Supplemental Information

Sema3E-PlexinD1 signaling selectively suppresses disoriented angiogenesis in ischemic retinopathy in mice

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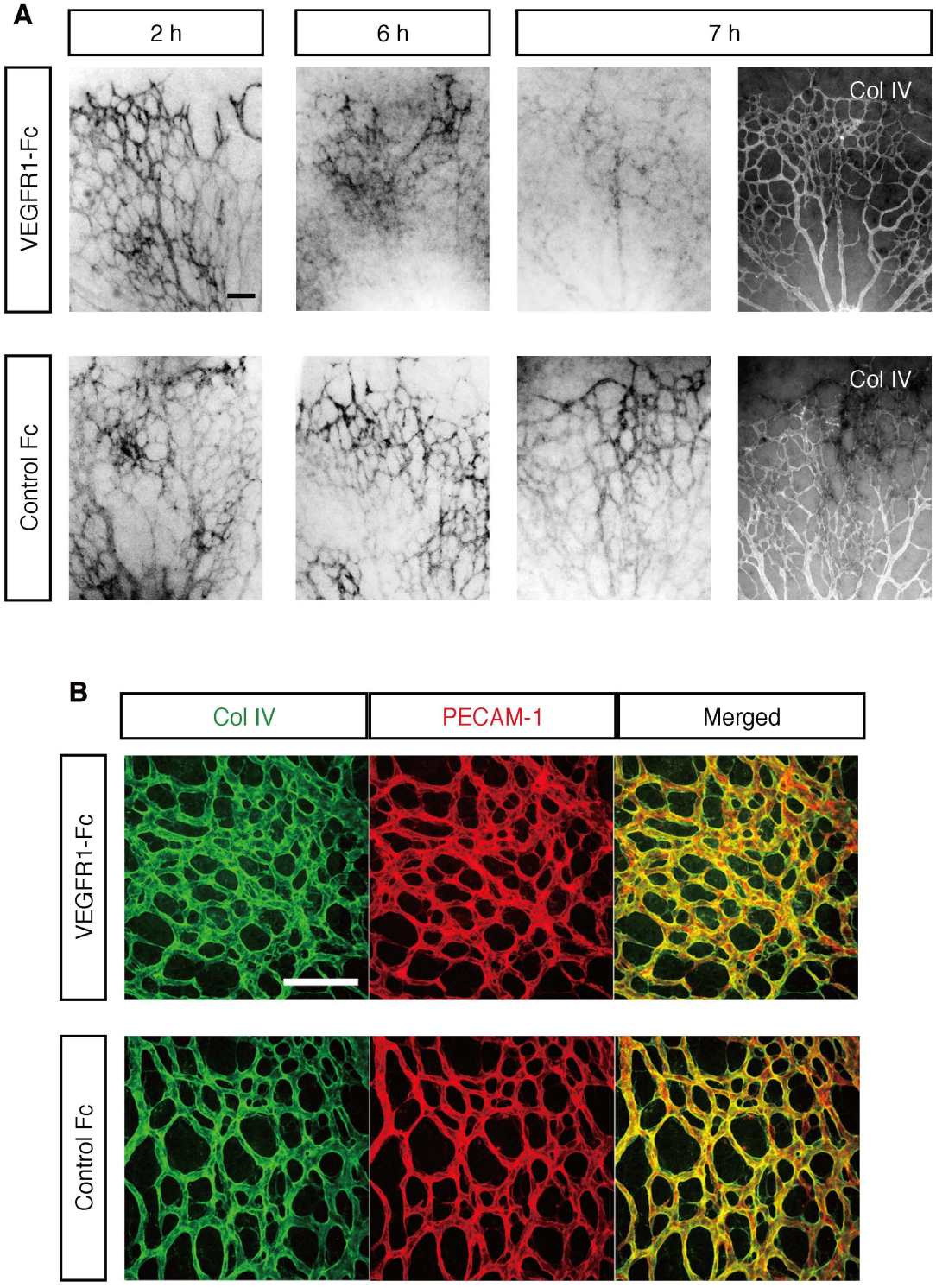
Supplemental Methods

Cell culture. To form the concentration gradients of VEGF or Sema3E, HUVECs cultured at a density of 5×10^3 cells/cm^2 in gelatin-coated μ-Slide VI (Ibidi) were serum-starved with EBM-2 containing 0.5% BSA for 12 hours, and then stimulated with 50 ng/ml human VEGF (HumanZyme) or 500 ng/ml human Sema3E (R&D) according to the manufacturer's protocol. After 30 minutes, cells were fixed, blocked, and stained with Alexa488-phalloidin, anti-RhoJ Ab, and TO-PRO-3.

Live-cell imaging. HUVECs cultured on a 0.1% gelatin-coated culture dish (Iwaki) were imaged using an Olympus IX-81 inverted microscope. To monitor the cell shape, a series of time-lapse images were converted into video format using MetaMorph software (Molecular Devices). For monitoring HUVECs ectopically expressing EGFP and RhoJ-WT, images were obtained every 3 minutes 12 hours after the transfection of plasmid vectors. For HUVECs stimulated with Sema3E (500 ng/ml) after 12 hours' serum starvation, images were obtained every 30 seconds. Stealth siRNA was transfected 48 hours prior to the serum starvation. Scale bar: 50 μm.

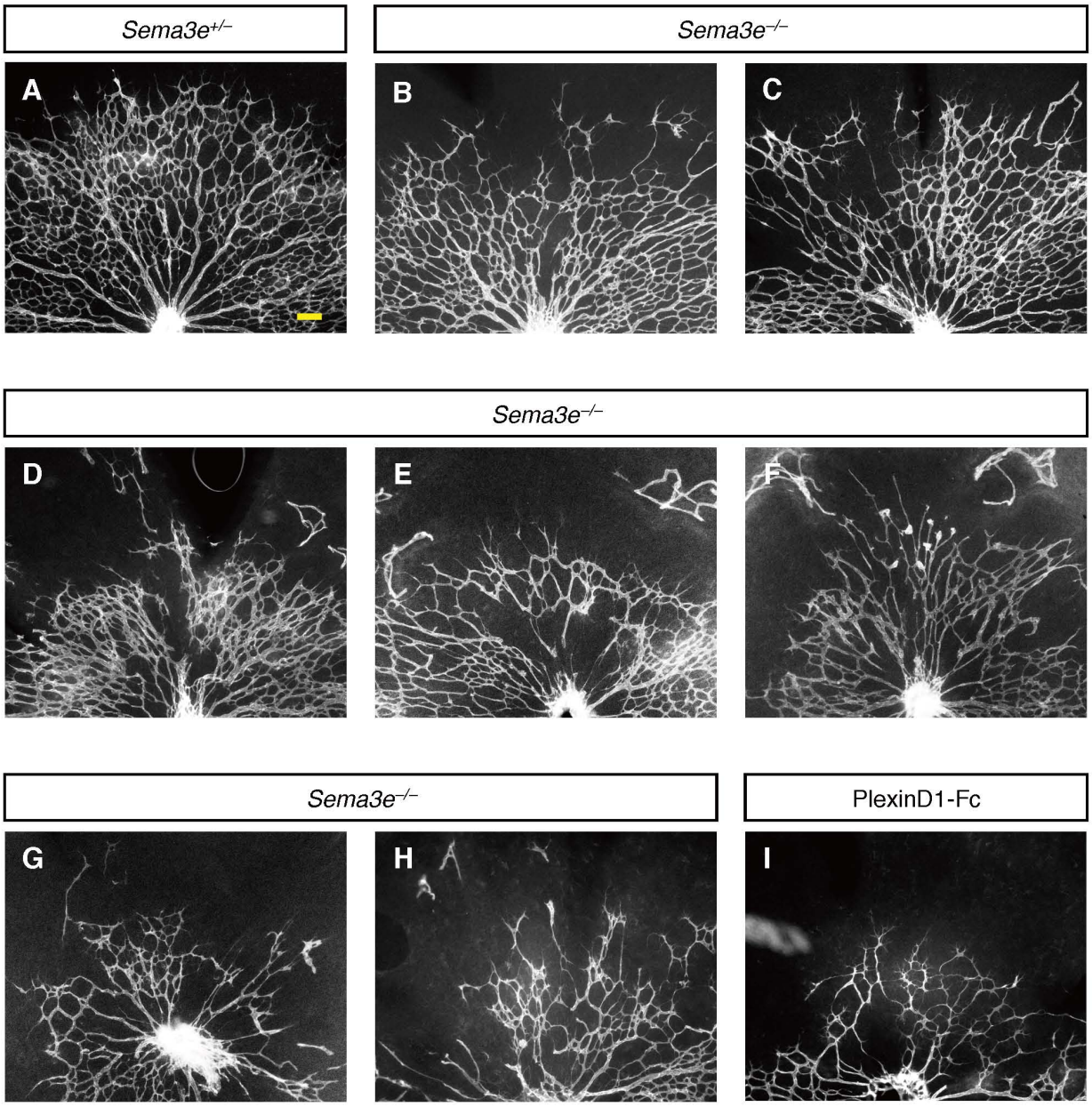
Supplemental Movies

S1 Time-lapse monitoring of merged DIC and fluorescence microscopic images of HUVECs transfected with plasmid vectors expressing RhoJ-WT and EGFP.
S2 Time-lapse monitoring of serum-starved HUVECs.
S3 Time-lapse monitoring of Sema3E-stimulated HUVECs after serum starvation.
S4 Time-lapse monitoring of Sema3E-stimulated HUVECs pretreated by RhoJ siRNA.

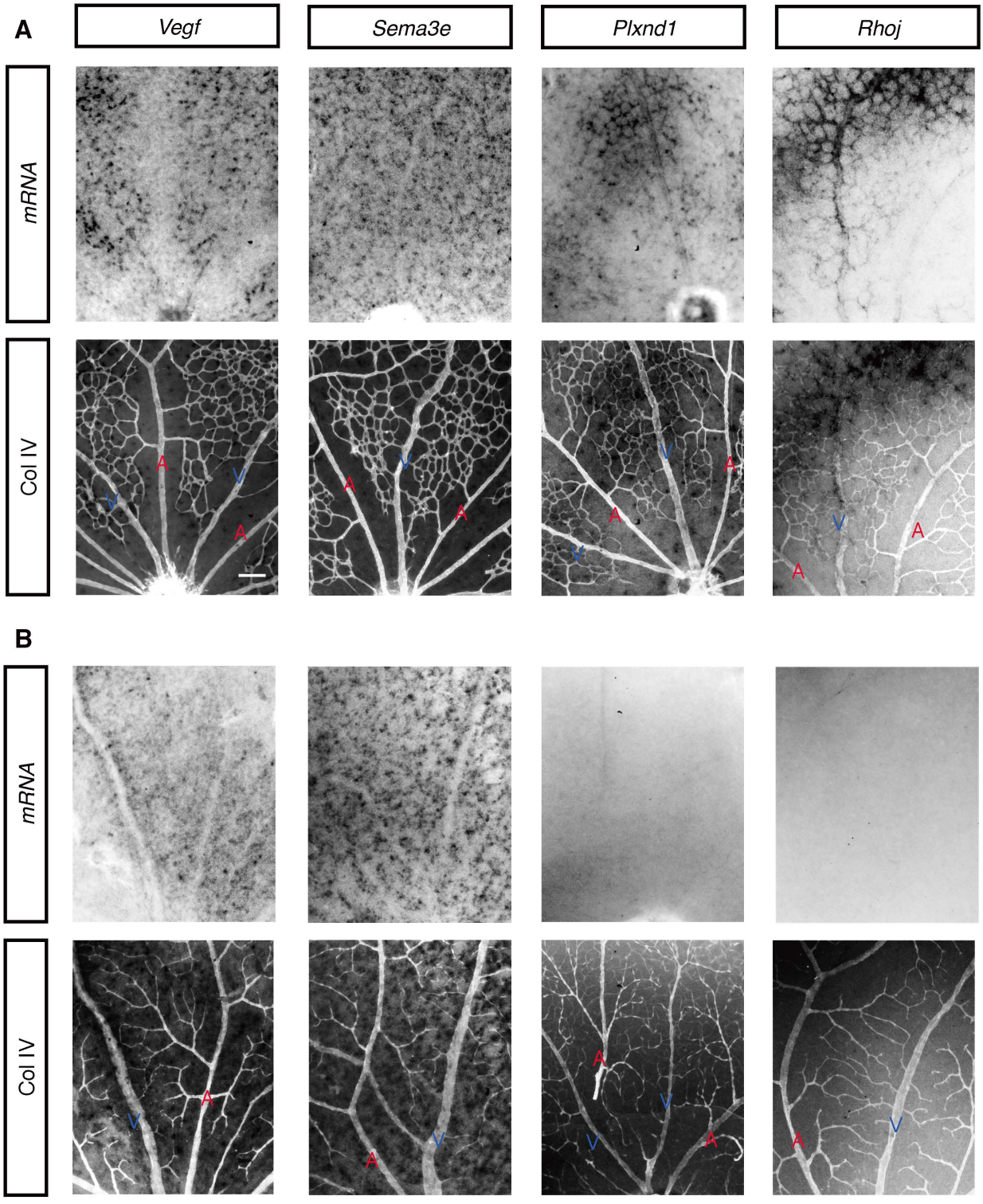


Supplemental Figure S1

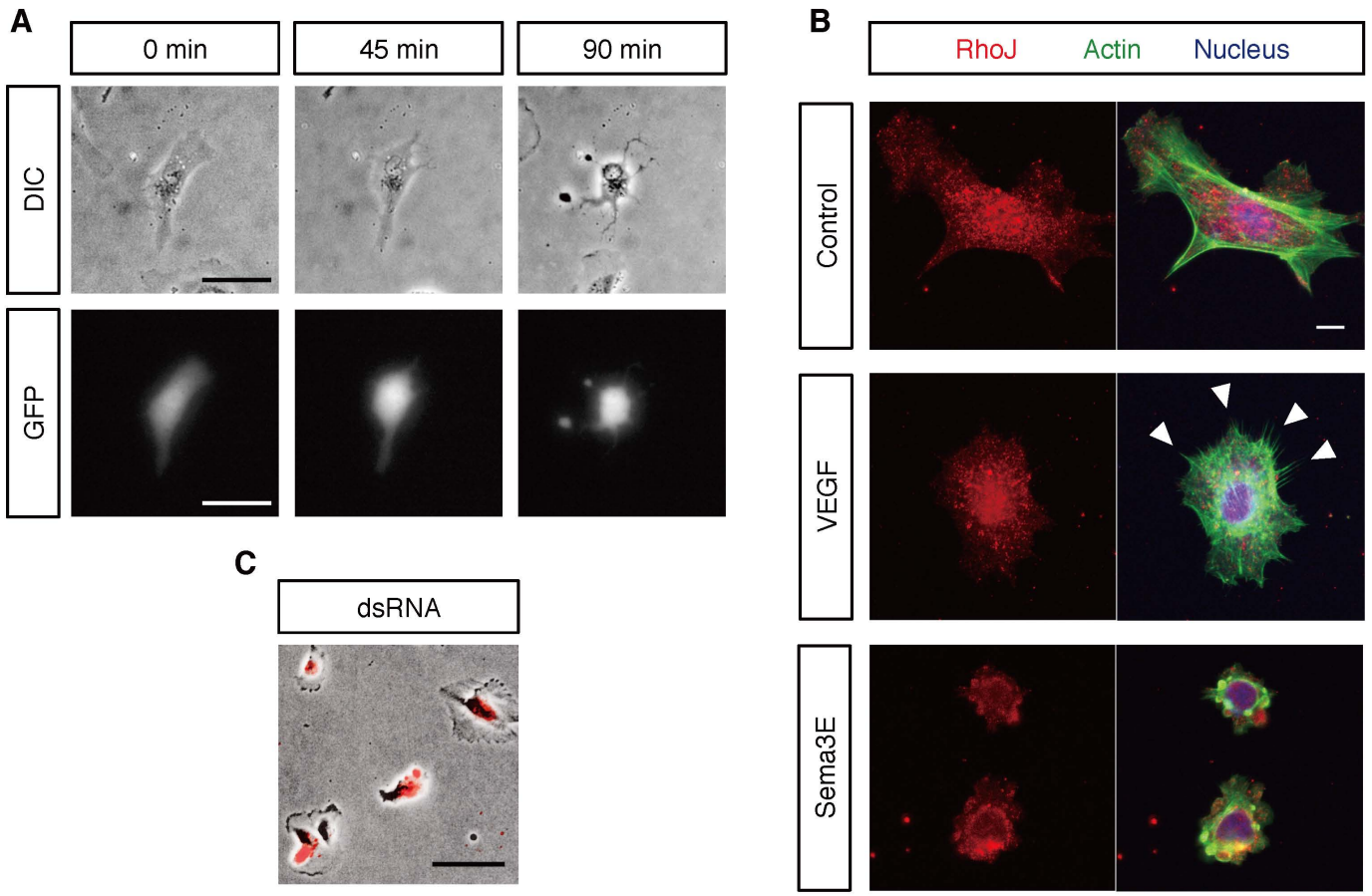
Downregulation of endothelial PlexinD1 expression after blocking VEGF signaling. (**A**) Whole-mount in situ hybridization (ISH) for *Plxnd1* and immunohistochemistry (IHC) for type IV collagen (Col IV) at 2, 6, and 7 hours after intravitreal protein injection in P4 retinas. After VEGFR1-Fc injection, endothelial *Plxnd1* expression was downregulated at around 7 hours, and became undetectable at 8 hours as demonstrated in Figure 1E. (**B**) Double IHC for Col IV and PECAM-1 at 8 hours after intravitreal protein injection in P4 retinas. Maintenance of endothelial networks after VEGFR1-Fc injection excludes the possibility that vessel regression is responsible for the *Plxnd1* downregulation. Scale bar: 100 μm.



Supplemental Figure S2
Defective vascular development in *Sema3e*-deficient retinas. (**A**-**I**) Whole-mount IHC for PECAM-1 in P4 retinas. *Sema3e*$^{-/-}$ retinas display a variety of vascular defects, including solitary clusters of retinal ECs isolated from the advancing vascular fronts (**B** and **C**), attachment of hyaloid vessels to the retinal surfaces (**C**-**H**), intraretinal vascular tufts (**F**), and poor development of morphologically distinct arteries and veins (**D**-**H**). Note that the disorganized capillary networks in *Sema3e*$^{-/-}$ retinas (**G** and **H**) are recapitulated by those in P4 retinas injected with PlexinD1-Fc at P1 (**I**). Scale bar: 100 μm.

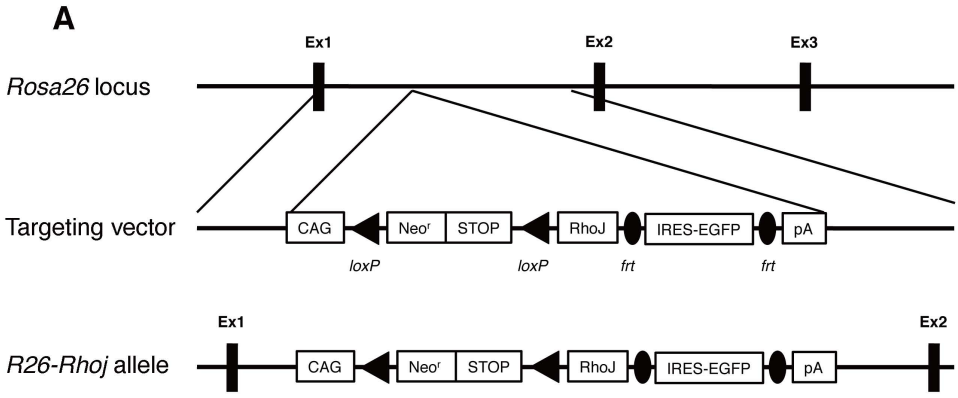


Supplemental Figure S3
Expression of endothelial PlexinD1 and RhoJ at later postnatal stages. (**A** and **B**) Whole-mount ISH in conjunction with IHC for Col IV in P7 (**A**) and P16 (**B**) retinas. At P7, endothelial expression of the *Plxnd1* and *Rhoj* genes is maintained in veins and capillaries, whereas their expression is almost undetectable at P16. Note the constitutive *Sema3e* expression in neurons at P16. A, artery; V, vein. Scale bar: 100 μm.



Supplemental Figure S4

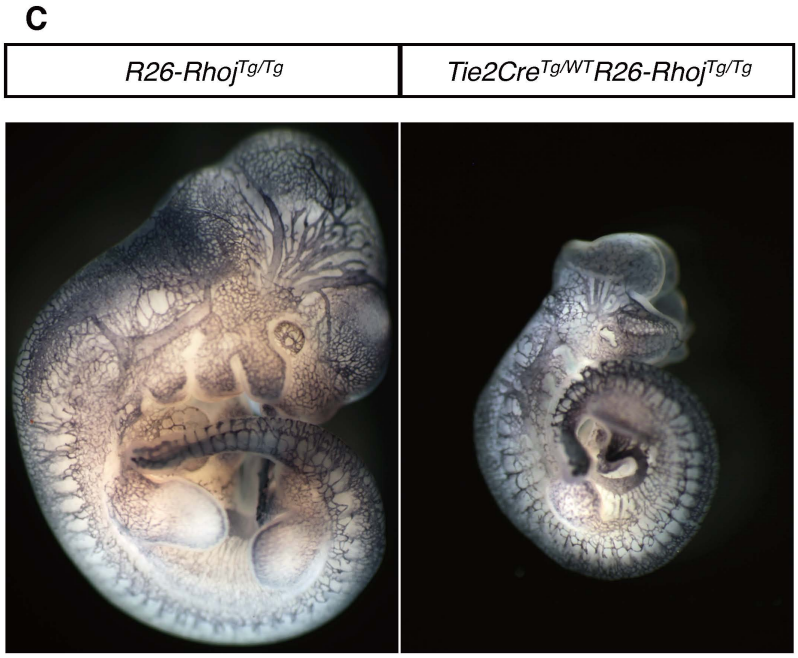
RhoJ expression in cultured HUVECs. (**A**) Sequential time-lapse images of cell contraction 12 hours after co-transfection of plasmid vectors expressing RhoJ-WT and EGFP. DIC, differential interference contrast microscope. (**B**) Intracellular localization of RhoJ proteins. By contrast to the cytoplasmic localization in the control and VEGF-stimulated HUVECs, RhoJ proteins are localized in the membrane blebs after Sema3E stimulation. Note the absence of RhoJ proteins in VEGF-induced filopodia (arrowheads) projecting toward the higher VEGF concentration. (**C**) Merged image of DIC and fluorescence microscopes 24 hours after transfection of BLOCK-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen) in cultured HUVECs. Note the ubiquitous transfection of double-stranded RNA (dsRNA). Scale bar: 50 μm (**A**); 10 μm (**B**); 100 μm (**C**).

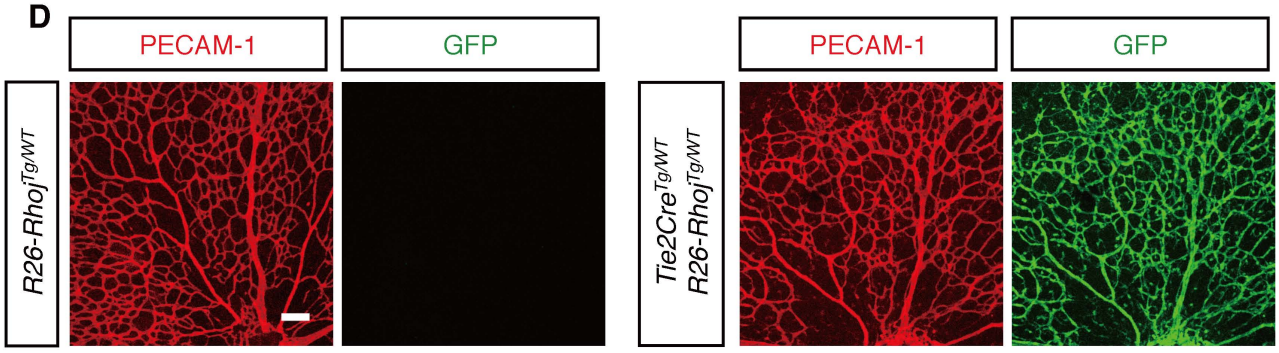


B

Offsprings of *Tie2Cre*$^{Tg/WT}$*R26-Rhoj*$^{Tg/WT}$ male and *R26-Rhoj*$^{Tg/Tg}$ female mice

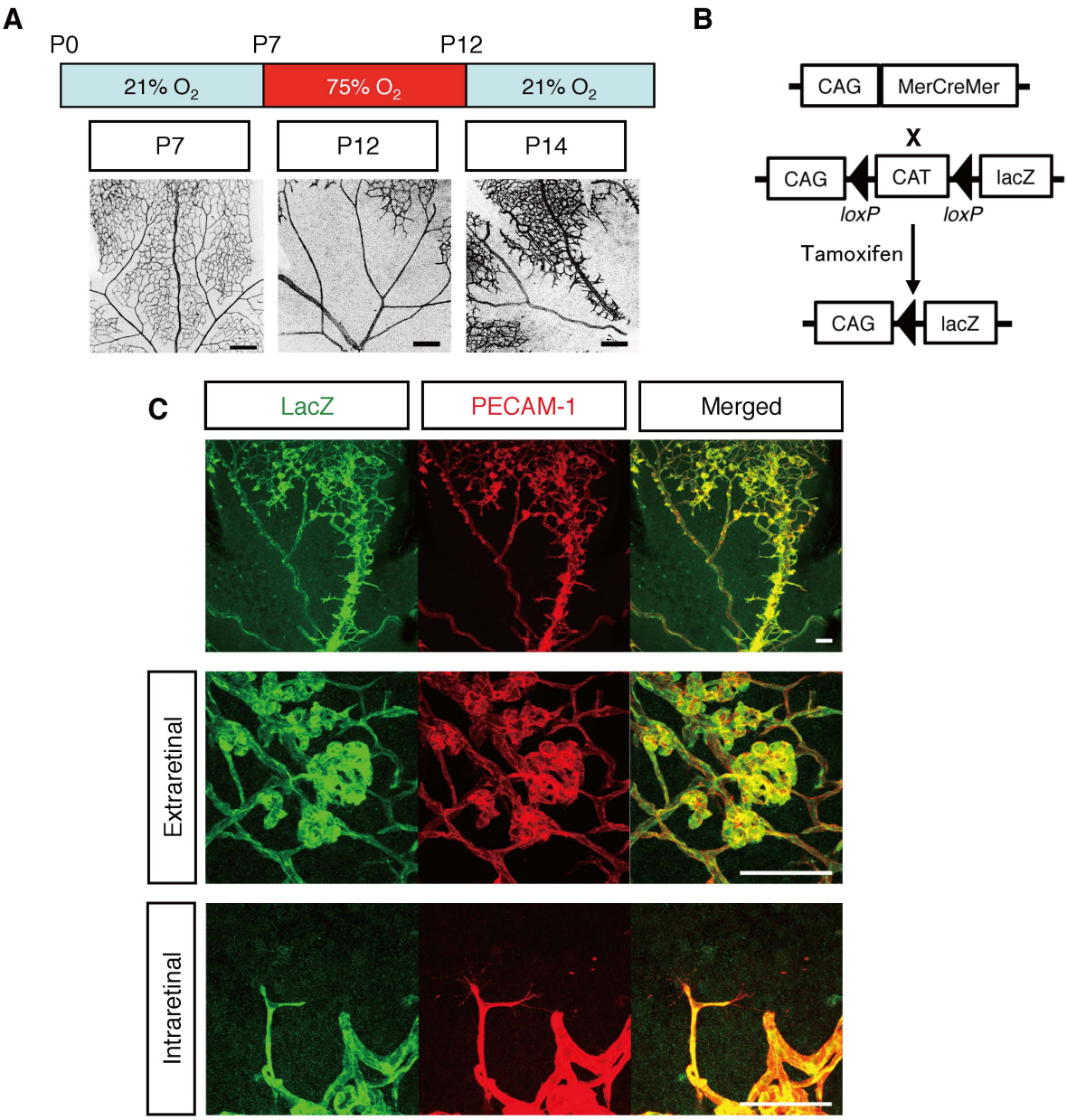
Genotype	*n*	%
R26-Rhoj$^{Tg/WT}$	45	41
R26-Rhoj$^{Tg/Tg}$	48	44
Tie2Cre$^{Tg/WT}$*R26-Rhoj*$^{Tg/WT}$	16	15
Tie2Cre$^{Tg/WT}$*R26-Rhoj*$^{Tg/Tg}$	0	0
Total	109	100





Supplemental Figure S5

***R26-Rhoj* conditional Tg mouse.** (**A**) Schematic representation of the targeting strategy for the *R26-Rhoj* allele. RhoJ and EGFP are expressed under control of the *CAG* promoter after Cre-*loxP*-mediated deletion of the Neo^r-STOP cassette. (**B**) Genotypes of newborn pups obtained from mating *Tie2Cre*$^{Tg/WT}$*R26-Rhoj*$^{Tg/WT}$ male with *R26-Rhoj*$^{Tg/Tg}$ female mice. Out of 16 *Tie2Cre*$^{Tg/WT}$*R26-Rhoj*$^{Tg/WT}$ newborns, 5 pups (31%) demonstrated general growth retardation. (**C**) Whole-mount IHC for PECAM-1 in *R26-Rhoj* conditional Tg embryos at 10.5 dpc. (**D**) Whole-mount IHC for PECAM-1 and GFP in P5 retinas of *R26-Rhoj* mice. GFP is uniformly expressed in ECs of the *Tie2Cre;R26-Rhoj* double Tg mice. Scale bar: 100 μm.

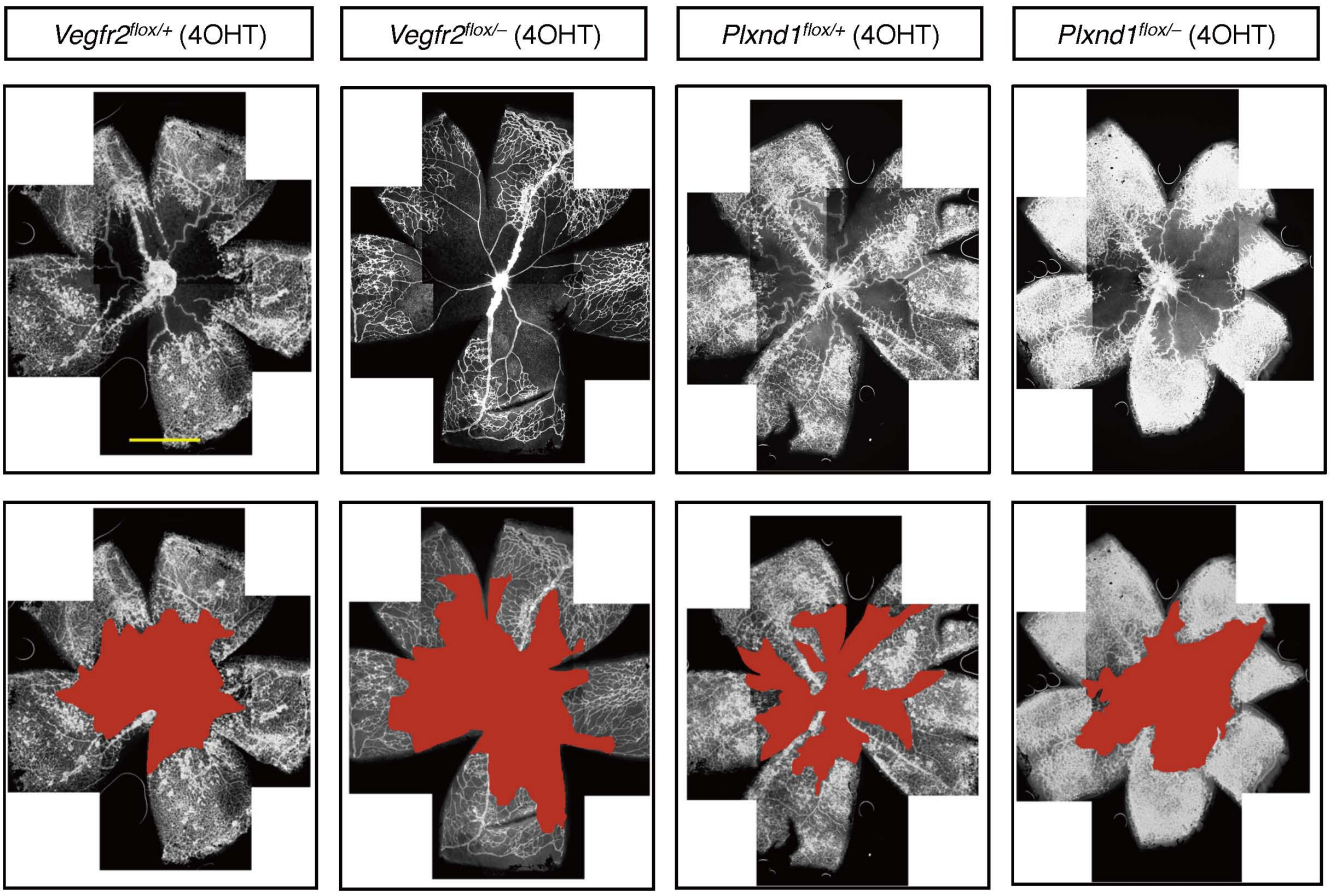


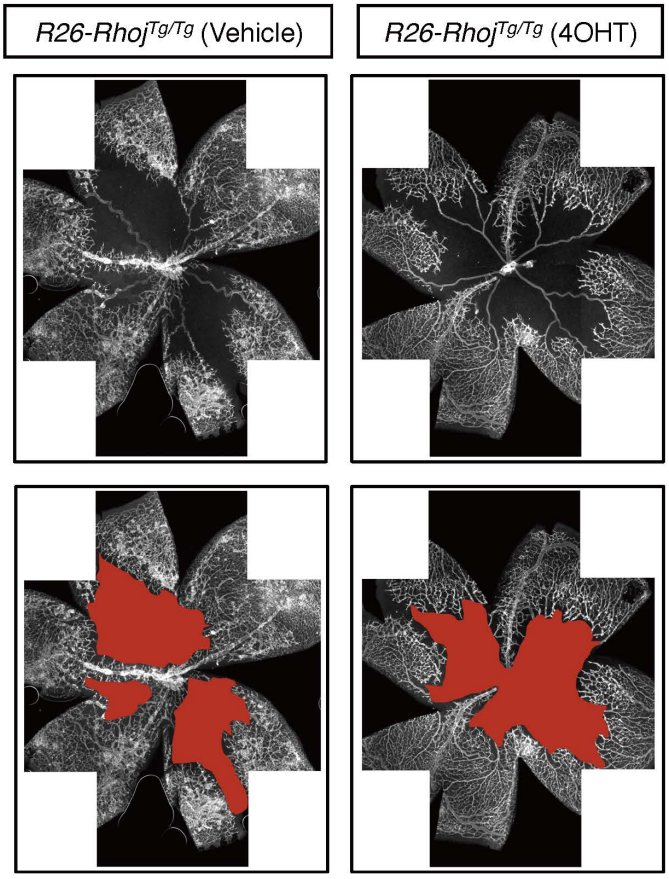
Supplemental Figure S6

Tamoxifen-inducible Cre-*loxP*-mediated genetic recombination in the OIR model. (**A**) Whole-mount IHC for PECAM-1 illustrating the establishment of the OIR model. Exposure to 75% oxygen from P7 to P12 leads to the regression of preformed retinal capillaries followed by vascular regrowth to the ischemic areas. (**B**) Representation of the tamoxifen-inducible *loxP* recombination in the *CAG-MerCreMer* Tg mouse bred with the *CAG-CAT-lacZ* Tg mouse (1). MerCreMer contains two mutated estrogen receptor-binding domains, which confers dependency on binding to tamoxifen but not 17β-estradiol for translocation to the nucleus and recombinase activity (2). The *CAG-CAT-lacZ* transgene has a *chloramphenicol acetyltransferase* (*CAT*) cassette which is flanked by two *loxP* sites, followed by a *lacZ* cassette. The transgenes are under control of the *CAG* promoter; however, lacZ is expressed only after Cre-*loxP*-mediated genetic recombination. (**C**) Whole-mount IHC for lacZ (Millipore) and PECAM-1 in P18 OIR retinas of the *CAG-MerCreMer*;*CAG-CAT-lacZ* double Tg mice after daily i.p. injections of 4-hydroxytamoxifen (4OHT) from P12. Note the uniform *loxP* recombination represented by lacZ expression in the ECs of the extraretinal and intraretinal vessels. Scale bar: 200 μm (**A**); 100 μm (**C**).

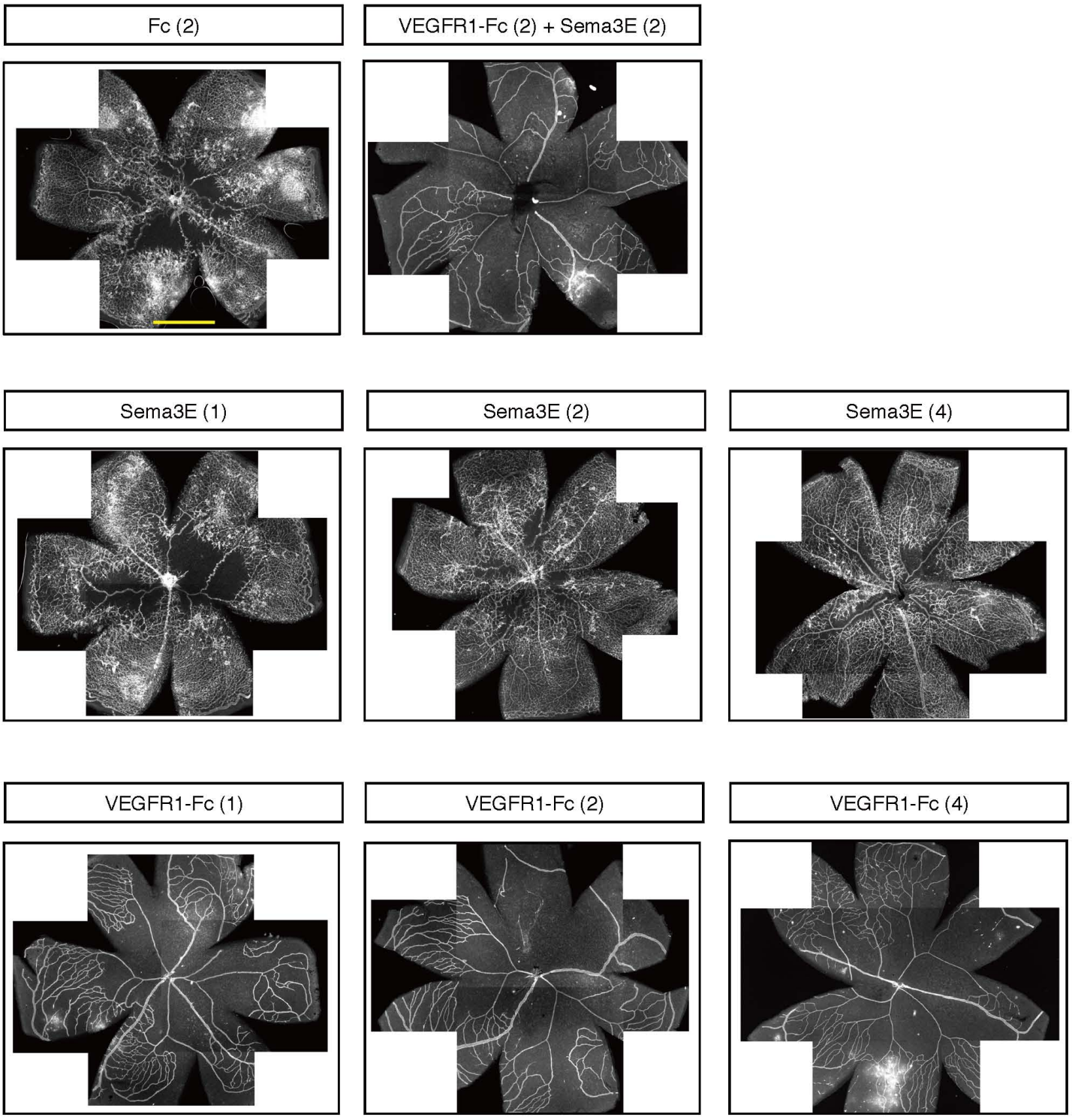
References

1. Araki K, Araki M, Miyazaki J, Vassalli P. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. *Proc Natl Acad Sci U S A.* 1995;92(1):160-164.
2. Zhang Y, Riesterer C, Ayrall AM, Sablitzky F, Littlewood TD, Reth M. Inducible site-directed recombination in mouse embryonic stem cells. *Nucleic Acids Res.* 1996;24(4):543-548.

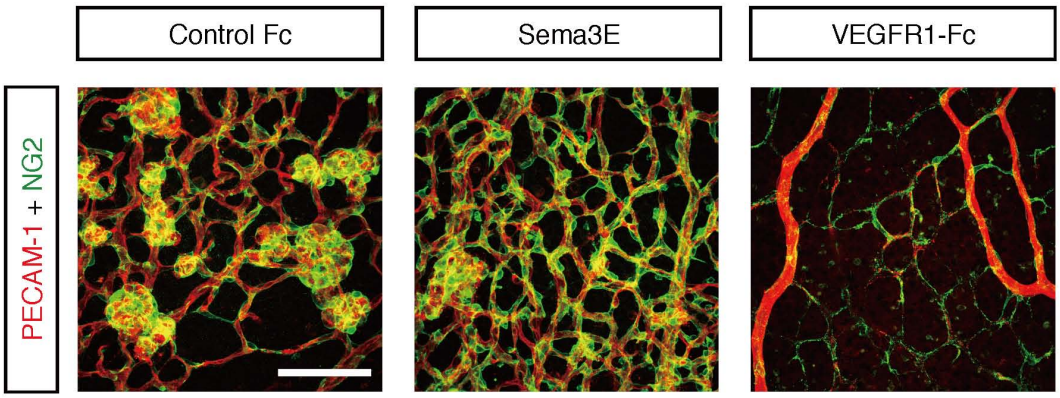




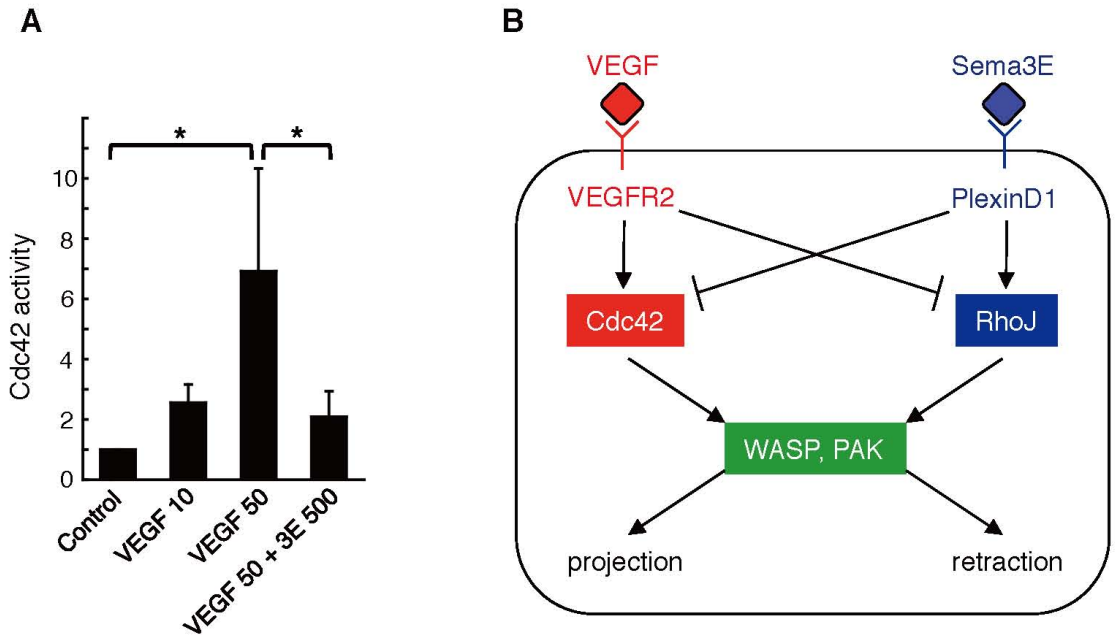
Supplemental Figure S7
Unvascularized retinal areas after tamoxifen-inducible Cre-*loxP* genetic recombination in the OIR model. Whole-mount PECAM-1 IHC in P18 OIR retinas of *CAG-MerCreMer* mice carrying *Vegfr2-flox*, *Plxnd1-flox*, or *R26-Rhoj* alleles after daily i.p. injections of 4OHT or vehicle from P12. The unvascularized retinal areas represented in red were measured for the morphometric analyses. Scale bar: 1 mm.



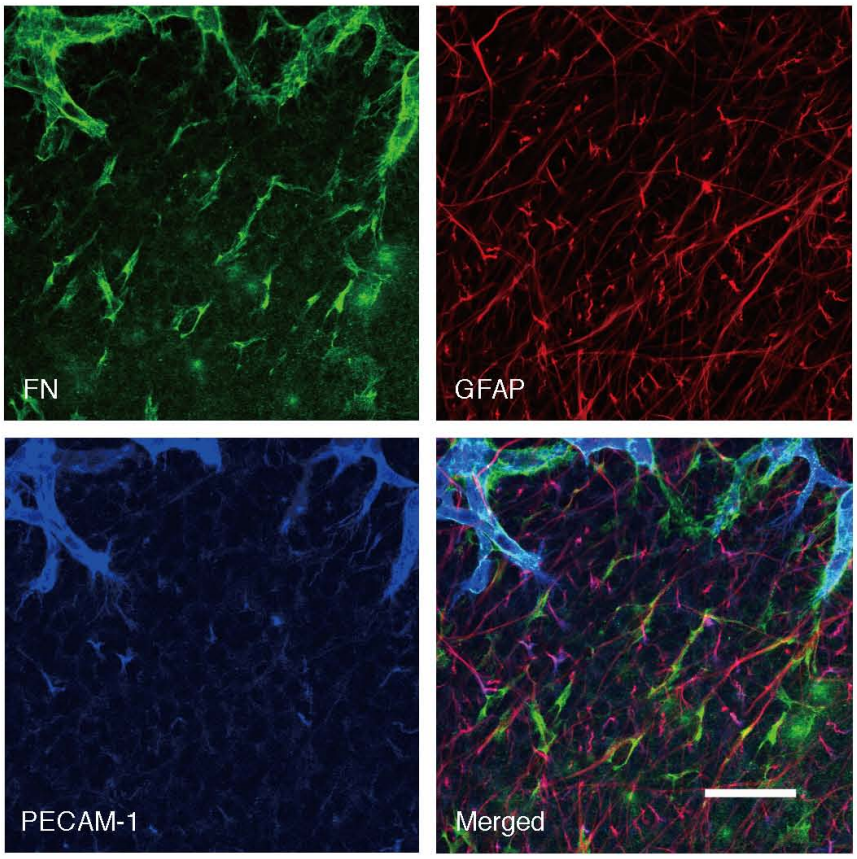
Supplemental Figure S8
Effects of intravitreal protein injections in the OIR model. Panoramic views of whole-mount PECAM-1 IHC in P17 OIR retinas after intravitreal protein injections at P14. The protein concentrations (μg/μl) are indicated in the parentheses. In retinas co-injected with VEGFR1-Fc and Sema3E, the vascular phenotypes were identical to those injected with VEGFR1-Fc alone. Scale bar: 1 mm.



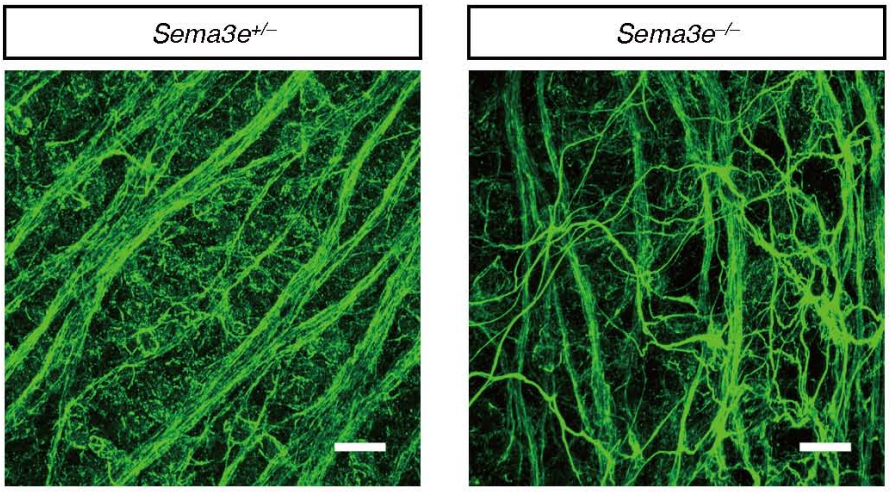
Supplemental Figure S9
Pericyte coverage in the OIR model. Double IHC for PECAM-1 and NG2 in P17 OIR retinas 72 hours after intravitreal injections of 2 μg/μl recombinant proteins. The photographed areas are the same as the lower panels in Figure 7B. After Sema3E injection, most of intraretinal vessels were associated with NG2-expressing periendothelial mural cells. By contrast, after VEGFR1-Fc injection, pericytes formed ghost vessels without PECAM-1-expressing ECs, implying endothelial degeneration. Scale bar: 100 μm.



Supplemental Figure S10
Cdc42 and RhoJ as the downstream targets of VEGF and Sema3E signals in endothelial filopodia formation. (**A**) G-LISA Cdc42 activation assay with anti-Cdc42 Ab (Cytoskeleton Inc.). VEGF-induced Cdc42 activation in cultured HUVECs is diminished by exposure to Sema3E. The values 10, 50, 500 indicate the protein concentrations (ng/ml). Error bars represent SEM; $*P < 0.05$. (**B**) Schematic diagram representing the signaling pathways involved in endothelial filopodia formation.



Supplemental Figure S11
Defective extracellular assembly of fibronectin matrices by retinal astrocytes in the OIR model. Whole-mount IHC for fibronectin (FN, Dako), glial fibrillary acidic protein (GFAP, clone G-A-5, Cy3 conjugated, Sigma), and PECAM-1 in P16 OIR retina. FN proteins are expressed only sporadically in GFAP-expressing astrocytes. Scale bar: 50 μm.



Supplemental Figure S12
Disordered axonal fibers in retinas of *Sema3e*-deficient mice. Whole-mount IHC for neurofilaments in retinas of P4 *Sema3e*-deficient mice. Note the transverse axonal bundling and/or branching in small areas of the *Sema3e*$^{-/-}$ retina compared with the parallel axon alignment in the *Sema3e*$^{+/-}$ retina. Scale bar: 20 μm