Cell Reports

Supplemental Information

The Orphan Receptor Tie1 Controls Angiogenesis and Vascular Remodeling by Differentially Regulating Tie2 in Tip and Stalk Cells

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Supplemental Figures

Figure S1

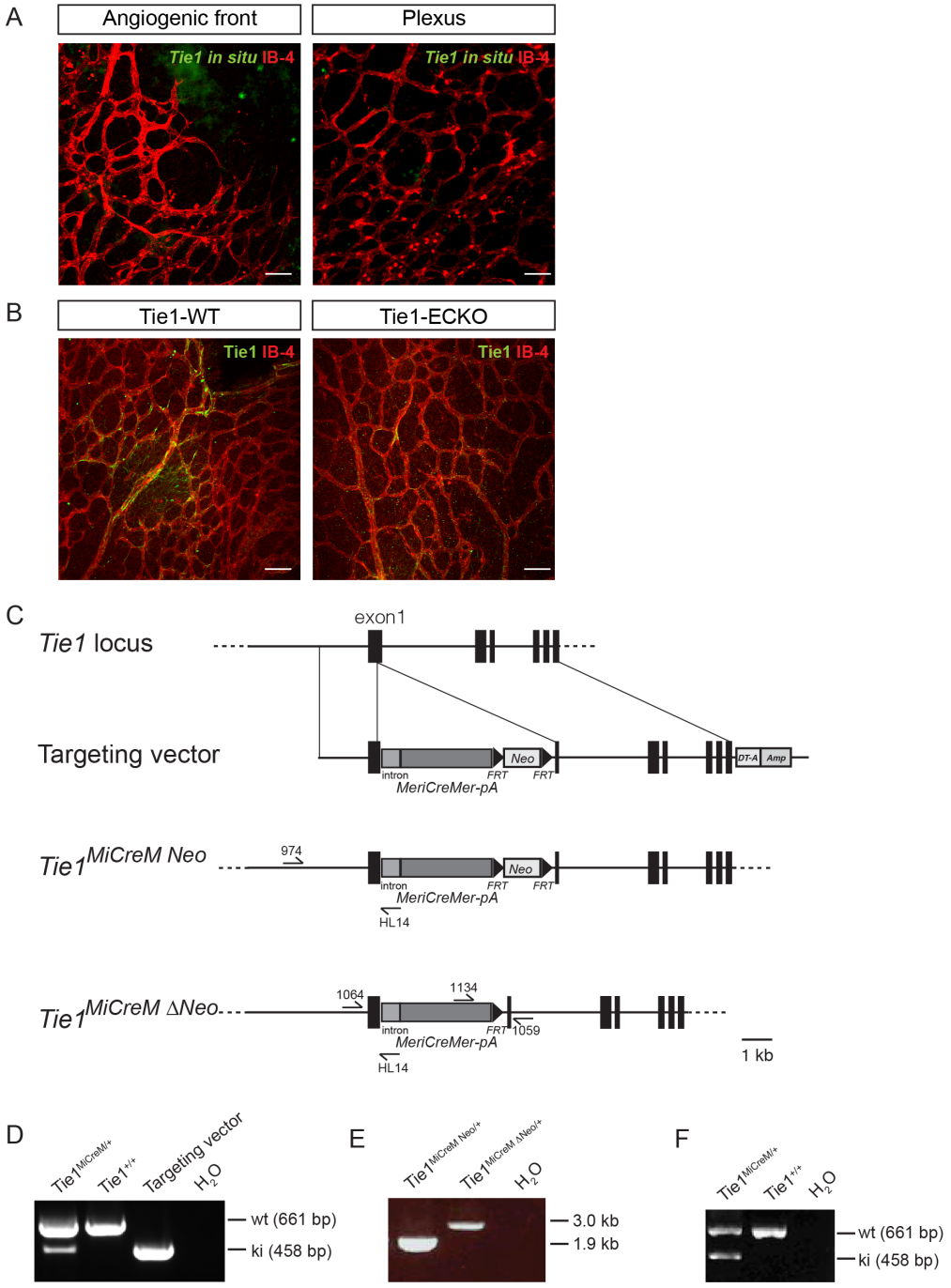

Figure S1. Controls for Tie1 expression and generation of the *Tie1*^{MiCreM} knockin mouse (related to Figure 1)

(A) Control in situ hybridization with a sense probe against Tie1 mRNA in the P6 whole-mounted retina vasculature (IB-4) showing negative staining with the sense probe. (B) Representative images showing the specificity of Tie1 mab 5F5 immunostaining in wild-type (WT) P6 retina vasculature (IB-4) and less staining in retina isolated from EC-specific Tie1

knockout (Tie1-ECKO) mice post-tamoxifen treatment. Scale bar 50 µm. (C) Schematic depiction of the endogenous *Tie1* locus, the targeting vector, the targeted allele with (*Tie1*$^{MiCreM\ Neo}$), and without Neo cassette (*Tie1*$^{MiCreM\ \Delta Neo}$). Homologous recombination resulted in replacement of the start codon of exon1 by the MeriCreMer cassette. Oligonucleotides for ES cell screening (974, HL14), Neo deletion (1134, 1059) and genotyping (1064, HL14, 1059) are indicated (size of FRT sites not to scale). (D) Genotyping PCR of *Tie1*$^{MiCreM\ Neo}$ knockin (ki) and wild type (wt) alleles of gDNA of a correctly targeted ES cell clone and wild type E14 ES cells as control. (E) Screening PCR of *Tie1*^{MiCreM} mice before and after Neo deletion after breeding with a Flp deleter mouse. (F) Genotyping of mouse tail biopsies.

Figure S2

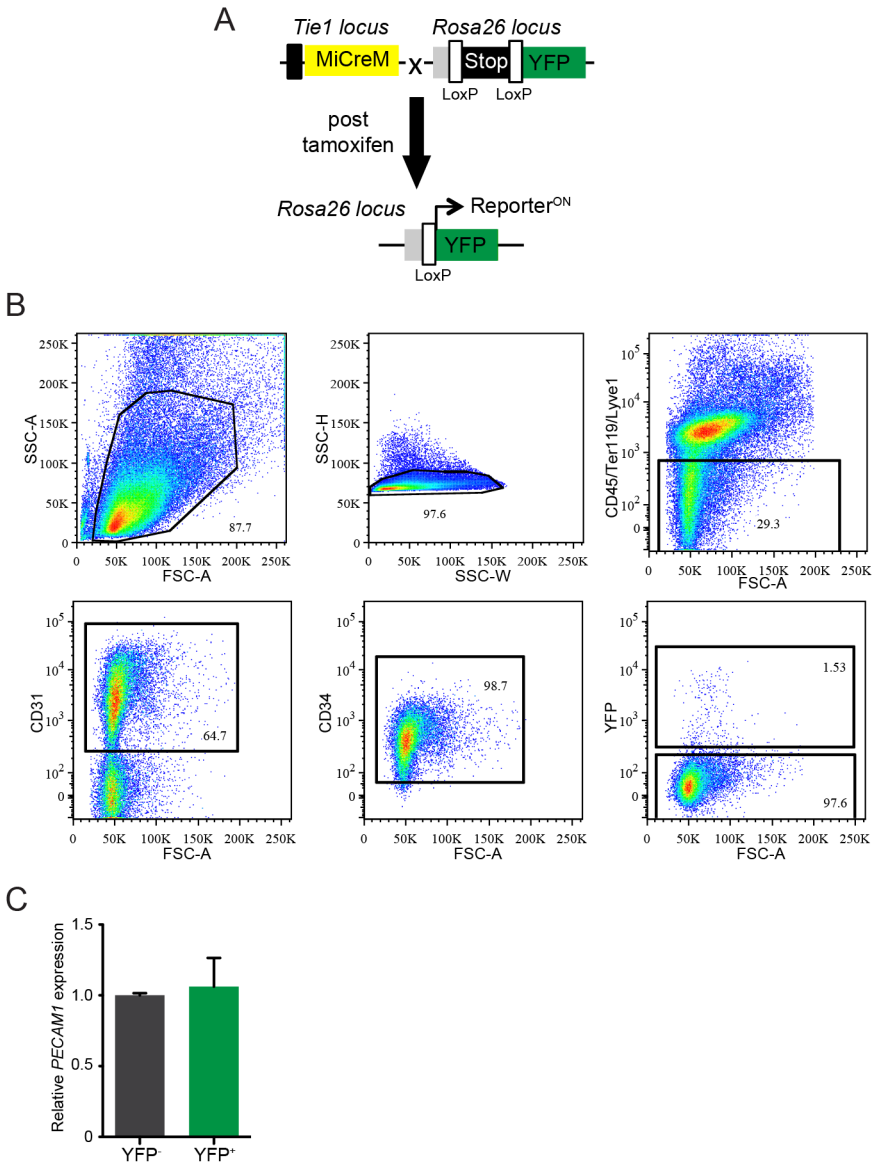

Figure S2. EC isolation from *Tie1*^{MiCreM} knockin mouse and gene expression analysis (related to Figure 1)

(A) Schematic depiction the *Tie1*$^{MiCreM/+}$ knockin-*Rosa26*^{YFP} reporter mouse model in which tamoxifen treatment leads to Cre-mediated recombination of the floxed Stop cassette in the *Rosa26* locus and YFP expression. (B) Representative FACS sorting scheme for the isolation of viable (PI^-) lung EC. $CD45^-Ter119^-Lyve1^-CD31^+CD34^+YFP^+$ and $CD45^-Ter119^-Lyve1^-CD31^+CD34^+YFP^-$ cells were sorted for gene expression analysis. (C) Relative *PECAM1* expression in YFP^+ EC compared to YFP^- EC of P6 reporter mice post-tamoxifen (EC from 5-7 littermates pooled, n=4 independent experiments).

Figure S3

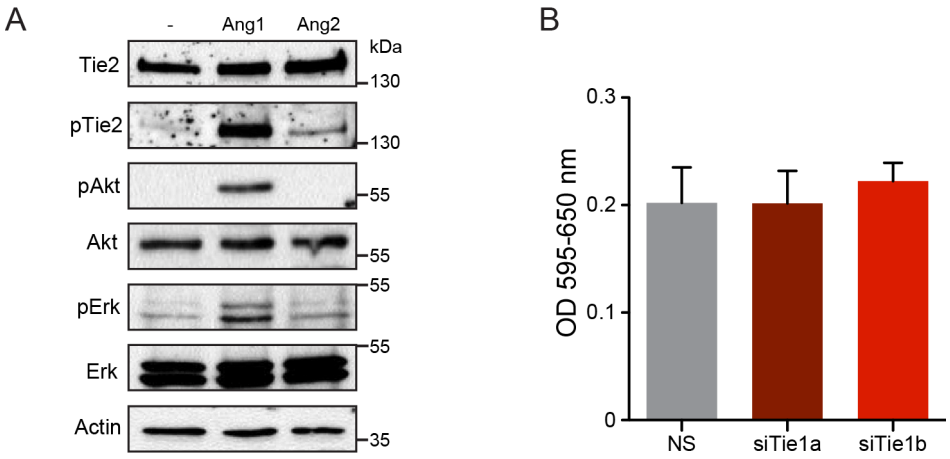

Figure S3. Ang-induced Tie2 signaling and effect of Tie1 deletion on EC proliferation (related to Figure 2)

(A) HUVEC were stimulated with Ang1 or Ang2 (400 ng/ml) for 20 min. Cell lysates were either immunoprecipitated with anti-Tie2 and immunoblotted sequentially with a pan-phosphotyrosine (pTie2) and Tie2 antibodies; or probed with antibodies against pAkt, total Akt, pErk, total Erk and actin. (B) Proliferation assay of serum starved Tie1-silenced HUVEC (siTie1a, siTie1b) compared to control showing no significant difference in proliferation after 24 hrs. (n=3).

Figure S4

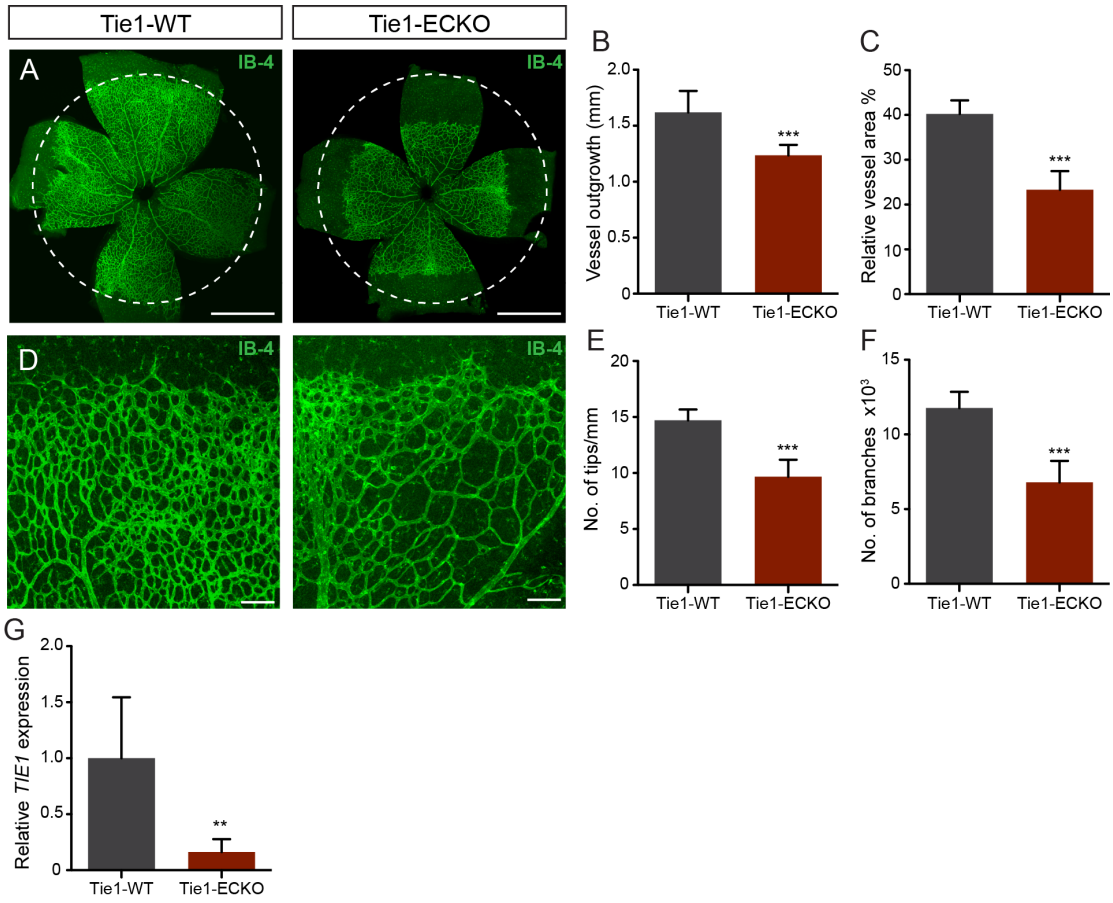

Figure S4. Impaired retinal vascularization in Tie1-ECKO mice (related to Figure 3)

(A) Representative images of IB-4 labeled P6 retinal vasculature of Tie1-WT and Tie1-ECKO mice post tamoxifen treatment. Vascular analyses showing significant decrease in (B) vascular outgrowth and (C) relative vessel area in Tie1-ECKO mice compared to Tie1-WT littermates (n=8-12 mice/group). (D) Vascular front of Tie1-WT and Tie1-ECKO mice. (E) Number of tips/mm vascular front and (F) number of branches were significantly reduced in Tie1-ECKO mice (n=8-12 mice/group). (G) Relative *TIE1* expression analyzed from total lung RNA of Tie1-WT and Tie1-ECKO P6 mice post-tamoxifen (n=8 mice/group). Scale bar 1 mm (A), 100 µm (D). **p<0.01 ***p<0.001. Images A and D are composite of tiled and automatically stitched images.

Figure S5

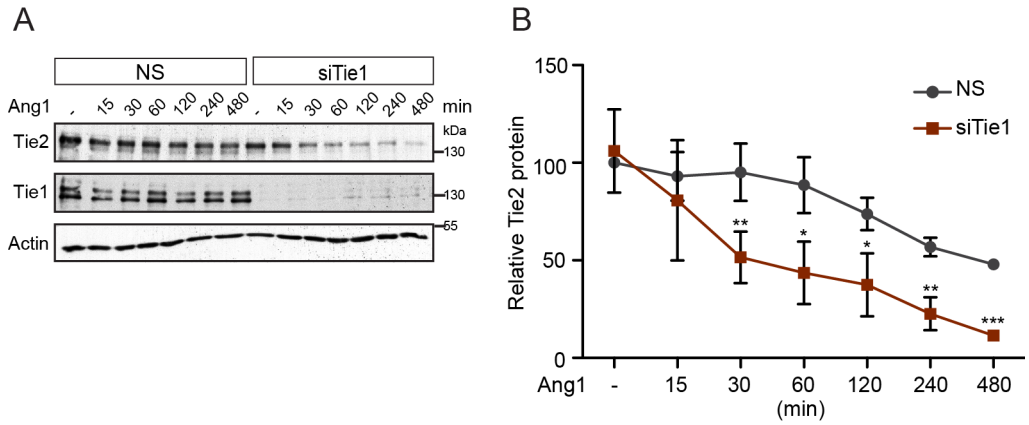

Figure S5. Tie1 regulates Tie2 protein turnover (related to Figure 5)

(A) Serum starved, confluent HUVEC were stimulated with Ang1 (400 ng/ml) at indicated time points (ranging from 15 to 480 min). Cell lysates were separated and immunoblotted with Tie2, Tie1 and actin antibodies. (B) Densitometric analyses of Tie2 showing increased loss of Tie2 protein in Tie1-silenced cells compared to corresponding controls (NS). (n=3), *p<0.05, **p<0.01, ***p<0.001.

Figure S6

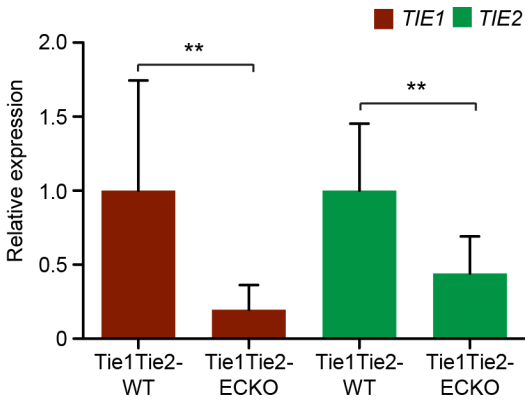

Figure S6. Tie1 and Tie2 deletion in Tie1Tie2-ECKO mice (related to Figure 6)

Relative *TIE1* and *TIE2* expression analyzed from total lung RNA of Tie1Tie2-WT and Tie1Tie2-ECKO P6 mice post-tamoxifen treatment (n=8 mice/group). **p<0.01.

Figure S7

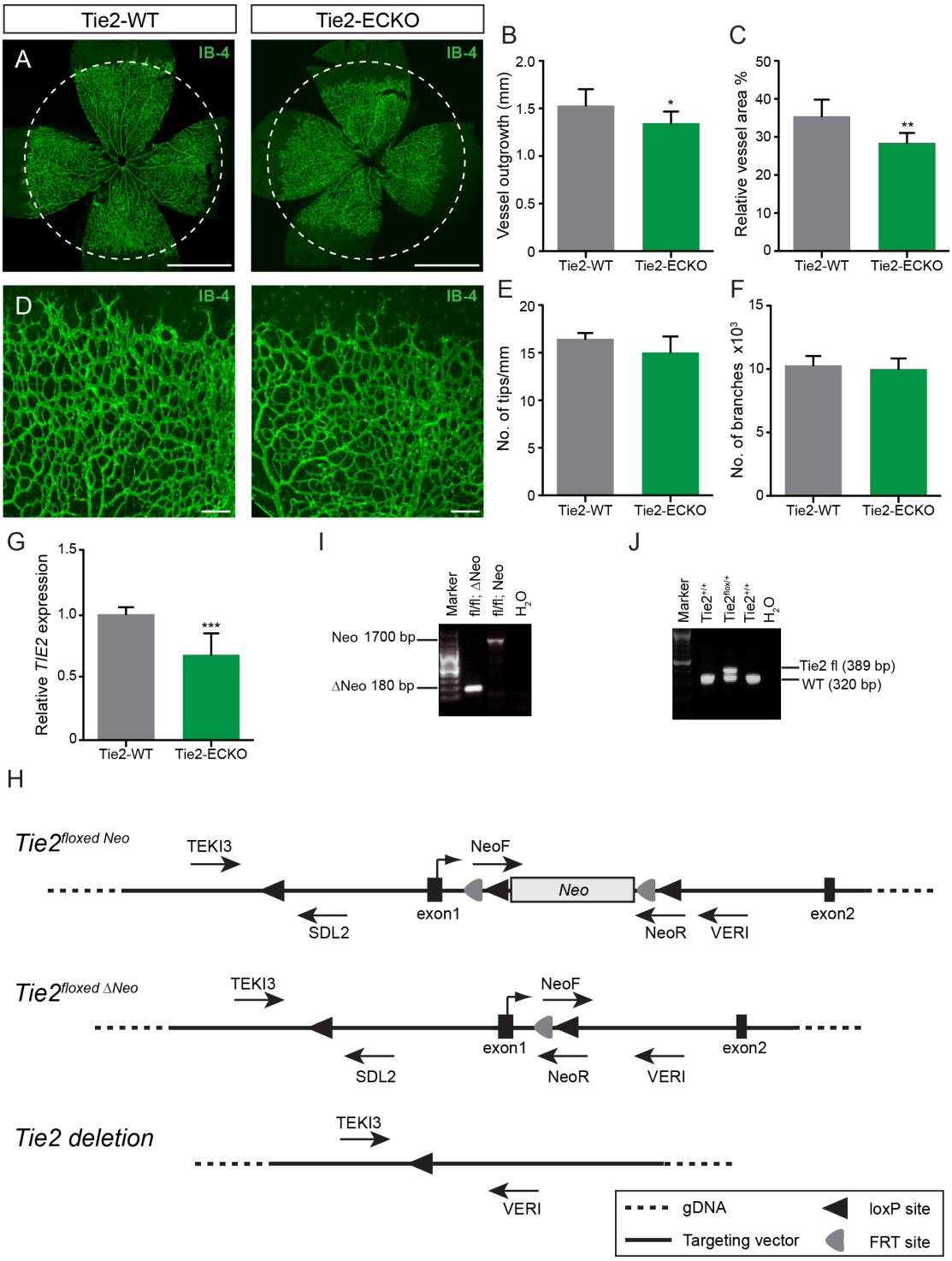

Figure S7. Impaired retinal vascularization in Tie2-ECKO mice and generation of the *Tie2*^{floxed} mice (related to Figure 6)

(A) Representative images of IB-4 labeled P6 retinal vasculature of Tie2-WT and Tie2-ECKO mice post tamoxifen treatment. Vascular analyses demonstrating a weak effect on the (B) vascular outgrowth and (C) relative vessel area in Tie2-ECKO mice compared to Tie2-WT

littermates (n=6-9 mice/group). (D) Vascular front of Tie2-WT and Tie2-ECKO mice showing no difference in (E) number of tips/mm vascular front and (F) number of branches in Tie2-ECKO mice (n=6-9 mice/group). (G) Relative *TIE2* expression analyzed from total lung RNA of Tie2-WT and Tie2-ECKO P6 mice post-tamoxifen showed on an average only about 25-33% Tie2 deletion (n=8 mice/group). Scale bar 1 mm (A), 100 μm (D). **p<0.01 ***p<0.001. Images A and D are composite of tiled and automatically stitched images. (H) Schematic depiction of the targeted *Tie2* allele with ($Tie2^{floxed\ Neo}$) and without Neo cassette ($Tie2^{floxed\ \Delta Neo}$) and post Cre mediated recombination of the floxed cassette. Oligonucleotides for Neo deletion (NeoF, NeoR) and genotyping (TEKI3, SDL2, VERI) are indicated. (I) Screening PCR of $Tie2^{floxed\ Neo}$ mice before and after Neo deletion by breeding with a FLP deletor mouse. (J) Genotyping PCR of mouse tail biopsies of $Tie2^{flox/+}$ and $Tie2^{+/+}$ alleles.

Supplemental Experimental Procedures

Generation of *Tie1*^{MiCreM} knockin mice

For the knockin approach, a gene targeting vector was constructed which upon homologous recombination would insert a MeriCreMer cDNA (Srinivas et al, 2001; Zhang et al, 1996) into the first exon of the *Tie1* locus. The targeting construct consisted, from 5' to 3', of a short homologous arm upstream of the ATG start codon of the *Tie1* gene, a splice donor site, an intron and a splice acceptor site (intron), all taken from the rabbit β-globin gene (Kouskoff et al, 1993), the coding sequence of codon-improved Cre (iCre) (Shimshek et al, 2002), flanked by two mutated estrogen receptor sites (Mer) (Zhang et al, 1996), a poly-adenylation signal (pA) from the rabbit β-globin gene (Kouskoff et al, 1993), a FRT-flanked Neomycin (Neo) resistance gene, a long homologous arm, and finally the diphtheria toxin subunit A gene (DT-A) to select against random integration events (Figure S1). Gene targeting experiments were performed in embryonic day (E) 14 embryonic stem cells. Correct homologous recombination of the targeting vector results in replacement of the start codon in the first exon of the *Tie1* locus with the inducible MeriCreMer cassette. Correctly targeted *Tie1* alleles were identified by PCR after screening a total of 240 Neomycin-resistant colonies. Embryonic stem cells of a Neo-resistant subclone (*Tie1* $^{MiCreMNeo/+}$*)* were injected into C57BL/6 blastocysts and chimeric mice were backcrossed to C57BL/6 mice to transmit the *Tie1*^{MiCreMNeo} allele. The FRT-flanked Neomycin selection cassette was removed by crossing *Tie1*$^{MiCreMNeo/+}$ mice with FLP deleter mice (Rodriguez et al, 2000). Site-specific integration was confirmed by PCR and Southern blotting before and after Neo deletion (Southern blots not shown). All *Tie1* gene sequences upstream and downstream of exon1 are preserved in the *Tie1*^{MiCreM} allele. Heterozygous *Tie1*$^{MiCreM/+}$ mice were fertile and showed no apparent abnormalities. Homozygous *Tie1*$^{MiCreM/MiCreM}$ embryos die between embryonic day E9.5 and E12.5 as previously described (Sato et al, 1995). Genotyping PCR of the *Tie1*^{MiCreM} allele was done with primers (1064: 5'-CAGAACAATGTCCTGCCTCCAAACC-3', 1059: 5'-CACCACCACACCCTAACTGAAATGC-3' and HL14: 5'-AATCAAGGGTCCCCAAACTCAC-3').

Generation of *Tie2*^{floxed} mice

Tie2 floxed mice were generated by the Ingenious targeting company (Ronkonkoma, NY, USA). Briefly, a targeting vector containing two loxP sites flanking exon1 of *Tie2* and a part of its promoter region; and a Neomycin selection cassette flanked with FRT sites was used (Figure S7). Embryonic stem cells of the targeted clone were injected into C57BL/6 blastocysts and chimeric mice were backcrossed to C57BL/6 mice to transmit the *Tie2*$^{flox/flox}$

alleles. Genotyping PCR of the floxed alleles was done with primers (TEKI3: 5'-CAGGCTATCACTGTGACACTGGTAC-3', SDL2: 5'-AAATACGCAGTTTCAGGGCTGGGA-3', VERI: 5'-ACCAATTCGGGGAATCCTATGGCA-3'). The Neomycin cassette was excised by crossing *Tie2*$^{flox/flox}$ with FLP deleter mice (Rodriguez et al, 2000).

Whole-mount retina staining

Mouse pups were sacrificed on day 6, eyeballs were fixed in either methanol or 4% paraformaldehyde (PFA). Retinas were isolated and blocking was performed with 0.5% Triton X-100/1% bovine serum albumin/10% goat serum in PBS for 1.5 hrs at room temperature (RT). The retina vasculature was either stained with FITC-conjugated IsolectinB-4 (IB-4) (Sigma, L9381) or CD31 (BD Pharmingen, 553370) and co-stained with primary antibodies; Collagen IV (Serotec, 2150-1470), Cleaved caspase3 (Cell Signaling, 9661), ERG1 (Abcam, ab92513), and Desmin (Abcam, ab15200) overnight at 4°C. Retinas were subsequently incubated with the appropriate Alexa-546 conjugated secondary antibodies (Invitrogen) for 1 h at RT and whole-mounted. Imaging was performed using the Zeiss LSM 710 confocal microscope and images were acquired using the tile scan and stitching function of the ZEN software (Zeiss).

Retina image analyses

Retina analyses were performed using the Fiji software (ImageJ). Vessel outgrowth was measured by analyzing the distance of the vascular front from the central optic nerve. Relative vessel area was calculated as IB-4^{+} area per retina area. For analysis of the tips, number of tips at the total retina front were counted and depicted as number of tips per mm vessel front. Branches in the vasculature were analyzed by skeletonizing the IB-4 stained images and counting the total number of branches. Regression analysis was performed by counting ColIV^{+}IB-4^{-} structures and correlating them to the vessel area. For the analysis of EC apoptosis, cleaved caspase3^{+} structures that co-localized with IB-4 staining were counted and correlated to the vessel area. ERG1^{+} nuclei co-localized with CD31 were counted. Relative pericyte coverage was analyzed by measuring desmin-positive area and correlating it to the vessel area. If relative values (ColIV and cleaved caspase3 analysis) are plotted then single values per mouse were normalized to the average of the WT littermates.

In situ hybridization (ISH) on whole-mounted retina

A plasmid containing mouse Tie1 cDNA region spanning the fibronectin domains 2 and 3 (FN2 and FN3) was used for synthesizing the ISH probes. Digoxigenin (DIG)-labeled sense and antisense RNA probes were synthesized using T7 and T3 polymerases, respectively. ISH was

performed with minor modifications as previously described (Powner et al, 2012). Briefly, eyes of P6 mouse pups were removed and fixed for 20 min in 4% PFA on ice. After isolation and dissection, retinas were stored in 100% methanol at -20° C overnight. Hybridization was performed at 66°C. RNA labeled probes were detected by an alkaline-phosphatase-coupled antibody (Roche Applied Sciences) and nitroblue tetrazolium/5-bromo-4- chloro-3-indolyl phosphate (NBT/BCIP) was used as a chromogenic substrate for alkaline phosphatase (Promega). After color development, retinas were extensively washed with PBS. Subsequently, retinas were incubated with IB-4 conjugated to Alexa-568 (1:200) for blood vessel staining. Bright field and fluorescent images were acquired simultaneously using a Zeiss LSM 880 equipped with a 20x objective. Images were processed and background corrected with a rolling ball algorithm in the Fiji software (ImageJ). To better visualize colocalization, a pseudo color (green) was applied to the bright field image.

Lung EC isolation procedures

Mouse pups (P6) were sacrificed and lungs were minced into small pieces. Single-cell suspensions were prepared by digesting the tissue in Dulbecco's Modified Eagle's medium (DMEM) containing 1.25 mM $CaCl_2$, 200 U/ml Collagenase I (Sigma, C9891) and 10 µg/ml DnaseI (Sigma, D4527) at 37°C for 30 min and subsequently sieved through a 100 µm cell strainer using the plunger of a 19G cannula syringe. For isolating EC by FACS sorting, cell suspensions were stained with antibodies against CD45, Ter119, Lyve1 and podoplanin (Pdpn) (Table S1) to deplete blood cells and lymphatic EC for 30 min at 4°C in PBS/5% fetal calf serum (FCS). Cells were depleted by incubating with 500 µl magnetic Dynabeads (Invitrogen, 114.15D) in 750 µl PBS/5% FCS for 30 min at 4°C on the rotator. The remaining cells were positively stained with CD31 and CD34 antibodies (Table S1) in PBS/5% FCS for 30 min at 4°C. Dead cells were excluded by propidium iodide (PI) (Life technologies, P3566) staining (1:3000). $CD45^-Ter119^-Lyve1^-Pdpn^-CD31^+CD34^+$ cells were sorted with a BD Biosciences FACS Aria Cell Sorter. For isolating EC by magnetic cell selection, cell suspensions were incubated with rat anti-mouse CD31 (BD biosciences, 553370) at 4°C for 1.5 hrs followed by incubation with prewashed sheep anti-mouse magnetic beads (Invitrogen, 11035) for 1 h at 4°C on a rotator. EC were selected using Dynal magnetic particle concentrator (Invitrogen) and plated on fibronectin coated 24-well plates.

Cell culture, siRNA transfection and lentivirus transduction

HUVEC were cultured in Endopan3 medium (PAN Biotech) supplemented with growth factors and serum. For silencing experiments, HUVEC were transfected with 100 nM Tie1

siRNA (687, s14140 s14142), Tie2 siRNA (s13984, s13983) or control siRNA (Life Technologies) using 5 µl Oligofectamin (Life Technologies, 12252-011) in Opti-MEM+ GlutaMAX-I (Life Technologies). A C-terminal EGFP tagged construct of human Tie1 (Tie1-GFP) was generated and used for transducing HEK293T cells to produce lentivirus using the pLenti6 expression vector (Invitrogen). Tie1-GFP lentivirus was subsequently used to transduce HUVEC for generating a Tie1-GFP expressing cell line.

HUVEC stimulation assay

To study angiopoietin induced signaling, HUVEC were starved overnight in EC basal medium (Endopan3) containing 0.5% FCS or for 4 hrs without FCS. Cells were stimulated with 400 ng/ml of human, recombinant Ang1 or Ang2 (R&D) in EC basal medium for indicated time. Cells were then washed with cold 1x PBS and lysed for protein extraction.

Protein isolation, immunoprecipitation and Western blotting

Cells were lysed on ice using lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, protease-inhibitor mix G (Serva) and 2 mM Na_3VO_4). Protein concentration was determined using the Protein Assay Reagent (BioRad Laboratories). For immunoprecipitation, cell lysates were incubated with protein G-sepharose beads (GE Healthcare) and 3 µg Tie2 antibody (Millipore, clone Ab33, 05-584) or Tie1 antibody (ReliaTech, clone 6f12, 101-M48) overnight at 4°C on a rotator. Beads were washed by centrifugation at 106 g, 4°C for 2 min with lysis buffer containing 2 mM Na_3VO_4 and boiled with 2x protein sample buffer at 95°C for 10 min. Immunoprecipitates and cell lysates were separated by SDS-PAGE followed by Western blotting. Blots were probed with Tie1 (R&D, AF619), Tie2 (R&D, AF313), pan phospho-Tyrosine (Millipore, 05-321), pAkt (Cell Signaling, 4060) and Akt (Cell Signaling, 2967), pErk1/2 (Santa Cruz, sc-7383), Erk1/2 (Santa Cruz, sc-94) and actin (Santa Cruz, sc-1616).

Flow cytometric analyses of HUVEC

Surface expression of Tie1 and Tie2 in HUVEC after siRNA transfection was analyzed by flow cytometry. Single cell suspensions were incubated with conjugated goat-anti-Tie1-PE (R&D) and mouse-anti-Tie2-APC (R&D) in basal medium containing 1% FCS for 30 min on ice. Corresponding isotype controls, goat IgG-PE and mouse IgG-APC (R&D), were used. Sample acquisition was performed with a BD FACS Canto II flow cytometer and subsequent analysis was done using FlowJo software.

Spheroid sprouting assay

The mosaic spheroid sprouting assays were performed with Tie1-GFP and wild-type (WT) HUVEC. For this, 1:1 mixture of WT and Tie1-GFP cells were resuspended in EC growth medium containing 20% methocel (Sigma). 25 µl cell suspension drops were pipetted on non-adherent plastic plates. Subsequently, plates were turned upside-down to form the so called hanging drops in which HUVEC spheroids are formed. Plates were incubated for 24 hrs at 37°C and spheroids were harvested by washing the plates with 10% FCS/PBS. Next, spheroids were centrifuged at 200 g for 5 min and resuspended in 20% FCS and 80% methocel. The collagen matrix was prepared on ice using Collagen type I (isolated from rat tail tendons), Medium 199 and NaOH (1 M) in 8:1:1 ratio. Additionally, 1x HEPES buffer was added to the mix for adjusting the pH. The Collagen solution was mixed with the spheroid solution in 1:1 ratio and transferred to a 24-well plate. For polymerization, gels were incubated for 30 min at 37°C and then stimulated with 75 ng/ml VEGF (R&D) in EC basal medium. Sprouting spheroids were imaged using the cell observer (Zeiss) and analyzed using the Fiji software (ImageJ).

Scratch wound assay

For a 2D migration assay, WT, Tie1-GFP or a 1:1 mixture of WT and Tie1-GFP HUVEC were seeded on 0.2% gelatin-coated cover slips. A scratch was made using the 100 µl pipette tip and cells were allowed to migrate for 5 hrs. Cells were then fixed with 4% PFA/PBS for 20 min at RT followed by staining with Alexa-546 conjugated Phalloidin (Invitrogen, A22283) to visualize the actin cytoskeleton of migrating cells. Nuclei were counterstained with Hoechst dye (1:3000). Cells in the wound area were imaged using the cell observer (Zeiss) and analyzed using the Fiji software (ImageJ).

Shear stress experiments

Shear stress experiments were performed using the ibidi flow chambers (ibidi cells in focus, 15 µ-Slide I 0.4 Luer, catalog no. 80176) connected to an IPS high precision peristaltic pump with a flow rate of 11 ml/min (IDEX corporation, ISM935). Laminar shear at 10 dynes/cm^2 was obtained by pumping EC media at 1.6 ml/min flow rate on Tie1-silenced and control cells seeded in the ibidi chambers. Shear stress experiments were performed for 48 hrs in a sterile, 5% CO2 incubator at 37°C.

Antibody uptake assay and immunofluorescence staining

Antibody uptake assay was performed to trace the intracellular trafficking of fluorescently labeled, surface presented Tie2 receptor following Ang1-mediated internalization. HUVEC were seeded on 0.2% gelatin-coated cover slips. Following overnight starvation, cells were incubated with 2 µg/mL Tie2 antibody (R&D, AF313) for 15 min on ice. Cells were washed with ice cold 1x PBS and incubated with Alexa-546 conjugated secondary antibody for 15 min on ice. After the labeling step on ice, HUVEC were stimulated with Ang1 (400 ng/ml) and incubated for desired time at 37°C. Cells were subsequently acid washed with ice cold 1x PBS (pH 2.5) to remove the non-internalized, surface bound antibody. Cells were fixed with methanol for 3 min followed by staining with LAMP1 antibody (Cell Signaling, 9091) for 1 h at RT and subsequently with Alexa-488 conjugated secondary antibody for 1 h at RT in the dark. Nuclei were counterstained with Hoechst dye (1:3000). Confocal images (Z-stacks) were acquired with the Laser Scanning Microscope LSM 700 (Zeiss). Tie2-LAMP1 co-localization analysis was performed using the Fiji software (ImageJ).

Cell apoptosis assay

Cell apoptosis in Tie1-silenced compared to control HUVEC was determined by the Caspase-Glo® 3/7 assay (Promega). Cells were seeded in 96-well plates, starved overnight in EC medium+ 0.5% FCS and then stimulated with 100 µl EC medium for 8 hrs. Subsequent steps were performed according to the manufacturer's protocol. Luminescence was detected using luminometer LUMIstar OPTIMA.

Cell proliferation assay

The MTT cell proliferation assay was performed to assess the proliferation in Tie1-silenced and control HUVEC according to the manufacturer's protocol (Roche). Briefly, starved cells were incubated with 10 µl MTT solution for 24 hrs in EC medium and subsequently in 100 µl SDS overnight. The amount of formazan produced was measured by an ELISA reader (OD of 550 nm and 650 nm).

RNA isolation and qRT-PCR

RNA of FACS-sorted mouse EC was isolated with Arcturus PicoPure RNA Isolation Kit (Life Technologies) and RNA of HUVEC was isolated with RNeasy Mini Kit (Qiagen). cDNA was synthesized with QuantiTect Reverse Transcription Kit (Qiagen). Subsequent qRT-PCR was performed with TaqMan gene expression assay (Life Technologies; Table S2), TaqMan Fast Advanced Mastermix (Life Technologies) and Roche Light Cycler 480.

Supplemental Tables

Table S1. List of antibodies used for EC isolation by FACS sorting (related to Figure 1).

Target protein	Species	Company
ms CD45-FITC	rat	BD Pharmingen
ms Ter119-FITC	rat	BD Pharmingen
ms Lyve1-FITC	rat	eBioscience
ms Podoplanin-A488	hamster	eBioscience
ms CD45-PE	rat	BD Pharmingen
ms Ter119-PE	rat	BD Pharmingen
ms Lyve1-PE	rat	eBioscience
ms CD31- APC	rat	BD Pharmingen
ms CD34- PCB	rat	eBioscience

Table S2. List of TaqMan assays (related to Figures 1-4).

Target gene	Species	Assay ID
TIE1	human	Hs00892696_m1
TIE2	human	Hs00945146_m1
DLL4	human	Hs00184092_m1
ANGPT2	human	Hs01048042_m1
VEGFR2	human	Hs00911700_m1
KLF2	human	Hs00360439_g1
BAX	human	Hs00180269_m1
NOS3	human	Hs01574659_m1
HPRT	human	Hs02800695_m1
GAPDH	human	Hs02758991_g1
B2M	human	Hs00984230_m1
TIE1	mouse	Mm00441786_m1
TIE2	mouse	Mm00443254_m1
PECAM1	mouse	Mm01242584_m1
BAX	mouse	Mm00432051_m1
STAT2	mouse	Mm00490880_m1
KLF2	mouse	Mm01244979_g1
ANGPT2	mouse	Mm00545822_m1
B2M	mouse	Mm00437762_m1
ACTB	mouse	Mm00607939_S1
HPRT	mouse	Mm00446968_m1

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