Supplemental material

Endothelial alpha-parvin controls integrity of developing vasculature and is required for maintenance of cell-cell junctions

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Running title: α-pv in vessel development

Detailed Methods

Antibodies.

The following antibodies were used: Anti-α-pv (Cell Signaling, 4026), Cy3-conjugated αSMA (Sigma, A2547), anti-β-catenin (Sigma, C2206), anti-BrdU (Invitrogen, 03-3900), anti-CD31 (PharMingen, 553370), anti-Claudin 5 (Invitrogen, 34-1600), anti-Collagen IV (BioRad, 2150-1470), anti-Erg1/2/3 (Santa Cruz, sc-353), anti-GAPDH (Millipore, MAB374), anti-ICAM2 (PharMingen, 553326), anti-Paxillin (BD Biosciences, 610051), anti-phospho-Paxillin (Cell Signaling, 2541), anti-NG2 (Millipore, AB5320), anti-Vinculin (Sigma, V9131), anti-VE-Cadherin (eBioscience, 14-1442-82), anti-VE-Cadherin (eBioscience, 14-1449-82) and anti-cleaved-caspase-3 (Cell Signaling, 9661). For secondary detection, species-specific Alexa Fluor-coupled secondary antibodies (Invitrogen) were used. Alexa-488-conjugated Isolectin-B4 (Life Technologies, I21411) was used to visualize the endothelium in the retinas. Alexa-546-conjugated Phalloidin (Invitrogen, A22283) was used to detect F-actin.

Whole embryo immunohistochemistry.

Staged embryos were dissected in PBS and their genotype determined by PCR. Yolk sacs and skin were fixed overnight in fixation buffer (80% methanol, 20% DMSO). Samples were rehydrated in 0.1% Tween-20 in PBS, incubated in blocking buffer (10% goat serum, 5% BSA in PBS) for 2 hours, and exposed to primary antibodies overnight at 4°C. After 5–7 hours of washing with 0.1% Tween-20 in PBS, samples were incubated with secondary antibodies overnight at 4°C.

Whole retina immunohistochemistry.

Dissection and labeling of retinas was performed as previously described[24]. Briefly, retinas were fixed for 2 hours on ice in 4% paraformaldehyde (PFA), incubated in 1% BSA and 0.3% Triton X-100, washed 2 times in Pblec (1% Triton X-100, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ PBS [pH 6.8]), and incubated overnight with isolectin-B4 and antibodies diluted in Pblec.

Proliferation assay.

Labeling of proliferating cells was performed as previously described[24]. Briefly, 300 μg of Bromodeoxyuridine (BrdU) per pup was injected intraperitoneally 4 hours before sacrifice. Following Erg1/2/3 and isolectin-B4 staining, retinas were fixed for 30 minutes in 4% PFA, washed 3 times with

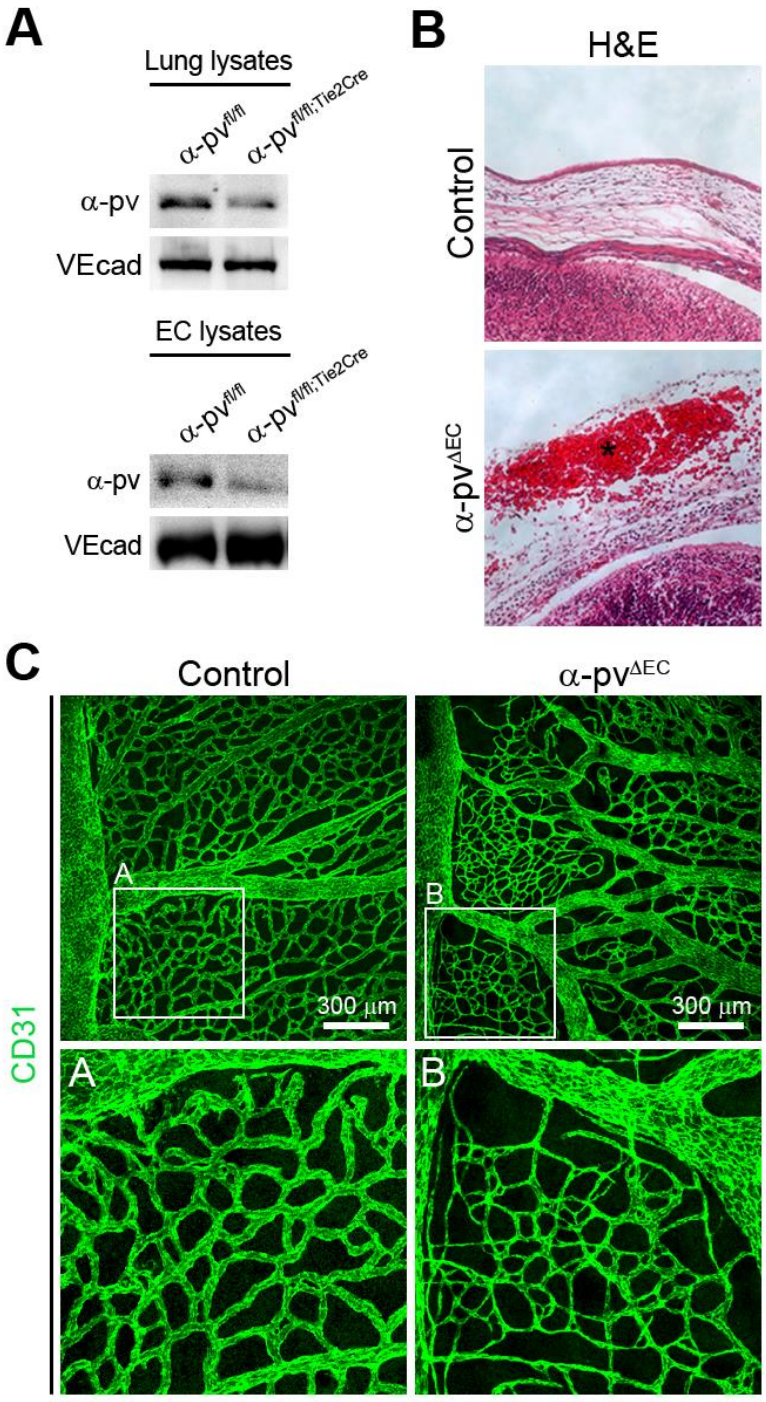
PBS, incubated for 1 hour in 6 M HCl and 0.1% Triton X-100, washed 5 times in PBS plus 0.1% Triton X-100, blocked, and incubated overnight with an anti-BrdU antibody.

SDS-PAGE and immunoblotting.
Tissues and cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris pH 7,4, 1 mM EDTA, 1% Triton X-100, supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Sigma)), homogenized in Laemmli sample buffer and boiled for 5 minutes. Lysates were resolved by SDS-PAGE gels. Proteins were then electrophoretically transferred from gels onto nitrocellulose membranes followed by incubation with antibodies. Bound antibodies were detected using enhanced chemiluminescence (Millipore).

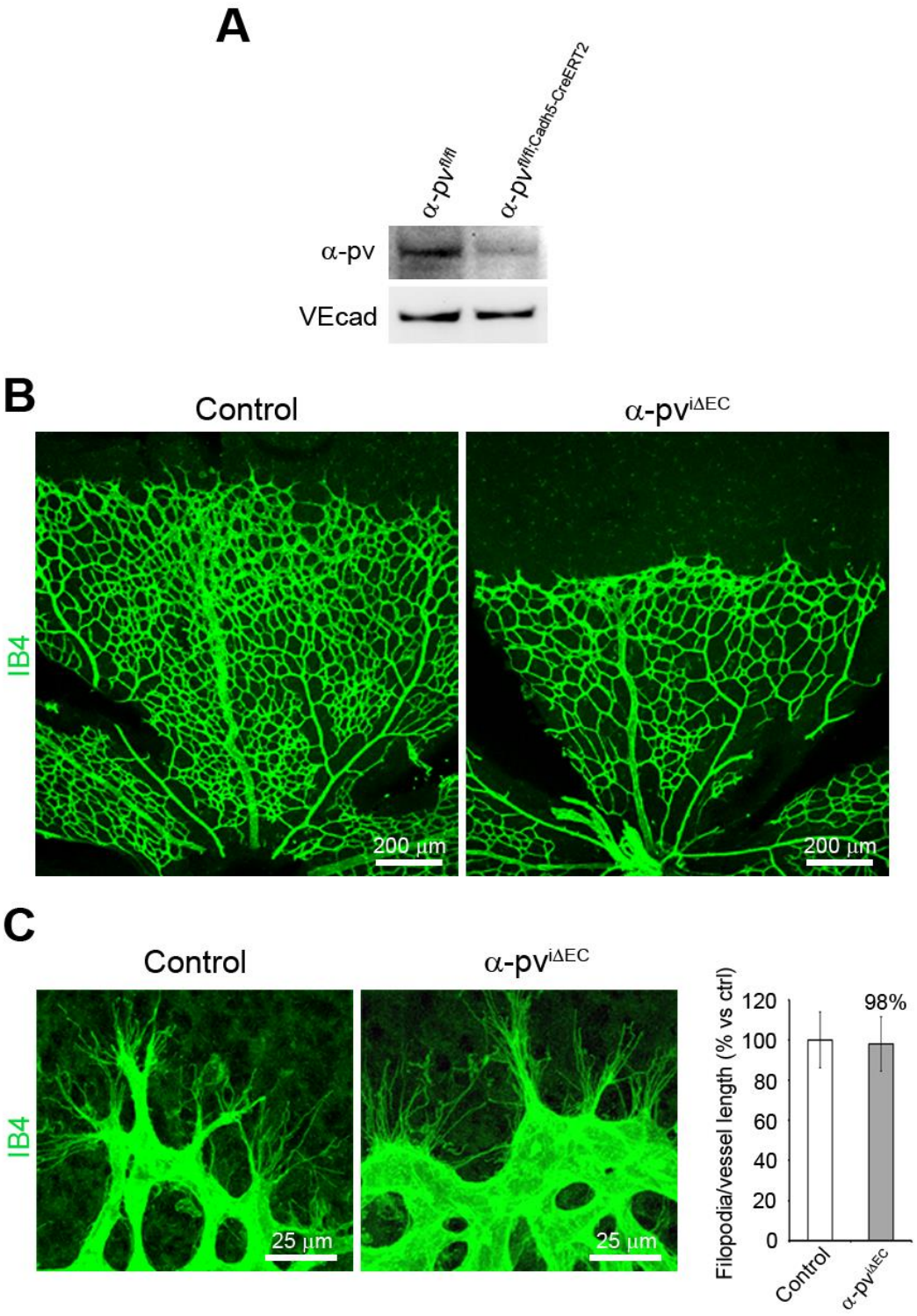
Rac activation assay.
Determination of Rac activity was performed as previously described[21].

Lentiviral gene transductions and live-cell fluorescence microscopy.
For lentiviral transductions α-pv was recloned from peGFP-c1 vector into the pLV-CMV-ires-puro vector using SnaBI and NheI restriction sites. Mouse α-catenin-mCherry was cut out of a pmCherry-c1 vector using NdeI and XbaI restriction enzymes and cloned into the pLV-CMV-ires-puro vector using NdeI and NheI restriction sites. Lentiviral particles were isolated from the supernatant of human embryonic kidney 293 cells (HEK293T) transiently transfected with third-generation packaging constructs and the lentiviral expression vectors. HUVECs, cultured to 80% confluency, were infected with supernatant containing lentiviral particles overnight.
For live microscopy cells were plated on Lab-Tek chambered 1.0 borosilicated coverglass slides coated with 5 μg/ml fibronectin and imaged within microscope incubators at 37°C and 5% CO2. Widefield imaging was performed on an inverted Zeiss widefield Observer.Z1 microscope equipped with a 63x 1.40 Plan Apochromat oil objective, definite focus system, and Hamamatsu Orca-R2 digital camera. Images were enhanced for display with an unsharp mask filter and adjusted for brightness/contrast in ImageJ.

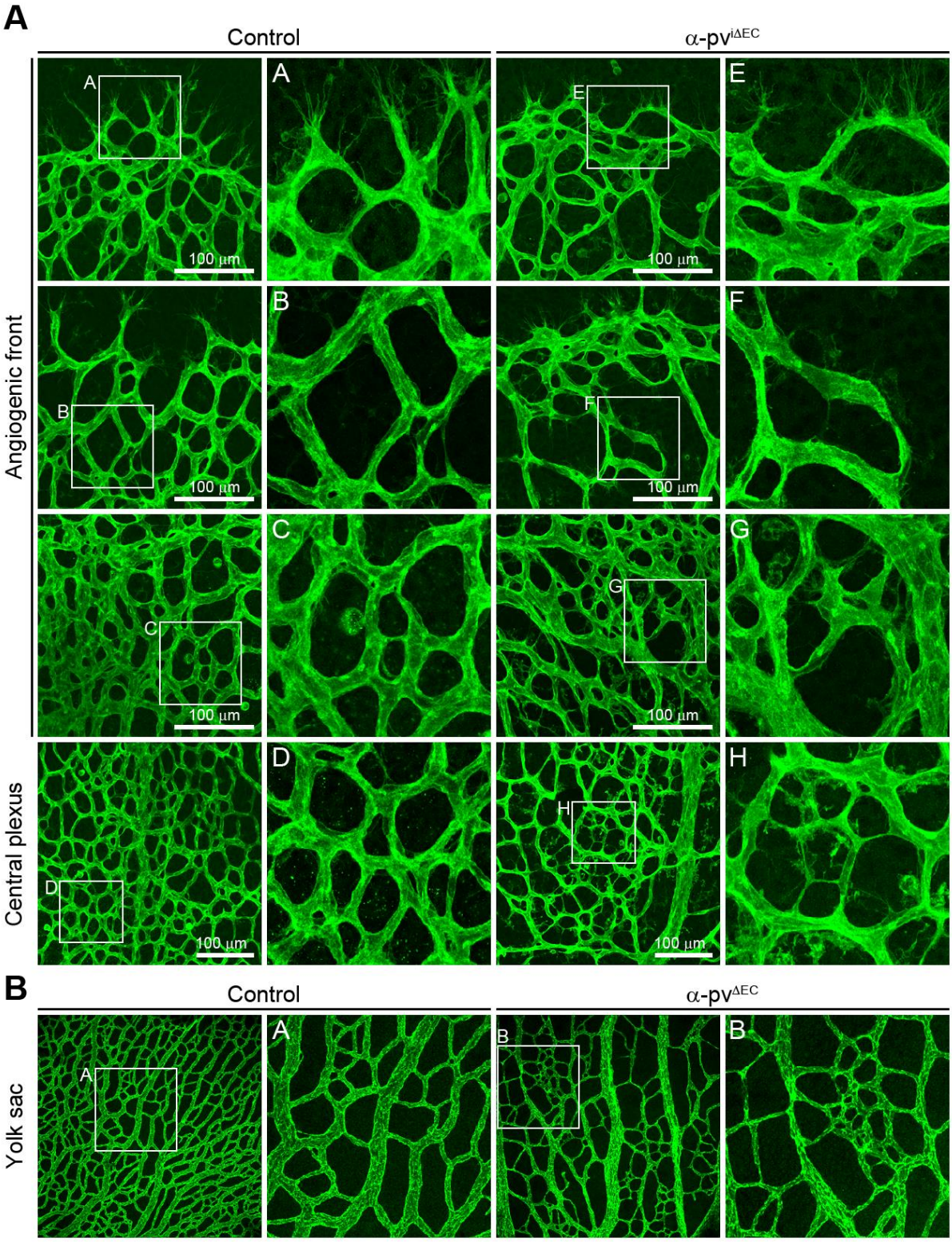
Online Figures



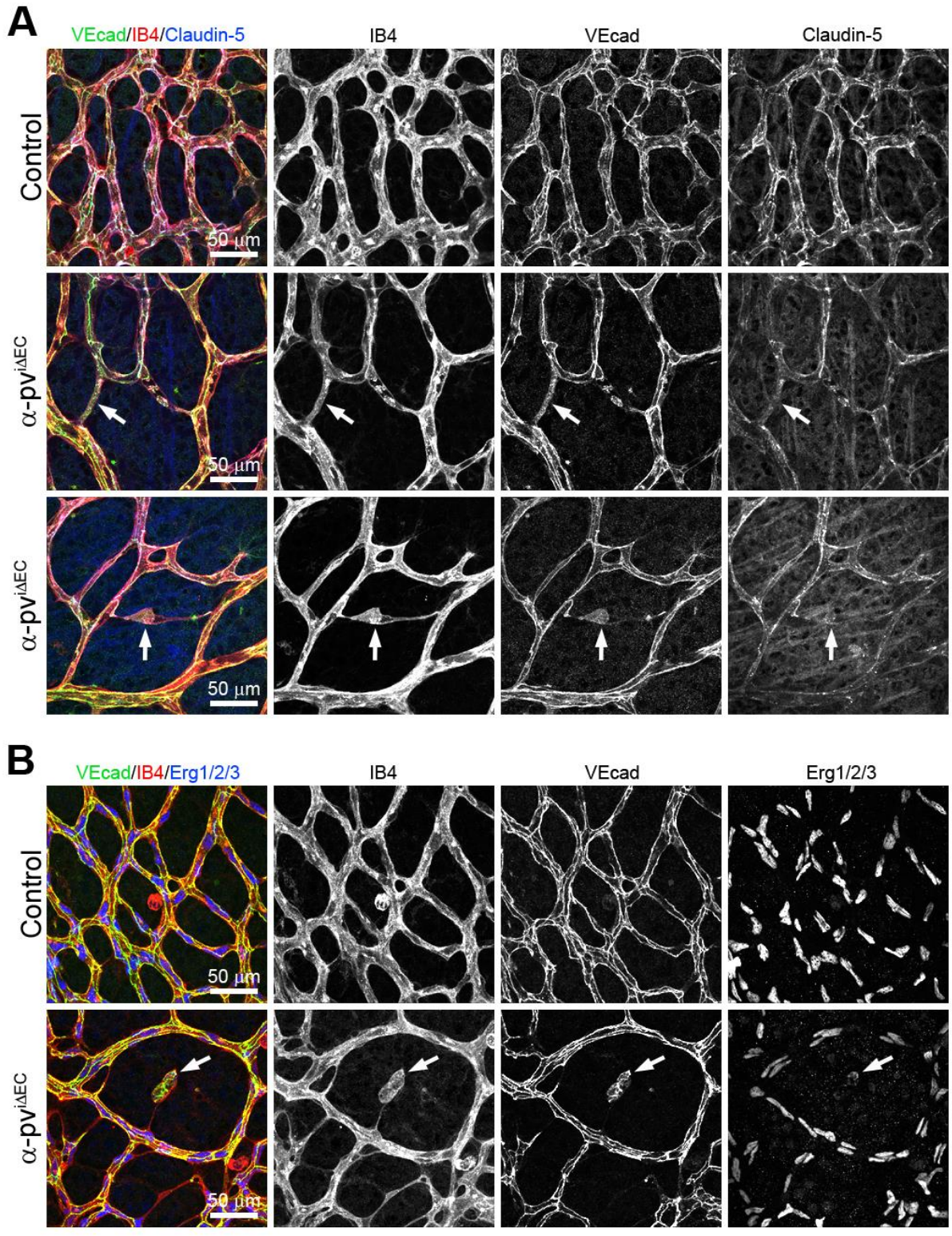
<u>Online Figure I.</u> Tie2-Cre-mediated deletion of *α-pv* gene. (A) Western blot of α-pv protein levels from lysates prepared from whole lungs and ECs isolated from E13.5 control and α-pv$^{\Delta EC}$ embryos. VE-cadherin was used as a loading control. (B) Hematoxylin and eosin staining of sagittal sections through the head region of E15.5 control and α-pv$^{\Delta EC}$ embryos. Hemorrhage (asterisk). (C) CD31 whole-mount immunostaining of E15.5 control and α-pv$^{\Delta EC}$ YS.



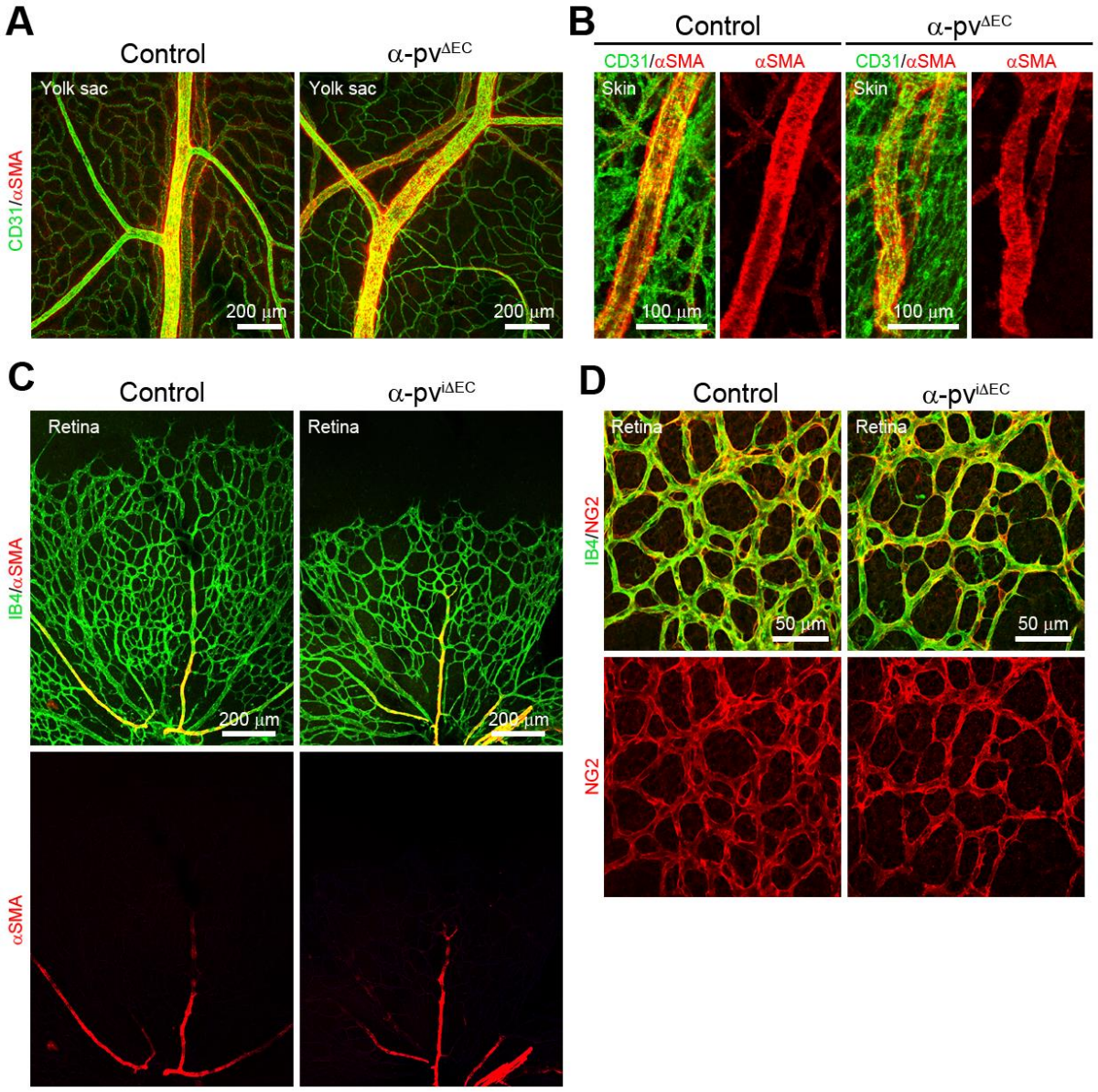
<u>Online Figure II.</u> Cadh5(PAC)-Cre^{ERT2}-mediated deletion of *α-pv* gene. (A) Western blot analysis of lung lysates from P6 control and α-pv^{iΔEC} mice 3 days after tamoxifen administration. VE-cadherin was used as a loading control. (B) P6 control and α-pv^{iΔEC} retinas labeled for IB4. (C) Quantification of number of filopodia per vessel length in the control and α-pv^{iΔEC} retinas. Values represent percentages of means versus controls ± s.e.m.



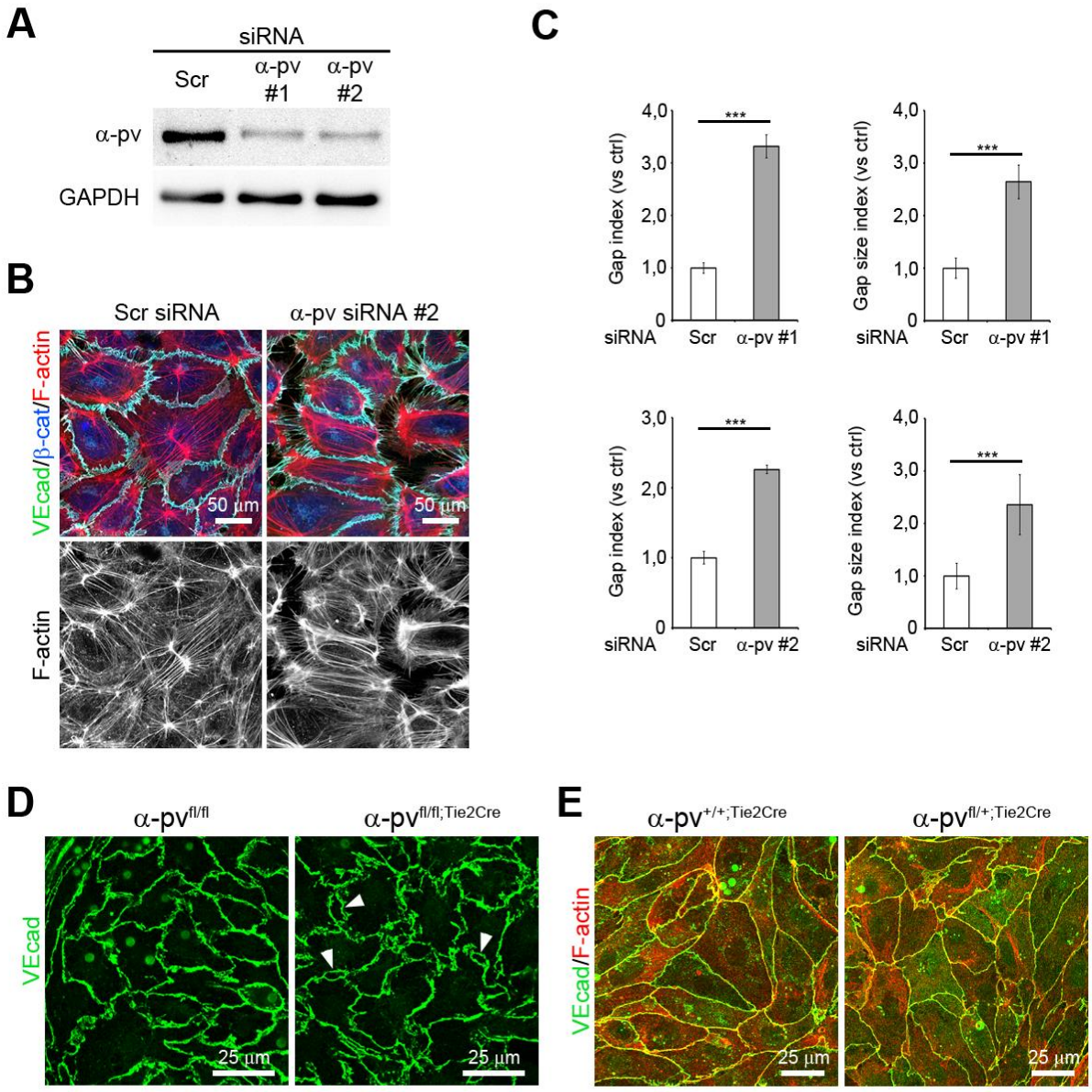
Online Figure III. Abnormal vessel morphology in α-pv$^{i\Delta EC}$ mice and α-pv$^{\Delta EC}$ embryos. (A) P7 control and α-pv$^{i\Delta EC}$ retinas labeled for IB4. (B) E15.5 control and α-pv$^{\Delta EC}$ YSs immunostained for CD31.



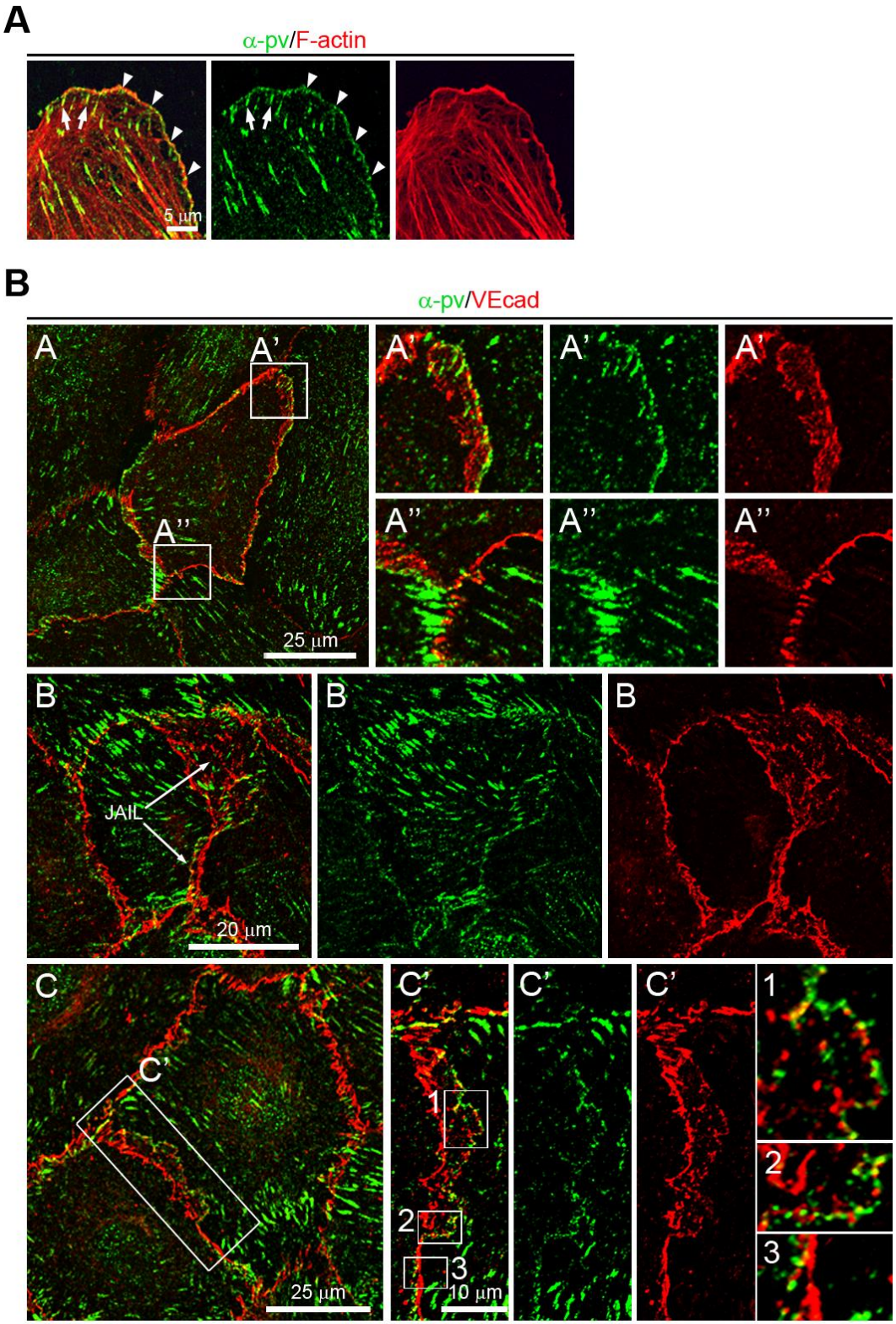
<u>Online Figure IV.</u> Altered cell junction morphology in α-pv^{iΔEC} mice. (A) P6 control and α-pv^{iΔEC} retinas labeled for VE-cadherin, IB4 and claudin-5. Arrows highlight vessel segments with diffuse punctuated VE-cadherin stain. (B) P6 control and α-pv^{iΔEC} retinas labeled for VE-cadherin, IB4 and Erg1/2/3. Arrows point to a fragmented vessel partially disconnected from the vascular bed.



<u>Online Figure V.</u> Mural cell coverage of embryonic and retinal vessels in α-pv$^{\Delta EC}$ and α-pv$^{i\Delta EC}$ mice. CD31 and αSMA whole-mount immunostaining of YS (A) and skin (B) of E15.5 control and α-pv$^{\Delta EC}$ embryos. Whole-mount labeling of P6 control and α-pv$^{i\Delta EC}$ retinas for IB4 and αSMA (C) and IB4 and anti-NG2 (D).



<u>Online Figure VI.</u> siRNA depletion of α-pv in HUVECs. (A) Western blot analysis of α-pv protein levels from lysates prepared from HUVECs transfected with two different siRNAs against α-pv and scrambled control. GAPDH was used as a loading control. (B) Triple-fluorescent labeling for VE-cadherin, β-catenin and F-actin of control and α-pv depleted HUVECs cultured on gelatin for 24 hours. (C) Quantification of the gap index and the gap size index in control and α-pv depleted HUVECs. Values means versus controls ± s.e.m. P values are ≤ 0.001. (D) VE-cadherin immunostaining of primary ECs isolated from α-pv$^{fl/fl}$ and α-pv$^{\Delta EC}$ embryos cultured on gelatin-coated slides for 48 hours. Arrowheads highlight intercellular gaps. (E) Double-fluorescent labeling for VE-cadherin and F-actin of primary ECs isolated from α-pv$^{+/+;Tie2Cre}$ and α-pv$^{fl/+;Tie2Cre}$ embryos cultured on gelatin-coated slides for 48 hours.



Online Figure VII. α-pv localizes at junction-associated intermittent lamellipodia. (A) Double-fluorescent labeling for α-pv and F-actin of HUVECs cultured under sparse conditions on gelatin-coated slides for 24 hours. Arrows point to FAs and arrowheads indicate FXs. (B) Double-immunostaining of α-pv and VE-cadherin of HUVECs cultured under subconfluent conditions on gelatin for 24 hours and treated with sphingosine-1-phophate (0.5 μM) for 10 minutes.

Online Tables

		Genotype (%)					
					Tie2-Cre		
Stage	Total	α-pv$^{+/+}$	α-pv$^{fl/+}$	α-pv$^{fl/fl}$	α-pv$^{+/+}$	α-pv$^{fl/+}$	α-pv$^{fl/fl}$
P1	151	17%	33%	12%	14%	23%	1%*
P21	149	17%	33%	13%	14%	23%	—

* death at birth

Online Table I. **Genotypes of the progeny from α-pv$^{fl/+}$;Tie2-Cre males and α-pv$^{fl/+}$ females intercrosses.**

			Genotype (%)			
					Tie2-Cre	
Stage	Total	Resorb	α-pv$^{fl/+}$	α-pv$^{fl/fl}$	α-pv$^{fl/+}$	α-pv$^{fl/fl}$ (alive)
E13.5	136	4	17%	22%	36%	25% (100%)
E14.5	52	1	25%	21%	31%	23% (91%)
E15.5	128	7	21%	20%	36%	24% (70%)
E17.5	32	2	25%	20%	43%	12% (40%)
E18.5	33	3	27%	25%	33%	15% (40%)

Online Table II. **Genotypes of the progeny from α-pv$^{fl/+}$;Tie2-Cre males and α-pv$^{fl/fl}$ females intercrosses.**

Online Videos

Online Video I. **α-pv is recruited to JAIL.**

Online Video II. **α-pv is required for JAIL formation.**