SUPPLEMENTAL INFORMATION FOR

Tie1 deletion inhibits tumor growth and improves angiopoietin antagonist therapy

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SUPPLEMENTAL INFORMATION

Legends to Supplemental Figures

Supplemental Figure 1. Conditional targeting of the Tie1 locus. (**A**) Photomicrographs of control and Tie1 deleted (*Tie1*$^{\Delta/\Delta}$, *PGK-Cre* deletor) embryos. At E14.5, oedematous (arrows) and hemorrhagic (stars) phenotypes were observed in Tie1-deleted but not in control embryos. Scale bar: 2 mm. $n > 4$ embryos/genotype. (**B**) Ultramicroscopy 3D reconstructions of images obtained by optical sectioning of control and Tie1-deleted (*Tie1*$^{iEC\Delta/\Delta}$, *Pdgfb-icre/ERT2* deletor) embryos stained for Pecam1 and Prox1. PLLV, peripheral longitudinal lymphatic vessel. pTD, primordial thoracic duct. sLECs, superficial lymphatics. CV, cardinal vein. Scale bars: 100 μm. $n = 2$ embryos/genotype. (**C**) The homologous recombination event deletes sequences encoding the Tie1 translation initiation site and exon 1 (E1). Red arrowheads, *lox*P sites; yellow arrowheads, FRT sites; N, *Nsi*I cleavage sites; *neo*, neomycin resistance cassette; c-knockout, conditional knockout. (**D**) Southern blot analysis of DNA from a litter at embryonic day (E) 14.5 after *Nsi*I digestion, hybridized with the 5' external

probe indicated by the green bar in panel (A). Right margin, molecular sizes of the digested fragments. (**E**) *Tie1* mRNA expression in embryos of the same litter analyzed by Northern blotting. Ribosomal 28S RNA staining was used as a loading control. (**F**) Tie1 expression analyzed by Western blotting of whole E14.5 embryo lysates. Hsc70 was used as a loading control. Representative data from 3 independent experiments, $n > 2$ embryos/genotype/experiment.

Supplemental Figure 2. PCR genotyping of the Tie1 conditional mice. (**A**) A scheme (not in scale) showing the wild-type, conditional (flox), and Tie1 deleted allele after Cre-mediated recombination (Tie1 Δ). The colored arrows indicate the PCR forward (black and cyan) and reverse (green and red) primer binding sites. Primer sequences are described in the Supplemental Methods. Double-arrowheads indicate the expected PCR product sizes in base pairs (bp). E1, exon 1. (**B**) Photographs of representative electrophoresis gels containing the PCR products of the wild type (+), conditional (flox), and the conditional Tie1 knockout (Δ) alleles as well as the recombinase transgene (Cre). DNA samples used for the PCRs were extracted from ear biopsies of wild-type, *Tie1*$^{flox/flox}$, *Pdgfb-icre/ERT2;Tie1*$^{+/flox}$ and *Pdgfb-icre/ERT2;Tie1*$^{flox/flox}$ mice, and Cre recombinase activity was induced by administration of tamoxifen.

Supplemental Figure 3. Dysmorphic lymph sacs in Tie1-deleted embryos. Analysis of blood and lymphatic vasculature in (**A-B**) *Tie1*$^{flox/flox}$ (control) and (**C-D**) *PGK-cre;Tie1*$^{flox/flox}$ (*Tie1*$^{\Delta/\Delta}$) embryos at E14.5. Immunofluorescence staining for endomucin (red), Prox1 (red) and Lyve1 (green) of comparable thoracic transverse sections identifies blood vessels and lymph sacs, respectively. Note dysmorphic lymph sacs in *Tie1*$^{\Delta/\Delta}$ but not in control embryos. Nuclei were stained with DAPI (blue). Ao, dorsal aorta. Cv, cardinal vein. Ls, lymph sacs.

Scale bar: 100 μm. n > 4 embryos/genotype.

Supplemental Figure 4. Tie1 expression in LLC tumor vasculature. (**A**) Tie1 expression (X-gal staining, blue) in tumor vessels (endomucin, brown) in *Tie1*$^{lacZ/+}$ mice. (**B**) Immunochromogenic staining for Tie1 (brown) in the tumor vasculature of the *Tie1*$^{+/+}$ mice. (**C**) Immunofluorescence for Tie1 (green), endomucin (red), and DAPI (blue) in tumor sections from control and *Tie1*$^{iEC\Delta/\Delta}$ mice (*Pdgfb-icre/ERT2* deletor). Maximum projections from confocal microscopy z-stack images are shown. (**D**) Tie1 staining only is shown for better visualization. Note the lack of Tie1 antigen in vessels in tumors grown in *Tie1*$^{iEC\Delta/\Delta}$ mice. Scale bars: 50 μm.

Supplemental Figure 5. Endothelial cell death and intravascular fibrin deposition frequency in control and *Tie1*$^{iEC\Delta/\Delta}$ tumor vessels. Quantification of endothelial cell death and intravascular fibrin deposition frequency in control and *Tie1*$^{iEC\Delta/\Delta}$ tumors. Error bars indicate SEM. n = 84-144 vessels from 5-7 LLC tumors/genotype (*Pdgfb-icre/ERT2* deletor).

Supplemental Figure 6. Endothelial cell proliferation in control and *Tie1*$^{iEC\Delta/\Delta}$ tumor vessels. Endothelial cell proliferation indices calculated as the ratio of (**A**) Ki67 and (**B**) 5-bromodeoxyuridine (BrdU) positive endothelial cells to the total number of endomucin-positive endothelial cells in the LLC tumor blood vessels. Error bars indicate SEM. n = 3-6 tumors/genotype (both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors).

Supplemental Figure 7. Vessel coverage by mural cells in tumors of control and *Tie1*$^{iEC\Delta/\Delta}$ mice. Immunofluorescence for endomucin (green) and (**A-B**) NG2 and (**D-E**) α-SMA (SMA, red) to identify blood vessels, pericytes and smooth muscle mural cells, respectively in LLC tumor sections. Confocal microscopy z-stack images are shown. Scale

bars: 50 μm. (**C, F**) Quantification of the % of total NG2- or SMA-positive area relative to total endomucin-positive area per field. Error bars indicate SEM. *n* = 5-8 tumors/genotype (both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors).

Supplemental Figure 8. Expression of leukocyte adhesion molecules in tumor vessels. Immunofluorescence for (**A-B**) P-selectin (red) and endomucin (green) and (**D-E**) Icam1 (red) and Pecam1 (green) in LLC tumor sections. (**A-B**) Inset, P-selectin only. (**C**) % of P-selectin-positive area relative to total endomucin-positive vessel area. (**D-E**) Inset, Icam1 only. (**F**) % of Icam1-positive area relative to total Pecam1-positive vessel area. Error bars, SEM. *n* = 3 LLC tumors/genotype (*Cdh5(PAC)-cre/ERT2* deletor). **P*<0.05. Scale bars: 100 μm.

Supplemental Figure 9. Leukocyte recruitment to tumor stroma. (**A**) Shown are stainings for total leukocytes (CD45), macrophages (F4/80) and neutrophils (Ly6G/Gr-1) in LLC tumor sections. (**B**) % of the indicated leukocytes per total number of cells (nuclei, DAPI or hematoxylin) in LLC tumor sections. Error bars, SEM. *n* = 5-6 tumors/genotype (both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors). Scale bars: 100 μm.

Supplemental Figure 10. Analysis of the vasculature in organs of adult mice lacking Tie1. (**A**) Representative confocal microscopy images of vascular beds from lungs, kidney glomeruli, cardiac muscle, thyroid gland, small intestinal villi, tracheal mucosa and ear skin of 8-10 week-old control and Tie1-deficient adult mice in which the genetic deletion was induced for 3 weeks (*Tie1*$^{iEC\Delta/\Delta}$, *Pdgfb-icre/ERT2* deletor) stained for endomucin or Pecam1. (**B**) Quantification of vascular density in control and *Tie1*$^{iEC\Delta/\Delta}$ tissues shown in (A). Error bars, SEM. *n* = 4 mice/genotype. (**C**) Analysis of the tracheal vasculature (Pecam1, green) by tile scanning confocal microscopy. (**D-E**) High magnification images of postcapillary

venules stained for Pecam1 and for (**F-G**) Cdh5 (VE-cadherin), depicting endothelial cell junctions. (**H**) Quantification of tracheal capillary branching and diameter. Error bars, SEM. *n* = 4 mice/genotype. (**I**) Analysis of the morphological appearance of the left lobe of the lungs in H&E stained sections. (**J**) Masson's trichrome stained alveolar interstitial areas at low (upper) and higher (bottom) magnifications. (**K**) Quantification of the interstitial lung area (as % of control). Error bars, SEM. *n* = 4 mice/genotype. (**L-M**) Transmission electron micrographs of control and *Tie1*$^{iEC\Delta/\Delta}$ lung alveolar septum microvasculature. Black arrowheads, endothelial adherens junctions (AJs). Stars, basement membrane (BM). EC, endothelial cell. RBC, red blood cell. Ep, epithelium. *n* = 2 mice/genotype. Scale bars: 50 μm, except for (**L-M**) 1 μm.

Supplemental Figure 11. Renal function in control and Tie1-deficient mice. (**A**) Representative transmission electron micrographs from control and *Tie1*$^{iEC\Delta/\Delta}$ (*Pdgfb-icre/ERT2* deletor) kidney glomerular capillary vasculature. Black arrowheads, endothelial cell fenestrations. Ubiquitously Tie1-deleted (*Tie1*$^{i\Delta/-}$) mice were also examined (*Rosa26*/*creERT2* deletor, see Supplemental Table 1 and Supplemental Methods for details). White arrowheads, basement membrane. EC, endothelial cell. RBC, red blood cell. Pd, podocyte. Scale bars: 1 μm. (**B**) Blood urea nitrogen (BUN) and creatinine analysis in serum from control (*n* = 6), *Tie1*$^{i\Delta/-}$ (*n* = 4) and *Tie1*$^{iEC\Delta/\Delta}$ (*n* = 2) mice. Tie1-deficient mice (*Tie1*$^{i\Delta}$), *Tie1*$^{i\Delta/-}$ + *Tie1*$^{iEC\Delta/\Delta}$ mice. Error bars, SEM. (**C**) Representative Coomassie blue stain of urine samples fractioned in a 4.0 - 7.5% SDS-PAGE. *n* > 3 for control and *Tie1*$^{i\Delta/-}$ mice, and *n* = 2 for *Tie1*$^{iEC\Delta/\Delta}$ mice.

Supplemental Figure 12. Analysis of tumor growth and vasculature in control and *Tie1*$^{iEC\Delta/\Delta}$ mice treated with anti-VEGF or anti-VEGFR-2 antibodies. (**A**) LLC, EL4 and

B16F1 tumor growth curves and tumor volumes (mm^3) at 17 and 18 (LLC), 16 (EL4) or 13 (B16F1) days in control and *Tie1*$^{iEC\Delta/\Delta}$ mice treated with IgG control, placebo, DC101 (800 μg/mouse) or mrc84 (200 μg/mouse) antibodies. (**B**) Tumor weights (g). (**C**) Vessel density (area % relative to control + IgG or control + placebo). (**D**) Endothelial staining of LLC and (**E**) EL4 tumors (endomucin or Pecam1, green) in control and *Tie1*$^{iEC\Delta/\Delta}$ mice (both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors). Error bars, SEM. The numbers in the box plots represent *n* mice or tumors/group. *P* values were obtained by ANOVA one-way test. Scale bars: 100 μm.

Supplemental Figure 13. VEGFR-2 protein analysis of Tie1-deficient mice stimulated with VEGF *in vivo*. Adult *Pdgfb-icre/ERT2;Tie1*$^{flox/lacZ}$ mice were treated with tamoxifen (*Tie1*$^{iEC\Delta/-}$, see Supplemental Table 1) and injected i.v. tail with PBS or VEGF (10 μg), followed by analysis after 10 min. Lung lysates were immunoblotted for VEGFR-2. Hsc70 was used as a loading control. See immunoblot for Tie1 in Supplementary Figure 15 for efficiency of deletions. Ctrl, control. *iEC*Δ/-, *Tie1*$^{iEC\Delta/-}$ mice. *n* = 2 mice/group, two independent experiments.

Supplemental Figure 14. Kidney histopathology in control and *Tie1*$^{iEC\Delta/\Delta}$ mice treated with the anti-VEGF blocking antibody mrc84. (**A**) Endomucin staining of formalin-fixed, paraffin-embedded kidney sections from control and *Tie1*$^{iEC\Delta/\Delta}$ tumor bearing mice (EL4 tumors, both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors) treated with placebo or mrc84 antibody, as described in Supplemental Methods. Renal cortex (upper panels) and glomeruli (bottom panels) are shown at low and high magnifications, respectively. Quantification of (**B**) glomerular perimeter (relative to control + placebo, %), (**C**) glomerular

blood vessel density (BVD, relative to control + placebo %), and (**D**) Bowman's space area (relative to control + placebo, %). Error bars, SEM. Scale bars: 100 μm.

Supplemental Figure 15. Phospho-Tie2 and Tie2 analysis of Tie1-deleted mice stimulated with VEGF, Comp-Angpt1 and Angpt2 *in vivo*. (**A**) 8-week old *Pdgfb-icre/ERT2;Tie1*$^{flox/flox}$ and *Pdgfb-icre/ERT2;Tie1*$^{flox/lacZ}$ progeny were treated with tamoxifen (iECΔ, see Supplemental Table 1) and injected i.v. tail with PBS, VEGF-A (VEGF, 10 μg) (**B**) COMP-Angpt1 (CA1, 20 μg) or Angpt2 (20 μg), followed by analysis after 10 min. Note that in (A) the lungs were subjected to Tie2 immunoprecipitation (IP), followed by phosphotyrosine (pY) immunoblotting. The membrane was stripped and re-probed for Tie2. In (B), a specific phosphotyrosine of Tie2 (pTie2, Y1106) immunoblotting was done. The membrane was stripped and re-probed for Tie2. Western blotting for Tie1 shows the efficiency of the deletions and immunoblotting for Flag confirms that CA1 and Angpt2 have reached the target organ. Hsc70 was used as a loading control. Ctrl, control mice. n = 2 mice/group, two independent experiments for (A). n = 2 mice/group, one experiment for (B).

Supplemental Figure 16. ß-galactosidase staining of retinas from *Pdgfb-icre/ERT2;Rosa26-R* and *Tie1*$^{+/lacZ}$ mice. For the staining shown in (**E-H**) the *Pdgfb-icre/ERT2;Rosa26-R* (*Pdgfb-icre;Rosa26*) pups were treated daily with tamoxifen injections (intragastric; 50 μg/day) from postnatal day (P)1 to P4, and retinas were dissected at P5. (**B-C, F-G**) Panels show the boxed areas in (**A**) and (E), and (**D, H**) in (C) and (G) at a higher magnification. (a) arteriole, (v) venule. Scale bars: 100 μm. n > 4 retinas/genotype.

Supplemental Figure 17. Staining and quantification of retinal vasculature in control and *Tie1*$^{iEC\Delta/\Delta}$ mice at P5-6. Newborn *Pdgfb-icre/ERT2*;*Tie1*$^{flox/flox}$ pups were treated with tamoxifen, as in the experiment of Supplemental Figure 16, to induce Tie1 deletion

(*Tie1*$^{iEC\Delta/\Delta}$). (**A**) Confocal microscopy images of flat-mounted retinas at P5-6 stained for IsolectinB4 (IB4). Scale bar: 100 μm. (**B**) Tie1 protein analyzed by Western blotting of lung lysates from the pups. Hsc70 was used as a loading control. Bar graph of densitometric quantification of Tie1 relative to Hsc70. **P*<0.001. Ctrl, control. *iEC*Δ/Δ,*Tie1*$^{iEC\Delta/\Delta}$ pups. (**C**) % of IB4-positive staining/total area (vascular area), (**D**) number of branchpoints/vascular area, and (**E**) radial distance (distance between the average edge of the sprouting vessel front and the optic nerve). Error bars, SEM. *n* = 11-12 retinas/genotype.

Supplemental Figure 18. Pericyte coverage of retinal vasculature in control and *Tie1*$^{iEC\Delta/-}$ mice at P5-6. (**A**) Confocal microscopy images of flat-mounted control and Tie1-deleted (*Tie1*$^{iEC\Delta/-}$, *Pdgfb-icre/ERT2* deletor) angiogenic front, (**B**) artery (a) and (**C**) vein (v) retinal vessels at P5-6, stained for pericytes (NG2, red) and endothelium (IB4, green). Scale bar: 100 μm. Arrowheads: blunted angiogenic front, arrows: detached pericytes.

Supplemental Figure 19. Angiogenesis-related RNA and protein analysis in lungs from the control, *Tie1*$^{iEC\Delta/\Delta}$ and *Tie1*$^{iEC\Delta/-}$ pups at P6. (**A**) qRT-PCR analysis of lung biopsies. mRNA levels were normalized to *Gapdh* as a housekeeping gene. Error bars, SD. $^{*}P < 0.05$; *n* = 4 control and 5 either *Tie1*$^{iEC\Delta/\Delta}$ or *Tie1*$^{iEC\Delta/-}$ (together called *Tie1*$^{iEC\Delta}$, both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors) pups. (**B-C**) Western blot analysis of lungs. Percentage of protein levels normalized to Hsc70 control. Error bars, SEM. $^{*}P < 0.05$; *n* = 5-8 control (Ctrl) and 6-14 *Tie1*$^{iEC\Delta}$ (*iEC*Δ) pups.

Supplemental Table 1. Summary of tamoxifen treatments to induce Tie1 deletion in embryonic, postnatal and adult Tie1 conditional mice.

Strain	Age	Tamoxifen	Treatment	Analysis
Pdgfbicre/ERT2; *Tie1*$^{flox/flox}$ & *Pdgfbicre/ERT2*; *Tie1*$^{flox/lacZ}$	6-9 weeks	2 mg/day (4-OHT)	2 times gavage (in sunflower oil), consecutive days (at E7.5 & E8.5)	Embryonic developmental (lymph)angiogenesis
Cdh5(PAC)cre/ERT2 & *Pdgfbicre/ERT2*; *Tie1*$^{flox/flox}$	6-9 weeks	15 mg (free base)	Slow release (21-days) pellets, implanted s.c.	Tumor angiogenesis and unchallenged condition
Pdgfbicre/ERT2; *Tie1*$^{flox/flox}$	6-9 weeks	2 mg/day (4-OHT)	3 times gavage (in sunflower oil), every second day	Unchallenged condition
Pdgfbicre/ERT2; *Tie1*$^{flox/flox}$	6-9 weeks	2 mg/day (tamoxifen)	4 times i.p. (in sunflower oil), every second day	Unchallenged condition
Rosa26/creERT2; *Tie1*$^{flox/lacZ}$	4 weeks	200 mg/kg/day (tamoxifen)	5 times i.p. (in sunflower oil), consecutive days	Unchallenged condition
Pdgfbicre/ERT2; *Tie1*$^{flox/lacZ}$	6-9 weeks	2 mg/day (4-OHT)	3 times gavage (in sunflower oil), every second day or 5 times i.p. (in corn oil)	*In vivo* growth factor stimulations
Pdgfbicre/ERT2; *Rosa26-R*	newborn	50 µg/day (4-OHT)	P1-4, daily intragastric injections (in ethanol)	Cre reporter activity
Pdgfbicre/ERT2; *Tie1*$^{flox/flox}$, *Cdh5(PAC)cre/ERT2* & *Pdgfbicre/ERT2*; *Tie1*$^{flox/lacZ}$	newborn	50 µg/day (4-OHT)	P1-4, daily intragastric injections (in ethanol)	Postnatal angiogenesis

Supplemental Methods

Mice, genotyping, Cre induction and procedures. Mice were anaesthetized with intraperitoneal injections of xylazine (10 mg kg^{-1}) and ketamine (80 mg kg^{-1}). We used *Tie1*$^{+/lacZ}$, *Tie1*$^{flox/flox}$, *ROSA26-R*, *PGK-Cre*, *Cdh5(PAC)-Cre/ERT2*, *Pdgfb-iCre/ERT2* and *Rosa26/CreERT2* mouse lines, which have been published previously (for references see PCR genotyping section). All embryos, newborn pups and adult mice analyzed were in a congenic C57Bl6J background. Inducible endothelial-specific inactivation of Tie1 was obtained by administration of tamoxifen to *Cdh5-Cre/ERT2;Tie1*$^{flox/flox}$, *Cdh5-Cre/ERT2;Tie1*$^{flox/lacZ}$, *Pdgfb-iCre;ERT2/Tie1*$^{flox/flox}$, *Pdgfb-iCre/ERT2;Tie1*$^{flox/lacZ}$ and *Rosa26/CreERT2;Tie1*$^{flox/lacZ}$ mice. The appropriate concentration of tamoxifen was administered to newborn pups and adult mice via intragastric or intraperitoneal injection, gavage or implantation of subcutaneous pellets, at the specified time points (see **Supplemental Table 1**). Deletion of the *Tie1* gene in the above mentioned strains was validated by Southern blotting, PCR genotyping, Northern blotting, qRT-PCR and Western blotting, except for the *Rosa26/CreERT2;Tie1*$^{flox/lacZ}$ mice, where only PCR genotyping and Western blotting were performed (**Supplemental Figures 1** and **2**). LLC, B16F1 and B16F10 melanoma and EL4 leukemia/lymphoma syngeneic grafts were made by injecting 1–3 × 10^6 cells into the subcutaneous space of the abdominal flank of *Cdh5-Cre/ERT2*;*Tie1*$^{flox/flox}$ and *Pdgfb-iCre/ERT2*;*Tie1*$^{flox/flox}$ mice. For combinatorial inhibition studies, anti-VEGF (mrc84, 200 μg in 100 μL of PBS), anti-VEGFR-2 (DC101, 800 μg in 100 μL of PBS), placebo or control antibodies were injected intraperitoneally (i.p.) starting four days after the tumor cell implantations. The injections were repeated every 3.5 days (mrc84) or every 2 days (DC101) until the experiments were terminated. The recombinant adeno associated virus serotype 9

(AAV9) encoding mTie2-ECD or mFc were i.p. injected ($1x10^{12}$ vector genomes/mouse) one day before the tumor cell implantations. Pharmacological Notch inhibition was induced by injecting DAPT (Sigma) or vehicle subcutaneously into the neonatal pups, 48 h before analysis. Angpt2 blockers (1, 2) were administered subcutaneously at 30 mg kg−1 from P1-P4. After sacrificing the mice, the tissues were immersed in 4% paraformaldehyde, washed in phosphate buffered saline (PBS) and processed for whole-mount staining, or immersed in OCT medium (Tissue Tek) or embedded in paraffin.

PCR genotyping of mice. We used the *Tie1*$^{+/lacZ}$ (3), *Tie1*$^{flox/flox}$ (4), *ROSA26-R* (5), *PGK-Cre* (6), *Cdh5(PAC)-Cre/ERT2* (7), *Pdgfb-iCre/ERT2* (8) and *Rosa26/CreERT2* (9) mouse lines. Ear or tail biopsies were digested overnight with proteinase K, and genomic DNA was isolated using standard procedures. For the PCR genotyping of Tie1 wild-type and conditional alleles (Tie1c-KO PCR), the following primer pairs were used: Tie1_v3393 (5' CATTGGACAGACAAGCATGG 3') and Tie1_v3394 (5' CCACCAGACAGAACCACTCA 3'). PCR was performed in a 20 µl volume according to GoTaq Green Master Mix (Promega) for 2 minutes at 95°C followed by 31 cycles of 30 seconds at 95°C, 30 seconds at 61.5°C and 30 seconds at 72°C. For the evaluation of Cre-mediated recombination by PCR genotyping (Cre-del PCR), the Tie1-deleted allele was amplified by performing nested PCR using the following primer pairs for the first PCR: Tie1_v3358 (5' TGGGGGTGTCCTTGTACTTC 3') and Tie1_v3359 (5' CTCATTCCTGGCCTGTGTTT 3') and Tie1_v3399 (5' TAAAGGGTCGTCACGCTCCTAATTT 3') and Tie1_v3400 (5' TTTTCTGGTCCTGTGGCAGAGTAAG 3') for the second PCR. PCRs were performed in 20 µl volume according to Phusion High-Fidelity DNA Polymerase reaction mix protocols (Finnzymes), for 30 seconds at 98°C followed by 15 cycles of 5 seconds at 98°C and 10 seconds at 72°C for the first PCR. The thermal cycles for the second PCR were: 30 seconds

at 98°C, followed by 33 cycles of 5 seconds at 98°C, 5 seconds at 65°C and 6 seconds at 72°C. The location of the primers and the expected molecular sizes of the amplified PCR fragments from the wild type (+), conditional (flox) and Cre-mediated deleted (Tie1c-knockout (Δ) Tie1 alleles are indicated in **Supplemental Figure 2A**. The PCR primers and conditions for genotyping of all other mouse strains used have been published previously (3, 5-8)

Southern blotting. For the preparation of nonradioactive digoxigenin-labeled DNA 5' external hybridization probe for Tie1 Southern blot analysis, we cloned into the pCRII plasmid (Invitrogen) a 470 bp DNA insert containing base pairs -2008 to -1579 upstream of the start codon of the wild-type Tie1 allele in the sv129 mouse genetic background, and base pairs -8462 to -8013 of the wild-type Tie1 allele from the C57Bl mouse genetic background. The resulting plasmid was EcoRI digested, gel purified and PCR digoxigenin-dUTP labeled (PCR DIG Probe Synthesis kit, Roche) using the following PCR primer pairs: Tie1_Nsil_v2608 (5' GCGGCTGCAAGTTCCTGGGT 3') and Tie1_Nsil_v2609 (5' TGACCGATCAGAGCAGGCCG 3'). Genomic DNA samples from halved E14.5 embryos from the *PGK-Cre;Tie1*$^{flox/flox}$ mouse progeny were prepared by digesting with proteinase K overnight and employing standard procedures for DNA extraction. The DNA samples (10 μg) were *Nsi*I digested, electrophoresed in a 1% (w/v) Tris-Acetate-EDTA (TAE) agarose gel at 80V for 2.5-3 h, transferred overnight to a nylon membrane (Hybond-N+, GE Healthcare Life Sciences) and fixed by UV cross-linking. Hybridization was performed using the DIG Easy Hyb hybridization reagent (Roche) and employing standard procedures for non-radioactive nucleic acid probes. Chemiluminescent detection was performed using the anti-Digoxigenin-AP Fab fragments (Roche) and the CDP-Star reagent (New England BioLabs). The expected molecular sizes of the digested fragments from the wild-type, conditional (flox)

and Cre-mediated deleted (c-knockout, Δ) Tie1 alleles are indicated in **Supplemental Figure 1C-D**.

Northern blotting. For the generation of nonradioactive digoxigenin-labeled RNA hybridization probes for Tie1 Northern blot analysis, we cloned into pCRII plasmids (Invitrogen): a 474 bp cDNA insert containing exons 1-2 of the Tie1 gene (base pairs 44-517 of the NCBI Reference Sequence NM_011587.2) (PCRII-Tie1-ex1-2), and a 689 bp cDNA insert, encoding exons 5-9 of the Tie1 gene (base pairs 768-1456 of NM_011587.2) PCRII-Tie1-ex5-9. The cDNA fragments were amplified using the following PCR primer pairs: Tie1_v2718 (5' AGCACCGAGGTGCACTGAGC 3') and Tie1_v2719 (5' GGTGTGCCCCTGGGCTGTTG 3'); and Tie1_v2720 (5' CATCGTGCGAGGCTGTGGGG 3') and Tie1_v2721 (5' TGAAGCGCCGGCTGTCTTGG 3'), respectively. These were digested with the appropriate restriction enzymes (see below) and, after RNA extraction of the digests, labeled with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerases (DIG RNA Labeling Kit, Roche). Briefly, for the preparation of the anti-sense and sense DIG-RNA Tie1 ex1-2 probes HindIII/T7 and XbaI/SP6 restriction enzymes/RNA polymerases were used, respectively, whereas for the generation of anti-sense and sense DIG-RNA Tie1 ex5-9 probes, we employed XbaI/SP6 and HindIII/T7 restriction enzymes/RNA polymerases, respectively. Total RNA samples from halved E14.5 embryos from the *PGK-Cre;Tie1*$^{flox/flox}$ mice progeny were extracted using the NucleoSpin RNA II Kit (Macherey-Nagel). The RNAs (10 μg) were fractionated in a 1% (w/v) agarose gel containing 0.22 M formaldehyde at 135V for 1.5-2 h, transferred overnight to a nylon membrane (Hybond-N+, GE Healthcare Life Sciences) and fixed by UV cross-linking. Hybridization was performed employing standard procedures for non-radioactive nucleic acid probes. Chemiluminescent detection was performed using the anti-Digoxigenin-AP Fab fragments (Roche) and the

CDP-Star reagent (New England BioLabs). For the data shown in **Supplemental Figure 1E**, we used the DIG-RNA probes directed to Tie1 ex5-9; however, we also obtained the same results using the DIG-RNA probes directed to Tie1 ex1-2 (*data not shown*).

Tumor cell lines, xenografts and combinatorial antibody or AAV treatment studies. The mouse Lewis lung carcinoma (LCC) cells (ATCC) and B16F1 and B16F10 melanoma cells (Caliper Life Sciences) were maintained in DMEM, supplemented with 2 mM L-glutamine, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% fetal calf serum (Promo Cell). The mouse EL4 leukemia/lymphoma cells (a kind gift from Prof. Sirpa Jalkanen, University of Turku) were maintained in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 1 mmol/L sodium pyruvate (Gibco), MEM vitamin solution (Gibco), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), and 10% fetal calf serum. The mice were anesthetized as above and LLC, B16F1, B16F10 or EL4 syngeneic grafts were made by injecting 1–3 × 10^6 cells into the subcutaneous space in the hind flanks of the *Cdh5-Cre/ERT2;Tie1*$^{flox/flox}$ and *Pdgfb-iCre/ERT2;Tie1*$^{flox/flox}$ mice. Cre recombinase was induced by subcutaneous implantation of the sustained tamoxifen-release pellets (free base, 15 mg, 21-day, Innovative Research of America) two days (LLC and B16F10) and one week (B16F1 and EL4) before tumor cell implantation. After the establishment of the tumors during the first few days, growth rates were followed by measurements of the width (w), height (h) and depth (d) of the tumors with a digital caliper every second day. Tumor volumes (in cubic centimeters) were calculated according to the formula: w x h x d/2 (10). Mice were sacrificed at day 13 (B16F1), 14 (B16F10), 16 (EL4), 17 or 18 (LLC), or when the first tumor within an experimental group had grown to the permission-limiting size of 2 cm^3. Tumors were excised, weighed, measured in three dimensions with a digital caliper, photographed and processed for histological analysis.

For combinatorial inhibition studies, anti-VEGF (mrc84, 200 μg in 100 μL of PBS), anti-VEGFR-2 (DC101, 800 μg in 100 μL of PBS) or control antibodies were injected intraperitoneally starting four days after tumor cell implantation and repeated every 3.5 days (mrc84) or every two days (DC101) until the experiments were terminated. Alternatively, to induce the systemic overexpression of a soluble form of mouse Tie2 extracellular domain (mTie2-ECD, see ref.(1) for vector production) or mouse immunoglobulin 1 gamma Fc part (mFc) as a negative control, the recombinant adeno associated virus serotype 9 (AAV9) encoding mTie2-ECD or mFc was injected intraperitoneally at the dose of $1x10^{12}$ vector genomes/mouse. One day after the AAVs injections, the LLC tumor cells ($1x10^{6}$) were implanted into the subcutis of the abdominal flank. Serum was collected at day 5 after vector administration and at the termination of the experiments to examine for mTie2-ECD expression by Western blot analysis, as previously described in ref.(1).

For tumor vascular perfusion studies, 100 μl of biotinylated *Lycopersicon esculentum* (Tomato) lectin (1 mg/ml, Vector Laboratories) was injected through the tail vein of the tumor-bearing mice 10 min before sacrifice. A perfusion index was quantified as the percentage of the lectin-positive vessel area per endomucin-positive vessel area in each microscopic field.

Hypoxic areas in the tumor were visualized by i.p. injection of pimonidazole hydrocloride (Hypoxyprobe kit, Millipore) to the tumor-bearing mice (60 mg/kg), 1.5 h before sacrifice. Staining was performed with a FITC-conjugated monoclonal antibody, which recognizes pimonidazole adducts in hypoxic tissue proteins (Hypoxyprobe-1 Mab1, Millipore). Hypoxic

areas were calculated as the percentage of hypoxyprobe-positive tumor area in each microscopic field.

For the assessment of cell proliferation *in vivo*, 100 μl of bromodeoxyuridine (BrdU, 10 mg/ml, Sigma) was i.p injected into the tumor-bearing mice two hours before sacrifice. The proliferating endothelial or tumor cell index was quantified as the percentage of BrdU-positive endothelial cells per endomucin-positive vessel area or the percentage of BrdU-positive tumor cells per total area in each microscopic field. Cells were identified based on their nucleus, stained with 4',6-diamidino-2-phenylindole (DAPI).

Analysis of angiogenesis in the postnatal retinas. Newborn *Cdh5-Cre/ERT2;Tie1*$^{flox/flox}$, *Pdgfb-iCre/ERT2;Tie1*$^{flox/flox}$, *Cdh5-Cre/ERT2;Tie1*$^{flox/lacZ}$, *Pdgfb-iCreERT2;Tie1*$^{flox/lacZ}$, *Pdgfb-iCre/ERT2;ROSA26-R* or control pups were injected intragastrically with 2 μl of 4-OHT (Sigma) dissolved in 97% ethanol, from P1 to P4 using a 10 μl Hamilton syringe. Induction resulted in strong Cre-mediated β-galactosidase activity in all retinal vessels at P6 (as shown for the *Pdgfb-icre/ERT2;ROSA26-R* reporter strain progeny, **Supplemental Figure 12A-D**). The pups were killed either on P5 or P6, and their eyes were collected for analysis. The Notch inhibitor DAPT (Sigma) was dissolved in 50% dimethylsulphoxide and 50% sterile water, and administered subcutaneously at 10 mg kg^{-1} (ref.(11)) on P4 and P5, at 12 hour intervals, after which the tissues were collected for processing at P6. The Angpt2 blocking antibodies (1, 2) and human IgG isotype antibody used as a control were dissolved in sterile phosphate buffered saline (PBS) and administered subcutaneously at 30 mg kg^{-1} from P1-P4. The pups were euthanized on P5-6 and their eyes were enucleated for analysis.

Immunohistochemistry. After sacrificing the mice, the tissues were immersed in 4% paraformaldehyde, washed in phosphate buffered saline (PBS) and processed for whole-mount staining, immersed in OCT medium (Tissue Tek) or embedded in paraffin. 10 and 50 μm frozen sections of tumors were fixed with cold methanol or acetone. 6 μm paraffin sections of the embryos, tumors, and organs described in Supplemental Figure 10 were deparaffinized in xylene, rehydrated through graded ethanol series (100%, 95%, 70% and 50%) and citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate, pH 6.0), High pH buffer (S-2367, Dako or 10mM Tris base 1mM EDTA, 0.05% Tween-20, pH 10) or trypsin solution (0.1% trypsin, 0.1% $CaCl_2$, pH 7.8) was used for the antigen retrieval treatment. Blocking of sections was done with TNB (Perkin Elmer) or 5% normal donkey serum (Millipore) in 1% (v/w) BSA in PBS for 30 min. The following primary antibodies were used for immunostaining of mouse tissues: rat anti-mouse endomucin (sc-65495, Santa Cruz Biotechnology, 1:500), rabbit anti-mouse Lyve1 (ref(12), 1:1000), polyclonal goat anti-human Prox1 (AF-2727, R&D systems, 1:500), Armenian hamster anti-mouse Pecam1 (clone 2H8, MAB1398Z, Millipore, 1:200), unconjugated or fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Pecam1 (clone MEC 13.3, 553370, BD Pharmingen, 1:500, 1:800), polyclonal rabbit anti-FITC (Zymed/Invitrogen, 1:500), polyclonal rabbit anti-Ki67 (NB110-89719, Novus Biologicals, 1:300), Alexa-Fluor-594-conjugated mouse anti-BrdU monoclonal antibody (clone MoBU-1, B35132, Invitrogen, 1:500), polyclonal goat anti-active Caspase-3 (AF-835, R&D systems, 1:200), mouse anti-α-SMA-Cy3 (clone 1A4, C6198, Sigma, 1:500), rabbit anti-mouse NG2 (MAB5384, Millipore, 1:200 or 1:1000 for retina whole-mounts), polyclonal goat anti-P-selectin/CD62P (AF-737, R&D systems, 1:200), rat anti-mouse Icam1/CD54 (clone eBioKAT-1, 16-0542-82, eBioscience, 1:100), rat anti-mouse CD45 (550539, BD Pharmingen, 1:200), rat anti-mouse F4/80 (clone CI:A3-1,

MCA497R, AbD serotec, 1:500), rat anti-mouse Ly-6G and Ly-6C (Gr-1) (clone RB6-8C5, 550291, BD Pharmingen, 1:200), and polyclonal goat anti-mouse Dll4 (AF-1389, R&D systems, 1:200 or 1:500 for retina whole-mounts). Sections were washed with TNT buffer (100 mM Tris pH 7.4, 150 mM NaCl, 0.05% Tween-20) and the primary antibodies were detected with the appropriate Alexa 488, 594 or 647 secondary antibody conjugates (Molecular Probes/Invitrogen). Alternatively, the primary antibodies were detected with a chromogenic visualization method by using the Tyramide Signal Amplification kit (Perkin Elmer) following the manufacturer's instructions. The mounting of the slides was by using Permount (Fisher Scientific).

For the detection of Tie1 in LLC tumors grown in *Tie1*$^{+/+}$, control and Tie1-deleted mice the staining was performed in 15 μm fresh un-fixed frozen sections. The sections were allowed to dry for one hour at room temperature and High pH buffer (10mM Tris base 1mM EDTA, 0.05% Tween-20, pH 10) was used as antigen retrieval treatment. Blocking of sections was done in TNB (chromogenic method) or 10 % normal donkey serum in 1% (v/w) BSA in PBS (immunofluorescent method) for 30 min at room temperature. Detection of Tie1 protein in the tumor vessels was done using the polyclonal goat anti-human Tie1 antibodies (AF-619, R&D systems, 1:200) and co-incubating with rat anti-mouse endomucin (as above) when performing the immunofluorescence procedure. Incubation with primary antibodies was performed at 4°C overnight and the primary antibodies were detected with the appropriate Alexa 488 or 594 secondary antibody conjugates (as above). Alternatively, the Tie1 antibodies were detected by chromogenic visualization method using the Tyramide Signal Amplification kit (as above, but 15 min for BT time).

For the detection of apoptotic cell death in tumor sections, we used the TUNEL method with the *In Situ* Cell Death Detection Kit (TMR red, 12156792910, Roche), following the manufacturer's instructions. The index of apoptotic endothelial or tumor cell death was calculated as percentage of TUNEL-positive endothelial cells per endomucin-positive vessel area or the percentage of TUNEL-positive tumor cells per total area in each microscopic field. Individual cells were identified based on their DAPI-positive nuclei.

For the detection of *Tie1* promoter driven β-galactosidase activity in the tumors grown in the *Tie1*$^{+/lacZ}$ reporter mice, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, Sigma) staining was performed in frozen tumor sections. Briefly, 10 μm sections were fixed with cold 4% paraformaldehyde for 10 min, washed in PBS and incubated in X-gal working solution at 37°C overnight, as previously described (4). Sections were washed in PBS, post-fixed in 1% paraformaldehyde and processed for immunochromogenic staining for endomucin.

Blood vessels in the retinas were visualized by whole-mount staining with biotinylated *Griffonia simplicifolia* lectin (IsolectinB4, Vector Laboratories), followed by immunostaining as described previously (11). To detect β-galactosidase activity, the eyes were processed as before (11). After the staining, the retinas were washed and mounted in Vectashield (Vector Laboratories) or post-fixed in 1% paraformaldehyde and processed for whole-mount immunofluorescence staining. All fluorescently labelled samples were mounted with Vectashield containing DAPI (Vector Laboratories).

Microscopy. Fluorescently labelled samples were analysed with an upright epifluorescent microscope (Zeiss 2, Carl Zeiss; × 20 objective with numerical aperture (NA) 0.30, ×40

objective (NA) 0.50 or a confocal microscope (Zeiss LSM 510Meta, objectives ×10 with NA 0.45, oil objectives ×40 with NA 1.3 and ×63 with NA 1.4; or Zeiss LSM 5 Duo, objectives ×10 with NA 0.45, oil objectives ×40 with NA 1.3 and ×63 with NA 1.4) using multichannel scanning in frame mode, as previously described (11). Three-dimensional projections were digitally reconstructed from confocal z-stacks. Co-localization of signals was assessed from single confocal optical sections. Images of whole retinas were acquired using tile scanning with a pinhole diameter >3.0 Airy units. X-gal staining of LacZ reporter mice was analysed with a Leica DM LB camera (objectives ×2.5 with NA 0.07 and ×20 with NA 0.4, ×40 with NA 0.65, ×63 with NA 0.75). Alternatively, when X-gal chromogen staining was combined with immunofluorescence, co-localization of the signals was detected as described previously (13).

Quantitative analysis of the vasculature. Endomucin and Pecam1-positive tumor vessels were quantified from 6-8 randomly selected × 20 epifluorescence micrographs from peripheral regions and 4-6 randomly selected micrographs from central regions of the tumor with uniform staining intensity, using the Image J software. Quantifications of vasculature in healthy organs from adult mice were performed in similar manner to that described in (14). The vascular surface area in retinas was quantified as the isolectin-B4-positive area from ×10 confocal micrographs acquired from all intact quarters of the processed retinas and at a similar distance from the optic stalk, using the Image J software. Vessel branching points, sprouts and filopodia were counted manually from fluorescence micrographs of tumors and retinas, as described previously (15). Endothelial Dll4 expression was quantified by threshold of the area of the Dll4 signals that were IB4 positive from ×25 confocal micrographs acquired from the angiogenic fronts of the retinas. These were expressed relative to the total vascular

surface area quantified as above by IB4. Images were edited using Photoshop software (Adobe).

Transmission electron microscopy (TEM). LLC tumor, lung and kidney specimens from control and Tie1-deleted mice were fixed in 1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4, then post-fixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Epon LX112 (Ladd Research Industries). 1 μm sections were stained with toluidine blue for histological analysis, and 80 nm sections were cut with a Leica Ultracut UCT microtome. Two grids from each specimen were examined in a Tecnai Spirit transmission electron microscope (Fei Europe). Images were captured with a Quemesa CCD camera (Olympus Soft Imaging Solutions GMBH). *n* for tumor, lung and kidney specimens > 3/genotype.

Protein measurement in urine. Urine samples were collected from mice placed on a Petri dish (16), 15 μl was fractionated in SDS-PAGE and proteins were stained with Coomassie blue. The urine of ubiquitously Tie1-deleted mice (*Rosa26/creERT2* deletor) was collected 3-4 weeks after first tamoxifen injection and/or before termination of the experiment at four or six months of age, whereas the urine of the endothelial-specific Tie1-deleted mice (*Pdgfb-icre/ERT2* deletor) was collected 10 days after the first tamoxifen administration and before termination of the experiment at 3 weeks after Cre recombinase induction.

Blood urine nitrogen and creatinine measurement from mouse serum. Blood was collected by cardiac puncture. After centrifugation (2500 *g*, for 10 min room temperature),

nitrogen and creatinine were determined using Siemens ADVIA 1800 Chemistry System (Nordlab, Oulu University Hospital, Finland).

RNA isolation and qRT-PCR analysis. Tissues were collected in RNAlater (Qiagen). Total RNA from postnatal retinas and lungs, collected at P6, and lungs from adult mice, was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel). Homogenization was carried out using rotor-stator homogenization, followed by on-column DNase digestion (RNase-Free DNase Set, 79254). Quality control of samples was carried out using a Nanodrop ND-1000 spectrophotometer. RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. qRT-PCR reactions were carried out in triplicate from every *in vitro* transcription reaction using the iQ Supermix Kit (Bio-Rad). qRT-PCR was performed using a BIO-RAD C1000 Thermal cycler according to a standardized protocol. The TaqMan Gene Expression Assays used for mouse mRNA were: *Gapdh* (4352932E), *Cdh5* (Mm00486938_m1), *Pecam1/CD31* (Mm01246167_m1), *Tie1* (Mm00441786_m1), *Tie2/Tek* (Mm00443242_m1), *Angpt2/Ang2* (Mm00545822_m1), *Vegfa* (Mm00437304_m1), *Dll4* (Mm00444619_m1), *Nrarp* (Mm00482529_s1) and *Hey1* (Mm00468865_m1). At least three retinas and lungs from *Pdgfb–iCre/ERT2;Tie1*$^{flox/flox}$, *Pdgfb–iCre/ERT2;Tie1*$^{flox/lacZ}$ and *Tie1*$^{flox/flox}$ littermates were used for analysis at P6. The data were normalized to the endogenous controls *Cdh5* and *Gapdh* for the retina and the lungs, respectively. At least three independent experiments per condition were analysed. Fold changes were calculated using the comparative CT (threshold cycle) method.

Preparation of protein lysates and Western blotting. Embryos and postnatal and adult lungs were rinsed with cold phosphate-buffered saline (PBS) and extracted with RIPA buffer

containing protease and phosphatase inhibitors (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/mL each aprotinin, leupeptin, 1 mM Na_3VO_4 and 1 mM NaF) using 1.4 mm Ceramic Bead Tubes (MO BIO Laboratories) to facilitate the break-down of the tissue. PowerLyzer® 24 Bench Top Bead-Based Homogenizer (MO BIO laboratories) was used to homogenize the tissue with 2 x 15 min cycles at 3500 rpm. Lysates were centrifuged for 10 min at 14000 rpm and supernatants were subjected to protein determination using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific). 10-100 μg of total tissue protein was solubilized in SDS sample buffer (reducing conditions), resolved by SDS-PAGE, transferred to nitrocellulose membranes, processed for immunoblotting and developed by standard ECL (SuperSignal® West Pico chemiluminescence Substrate from Thermo Fisher Scientific) procedures. For target protein detection, antibodies used were: polyclonal rabbit anti-Tie1 (C-18) (sc-342, Santa Cruz Biotechnology, 1:1000), polyclonal goat anti-mouse/rat Tie2 (AF-762, R&D systems, 1:1000), rat anti-mouse VE-Cadherin/CD144 (clone 11D4.1, 550548, BD Pharmingen, 1:1000), polyclonal rabbit anti-human Angpt2 (Zymed, 1:1000), polyclonal goat anti-mouse Dll4 (AF-1389, R&D systems, 1:1000) and rabbit anti-cleaved Notch1 (Val1744) (2412, Cell Signaling Technology, 1:1000). For a loading protein control, the membranes were immunoblotted for Hsc70 (B-6, sc-7298, Santa Cruz Biotechnology, 1:5000). Target protein levels were quantified by measuring the total intensity of the target protein and Hsc70 signals on films, and calculating the ratio between the two. Measurements of the signal intensities were carried out with the Photoshop software.

Growth factor stimulations and analysis of Tie2 phosphorylation *in vivo.* Growth factors hVEGF (gift from Dr. Michael Jeltsch), COMP-Angpt1 (CA1) and hAngpt2 (17) in 100 μl of

PBS were i.v. tail injected at a dose of 20 μg per mouse to anesthetized 7-9-week-old control and Tie1-deleted mice (*Pdgfb-icre/ERT2,Tie1*$^{flox/lacZ}$ progeny, **Supplemental Table 1)**. After 10 min, the lungs were isolated and flash-frozen in liquid nitrogen until use. For analysis of phosphorylated and total Tie2, the tissues were thawed and homogenized in ice-cold lysis buffer (RIPA or 0.5% Triton X-100 + 0.5% NP-40 in PBS) containing inhibitors. Tissue lysates were subjected to Western blotting (120 μg of total soluble protein, for phospho-Tie2 Tyr^{1108} analysis) or immunoprecipitation (2 mg of total soluble protein) followed by Western blotting (for phospho-Tie2 total Tyr analysis). Antibodies used were polyclonal goat anti-mouse/rat Tie2 (AF-762, R&D systems, 1:1000), polyclonal goat anti-human Tie1 (AF-619, R&D systems, 1:1000), rabbit anti-P-Tie2 (Y1108) (Biosource; cat. # 441313G, 1:1000, currently discontinued) rabbit anti-FLAG (F7425, Sigma, 1:1000) and anti-phosphotyrosine Antibody (clone 4G10, Millipore, 1:5000).

Statistical analysis. All results were expressed as mean ± SEM. unless otherwise indicated. All quantifications were performed in a strictly blinded fashion. Statistical analysis was carried out using PASW Statistics 18.0. A two-tailed paired or equal variance (homocedastic) Student *t*-test or one-way analysis of variance (ANOVA) was used for statistical analysis. A *P* value of less than 0.05 was considered to be statistically significant.

Supplemental References

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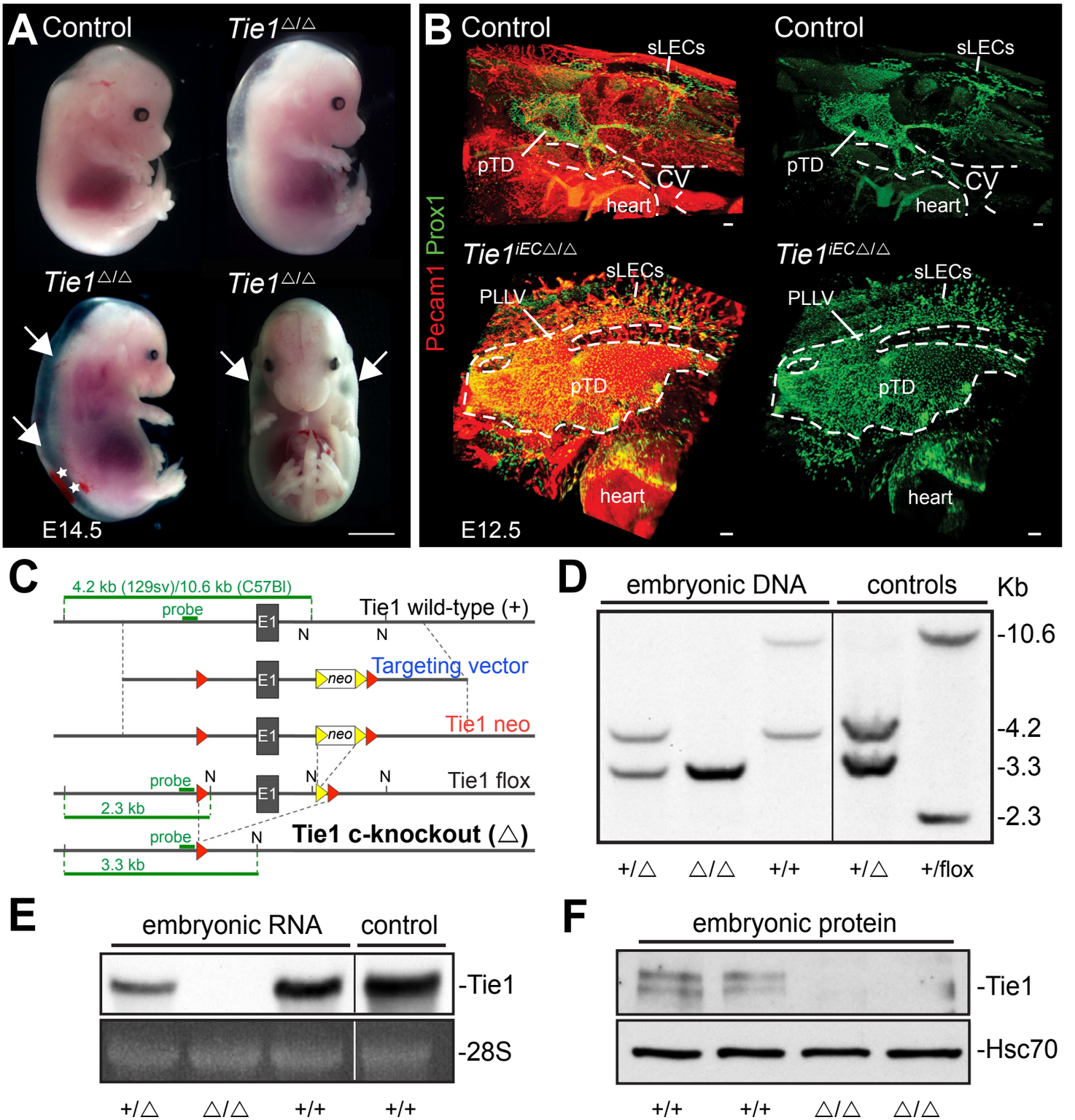
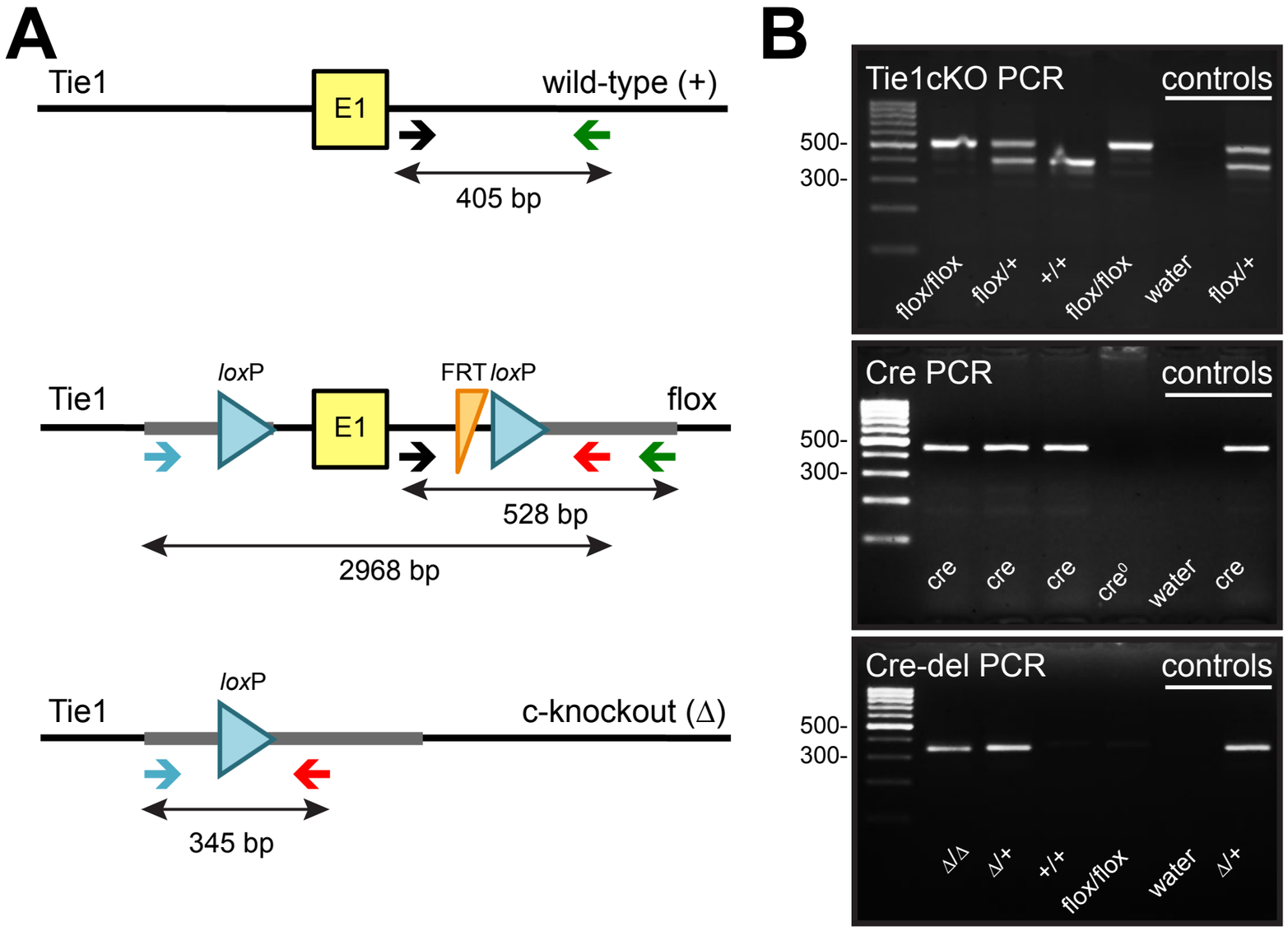
Supplemental Figure 1, D'Amico et al.



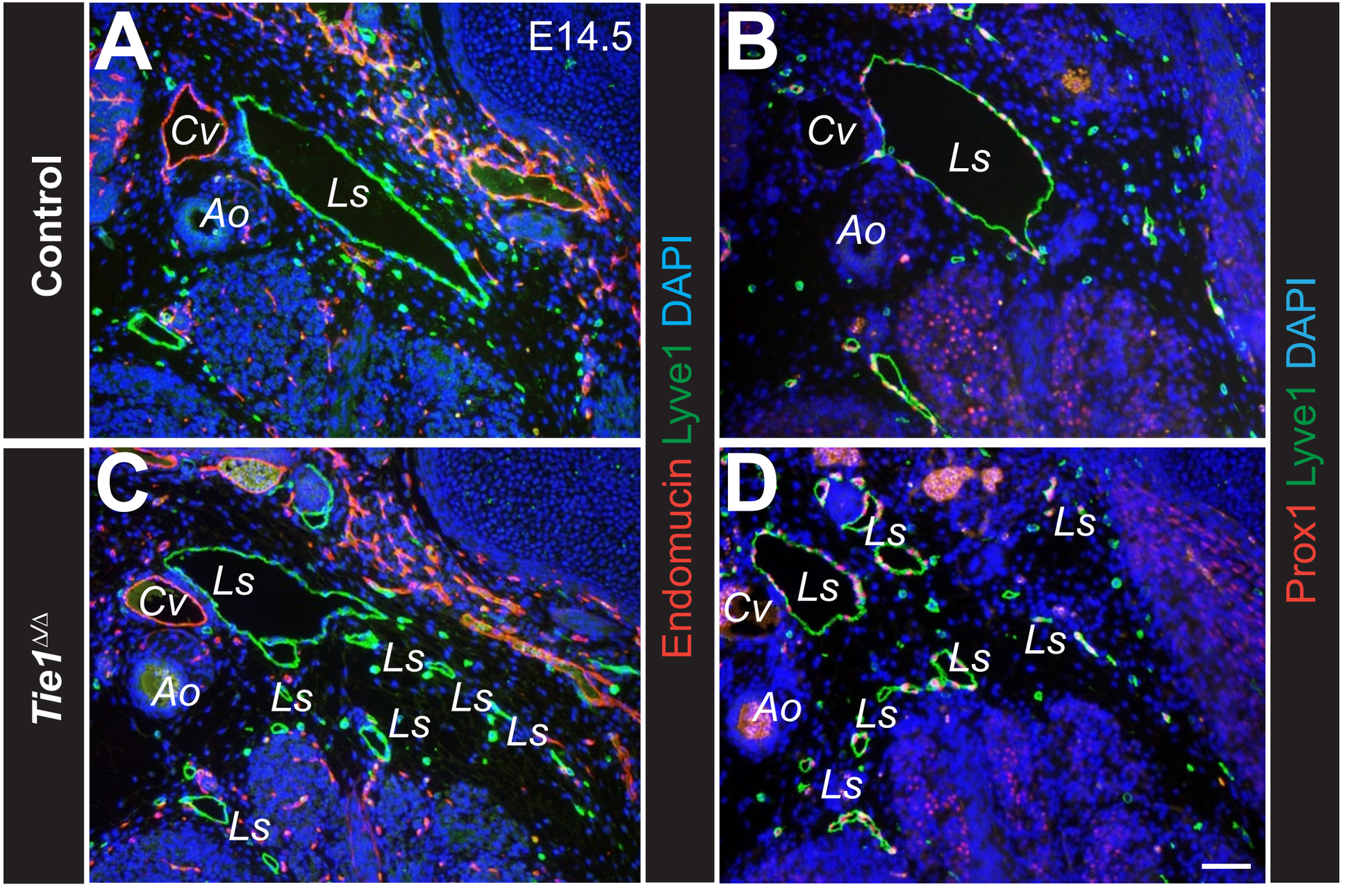
Supplemental Figure 1. Conditional targeting of the Tie1 locus. (**A**) Photomicrographs of control and Tie1 deleted (*Tie1*$^{\Delta/\Delta}$, *PGK-Cre* deletor) embryos. At E14.5, oedematous (arrows) and hemorrhagic (stars) phenotypes were observed in Tie1 deleted but not in control embryos. Scale bar: 2 mm. *n* > 4 embryos/genotype. (**B**) Ultramicroscopy 3D reconstructions of images obtained by optical sectioning of control and Tie1 deleted (*Tie1*$^{iEC\Delta/\Delta}$, *Pdgfb-icre/ERT2* deletor) embryos stained for Pecam1 and Prox1. PLLV, peripheral longitudinal lymphatic vessel. pTD, primordial thoracic duct. sLECs, superficial lymphatics. CV, cardinal vein. Scale bars: 100 µm. *n* = 2 embryos/genotype. (**C**) The homologous recombination event deletes sequences encoding the Tie1 translation initiation site and exon 1 (E1). Red arrowheads, *lox*P sites; yellow arrowheads, FRT sites; N, *Nsi*I cleavage sites; *neo*, neomycin resistance cassette; c-knockout, conditional knockout. (**D**) Southern blot analysis of DNA from a litter at embryonic day (E) 14.5 after *Nsi*I digestion, hybridized with the 5' external probe indicated by the green bar in panel (A). Right margin, molecular sizes of the digested fragments. (**E**) *Tie1* mRNA expression in embryos of the same litter analyzed by Northern blotting. Ribosomal 28S RNA staining was used as a loading control. (**F**) Tie1 expression analysed by Western blotting of whole E14.5 embryo lysates. Hsc70 was used as a loading control. Representative data from 3 independent experiments, *n* > 2 embryos/genotype/ experiment.

Supplemental Figure 2, D'Amico et al.



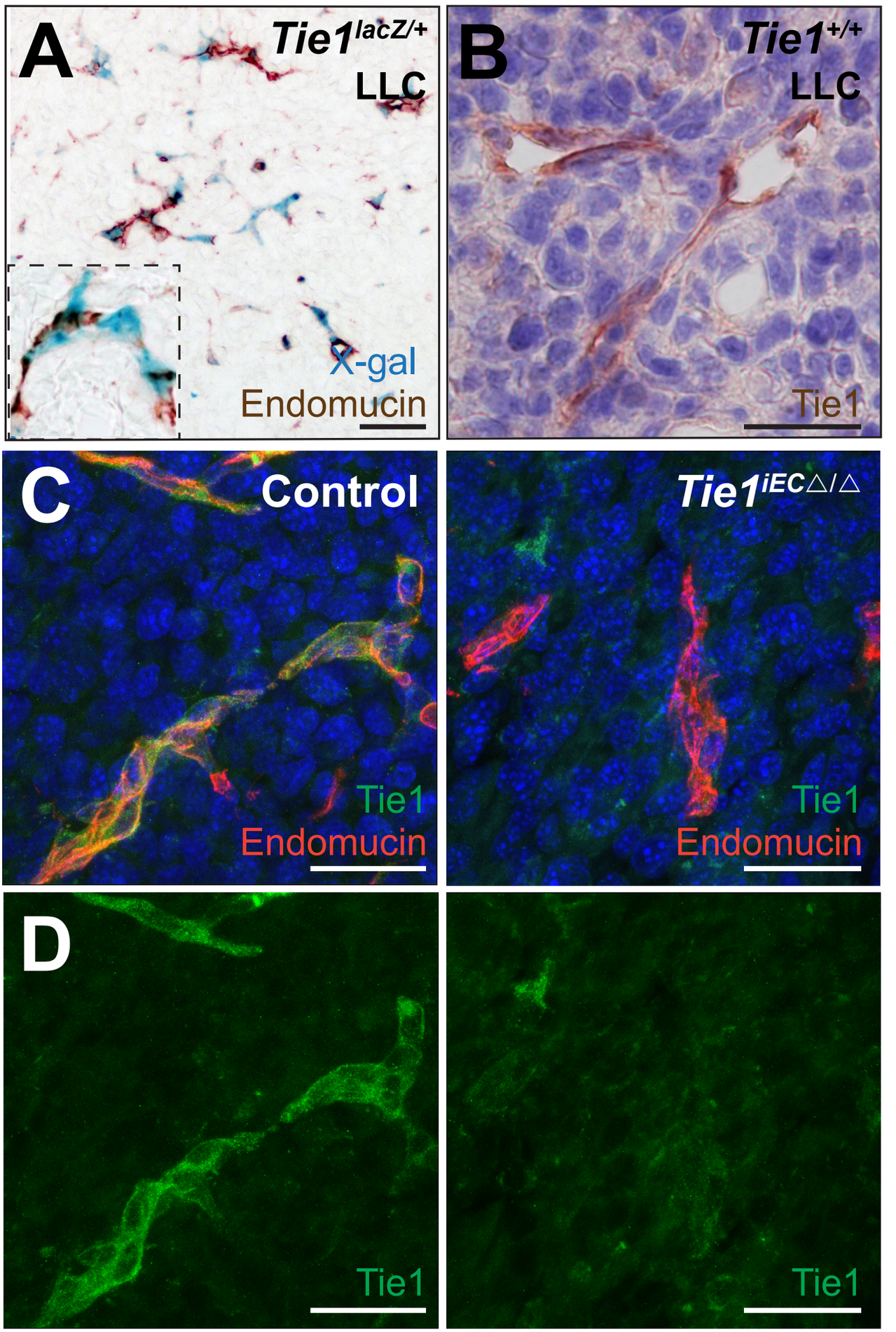
Supplemental Figure 2. PCR genotyping of the Tie1 conditional mice. (**A**) A scheme (not in scale) showing the wild-type, conditional (flox), and Tie1 deleted allele after Cre-mediated recombination (Tie1 Δ). The colored arrows indicate the PCR forward (black and cyan) and reverse (green and red) primer binding sites. Primer sequences are described in the Supplemental Methods. Double-arrowheads indicate the expected PCR product sizes in base pairs (bp). E1, exon 1. (**B**) Photographs of representative electrophoresis gels containing the PCR products of the wild type (+), conditional (flox), and the conditional Tie1 knockout (Δ) alleles as well as the recombinase transgene (Cre PCR). DNA samples used for the PCRs were extracted from ear biopsies of wild-type, *Tie1*$^{flox/flox}$, *Pdgfb-icre/ERT2;Tie1*$^{+/flox}$ and *Pdgfb-icre/ERT2;Tie1*$^{flox/flox}$ mice, and Cre recombinase activity was induced by administration of tamoxifen.

Supplemental Figure 3, D'Amico et al.



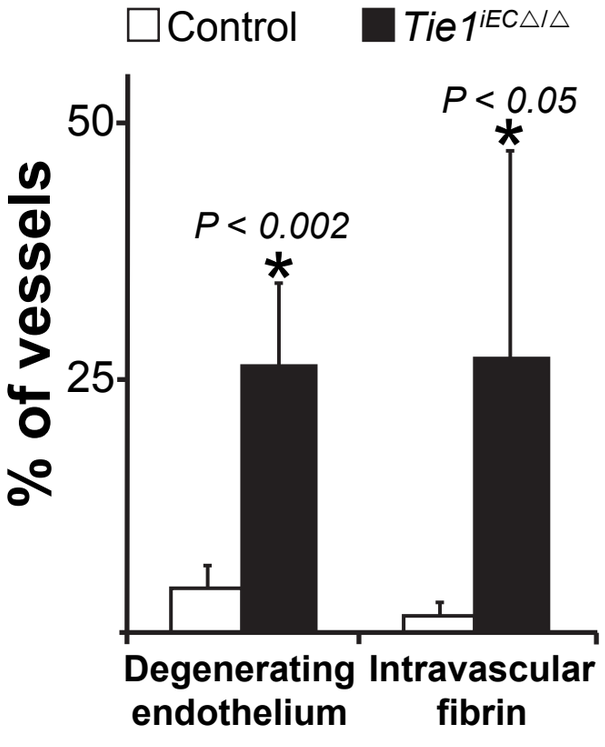
Supplemental Figure 3. Dysmorphic lymph sacs in Tie1 deleted embryos. Analysis of blood and lymphatic vasculature in (**A-B**) *Tie1*$^{flox/flox}$ (control) and (**C-D**) *PGK-cre;Tie1*$^{flox/flox}$ (*Tie1*$^{\Delta/\Delta}$) embryos at E14.5. Immunofluorescence staining for endomucin (red), Prox1 (red) and Lyve1 (green) of comparable thoracic transverse sections identifies blood vessels and lymph sacs, respectively. Note dysmorphic lymph sacs in *Tie1*$^{\Delta/\Delta}$ but not in control embryos. Nuclei were stained with DAPI (blue). Ao, dorsal aorta; Cv, cardinal vein; Ls, lymph sacs. Scale bar: 100 µm. *n* > 4 embryos/genotype.

Supplemental Figure 4, D'Amico et al.



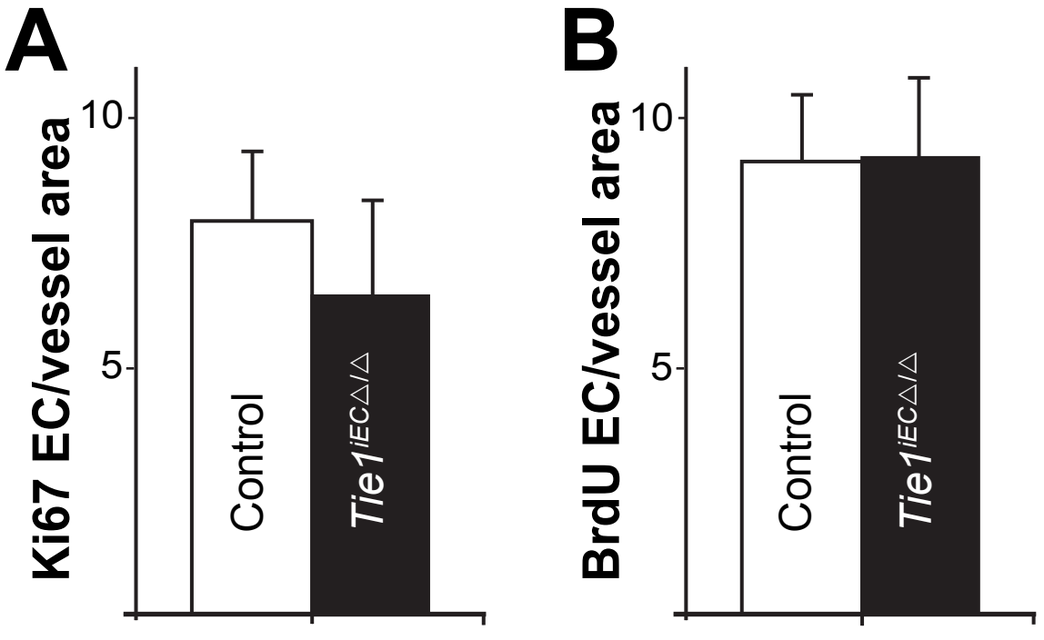
Supplemental Figure 4. Tie1 expression in LLC tumor vasculature. (**A**) Tie1 expression (X-gal staining, blue) in tumor vessels (endomucin, brown) in *Tie1*$^{lacZ/+}$ mice. (**B**) Immunochromogenic staining for Tie1 (brown) in the tumor vasculature of the *Tie1*$^{+/+}$ mice. (**C**) Immunofluorescence for Tie1 (green), endomucin (red), and DAPI (blue) in tumor sections from control and *Tie1*$^{iEC\Delta/\Delta}$ mice (*Pdgfb-icre/ERT2* deletor). Maximum projections from confocal microscopy z-stack images are shown. (**D**) Tie1 staining only is shown for better visualization. Note the lack of Tie1 antigen in vessels in tumors grown in *Tie1*$^{iEC\Delta/\Delta}$ mice. Scale bars: 50 μm.

Supplemental Figure 5, D'Amico et al.



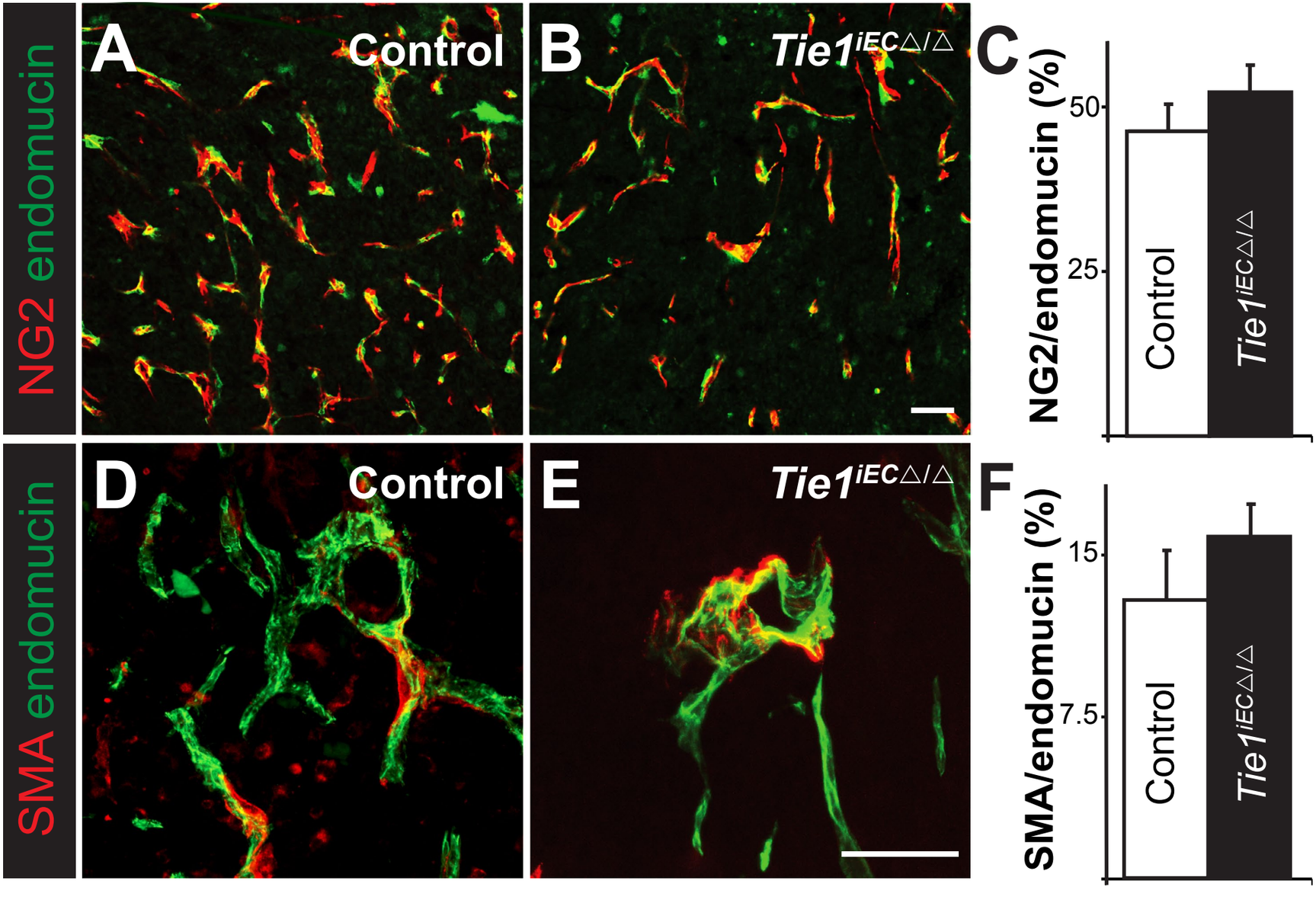
Supplemental Figure 5. Endothelial cell death and intravascular fibrin deposition frequency in control and *Tie1*$^{iEC\triangle/\triangle}$ tumor vessels. Quantification of endothelial cell death and intravascular fibrin deposition frequency in control and *Tie1*$^{iEC\triangle/\triangle}$ tumors. Error bars indicate SEM. *n* = 84-144 vessels from 5-7 LLC tumors/genotype (*Pdgfb-icre/ERT2* deletor).

Supplemental Figure 6, D'Amico et al.



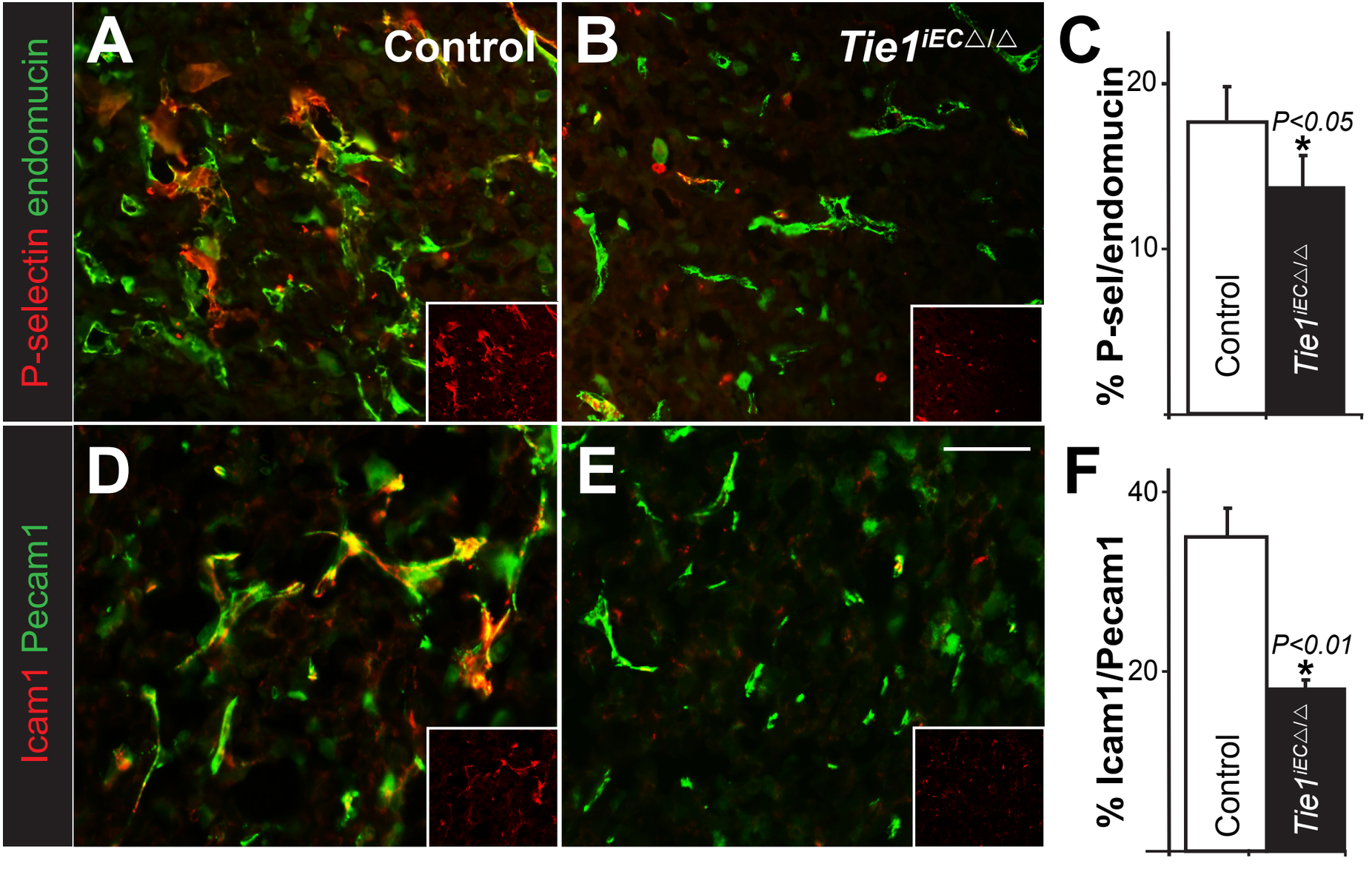
Supplemental Figure 6. Endothelial cell proliferation in control and *Tie1*$^{iEC\Delta/\Delta}$ tumor vessels. Endothelial cell proliferation indices calculated as the ratio of (**A**) Ki67 and (**B**) 5-bromo-deoxyuridine (BrdU) positive endothelial cells to the total number of endomucin-positive endothelial cells in the LLC tumor blood vessels. Error bars indicate SEM. *n* = 3-6 tumors/genotype (both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors).

Supplemental Figure 7, D'Amico et al.



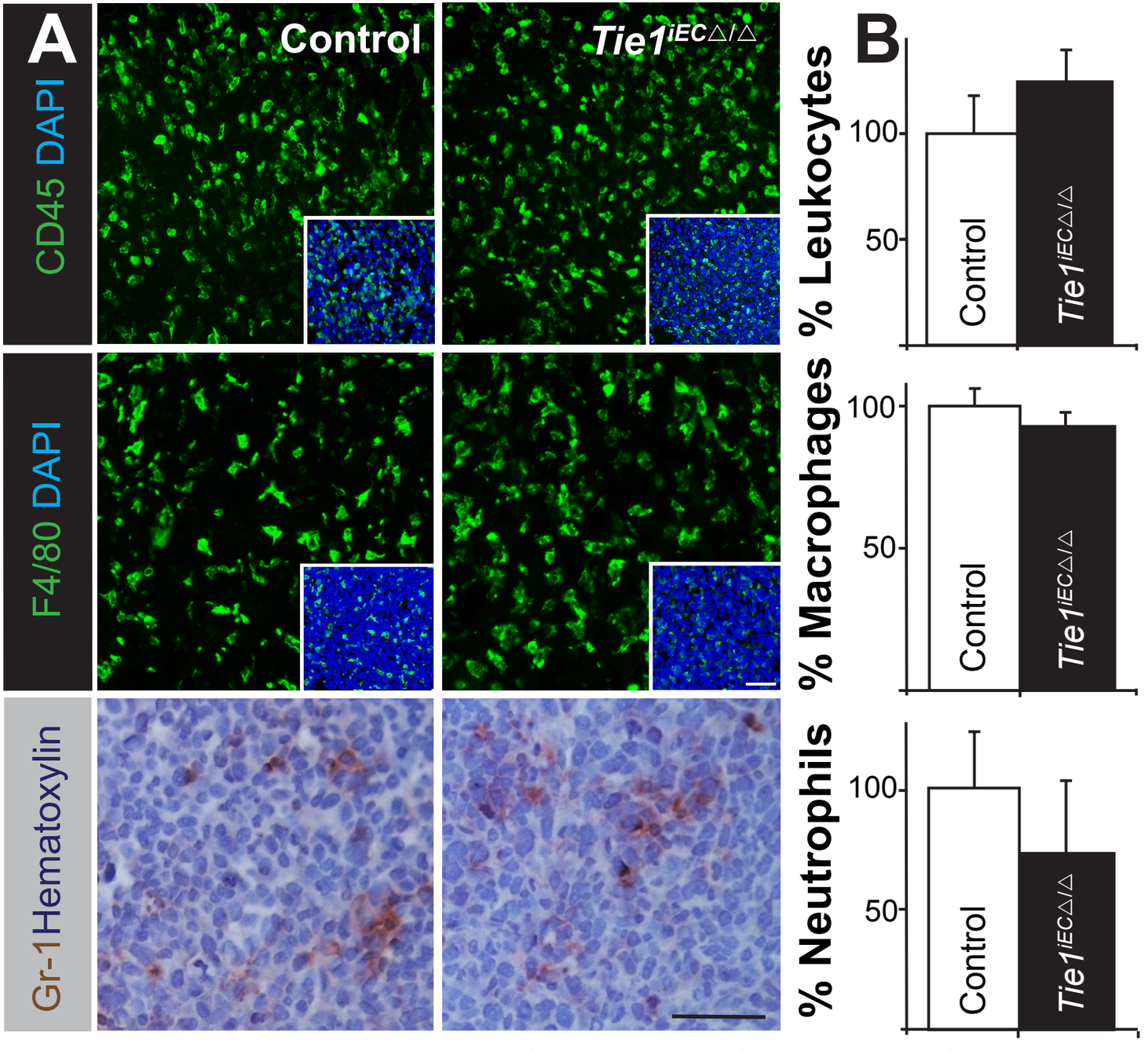
Supplemental Figure 7. Vessel coverage by mural cells in tumors of control and *Tie1*$^{iEC\Delta/\Delta}$ mice. Immunofluorescence for endomucin (green) and (**A-B**) NG2 and (**D-E**) α-SMA (SMA, red) to identify blood vessels, pericytes and smooth muscle mural cells, respectively in LLC tumor sections. Confocal microscopy z-stack images are shown. Scale bars: 50 μm. (**C, F**) Quantitation of the % of total NG2- or SMA-positive area relative to total endomucin-positive area per field. Error bars indicate SEM. *n* = 5-8 tumors/genotype (both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors).

Supplemental Figure 8, D'Amico et al.



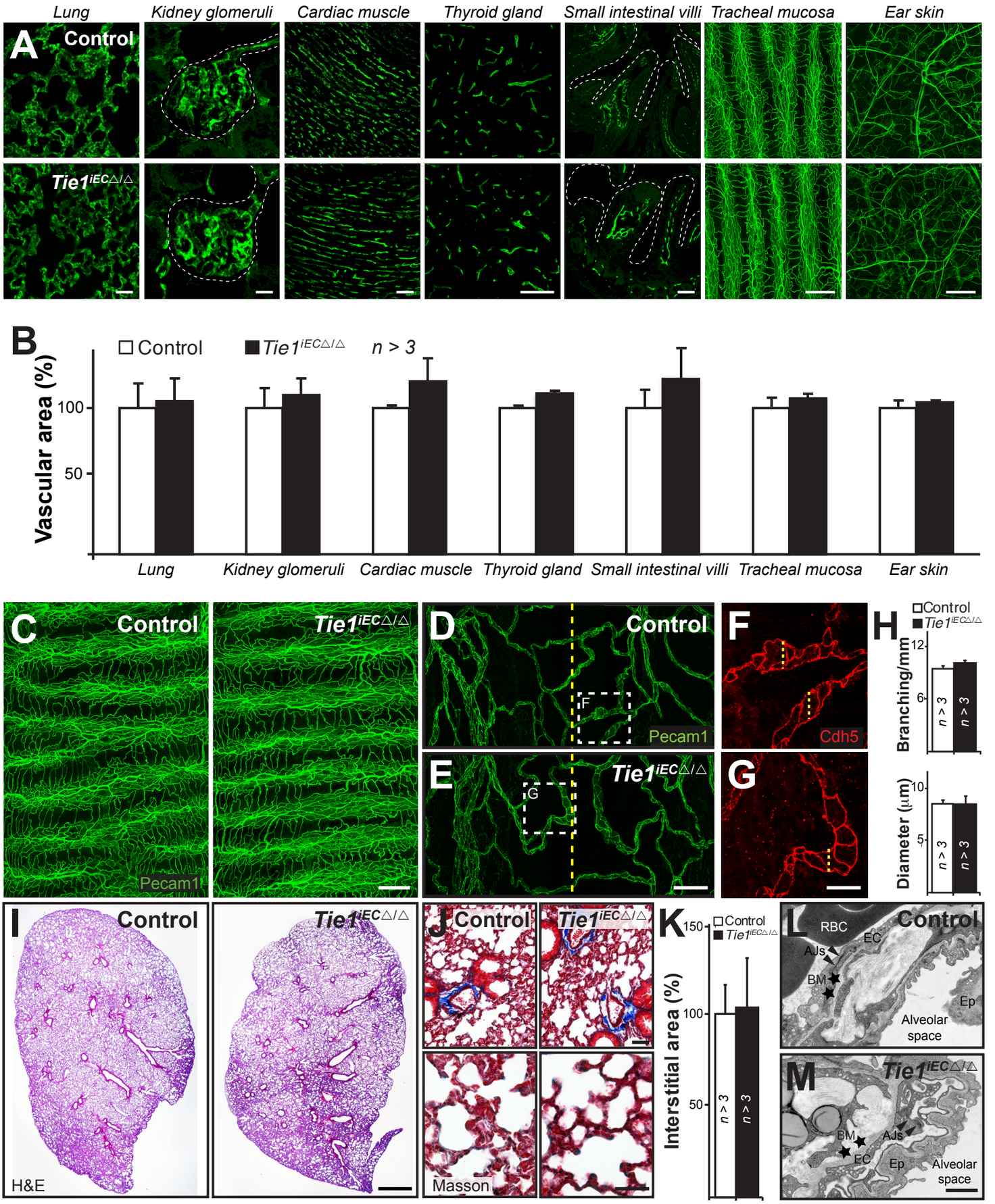
Supplemental Figure 8. Expression of leukocyte adhesion molecules in tumor vessels. Immunofluorescence for (**A-B**) P-selectin (red) and endomucin (green) and (**D-E**) Icam1 (red) and Pecam1 (green) in LLC tumor sections. (**A-B**) Inset, P-selectin only. (**C**) % of P-selectin-positive area relative to total endomucin-positive vessel area. (**D-E**) Inset, Icam1 only. (**F**) % of Icam1-positive area relative to total Pecam1-positive vessel area. Error bars, SEM. *n* = 3 LLC tumors/genotype (*Cdh5-cre/ERT2* deletor). **P*<0.05. Scale bars: 100 μm.

Supplemental Figure 9, D'Amico et al.



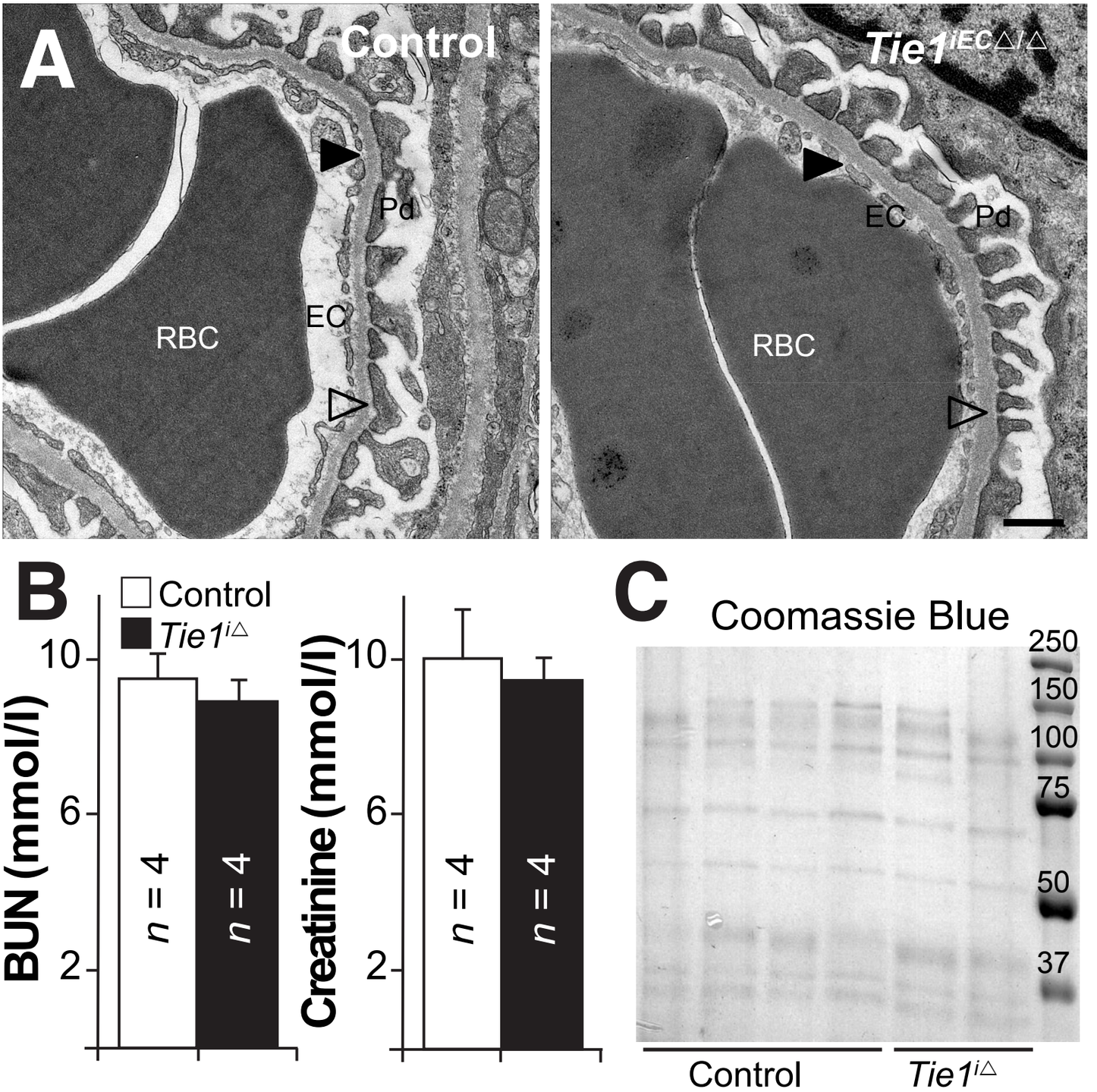
Supplemental Figure 9. Leukocyte recruitment to tumor stroma. (**A**) Shown are stainings for total leukocytes (CD45), macrophages (F4/80) and neutrophils (Ly6G/Gr-1) in LLC tumor sections. Scale bars: 100 μm. (**B**) % of the indicated leukocytes per total number of cells (nuclei, DAPI or hematoxylin) in LLC tumor sections. Error bars, SEM. *n* = 5-6 tumors/genotype (both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors). Scale bars: 100 μm.

Supplemental Figure 10, D'Amico et al.



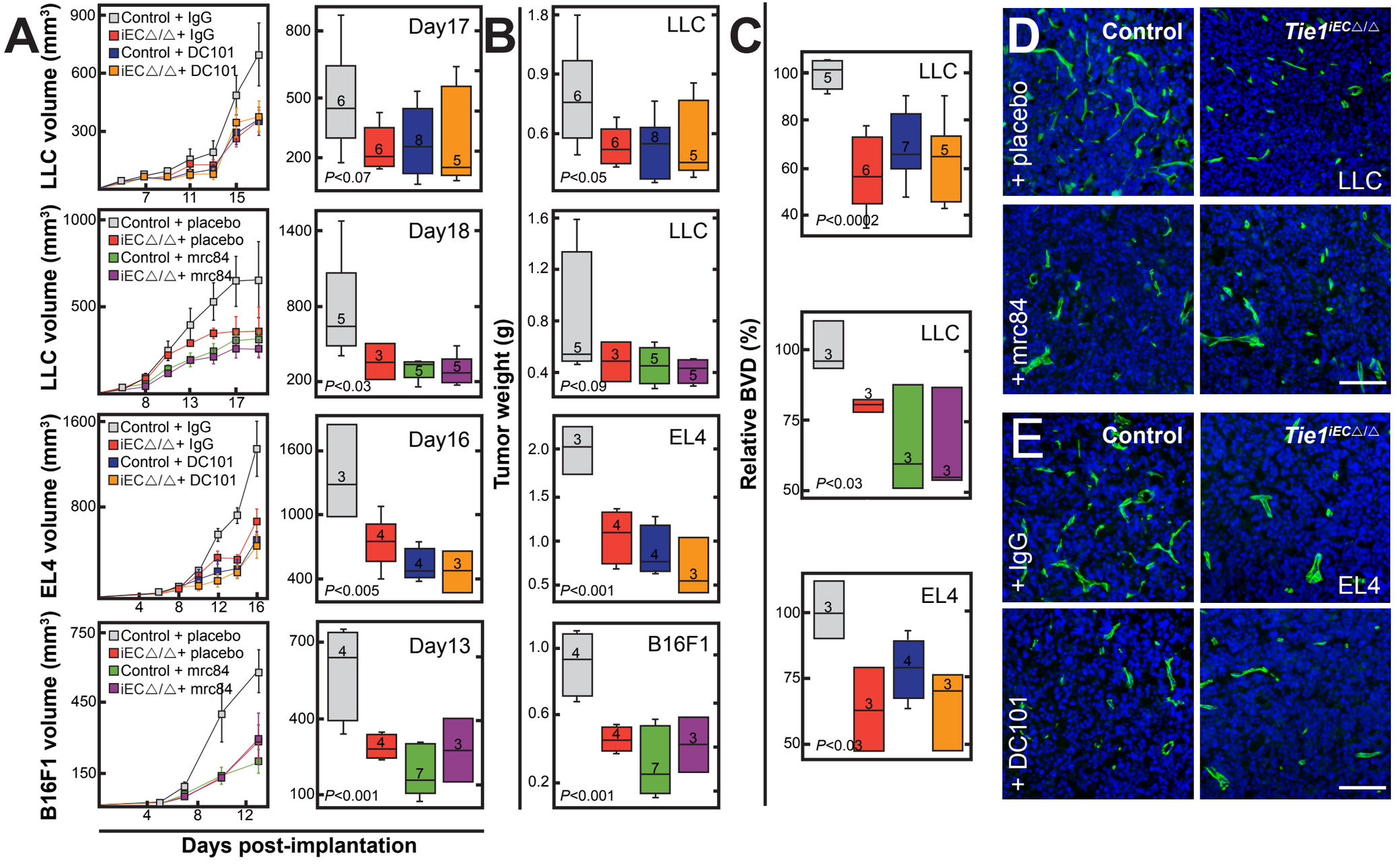
Supplemental Figure 10. Analysis of the vasculature in organs of adult mice lacking Tie1. (**A**) Representative confocal microscopy images of vascular beds from lungs, kidney glomeruli, cardiac muscle, thyroid gland, small intestinal villi, tracheal mucosa and ear skin of 8-10 week-old control and Tie1-deficient adult mice in which the genetic deletion was induced for 3 weeks (*Tie1*$^{iEC\Delta/\Delta}$, *Pdgfb-icre/ERT2* deletor) stained for endomucin or Pecam1. (**B**) Quantification of vascular density in control and *Tie1*$^{iEC\Delta/\Delta}$ tissues shown in (A). Error bars, SEM. *n* = 4 mice/genotype. (**C**) Analysis of the tracheal vasculature (Pecam1, green) by tile scanning confocal microscopy. (**D-E**) High magnification images of postcapillary venules stained for Pecam1 and for (**F-G**) Cdh5 (VE-cadherin), depicting endothelial cell junctions. (**H**) Quantification of tracheal capillary branching and diameter. Error bars, SEM. *n* = 4 mice/genotype. (**I**) Analysis of the morphological appearance of the left lobe of the lungs in H&E stained sections. (**J**) Masson's trichrome stained alveolar interstitial areas at low (upper) and higher (bottom) magnifications. (**K**) Quantification of the interstitial lung area (as % of control). Error bars, SEM. *n* = 4 mice/genotype. (**L-M**) Transmission electron micrographs micrographs of control and *Tie1*$^{iEC\Delta/\Delta}$ lung alveolar septum microvasculature. Black arrowheads, endothelial adherens junctions (AJs). Stars, basement membrane (BM). EC, endothelial cell. RBC, red blood cell. Ep, epithelium. *n* = 2 mice/genotype. Scale bars: 50 μm, except for (**L-M**) 1 μm.

Supplemental Figure 11, D'Amico et al.



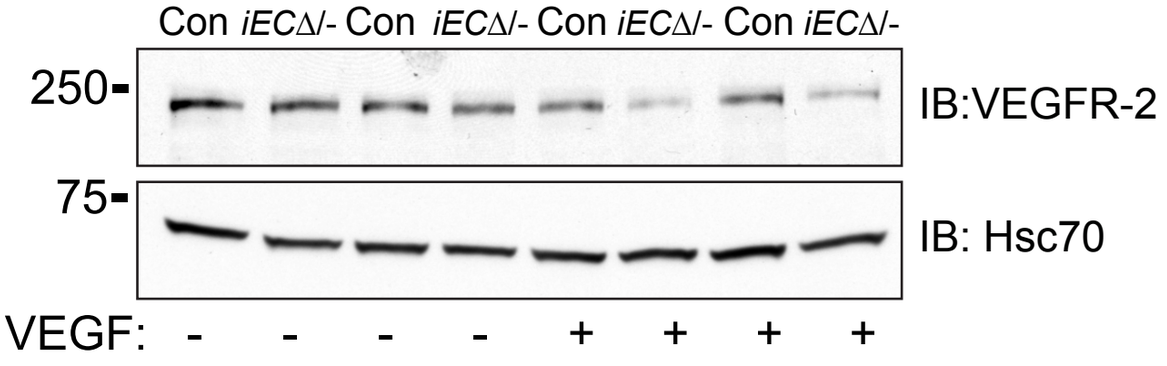
Supplemental Figure 11. Renal function in control and Tie1-deficient mice. (**A**) Representative transmission electron micrographs from control and *Tie1*$^{iEC\Delta/\Delta}$ (*Pdgfb-icre/ERT2* deletor) kidney glomerular capillary vasculature. Black arrowheads, endothelial cell fenestrations. Ubiquitously Tie1-deleted (*Tie1*$^{i\Delta/-}$) mice were also examined (*Rosa26/creERT2* deletor, see Supplemental Table 1 and Supplemental Methods for details). White arrowheads, basement membrane. EC, endothelial cell. RBC, red blood cell. Pd, podocyte. Scale bars: 1 µm. (**B**) Blood urea nitrogen (BUN) and creatinine analysis in serum from control (n = 6), *Tie1*$^{i\Delta/-}$ (n = 4) and *Tie1*$^{iEC\Delta/\Delta}$ (n = 2) mice. Tie1-deficient mice (*Tie1*$^{i\Delta}$), *Tie1*$^{i\Delta/-}$ + *Tie1*$^{iEC\Delta/\Delta}$ mice. Error bars, SEM. (**C**) Representative Coomassie blue stain of urine samples fractioned in a 4.0 - 7.5% SDS-PAGE. n > 3 for control and *Tie1*$^{i\Delta/-}$ mice, and n = 2 for *Tie1*$^{iEC\Delta/\Delta}$ mice.

Supplemental Figure 12, D'Amico et al.



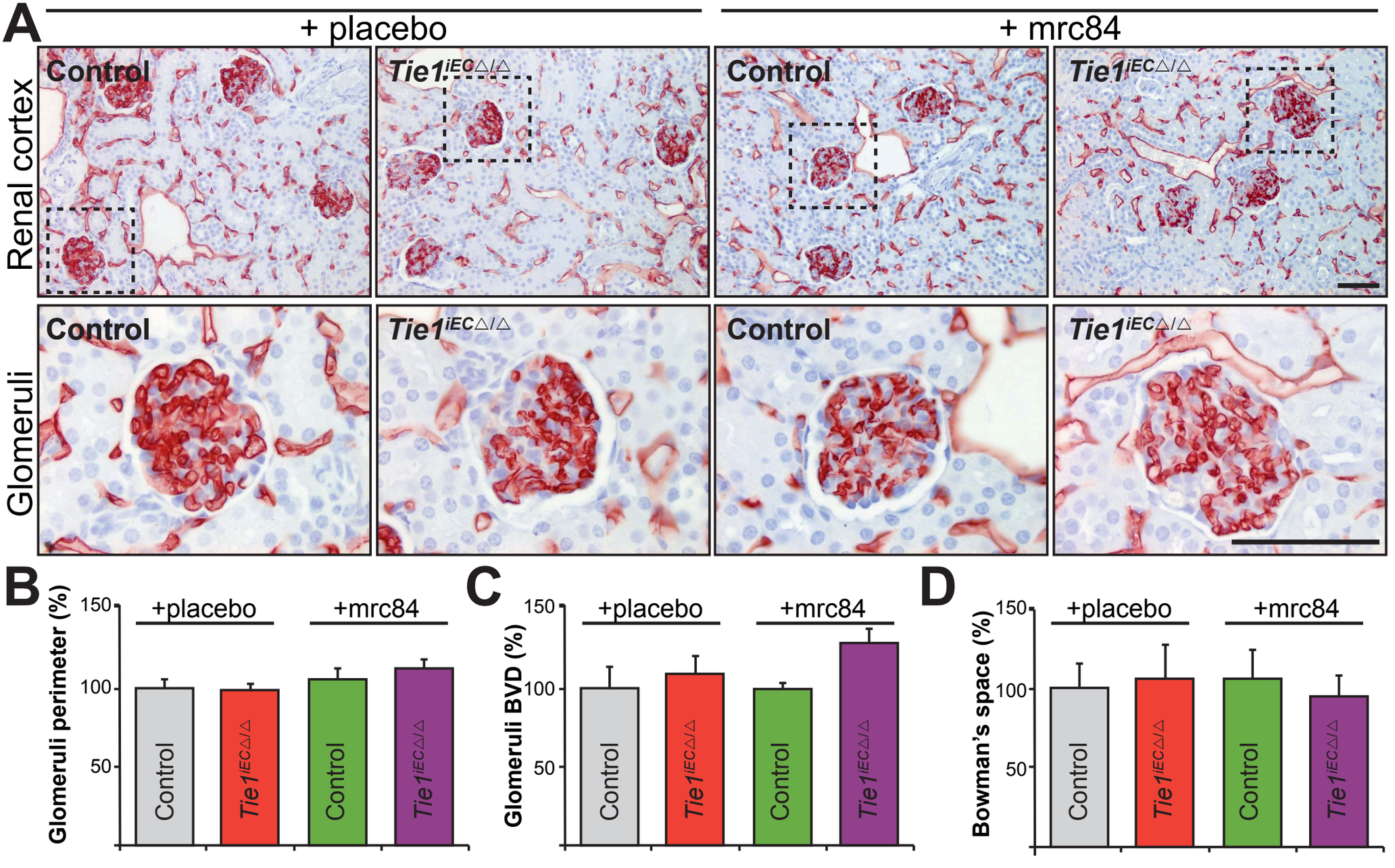
Supplemental Figure 12. Analysis of tumor growth and vasculature in control and *Tie1*$^{iEC\Delta/\Delta}$ mice treated with anti-VEGF or anti-VEGFR-2 antibodies. (**A**) LLC, EL4 and B16F1 tumor growth curves and tumor volumes (mm^3) at 17 and 18 (LLC), 16 (EL4) or 13 (B16F1) days in control and *Tie1*$^{iEC\Delta/\Delta}$ mice treated with IgG control, placebo, DC101 (800 µg/mouse) or mrc84 (200 µg/mouse) antibodies. (**B**) Tumor weights (g). (**C**) Vessel density (area % relative to control + IgG or control + placebo). (**D**) Endothelial staining of LLC and (**E**) EL4 tumors (endomucin or Pecam1, green) in control and *Tie1*$^{iEC\Delta/\Delta}$ mice (both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors). Error bars, SEM. The numbers in the box plots represent *n* mice or tumors/group. *P* values were obtained by ANOVA one-way test. Scale bars: 100 µm.

Supplemental Figure 13, D'Amico et al.



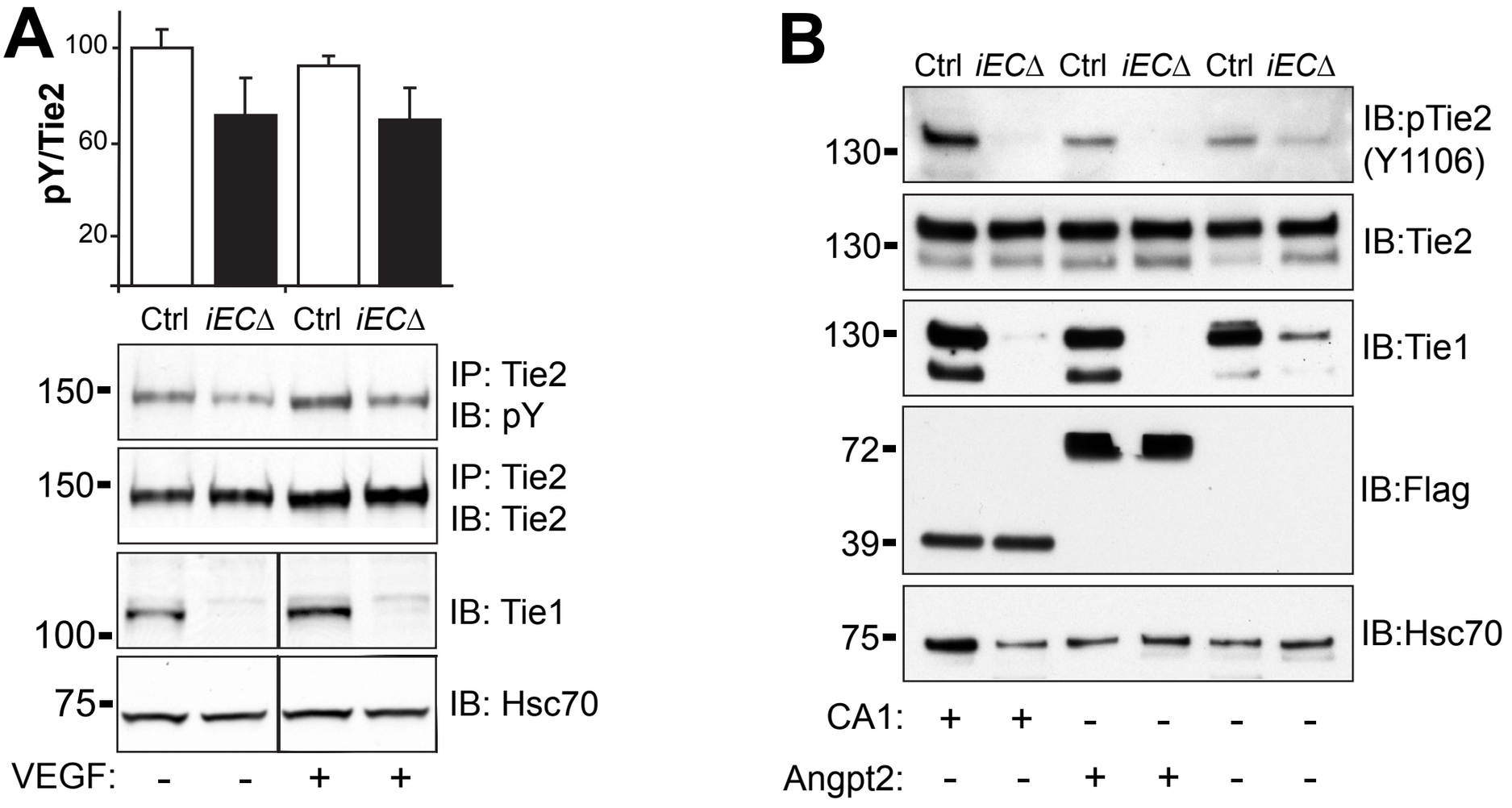
Supplemental Figure 13. VEGFR-2 protein analysis of Tie1-deficient mice stimulated with VEGF *in vivo*. Adult *Pdgfb-icre/ERT2;Tie1*$^{flox/lacZ}$ mice were treated with tamoxifen (*Tie1*$^{iECΔ/-}$, see Supplemental Table 1) and injected i.v. tail with PBS or VEGF (10 µg), followed by analysis after 10 min. Lung lysates were immunoblotted for VEGFR-2. Hsc70 was used as a loading control. See immunoblot for Tie1 in Supplementary Figure 15 for efficiency of deletions. Ctrl, control. *iECΔ/-*, *Tie1*$^{iECΔ/-}$ mice. *n* = 2 mice/group, two independent experiments.

Supplemental Figure 14, D'Amico et al.



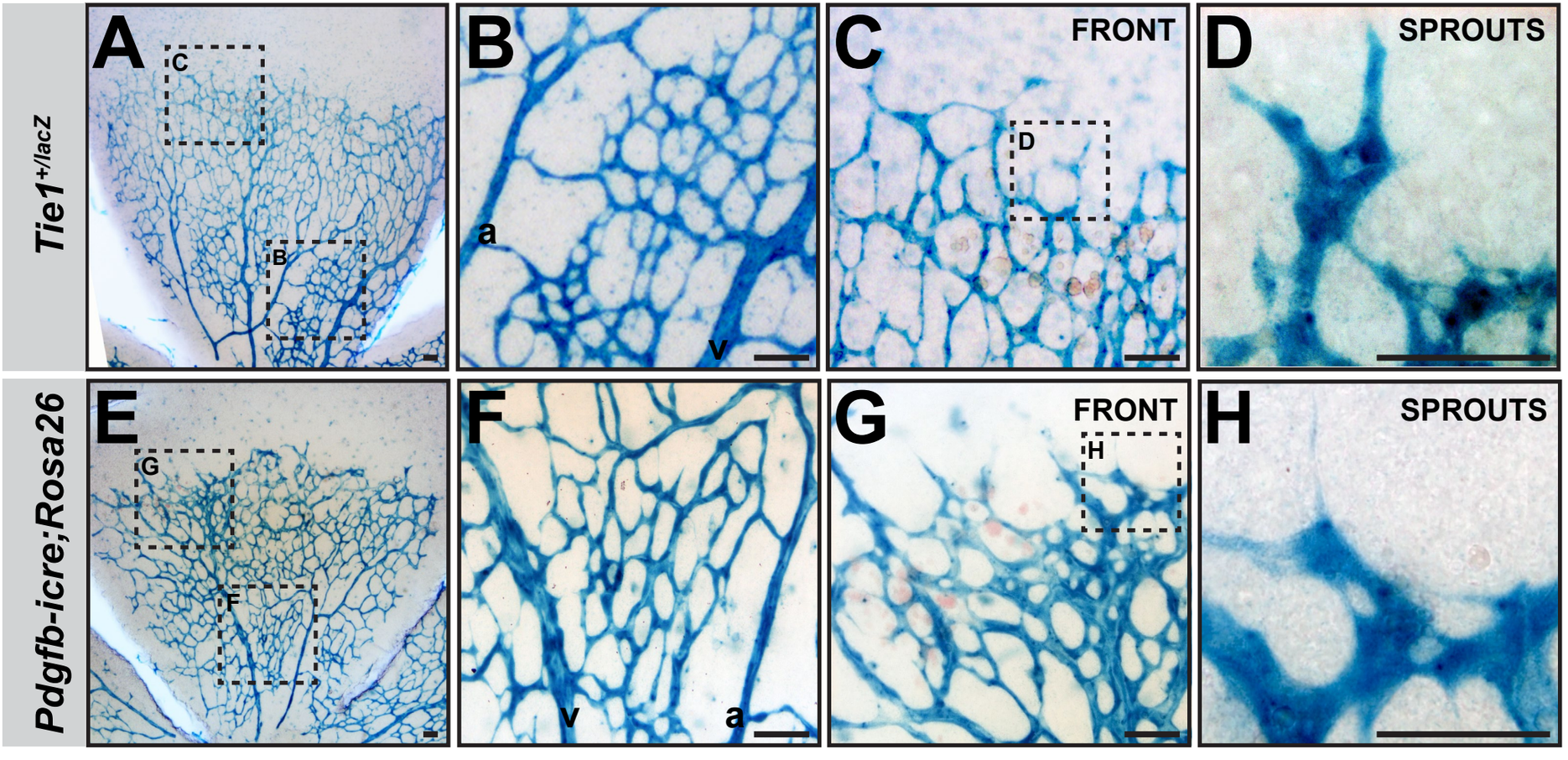
Supplemental Figure 14. Kidney histopathology in control and *Tie1*$^{iEC\triangle/\triangle}$ mice treated with the anti-VEGF blocking antibody mrc84. (**A**) Endomucin staining of formalin-fixed, paraffin-embedded kidney sections from control and *Tie1*$^{iEC\triangle/\triangle}$ tumor bearing mice (EL4 tumors, both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors) treated with placebo or mrc84 antibody, as described in Supplemental Methods. Renal cortex (upper panels) and glomeruli (bottom panels) are shown at low and high magnifications, respectively. Quantification of (**B**) glomerular perimeter (relative to control + placebo, %), (**C**) glomerular blood vessel density (BVD, relative to control + placebo %) and (**D**) Bowman's space area (relative to control + placebo, %). Error bars, SEM. Scale bars: 100 μm.

Supplemental Figure 15, D'Amico et al.



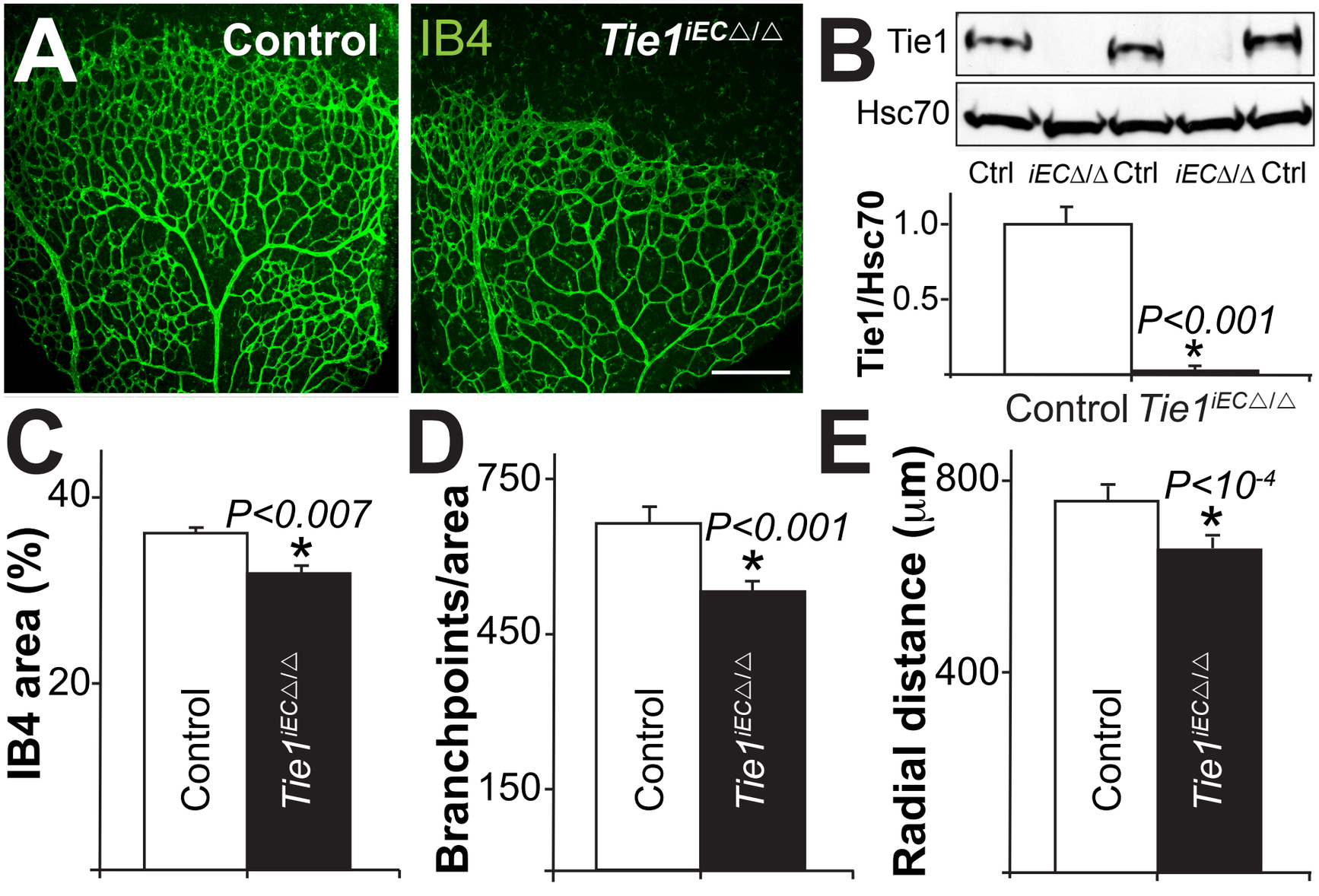
Supplemental Figure 15. Phospho-Tie2 and Tie2 analysis of Tie1-deleted mice stimulated with VEGF, Comp-Angpt1 and Angpt2 *in vivo*. (**A**) 8-week old *Pdgfb-icre/ERT2;Tie1*$^{flox/flox}$ and *Pdgfb-icre/ERT2;Tie1*$^{flox/lacZ}$ progeny were treated with tamoxifen (*iEC*Δ, see Supplemental Table 1) and injected i.v. tail with PBS, VEGF-A (VEGF, 10 µg) (**B**) COMP-Angpt1 (CA1, 20 µg) or Angpt2 (20 µg), followed by analysis after 10 min. Note that in (A) the lungs were subjected to Tie2 immunoprecipitation (IP), followed by phosphotyrosine (pY) immunoblotting. The membrane was stripped and re-probed for Tie2. In (B) a specific phosphotyrosine of Tie2 (pTie2, Y1106) immunoblot was done. The membrane was stripped and re-probed for Tie2. Western blotting for Tie1 shows the efficiency of the deletions and immunoblotting for Flag confirms the CA1 and Angpt2 have reached the target organ. Hsc70 was used as a loading control. Ctrl, control mice. *n* = 2 mice/group, two independent experiments for (A). *n* = 2 mice/group, one experiment for (B).

Supplemental Figure 16, D'Amico et al.



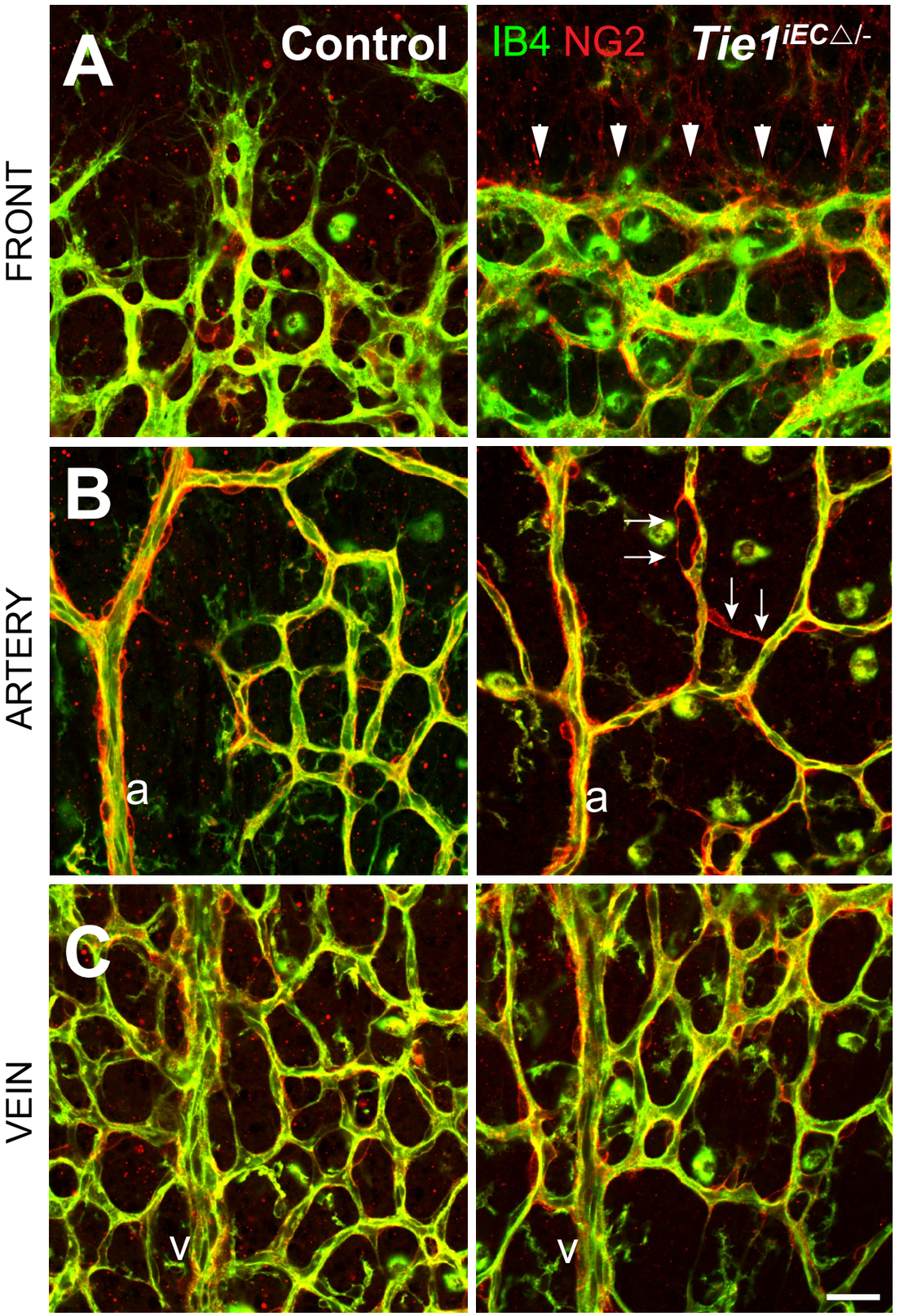
Supplemental Figure 16. ß-galactosidase staining of retinas from *Pdgfb-icre/ERT2;Rosa26-R* and *Tie1*$^{+/lacZ}$ mice. For the staining shown in (**E-H**) the *Pdgfb-icre/ERT2;Rosa26-R* (*Pdgfb-icre;Rosa26*) pups were treated daily with tamoxifen injections (intragastric; 50 µg/day) from postnatal day (P)1 to P4, and retinas were dissected at P5. (**B-C**, **F-G**) Panels show the boxed areas in (**A**) and (E), and (**D, H**) in (C) and (G) at a higher magnification. (a) arteriole, (v) venule. Scale bars: 100 µm. *n* > 4 retinas/genotype.

Supplemental Figure 17, D'Amico et al.



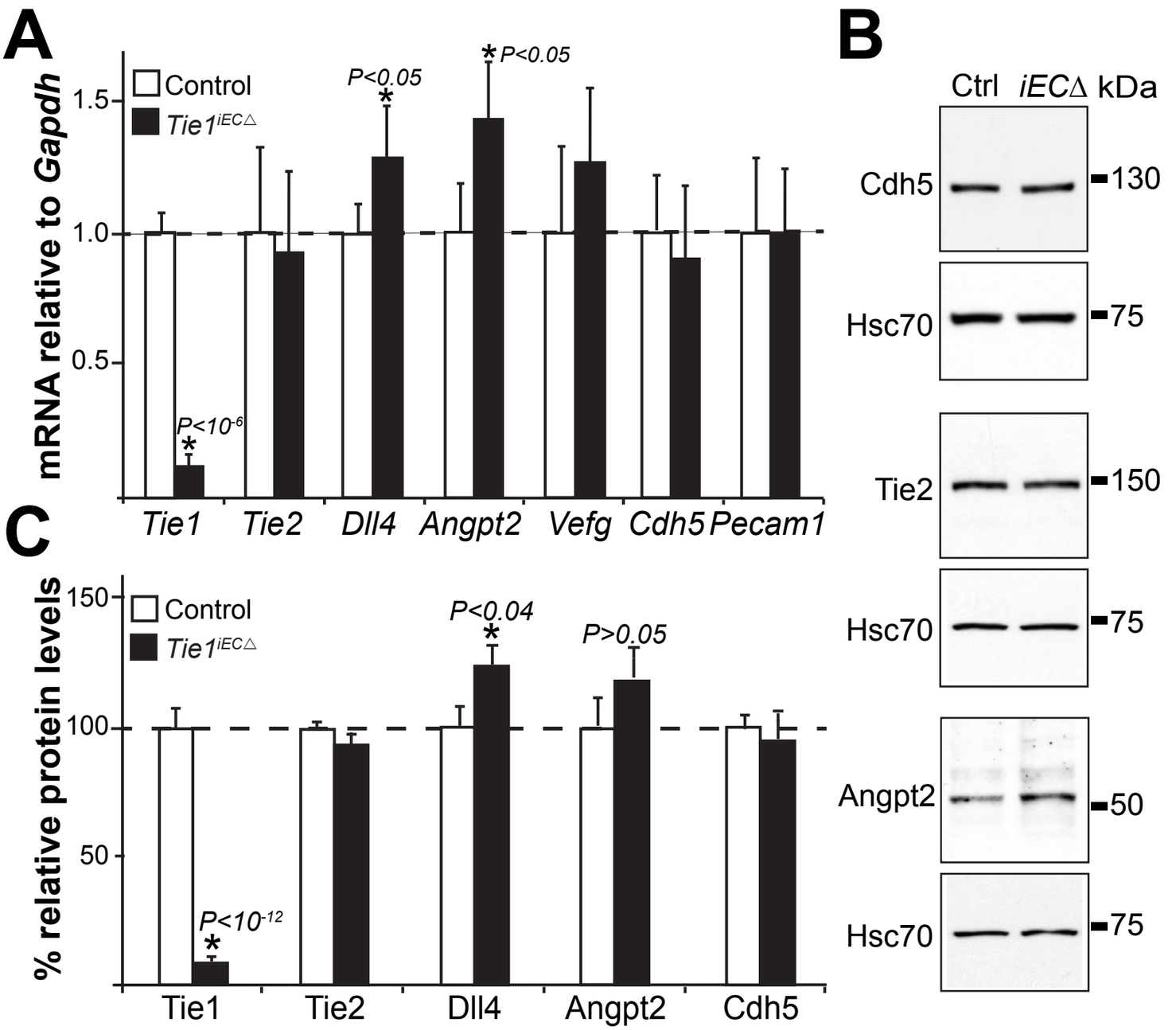
Supplemental Figure 17. Staining and quantification of retinal vasculature in control and *Tie1*$^{iEC\Delta/\Delta}$ mice at P5-6. Newborn *Pdgfb-icre/ERT2;Tie1*$^{flox/flox}$ pups were treated with tamoxifen, as in the experiment of Supplemental Figure 16, to induce Tie1 deletion (*Tie1*$^{iEC\Delta/\Delta}$). (**A**) Confocal microscopy images of flat-mounted retinas at P5-6 stained for IsolectinB4 (IB4). Scale bar: 100 μm. (**B**) Tie1 protein analyzed by Western blotting of lung lysates from the pups. Hsc70 was used as a loading control. Bar graph of densitometric quantification of Tie1 relative to Hsc70. **P*<0.001. Ctrl, control. *iEC*Δ/Δ,*Tie1*$^{iEC\Delta/\Delta}$ pups. (**C**) % of IB4-positive staining/total area (vascular area), (**D**) number of branchpoints/vascular area, and (**E**) radial distance (distance between the average edge of the sprouting vessel front and the optic nerve). Error bars, SEM. *n* = 11-12 retinas/genotype.

Supplemental Figure 18, D'Amico et al.



Supplemental Figure 18. Pericyte coverage of retinal vasculature in control and *Tie1*$^{iEC\triangle/-}$ mice at P5-6. (**A**) Confocal microscopy images of flat-mounted control and Tie1 deleted (*Tie1*$^{iEC\triangle/-}$, *Pdgfb-icre/ERT2* deletor) angiogenic front, (**B**) artery (a) and (**C**) vein (v) retinal vessels at P5-6, stained for pericytes (NG2, red) and endothelium (IB4, green). Scale bar: 100 µm. Arrowheads: blunted angiogenic front, arrows: detached pericytes.

Supplemental Figure 19, D'Amico et al.



Supplemental Figure 19. Angiogenesis-related RNA and protein analysis in lungs from the control, *Tie1*$^{iEC\Delta/\Delta}$ and *Tie1*$^{iEC\Delta/-}$ pups at P6. (**A**) qRT-PCR analysis of lung biopsies. mRNA levels were normalized to *Gapdh* as a housekeeping gene. Error bars, SD. **P* < 0.05; *n* = 4 control and 5 either *Tie1*$^{iEC\Delta/\Delta}$ or *Tie1*$^{iEC\Delta/-}$ (together called *Tie1*$^{iEC\Delta}$, both *Cdh5(PAC)-cre/ERT2* and *Pdgfb-icre/ERT2* deletors) pups. (**B**-**C**) Western blot analysis of lungs. Percentage of protein levels normalized to Hsc70 control. Error bars, SEM. **P* < 0.05; *n* = 5-8 control (Ctrl) and 6-14 *Tie1*$^{iEC\Delta}$ (*iEC*Δ) pups.