Inventory of Supplemental Data to Moya et al.: DEVELOPMENTAL-CELL-D-10-00883

Stalk cell identity depends on integration of Notch and Smad1/5 signaling cascades

A first file (Supplemental_Figs_Legends_Exp Proc_Refs DEVELOPMENTAL-CELL-D-10-00883.docx) contains:

1. **Figure S1** shows the gross morphology and a section of the heart of E9.5 control and dKO^{EC} littermates; shows the increasing apoptosis in non-endothelial cells of dKO^{EC} from 24 somites onwards, and the progressive (impaired) rostro-caudal formation of dorsal anastomozing vessels. This figure relates to Figure 1.

2. **Figure S2** shows the efficiency of Smad1/5 siRNA mediated knockdown in HUVECs and the consequences of this gene silencing on tubulogenesis; and shows pericyte recruitment to Smad1/5-deficient vessels. This figure relates to Figure 1-2.

3. **Figure S3** shows that polarity and (directed) migration are affected in Smad1/5 deficient tip cells or after Smad1/5 siRNA mediated knockdown in HUVECs . This figure is an extension of the data presented in Figure 2.

4. **Figure S4** shows nuclear p-Smad1/5/8 staining in arterial and venous endothelium and the *in vivo* monitoring of Id-expression during retinal angiogenesis. This figure illustrates also that knockdown of Smad1/5 in HUVECs results in increased branching in tubulogenesis assays, whereas overexpression of Id1 or Id3 has opposite effects. This figure relates to Figure 3.

5. **Figure S5** shows that BMP6 induces more efficiently Id target gene expression in HUVECs than BMP2. This figure relates to Figure 4.

6. **Figure S6** shows the effect of overproduction of NICD or Hes1 in HUVECs on the expression of tip and stalk cell markers. This figure relates to Figure 5.

7. **Figure S7** shows Hes1/Id heterodimer formation upon activation of Notch and BMP signaling pathways. This figure relates to Figure 6.

Supplementary Figure Legends are inserted immediately after every figure

8. **Supplemental Experimental Procedures**

9. **Supplementary References**

A second file (Compare revision 2011 and final version 2012.docx) is a file comparing revision 2011 and final submission 2012: this document allows to track all changes that have been implemented in the current final submission of the MS: *this file is intended solely for reviewing purposes, not to be uploaded to the journal's website*

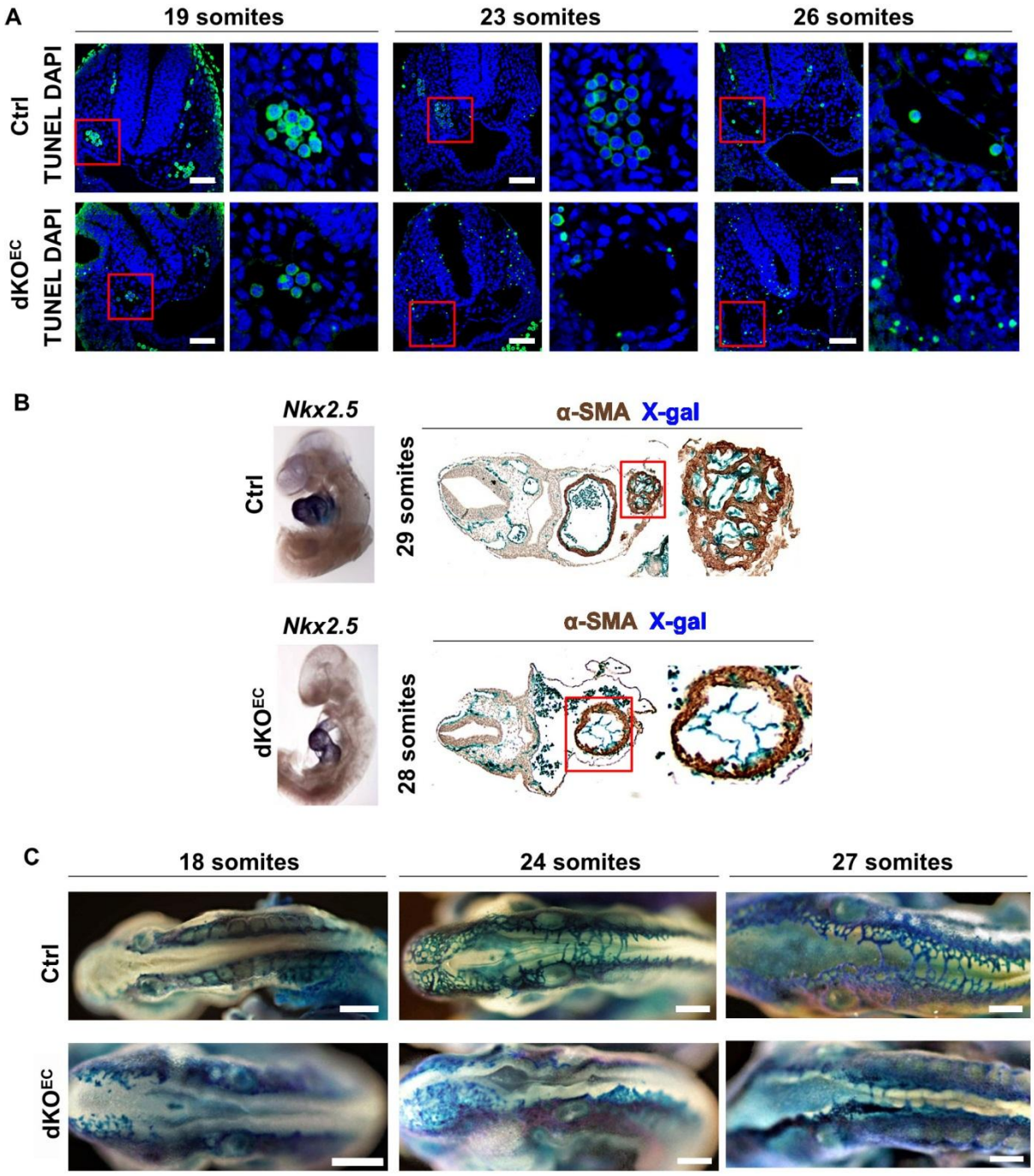


Figure S1, related to Figure 1. Apoptosis, heart looping and formation of dorsal anastomosing vessel. a, TUNEL assay on E9.5 mutant and control embryos. **b,** impaired heart looping as revealed by whole mount *Nkx2.5* expression and by sections of control and mutant E9.5 embryos stained with X-gal (blue) and smooth muscle actin antibody (brown). **c,** Dorsal view of whole-mount X-gal stained R26R reporter embryos show the progressive

rostro-caudal formation of dorsal anastomosing vessels. This process is severely impaired in dKO^{EC} embryos. Scale bars, 250 µm in (c).

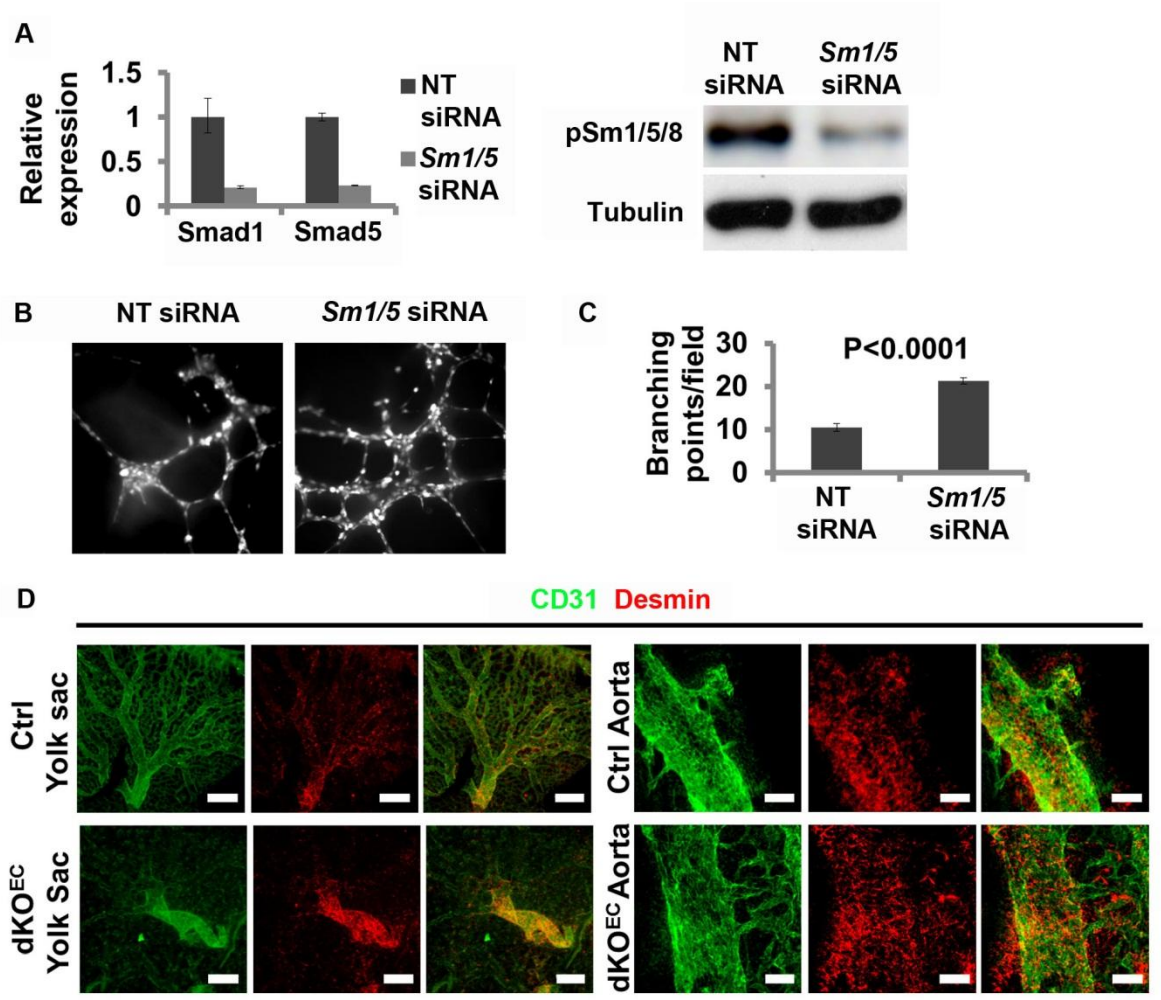


Figure S2, related to Figure 2. Hyperbranching and pericyte recruitment to Smad1/5-deficient vessels. a, *Smad1* and *Smad5* siRNA mediated downregulation (qPCR) results in reduced pSmad1/5/8 levels in HUVECs. **b,c,** Smad1/5^{KD} HUVECs show increased number of branching points (c: n≥20). **d,** Coverage of desmin-positive pericytes (red) in yolk sac vasculature (a) and dorsal aorta (b) of stage matched control and dKO^{EC} E9.5 embryos.

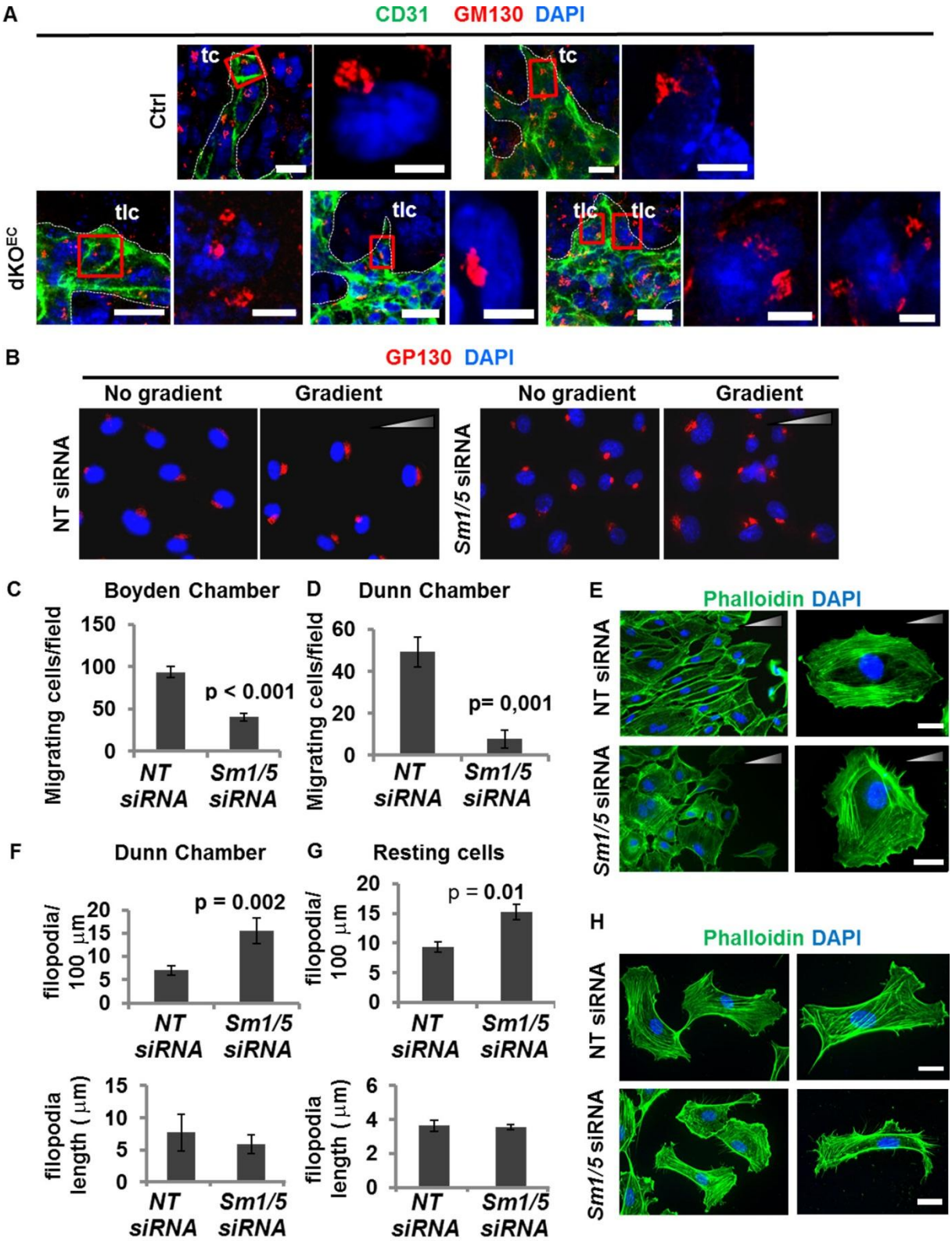
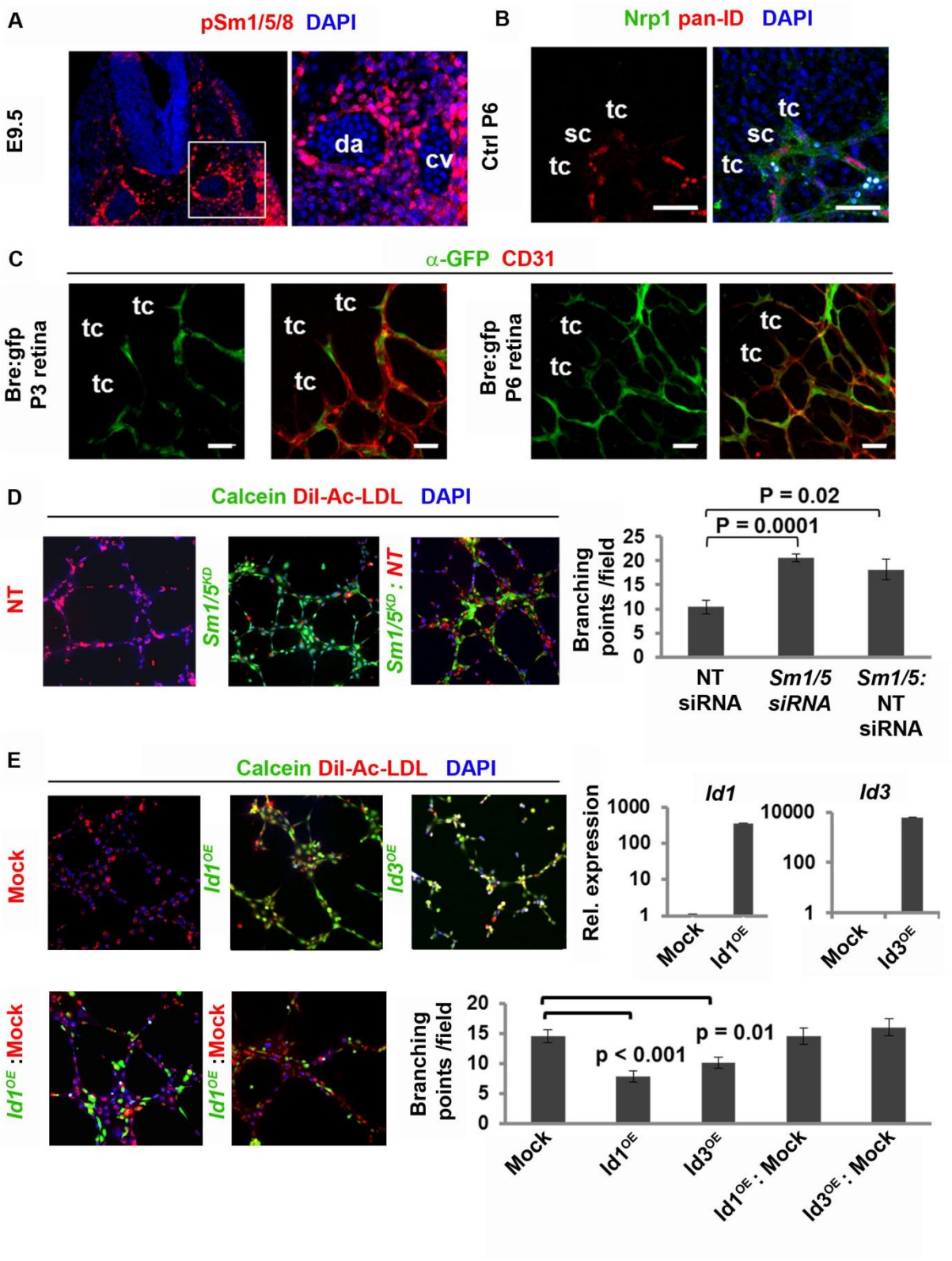

A
CD31 GM130 DAPI
Ctrl
dKO^EC
tc
tlc
B
GP130 DAPI
No gradient
Gradient
NT siRNA
Sm1/5 siRNA
C
Boyden Chamber
Migrating cells/field
p < 0.001
NT siRNA
Sm1/5 siRNA
D
Dunn Chamber
p= 0,001
E
Phalloidin DAPI
F
Dunn Chamber
filopodia/ 100 µm
p = 0.002
filopodia length (µm)
G
Resting cells
p = 0.01
H
Phalloidin DAPI

Figure S3, related to Figure 2. Smad1/5 signaling controls EC polarization and directed cell migration. Defective polarity of Smad1/5- deficient tip cells, as revealed by the random localization and lack of compaction of the Golgi apparatus (GM130). In tip cells of dKO^{EC} embryos, the Golgi apparatus can display these additional configurations: split in apical and basal portions (top left panels), laterally compacted (top right panels) or spread around the nucleus (lower panels). **b,** Random localization of Golgi apparatus (GP130) in *Sm1/5* siRNA transfected HUVECS when challenged with gradients of full medium in Dunn chambers. The gradient direction is indicated in the right upper corner (triangle). **c,b,** siRNA mediated downregulation of Smad1/5 impaired directed cell migration of HUVECS in Boyden (c) and Dunn (d) chambers (c: $n \geq 20$; d: $n \geq 15$). **e,** Control HUVECs align towards the source of the chemoattractant, while Sm1/5 deficient cells acquire a cobble stone morphology**. f-h,** Increased number of filopodia in HUVECs transfected with *Sm1/5* siRNAs (f, g: $n \geq 15$). *Abbreviations*: tc, tip cell; tcl, tip cell-like cell. Scale bars: 20 µm in left panels and 5 µm in right panels (boxed areas) in (a).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tc
sc
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Bre:gfp P6 retina
D
Calcein DiI-Ac-LDL DAPI
NT
Sm1/5KD
Sm1/5KD : NT
P = 0.02
P = 0.0001
Branching points /field
NT siRNA
Sm1/5 siRNA
Sm1/5: NT siRNA
E
Calcein DiI-Ac-LDL DAPI
Mock
Id1OE
Id3OE
Rel. expression
Id1
Id3
Id1OE :Mock
p < 0.001
p = 0.01
Mock
Id1OE
Id3OE
Id1OE : Mock
Id3OE : Mock

Figure S4, related to Figure 3. *In vivo* monitoring of pSm1/5/8 signaling and Id localization during angiogenesis. a, Cross section of control E9,5 embryos showing p-Smad1/5/8 localization in ECs of dorsal aorta and cardinal vein. **b,** Scattered distribution of Id proteins (pan-Id antibody) in endothelial stalk, and tip cells (Nrp1) in postnatal retina. **c,** GFP in BRE:gfp retinas recapitulates pan-Id pattern shown in (b). **d,e,** Quantification of branching points in chimaeric tube formation assays using HUVECs transfected with NT siRNA mixed equally with HUVECs transfected with *Smad1/5* siRNA (d), or with *Id1/3* overexpression and mock transfected cells (e)(d,e: n≥20). *Abbreviations*: sc, stalk cell; tc, tip cell; da, dorsal aorta; cv, cardinal vein. Scale bars: 35 µm in (a,b).

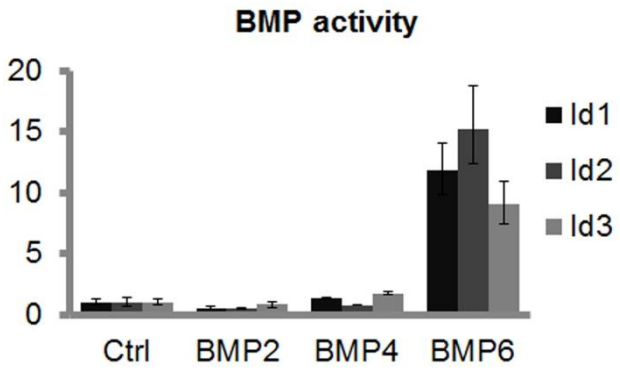


Figure S5, related to Figure 4. BMP signaling and Id expression in HUVECs. *Id1-3* expression in HUVECs treated with BMP2, BMP4 and BMP6.

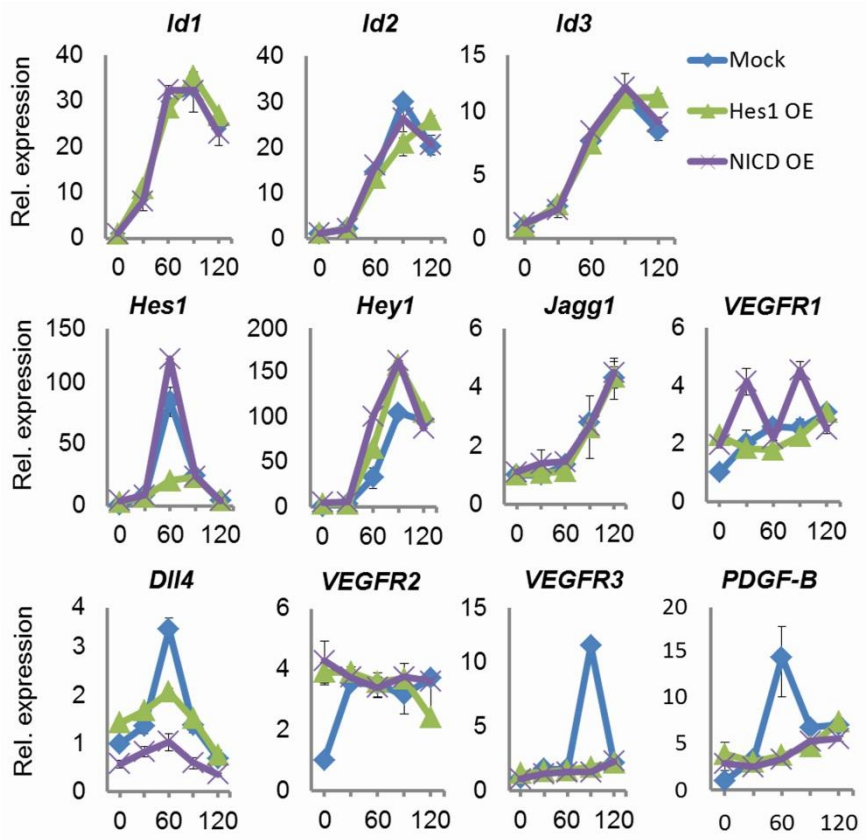
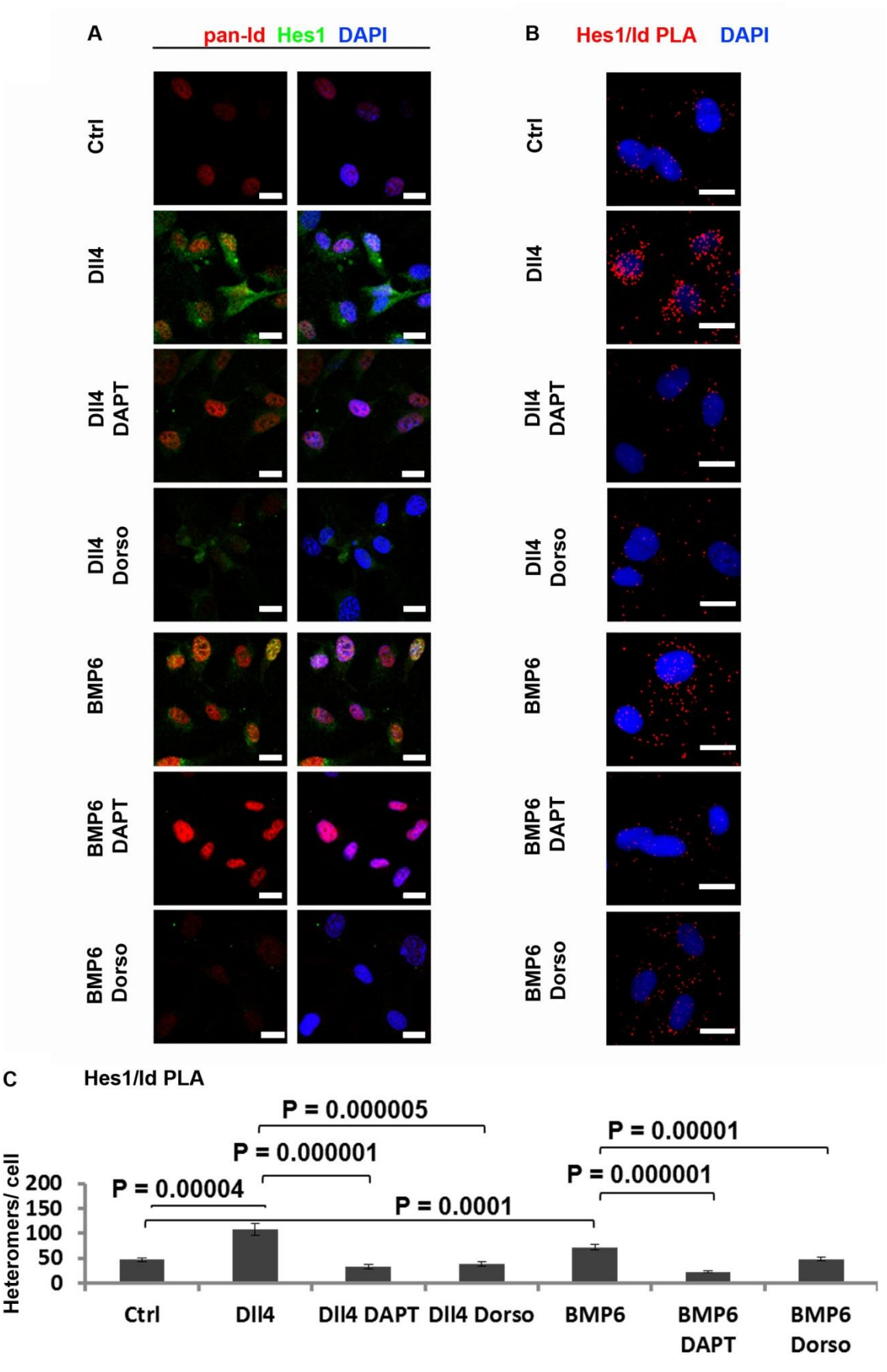


Figure S6, related to Figure 5. Endothelial stalk cell regulation of Dll4/Notch signaling.

a, *Hes1* or *NICD* overexpression in the regulation of tip and stalk cell enriched transcripts in HUVECs.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B
Hes1/Id PLA DAPI
Ctrl
Dll4
Dll4 DAPT
Dll4 Dorso
BMP6
BMP6 DAPT
BMP6 Dorso
C
Hes1/Id PLA
Heteromers/ cell
200
150
100
50
0
P = 0.00004
P = 0.000001
P = 0.000005
P = 0.0001
P = 0.000001
P = 0.00001
Ctrl
Dll4
Dll4 DAPT
Dll4 Dorso
BMP6
BMP6 DAPT
BMP6 Dorso

Figure S7, related to Figure 6. Hes1-Id heteromer formation upon activation of Notch or BMP signaling pathways. a, Localization of endogenous Hes1 and Id proteins in HUVECs stimulated with BMP6 (200 ng/ml) or Dll4 (1mg/ml, coated) in the presence or absence of DAPT (100 mM) or Dorsomorphin (10 mM). Id proteins are present in basal conditions. Dll4 stimulation causes increase of Hes1 levels. Notch inhibition results in decreased Hes1 levels but in increased amount of Id proteins. BMP6 stimulation resulted in increased colocalization of Hes1 with Id proteins, and inhibition of the BMP pathway with dorsomorphin effectively reduced Hes1 and Id production. **b,** Representative pictures showing Hes1/Ids heterodimers, which were detected by *in situ* PLA using a polyclonal pan-Id antibody and a monoclonal Hes1 antibody. Stimulation with Dll4 or BMP6 increased the number of PLA signals. **c,** Quantification of PLA signals. Images and data shown are representative of experiments repeated at least 3 times. P values were calculated using two-tailed Student's t-test ($n \geq 60$). Note: the data shown here belong to the same experimental data set of figure 4, hence control panels are identical. Error bars, s.e.m. Abbreviations: Dorso, dorsomorphin hydrochloride. Scale bars: 15 μm in (a,b).

Supplemental Experimental Procedure

Mice

Homozygous mice for *Smad1(*Tremblay et al., 2001) and *Smad5* (Umans et al., 2003) floxed alleles (*Smad1*$^{fl/fl}$;*Smad5*$^{fl/fl}$) were bred with transgenic mice that express Cre-recombinase in the endothelium (*Tie-2-Cre*$^{+/0}$)(Kisanuki et al., 2001). Resulting *Tie-2-Cre*$^{+/0}$;*Smad1*$^{fl/+}$;*Smad5*$^{fl/+}$ mice were mated with *Smad1*$^{fl/fl}$;*Smad5*$^{fl/fl}$ mice to obtain complete deletion of the four floxed alleles of *Smad1* and *Smad5* in endothelium (*Tie-2-Cre*$^{+/0}$;*Smad1*$^{fl/fl}$;*Smad5*$^{fl/fl}$ or dKO^{EC}). In addition, homozygous *Smad1*$^{fl/fl}$;*Smad5*$^{fl/fl}$;*ROSA26*$^{+/+}$ reporter mice (R26R)(Soriano, 1999) were crossed with *Tie-2-Cre*$^{+/0}$; *Smad1*$^{fl/+}$;*Smad5*$^{fl/+}$ mice to visualize the endothelium after X-gal staining. In resulting progeny, *Smad1*$^{fl/+}$;*Smad5*$^{fl/+}$, or the *Tie-2-Cre*$^{+/0}$;*Smad1*$^{fl/+}$;*Smad5*$^{fl/+}$;*R26R*$^{+/0}$ littermates where considered as controls. In addition, *BRE:gfp* mice were used to monitor BMP/Smad transcriptional activity (Monteiro et al., 2008). All mice used in the experiments have a variable mixed background (CD1, 129/ola, and C57BL6). Genotyping of *Smad1* and *Smad5* floxed or recombined alleles, and the transgenic BRE:gfp allele was done as described (Monteiro et al., 2008; Tremblay et al., 2001; Umans et al., 2007). All animal procedures were performed according to the guidelines of the Animal Welfare Committee of KU Leuven, Belgium.

Culture of dorsal midgestation embryo explants

E9.5 embryos were dissected in ice-cold 10% FCS in DMEM (Gibco). The forebrain, and the abdomen caudal from the forelimb bud, was transversally removed from the embryo. All ventral tissues including the heart were removed preserving the hindbrain and neural tube

roof. Dorsal explants were cultured on Millicell cell culture inserts (Millipore, PICM0RG50) in a 3.5 ml petri dishes containing 1 ml of EC medium (DMEM from Gibco with 20% Fetal Bovine Serum, 2 μM L-Glutamine, 100 U K-PenicillinG/ml, 100 μg Streptomycin sulfate/ml, 120 μg heparin/ml and 50 μg Endothelial Cell Growth Supplement/ml, from Millipore) in a humidified incubator at 37°C 5% CO_2 for 6 hours. Growth factors (100 ng BMP2/ml, gift from W. Sebald and J. Nickel) and/or N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-Butyl Ester, DAPT (100 μM, Calbiochem) or Dorsomorphin dihydrochloride (10 μM, Tolcris) were applied directly to the medium. Cultured dorsal explants of BRE:gfp E9.5 embryos were stained for α-GFP and PECAM. All experiments were repeated at least 3 times. The number of GFP-positive ECs was counted per confocal field in a total of 9 microscopic fields (aortae) for per condition. The nuclei (DAPI) of GFP positive ECs in the aortae were normalized with respect to the total number of ECs in each aorta and the data are presented as percentages. In order to quantify every cell or the aortae, each z-stack quantified independently per confocal field.

Matrigel tube formation assay and time course experiments

HUVECs (Lonza) were cultured in EGM®-2MV Microvascular Endothelial Cell Growth Medium-2 (full^{EC} medium, Lonza) and used between passages 6-9 (from purchase). HUVECs were seeded on 6-well plates at a density of 200.000 cells/well. The next day, the cells were transfected with 5nM of ON-TARGET*plus Smad1* siRNA and *Smad5* siRNA or non-targeting siRNA SMARTpools (Dharmacon technologies) in starvation medium (EBM medium with 0,1% FBS, no growth factors), using Lipofectamine 2000 (Invitrogen). Alternatively, HUVECS seeded on 6-well plates were transfected with 400ng/ml of pCDNA3-Id1, pBlueScript II-Ks-Id3 (kindly provided by P. ten Dijke), Hes1 or mouse pCD2-NICD (Raphael KOPAN

reference). After 18 hours, 40.000 cells were seeded on BD Matrigel™ (BD Biosciences) coated wells of 48-well plates and stimulated with full^{EC} medium, and cultured for 5 hours. For the chimaeric tube formation assay, HUVECs transfected with *Smad1/5* siRNAs or with *Id1-3* were labeled with Calcein AM (1μg/ml, Invitrogen) and NT siRNA or Mock transfected were labeled with DiI-Ac-LDL (10μg/ml, Biomedical Technologies). After 18 hours, these cells were mixed in a 1:1 proportion and seeded on Matrigel™ coated wells. Five hours after seeding, the cells were fixed with 2% PAF and nuclei stained with DAPI. Each experiment was repeated 3 times and each condition was done in triplicates. Quantification of the number of branching points or of tip cell positions calculated in 9 microscopic fields/condition.

For the time course experiments, HUVECs were seeded and trasfected in 6-well plates as described above starved for 12 hours and then stimulated with full^{EC} medium. Samples were collected every 30 minutes and processed for protein extraction and western blot analysis or for RNA isolation and qPCR analysis.

Cell migration assays

Circular guidemarks were drawn on the coverslip to mark the limit between the inner chamber and the annular bridge of the Dunn chamber. Within the marked region, 20.000 HUVECs were seeded in 30ul of full^{EC} medium forming a drop that corresponded to the size of the inner concentric well of the chamber. The cells were transfected (*Sma1/5* or NT siRNAs) on the coverslips the next day and starved for 18 hours. To set up gradient experiments, both concentric wells of the chamber were filled with starvation medium, and the coverslip seeded with cells was inverted onto the chamber in an offset position so that the seeded area matched with the inner well. All edges of the coverslips/chambers were sealed using hot wax except for a filling slit left at one edge of the outer well. The medium of the outer well was drained and replaced with full^{EC} medium (Chemoatractant). The slit was then sealed with hot wax

mixture. Both wells were filled with starvation medium in negative controls. After 5 hours, the cells were fixed with 2% PAF or with ice cold Methanol and stained with Rabbit Anti-GP130 Polyclonal Antibody (Cell Signaling Technology) and mounted with MOWIOL containing DAPI and Alexa Fluor 488 phalloidin (1:200, Invitrogen). All the migrating cells within the annular bridge were processed for confocal microscopy analysis (BioRad Radiance 2100). The cells within the inner well and outer well served as internal controls.

Boyden chamber assays were performed in 24-well plate transwell chambers of 8 μm pore size (Costar/Corning). After 18 hours, 50 000 cells were seeded into the top chamber in 100 ul starvation medium and allowed to settle for 2 hours. Then, the bottom well was filled with full^{EC} medium and the cells were incubated for 6 h at 37°C. The cells that remained in the topside of the transwell were carefully removed while the cells present on the lower surface were fixed with 2% PAF in PBS, the filters were removed and mounted on glass slides with MOWIOL containing DAPI. Each experiment was repeated 3 times and each condition was done in triplicates. A total of 15 fields per condition using a 40× objective lens was quantified using a fluorescence microscope.

Quantifications

Number of sprouts and medial anastomoses: We measured the angiogenic front of the growing vascular plexus and counted the number of sprouts along this length using ImageJ (NIH public domain software). We calculated the number of sprouts per 975 μm of vessel length from 20 microscopic fields picked from 20 E9.5 flat-mounted dorsal explants per group. The number of medial anastomoses was calculated as described above in 27 microscopic fields (embryos) for control embryos and 33 microscopic fields (embryos) for mutant embryos.

EC proliferation: Flat-mounted dorsal explants were stained for pH3 and PECAM. The number of proliferating pH3-positive ECs was counted per confocal field in a total of 11 microscopic fields (embryos) for control embryos and 12 microscopic fields (embryos) for mutant embryos.

Filopodia quantification: Transfected HUVECs with *Sm1/5* or NT siRNAs and starved over night for 18 hours. Then, the cells were trypsinized and cultured on gelatin-coated coverslips without gradient of full medium or in Dunn chamber with gradients of full medium to stimulate directed cell migration. After 4 hours, the cells were fixed in 2% PAF in PBS and mounted with MOWIOL containing DAPI and Alexa Fluor 488 phalloidin (1:200, Invitrogen) to visualize the cytoskeleton and the filopodia. We calculated the number of filopodia per 100 μm of cell surface length from 7-8 cells per group.The length of every individual filopodia was measured in all cells per condition.

All quantitative data were compiled and analyzed using unpaired two-tailed Student's t-test.

Gene expression analysis

Mouse primers: *Hes1*, 5'- GGTATTTCCCCAACACGCT-3' and 5'-GGCAGACATTCTGGAAATGA -3'; *Hey1,* 5'-TCCGATAGTCCATAGCCAGG-3' and 5'-TTGCAGATGACTGTGGATCA-3'; *Id1*, 5'-CCCTGAACGGCGAGATCA-3' and 5'-TTTTTTCCTCTTGCCTCCTGAA-3'; *Id2*, 5'-GAAAGCCTTCAGTCCGGTGA-3' and 5'-TGGTCCGACAGGCTGTTTTT-3'; *Id3*, 5'-CCACCCTGACTCAAGTCTAA-3' and 5'-CCAAGGAAACCAGAAGAACA-3'; *GAPDH*, 5'- AAGAAGGTGGTGAAGCAGGC-3' and 5'- GCCTCTCTTGCTCAGTGTCC-3'; and the following for human: *Hes1*, 5'-TACTTCCCCAGCACACTTGG-3' and 5'- CGGACATTCTGGAAATGACA-3'; *Hey1*, 5'-CGAAATCCCAAACTCCGATA-3' and 5'- TGGATCACCTGAAAATGCTG -3'; *Id1*, 5'-CTACGACATGAACGGCTGTTACTC-3' and 5'- CTTGCTCACCTTGCGGTTCT-3'; *Id2*,

5’-TCAGCACTTAAAAGATTCCGTG-3’ and 5’-GACAGCAAAGCACTGTGTGG-3’; *Id3,* 5’-CTTCCGGCAGGAGAGGTT-3’ and 5’-AAAGGAGCTTTTGCCACTGA-3’; and *GAPDH*, 5’- TGCACCACCAACTGCTTAGC-3’ and 5’-GGCATGGACTGTGGTCATGAG-3*; Dll4,* 5’- GTGCAGGTGTAGCTTCGCT -3’ and 5’-AGGCCTGTTTTGTGACCAAG -3’; *Jagg1*, 5’-ACTGTCAGGTTGAACGGTGTC-3’ and 5’-ATCGTGCTGCCTTTCAGTTT-3’; *VEGFR1*, 5’-TCCCTTCCTTCAGTCATGTGT-3’ and 5’-AAGAAGGAAACAGAATCTGCAA-3’; *VEGFR2*, 5’-CGGCTCTTTCGCTTACTGTT-3’ and 5’-CCTGTATGGAGGAGGAGGAA-3’; *VEGFR3,* 5’-GGTGTCGATGACGTGTGACT -3’ and 5’-AAAGGAGCTTTTGCCACTGA-3’; *PDGF-BB*, 5’-CTCTGCCTGGGACTCCTG-3’ and 5’-CTGGCATGCAAGTGTGAGAC-3’.

Proximity ligation assay

Coverslips with fixed and permeabilized HUVECs were blocked and incubated overnight at 4°C with rabbit-Id1z8 (pan-Id, Santa Cruz) and mouse-Hes1 (Abcam) or with rabbit-Hey1 (Abcam) and mouse-Id3 (Santa Cruz) primary antibodies. After several washes, HUVECs were incubated with α-mouse and α-rabbit secondary antibodies (PLA probes). Circularization and ligation of the oligonucleotides in the probes was followed by an amplification step. A complementary fluorescently labeled probe was used to detect the amplification products. The coverslips were mounted using Vectashield hard mounting set (Vector) containing DAPI for nuclear visualization. Six Z-stack micrographs per field were taken with 63× objectives using a Radiance 2100 confocal microscope (Bio-Rad). The number of heteromers was counted in a total of 7 fields/well containing at least 10 cells/field. All images obtained were exported into TIF format with ImageJ software (NIH) and further analyzed with Blob-Finder image analysis software (Centre for Image Analysis, Uppsala

University). Representative results of experiments repeated at least three times are shown. Quantifications are given as mean + s.e.m.

Statistical evaluation

All experiments were performed at least 3 times; Student t-test was performed for all statistical analyses. P values <0.05 were considered significant. *P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary references

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