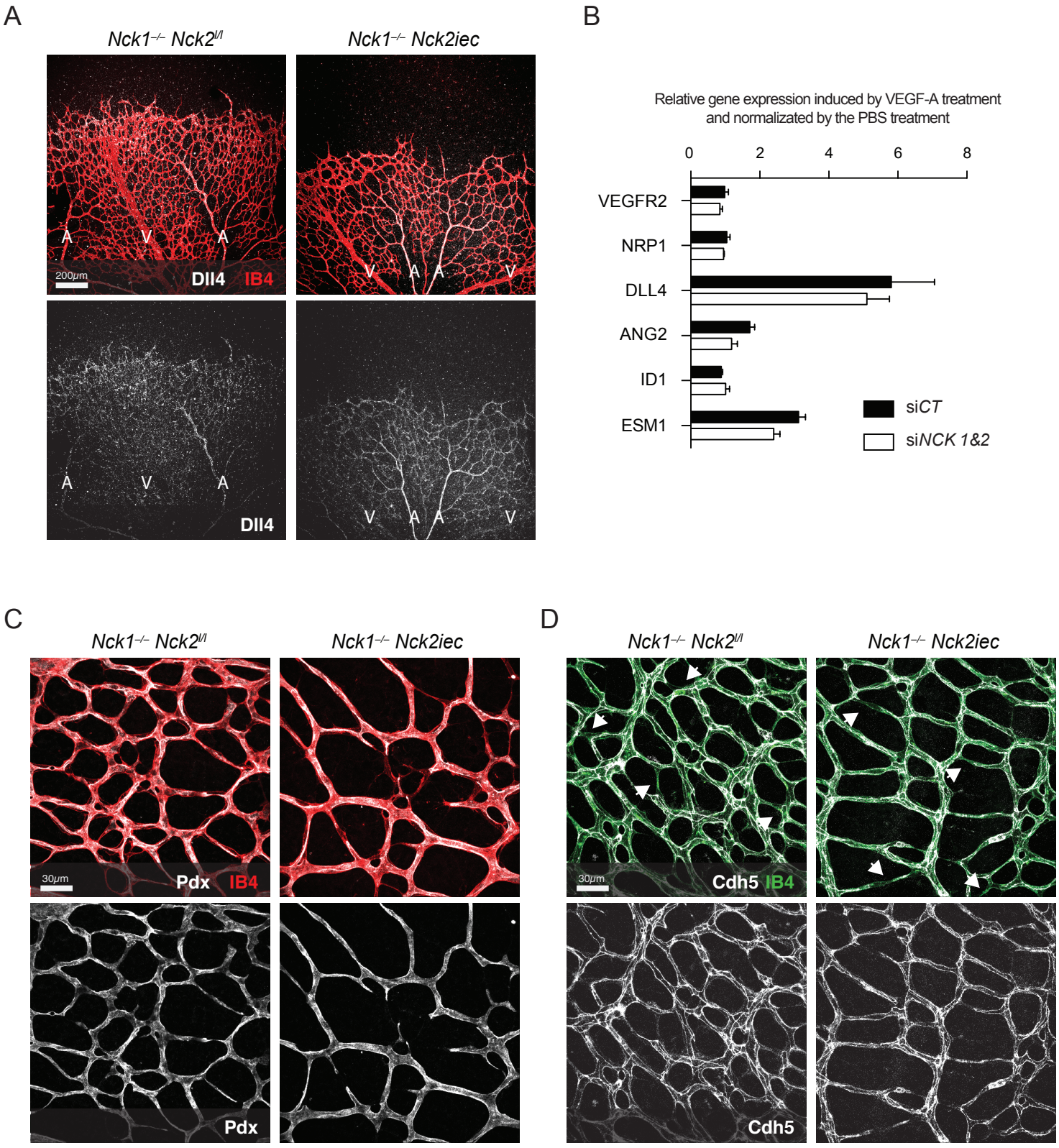

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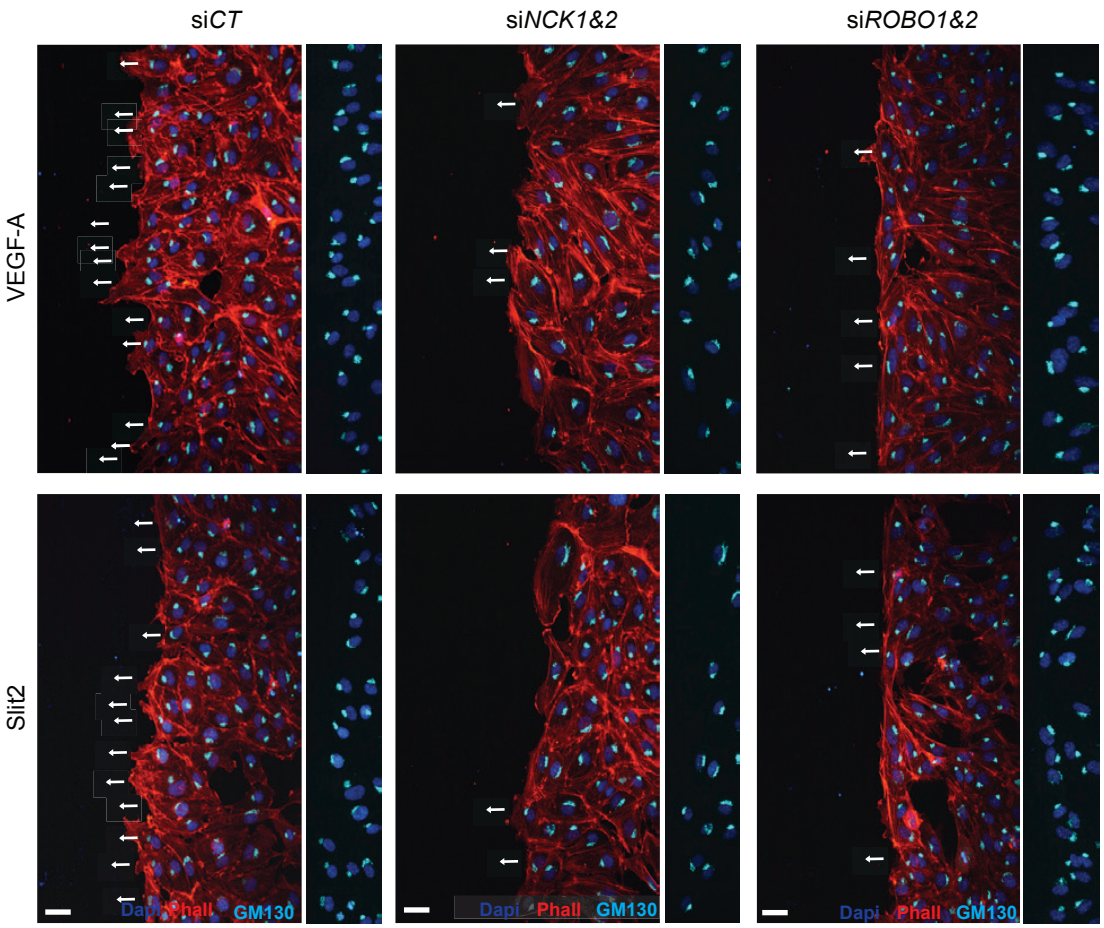

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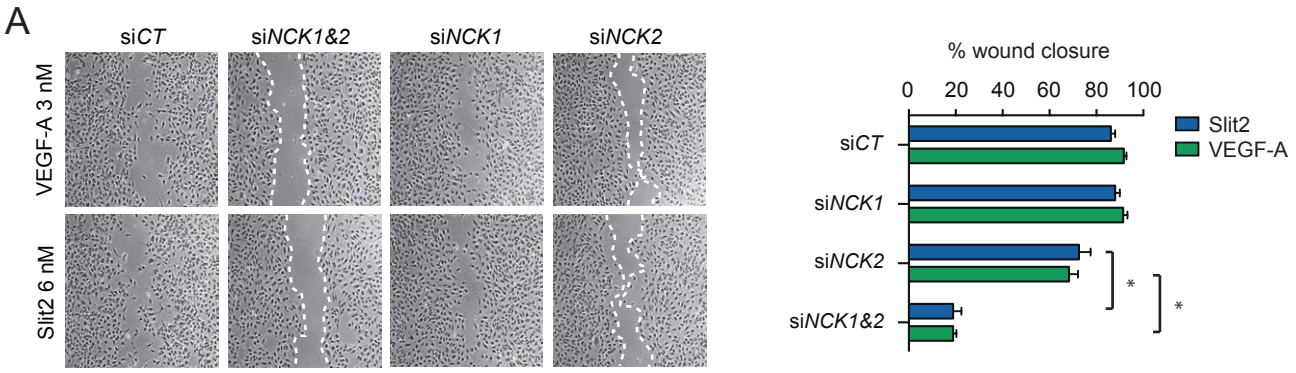

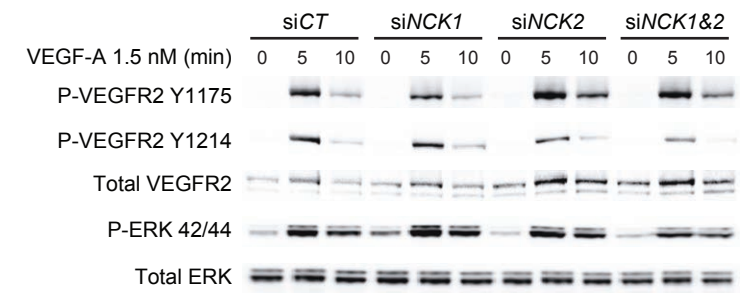

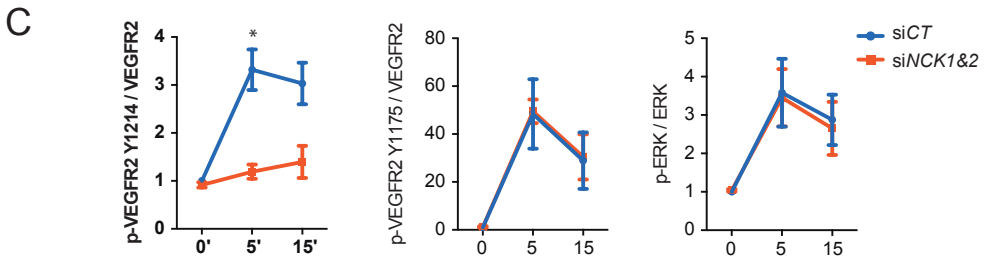

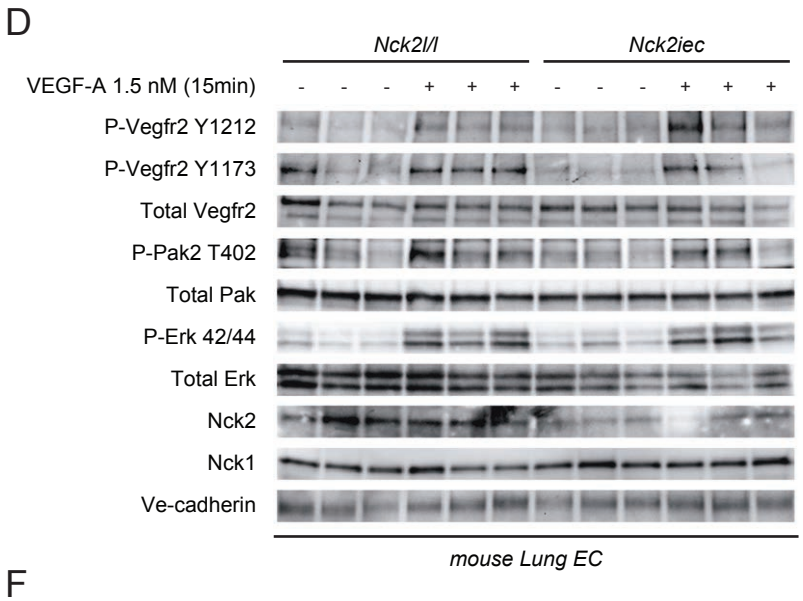

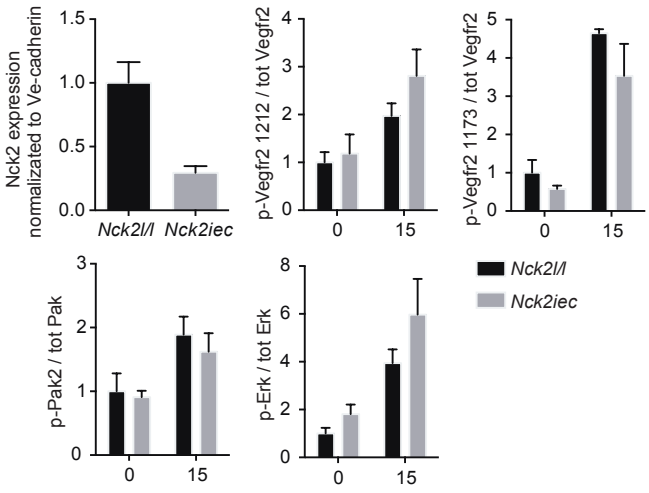

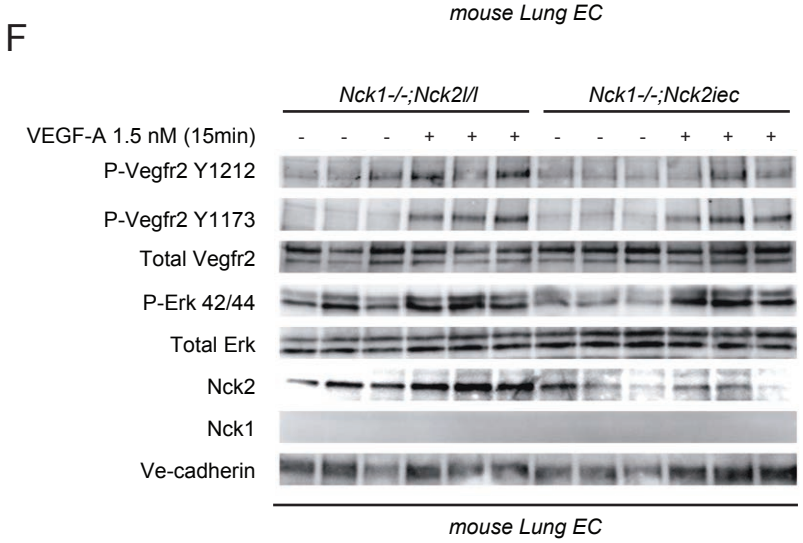

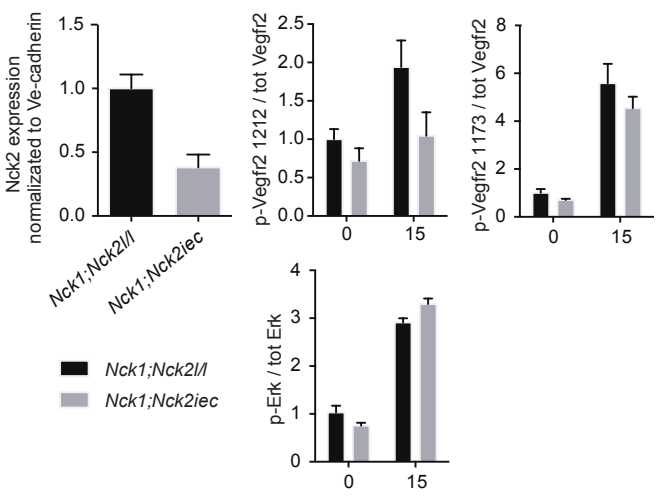

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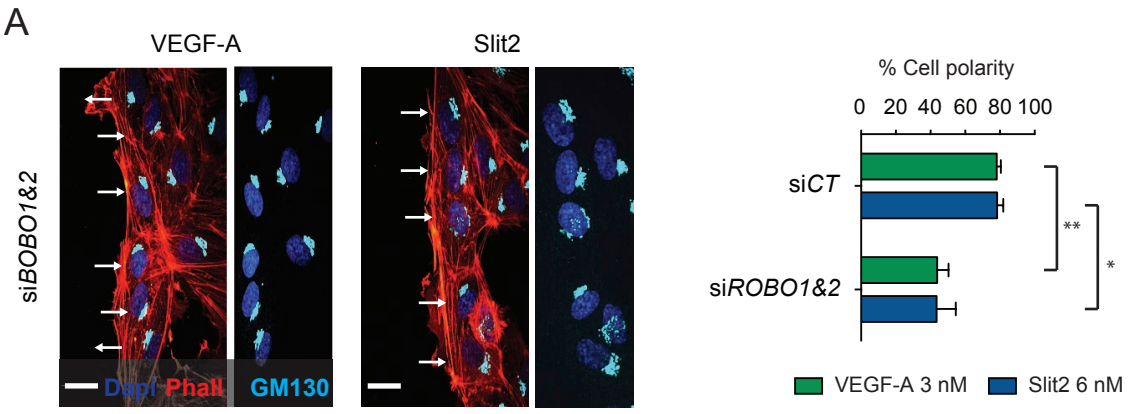

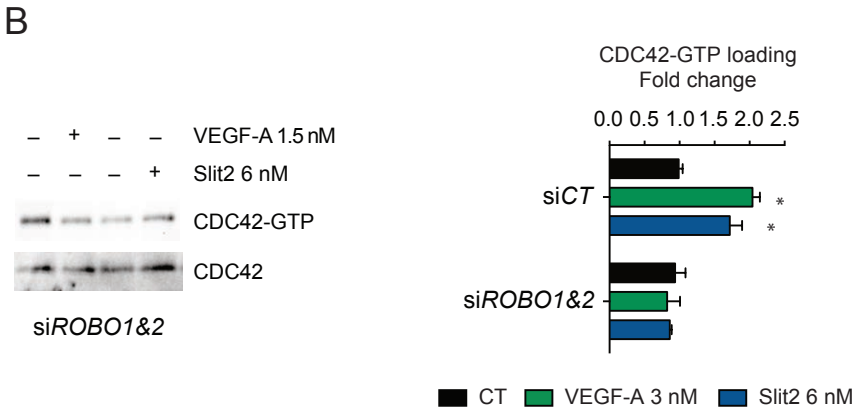

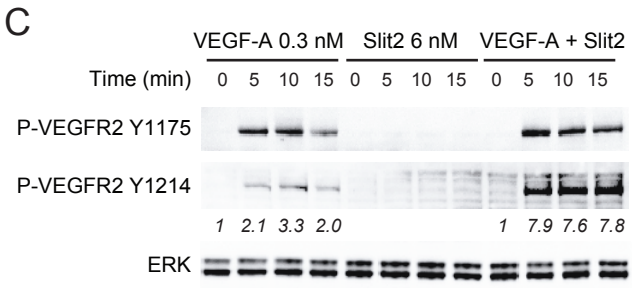

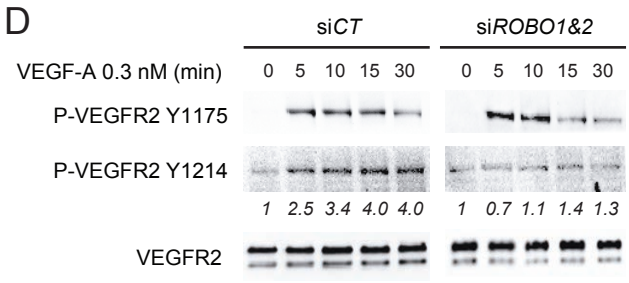

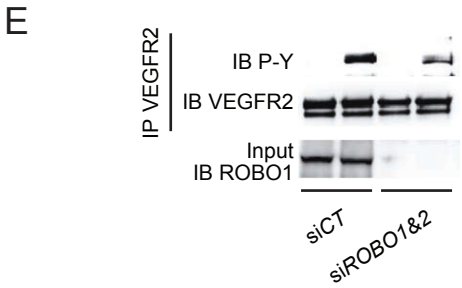

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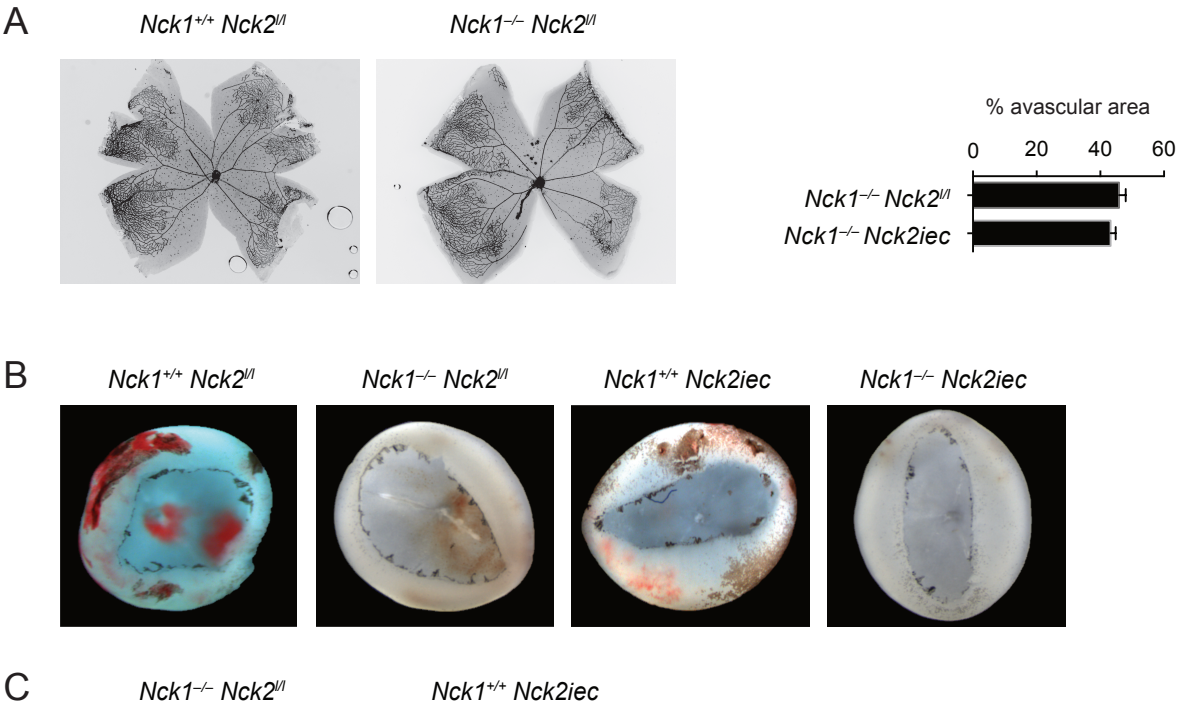

Dubrac et al. 2015 supp Figure 9

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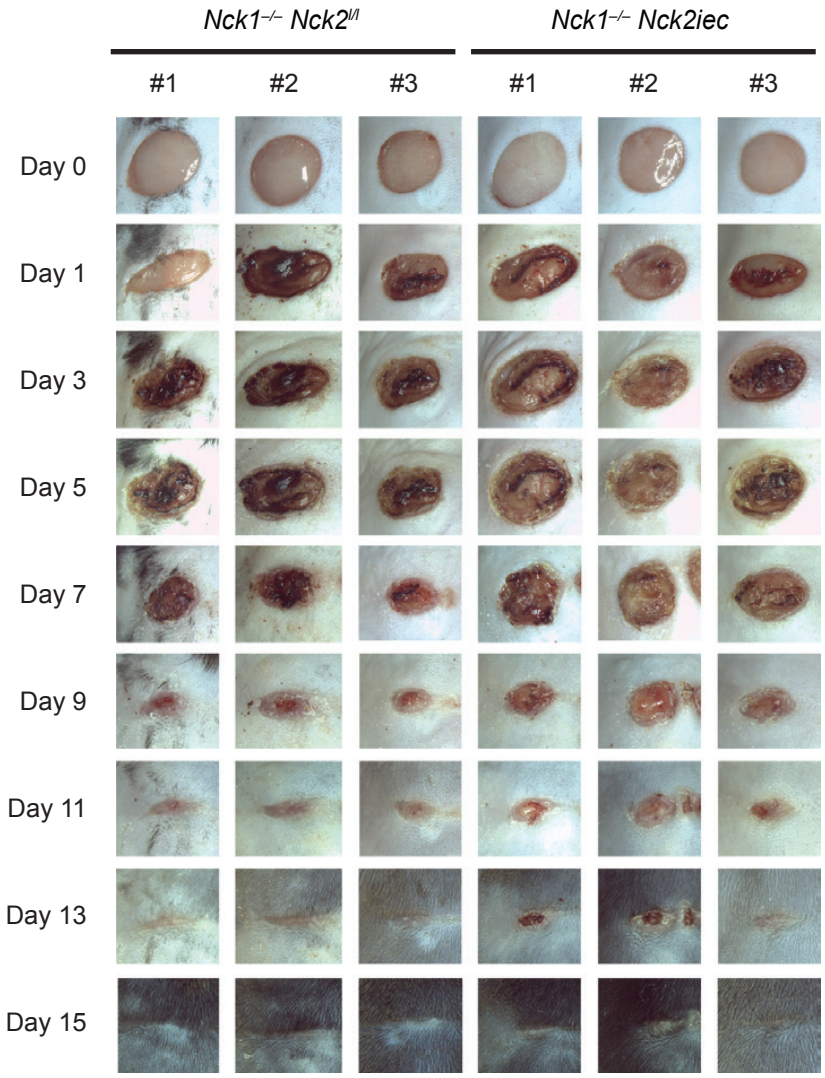

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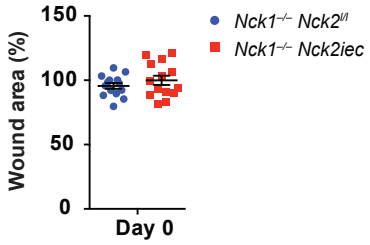

SUPPLEMENTAL FIGURES

Supplementary Figure 1. Nck is required for retinal angiogenesis.

A, qPCR measurement of *Nck2* levels in MLECs isolated from mice with the indicated genotypes. Graphs represent mean±SEM and statistical significance was assessed using Student's t-test. **P<0.01. **B,** CD31 staining shows purity of mLECs. **C,** Flat-mount of a P5 retina labeled with IB4 after processing with the Biologic CMM Analyser software[6]. Branch points are indicated by the red dots. D is the retina radius and d is the extent of the vascular front from the optic nerve. **D,** IsolectinB4-staining of retinal vessels from mice with the indicated genotypes. **E,** Whole mount Endomucin staining of E11 embryos shows vessel morphology in the head. Note that *Nck1*$^{+/+}$*Nck2iec* mutant embryos show immature and poorly organized vessels in the cranial vasculature (arrows) (n=5 embryos for *Nck1*$^{-/-}$*Nck2*$^{I/I}$ and n=3 embryos for *Nck1*$^{+/+}$*Nck2iec*). **F,** Quantification of vascular density and number of filopodia in hindbrain flatmounts. Results are presented as mean ± s.e.m and statistical significance was analyzed by Mann-Whitney *U* test. **P<0.01, NS: non significant.

Supplementary Figure 2. Nck deletion does not affect endothelial cell proliferation.

A, IsolectinB4 and Erg1/2/3 staining of P5 retinas from mice with the indicated genotypes. **B,** quantification of Erg1/2/3+, IsoB4+ endothelial cells per 50µm^2 and Erg1/2/3+, IsoB4+ endothelial cells normalized by the vascular area. **C,** IB4/Edu staining of P5 retinas from mice with the indicated genotypes. **D**, quantification of Edu+, IsoB4+ endothelial cells normalized by the vascular area. Number of retinas used for quantification in B and D is indicated. Graphs represent mean±SEM and statistical significance was assessed using a Mann-Whitney *U* test. ***P<0.001**,** NS: non significant. **E, F,** Western blot (E) and qPCR (F) detects NCK1 and 2 proteins in HUVECs (specific bands indicated by arrowheads), and combined siRNA against both *NCK1* and *2* abolishes NCK protein expression. **G,** HUVEC proliferation, measured by the Roche xCELLigence system, is not affected by *NCK1/2* knockdown. Right panel, quantification of proliferation at 48h. Graphs represent mean±SEM and statistical significance was assessed using a Student's t-test. ***P<0.001, NS: non significant.

Supplementary Figure 3. Effects of *Nck1,2* deletion on retinal angiogenesis.

A, IB4 and cleaved Caspase 3 (Cas3) staining of P5 retinas from mice with the indicated genotypes and quantification of Cas3+, IsoB4+ endothelial cells normalized by the vascular area. Number of retinas used for quantification is indicated. Bottom panel shows the Dapi and cleaved Caspase 3 (Cas3) staining of HUVEC transfected with control (si*CT*) or NCK1 and 2 siRNA (si*NCK1&2*) and quantification of Cas3+ cells. **B,** CollagenIV staining shows similar basement membrane coverage between control and *Nck1*$^{-/-}$*Nck2iec* mice. Bottom panel shows ratio of ColIIV+ area / IB4+ area. Number of retinas used for quantification is indicated. **C,** SMA staining shows similar smooth muscle coating in *Nck1*$^{-/-}$*Nck2*$^{l/l}$ control and *Nck1*$^{-/-}$*Nck2iec* mice. Bottom panel shows the quantification of the SMA coverage distance from the optic nerve normalized by the extent of the vascular front Number of retinas used for quantification is indicated. **D,** Pericyte coverage detected by NG2 staining is similar between control and *Nck1*$^{-/-}$*Nck2iec* mice. Bottom panel shows ratio of NG2+ area / IB4+ area. Number of retinas used for quantification is indicated. Graphs represent mean±SEM and statistical significance was assessed using a Mann-Whitney *U* test. NS: non significant.

Supplementary Figure 4. Effects of *Nck1,2* deletion on blood vessel lumen formation.

A, IB4/Dll4 staining of P5 retinas from mice with the indicated genotypes. A: artery, V: vein. **B,** Tip cell marker gene expression analysis of control (siCT) and *NCK1* and *2* si-RNA HUVECs treated with VEGF-A for 20h. **C,** IB4/Podocalyxin (Pdx) staining of P5 retinas from mice with the indicated genotypes. **D,** IB4/Cdh5 staining of P5 retinas from mice with the indicated genotypes.

Supplementary Figure 5. NCK and ROBO are required for VEGF-A and Slit2 induced front- rear polarity.

Low magnification images of Phalloidin-DAPI staining combined with Golgi labeling by GM130 (light blue) in HUVECs at the scratch wound edge (left) 2h after wounding. Arrows point to cells that have their Golgi apparatus positioned in the direction of migration.

Supplementary Figure 6. NCK is required for VEGF-A and Slit2 induced p-PAK2 and endothelial cell migration.

A, Scratch wound migration assay with HUVECs transfected with the indicated siRNAs (n=4). Note that combined *NCK1/2* siRNA decreases endothelial cell migration in response to Slit2 and VEGF-A, while individual *NCK1* or *NCK2* knockdown has no (*NCK1*) or little inhibitory effect (*NCK2*). Results are presented as mean ± s.e.m., statistical analyses were perfomed using Mann-Whitney *U* test. *P<0.05. **B,** Combined *NCK1/2* knockdown inhibits VEGF-A induced Y1214 activation. **C**,, Quantification (n=3). Results are presented as mean ± s.e.m., statistical analyses were perfomed using Kruskal-Wallis test with Dunn's multiple comparisons test. *P<0.05. **D and F,** Western-blot using MLEC from 3 different $Nck2^{l/l}$ and *Nck2iec* (D) or $Nck1^{-/-}Nck2^{l/l}$ and $Nck1^{-/-}$*Nck2iec* mice (F) stimulated with VEGF-A during 15min. **E and G,** Quantification. All values are mean ±SEM of at least 3 experiments.

Supplementary Figure 7. SLIT2-ROBO1/2 signaling cooperates with VEGF-A in activation of Y1214.

A, Left, Golgi polarity defects after *ROBO1/2* knockdown. Right, quantification of cell polarity (n=4, 120-150 cells were analyzed in each individual experiments). Results are presented as mean ± s.e.m., statistical analyses were perfomed using Student's t-test. *P<0.05, **P<0.01. **B,** Impaired CDC42 activation in *ROBO1/2* knockdown cells in response to VEGF-A and Slit2. Right, quantification (n=5). Results are presented as mean ± s.e.m., statistical analyses were perfomed using Mann-Whitney *U* test. *P<0.05. **C,** Western blot of HUVEC lysates with antibodies against VEGFR2 Y1175 and Y1214 following treatment with the indicated factors. Numbers represent fold increase in signal over control loading (total ERK). N=2 experiments. **D,** *ROBO1/2* siRNA reduces VEGFR2 Y1214 activation by VEGF-A. N=2 experiments. Numbers represent fold increase in signal over control loading (total VEGFR2). **E,** VEGFR2 IP of protein extracts from control- and *ROBO1/2* siRNA transfected HUVECs treated with VEGF (25µg/ml, 5min) followed by Western blot analysis with the indicated Abs. Note that *ROBO1/2*

siRNA decreases VEGFR2 phosphorylation.

Supplementary Figure 8. Effects of *Nck1,2* deletion on oxygen-induced retinopathy. **A,** Retinal vasculature of P12 mice after hyperoxia exposure between P7 and P12 (n=4 retina for *Nck1*$^{-/-}$*Nck2*$^{l/l}$ and n=4 retina for *Nck1*$^{+/+}$*Nck2iec*). Note no difference in vaso-obliteration before Tx administration. **B,** Freshly dissected P17 retinas. Note reduced hemorrhage in *Nck1*$^{-/-}$*Nck2iec* retinas. **C,** Avascular area in single *Nck1* and *2* mutants after OIR. Areas marked by arrows are shown below at higher magnifications.

Supplementary Figure 9. Effects of *Nck1,2* deletion on wound healing. **A**, Representative images of wound healing in *Nck1*$^{-/-}$*Nck2iec* and control littermate mice after wounding by punch biopsy. **B**, Quantification of wound area at day 0.

SUPPLEMENTAL REFERENCES

1. Zhang F, Michaelson JE, Moshiach S, Sachs N, Zhao W, Sun Y, Sonnenberg A, Lahti JM, Huang H and Zhang XA. Tetraspanin CD151 maintains vascular stability by balancing the forces of cell adhesion and cytoskeletal tension. *Blood*. 2011;118:4274-84.
2. Smith LE, Wesolowski E, McLellan A, Kostyk SK, D'Amato R, Sullivan R and D'Amore PA. Oxygen-induced retinopathy in the mouse. *Investigative ophthalmology & visual science*. 1994;35:101-11.
3. Connor KM, Krah NM, Dennison RJ, Aderman CM, Chen J, Guerin KI, Sapieha P, Stahl A, Willett KL and Smith LE. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. *Nature protocols*. 2009;4:1565-73.
4. Larrivee B, Prahst C, Gordon E, del Toro R, Mathivet T, Duarte A, Simons M and Eichmann A. ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. *Developmental cell*. 2012;22:489-500.
5. Koch AW, Mathivet T, Larrivee B, Tong RK, Kowalski J, Pibouin-Fragner L, Bouvree K, Stawicki S, Nicholes K, Rathore N, Scales SJ, Luis E, del Toro R, Freitas C, Breant C, Michaud A, Corvol P, Thomas JL, Wu Y, Peale F, Watts RJ, Tessier-Lavigne M, Bagri A and Eichmann A. Robo4 maintains vessel integrity and inhibits angiogenesis by interacting with UNC5B. *Developmental cell*. 2011;20:33-46.
6. Jones EA, Yuan L, Breant C, Watts RJ and Eichmann A. Separating genetic and hemodynamic defects in neuropilin 1 knockout embryos. *Development*. 2008;135:2479-88.
7. Lanahan AA, Lech D, Dubrac A, Zhang J, Zhuang ZW, Eichmann A and Simons M. PTP1b is a Physiologic Regulator of VEGF Signaling in Endothelial Cells. *Circulation*. 2014.