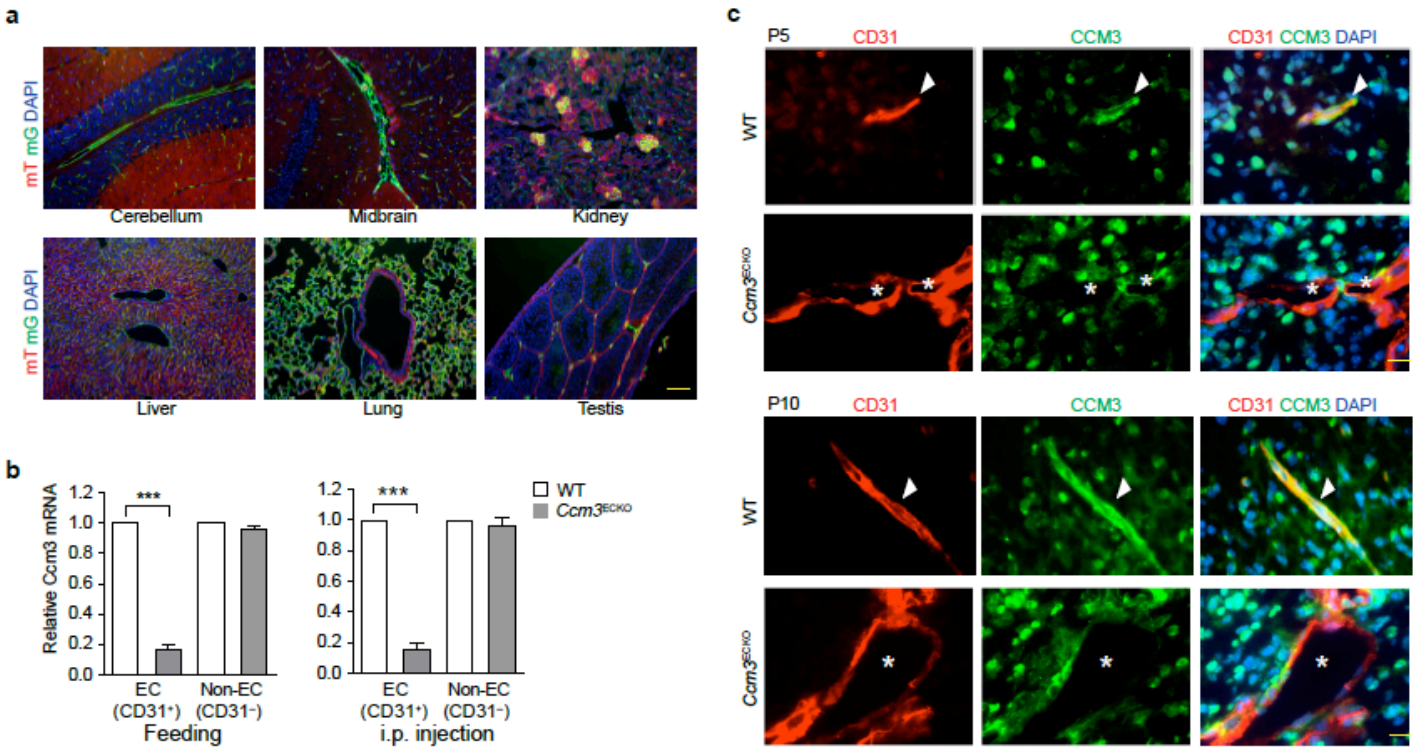
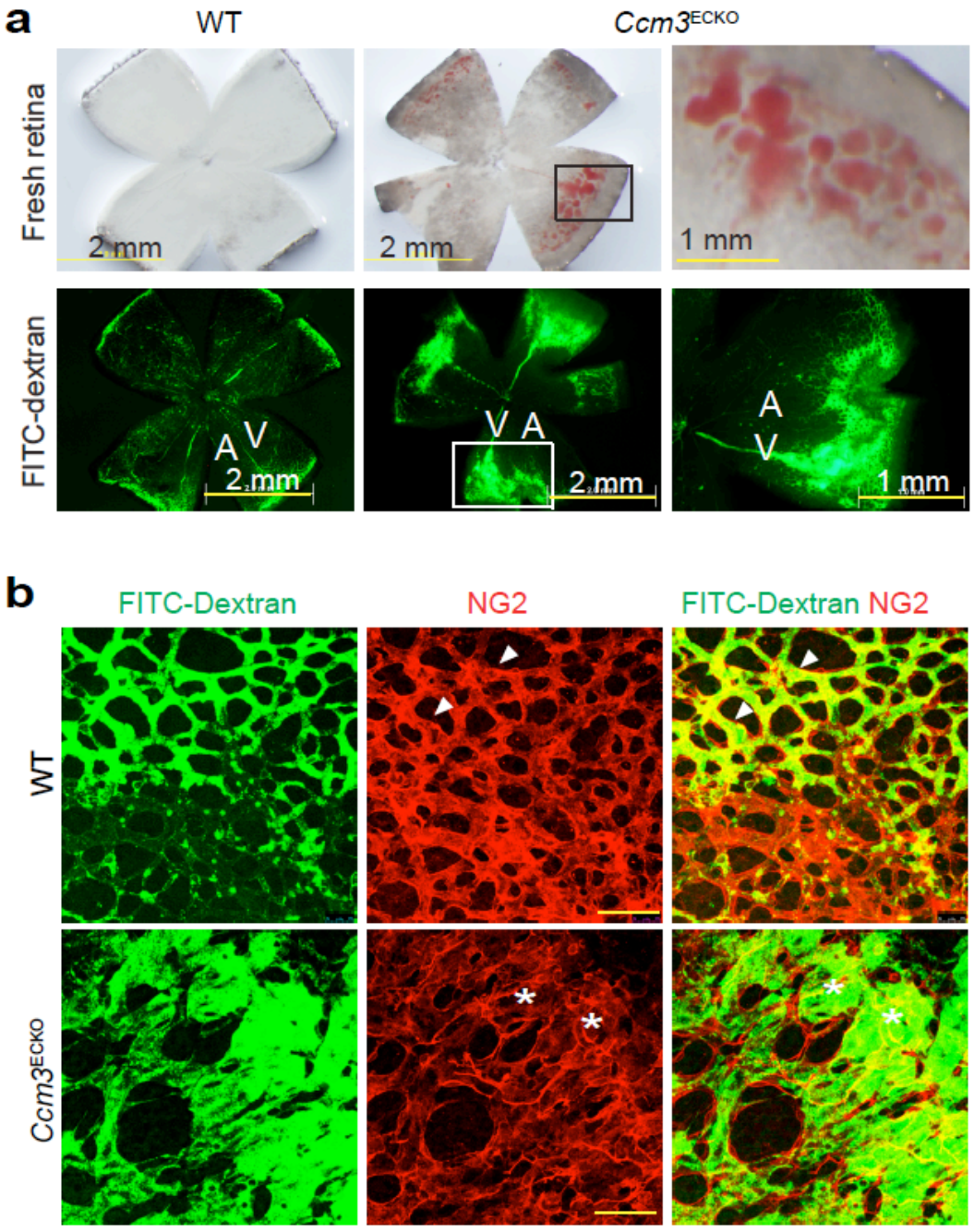


Supplementary Fig.1. EC-specific deletion of Ccm3 by Cdh5-CreERT2.

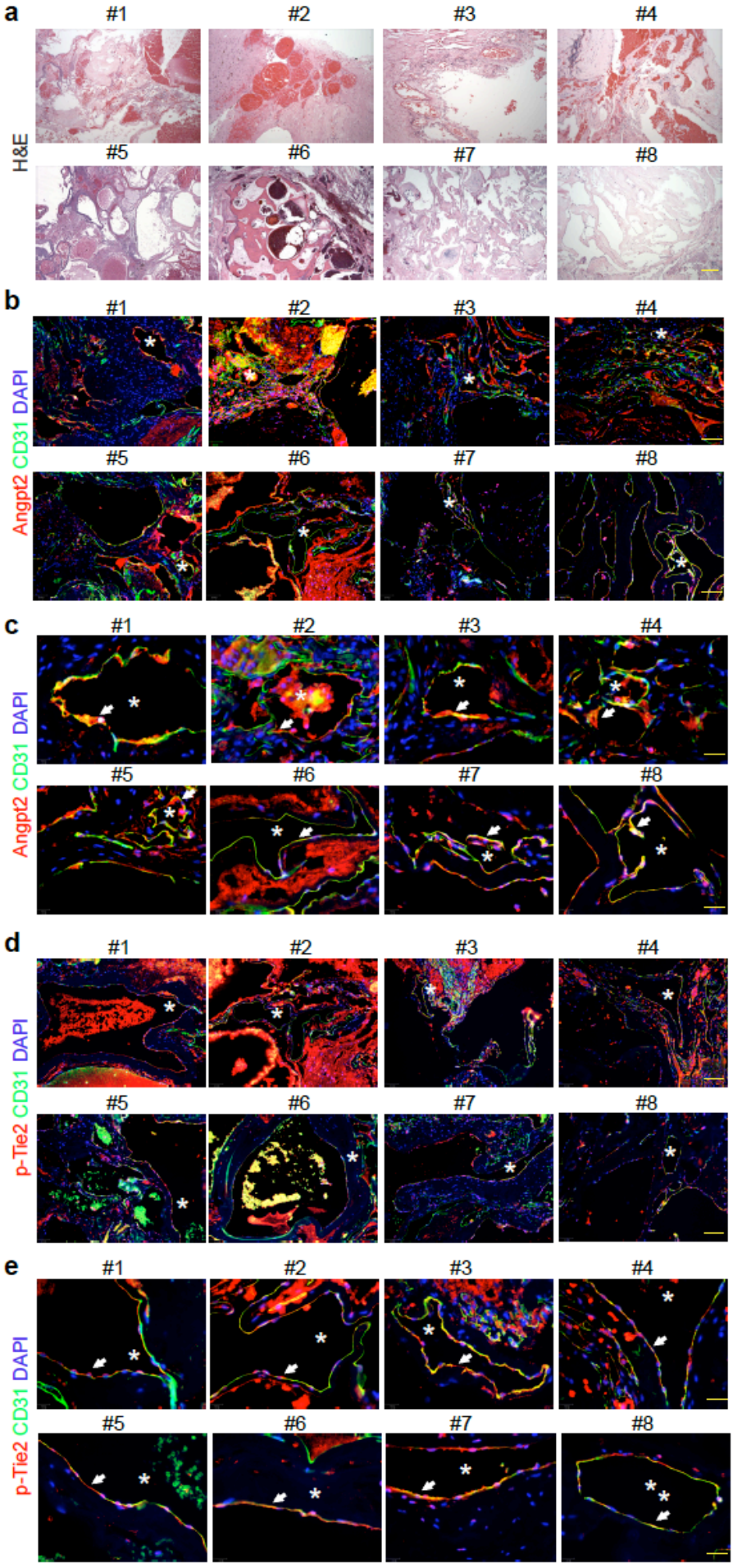
a. mT/mG reporter mice were bred with Cdh5CreERT2 deleter mice followed by tamoxifen feeding from P1 to P3. mG expression was specifically detected in the vasculature of various tissues, including cerebellum, midbrain, kidney, liver, lung and testis at P5. *n*=3 mice per group.

b. WT (*Pdcd10*$^{fl/fl}$) and *Ccm3*^{ECKO} (Cdh5CerERT2;*Pdcd10*$^{fl/fl}$) pups were administered with tamoxifen from P1 to P3 either by feeding or by i.p. injection. Brain tissues were harvested at P5. CD31^{+} mouse brain microvascular ECs and CD31^{-} non-EC cells from brain microvessels were isolated by FACS sorting. Ccm3 mRNA levels were determined by qRT-PCR with normalization by GAPDH, *n*=5 mice per group. ***P<0.001 by unpaired two-tailed Student's *t*-test. Error bars indicate s.e.m.

c. WT (*Pdcd10*$^{fl/fl}$) and *Ccm3*^{ECKO} (Cdh5CerERT2;*Pdcd10*$^{fl/fl}$) pups were fed with tamoxifen from P1 to P3 and brain tissues were harvested at P5 and P10. Cerebellum sections were co-stained by EC marker CD31 (red) together with CCM3 (green). CCM3 is detected in WT vessels (arrowhead) but is absent in vascular ECs of *Ccm3*^{ECKO} cerebellum with CCM lesions (asterisk). *n*=5 mice per group. (Scale bars: a: 100 μm; c: 25 μm).



Supplementary Fig.2. *Ccm3*^{ECKO} mice develop CCM lesions in retinas with increased vascular leakage.
a. P10 pups of WT and *Ccm3*^{ECKO} were perfused with FITC-dextran (2000 kDa) and whole mount retina images are shown. A: artery; V: vein. The boxed areas were shown in high power on the right side. Scale bar: 2 mm and 1 mm.
b. FITC-dextran perfused retinas were subjected to immunostaining with NG2 (red). Representative confocal images from *n*=10 mice are shown. Scale bar: 50 μm.


a
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Angpt2 CD31 DAPI
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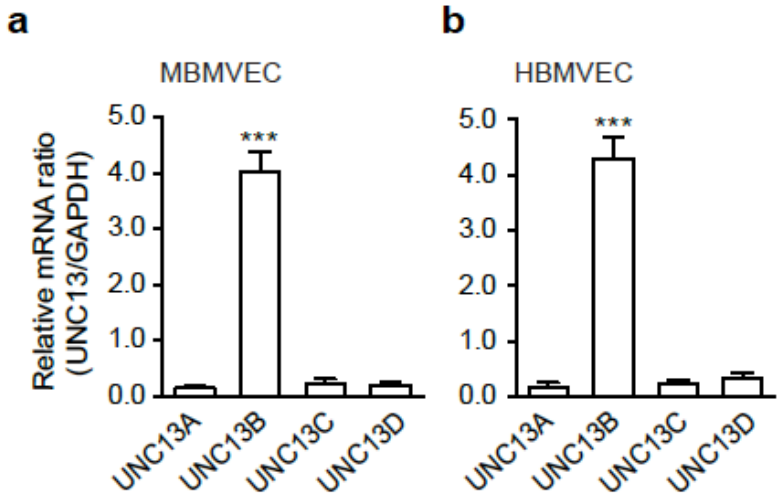
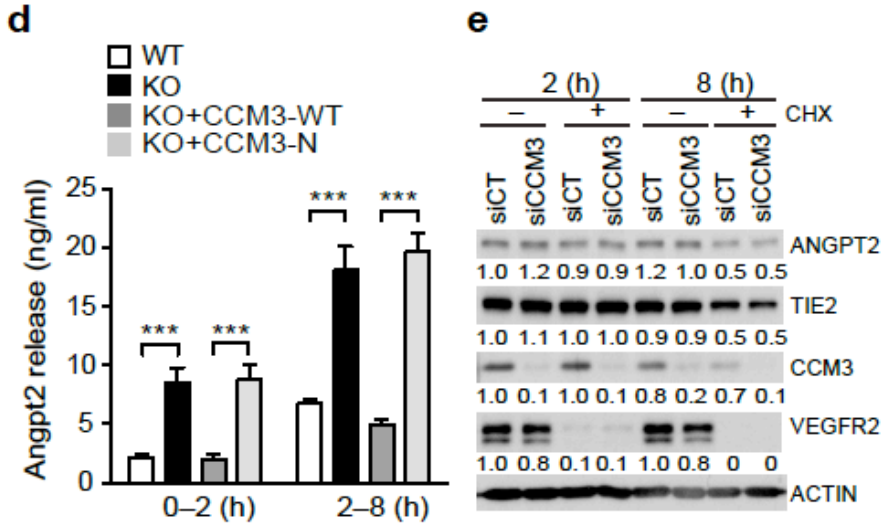
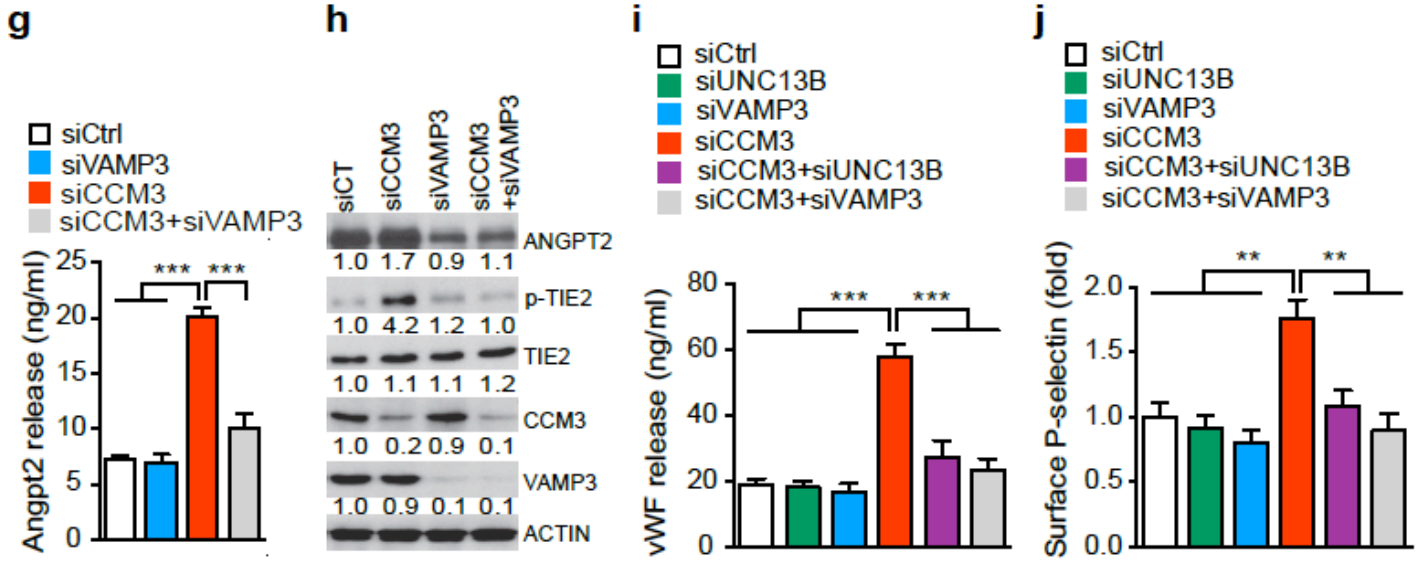
Supplementary Fig.3. Increased ANGPT2 expression and phosphor-TIE2 in human CCM3 lesions.
a. Representative images from H&E staining of 8 human CCM3 specimen. 6 samples contained typical CCM lesions (#1–6) and surrounding normal tissues, and the 2 cases (#7–8) showed severe vascular fibrosis.
b–c. ANGPT2 was upregulated in human CCM lesions. Human CCM specimens were immunostained for CD31 and ANGPT2. Representative images from eight human CCM3 samples are shown at low power (**b**) and high power (**c**). Asterisks indicate lesions whereas arrows indicate gain of ANGPT2 staining in lesions.
d–e. p-TIE2 was upregulated in human CCM lesions. Human CCM specimens were immunostained for CD31 and p-TIE2. Representative images from eight human CCM3 samples are shown at low power (**d**) and high power (**e**). Asterisks indicate lesions whereas arrows indicate gain of ANGPT2 staining in lesions.
(Scale bars: **a**, **b**, **d**: 100 μm; **c**, **e**: 25 μm).



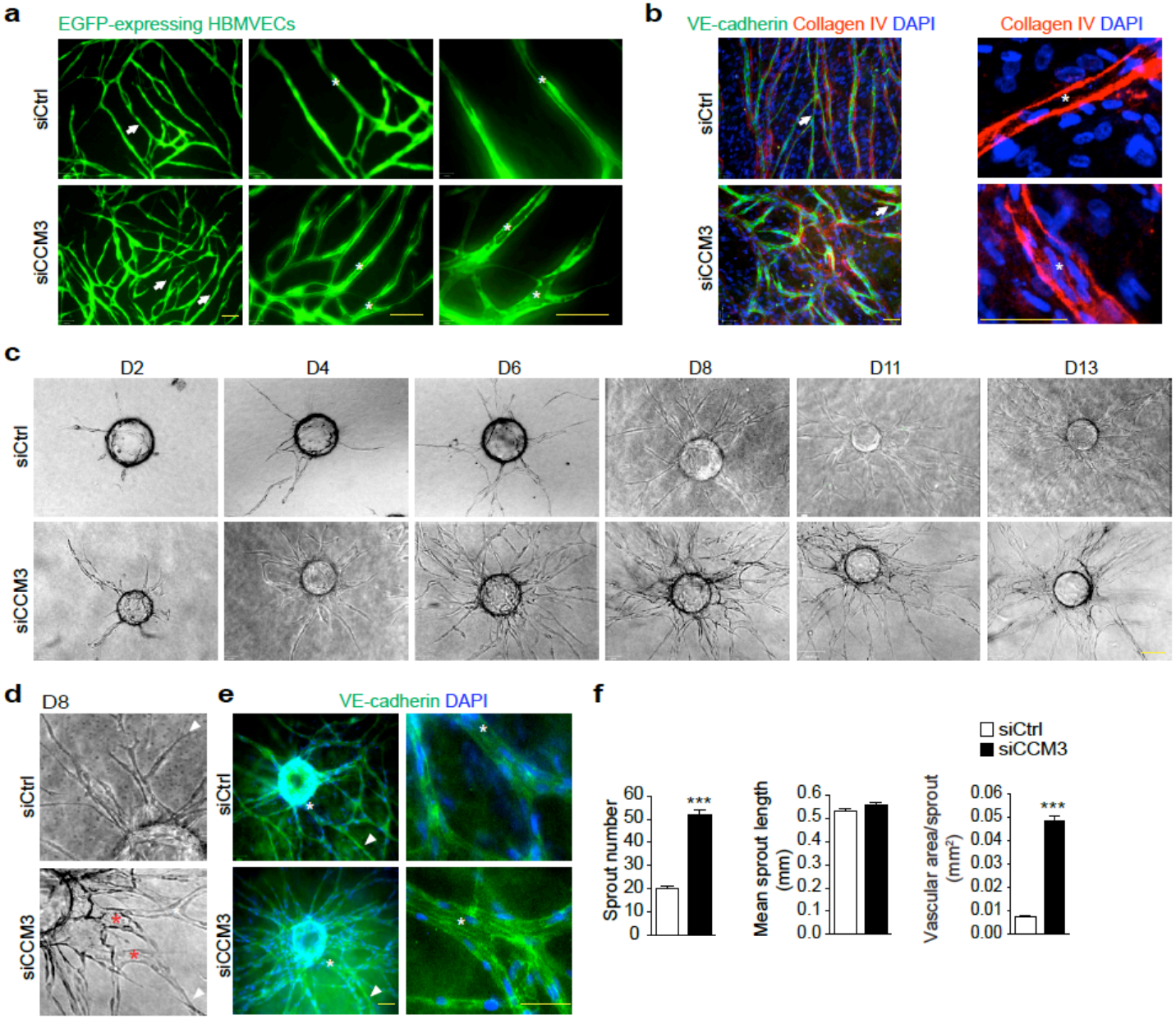
c

siRNA transfection	mRNA level		
	Angpt2	CCM3	UNC13B
Ctrl	100%	100%	100%
CCM3-1	103%	24%	
CCM3-2	99%	21%	
UNC13B-1	92%		22%
UNC13B-2	101%		18%
UNC13B+CCM3-1	104%	26%	19%
UNC13B+CCM3-2	97%	25%	15%

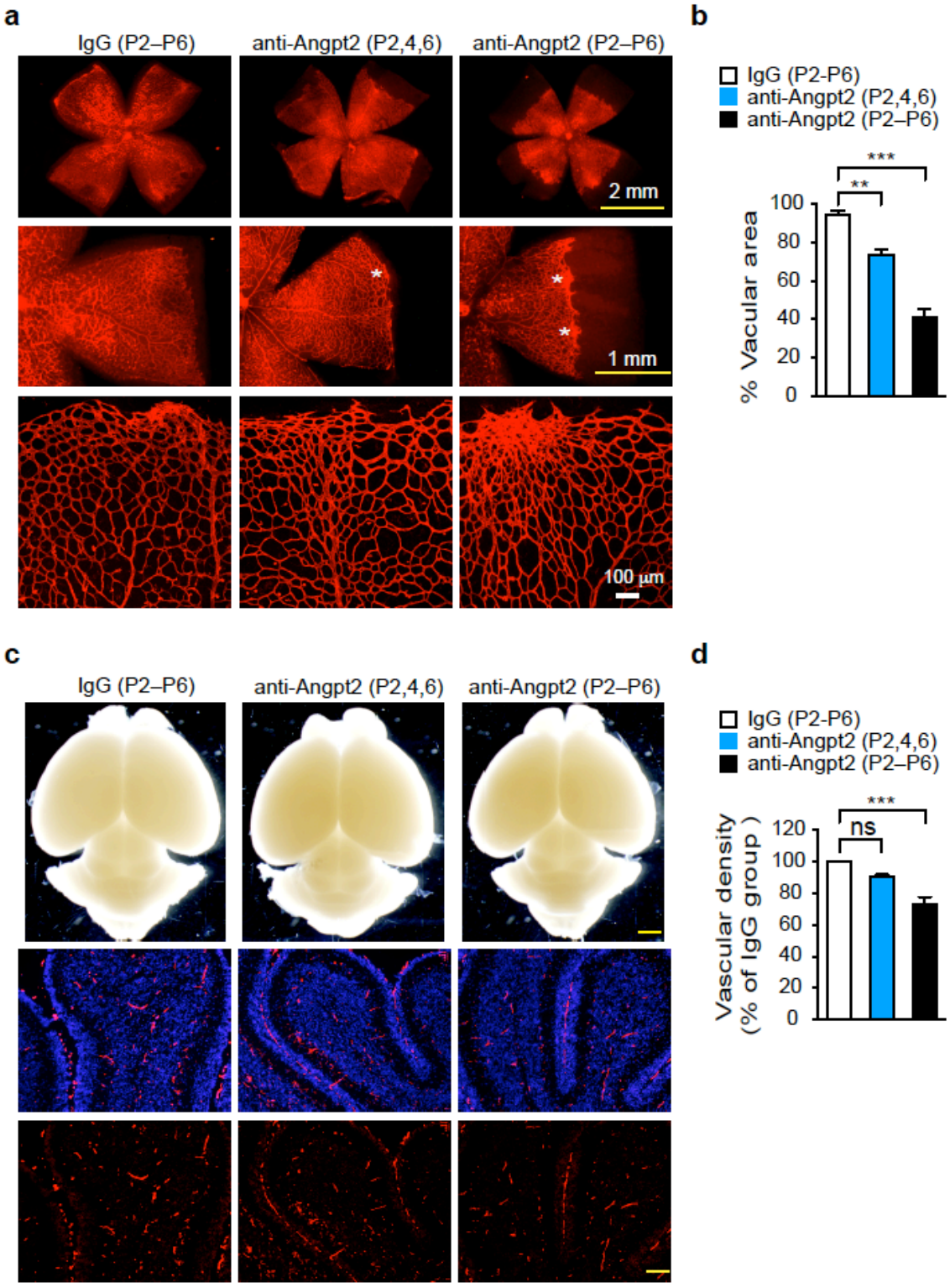




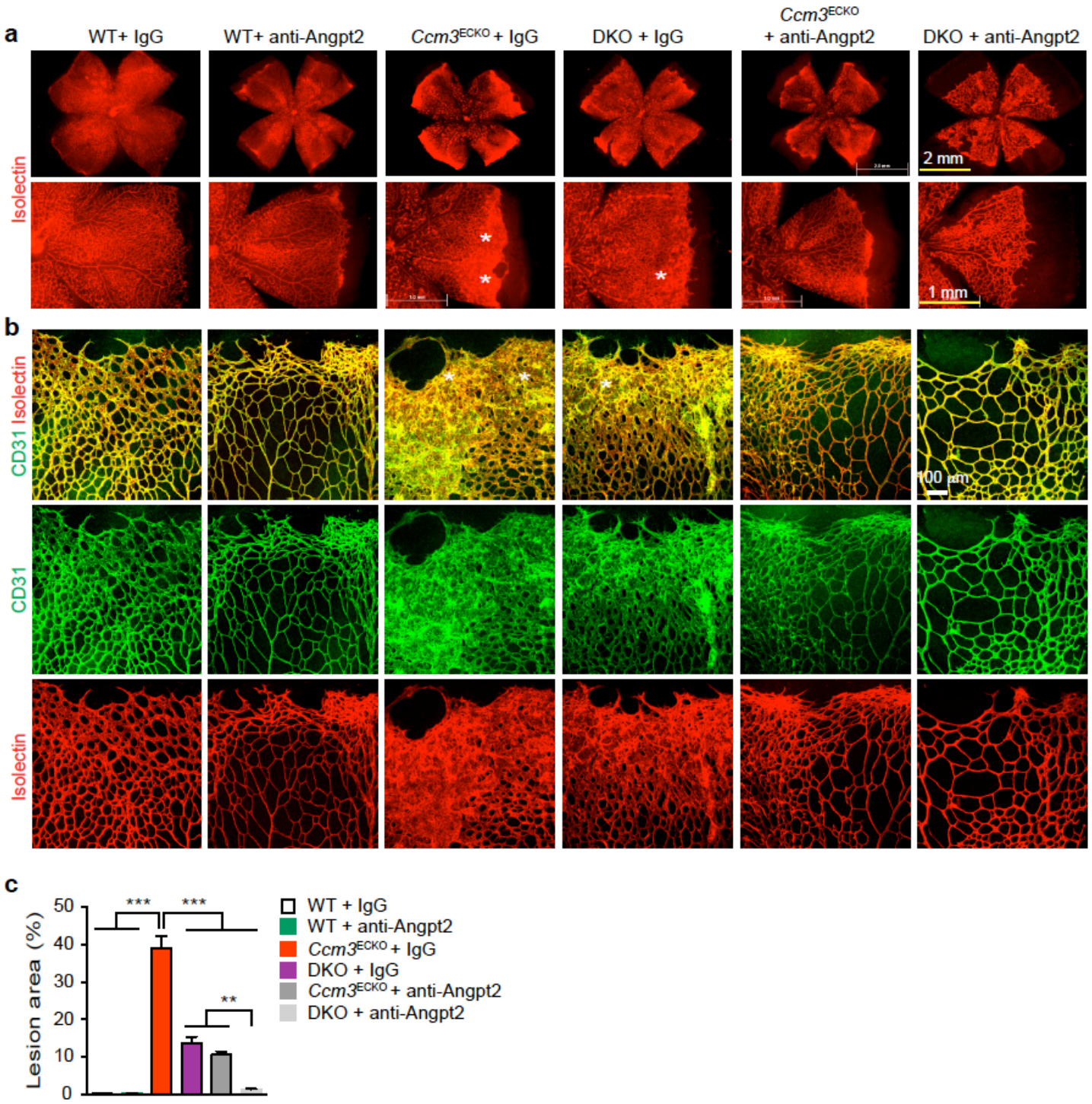
Supplementary Fig.4. CCM3-UNC13B/VAMP3 axis regulates EC exocytosis and ANGPT2 secretion.
a–b. UNC13B is the major isoform expressed in brain ECs. Gene expression of UNC13 isoforms in mouse brain microvascular ECs (MBMVECs) and human brain microvascular ECs (HBMVECs) was determined by qRT-PCR. Relative mRNA of UNC13 with normalization to GAPDH is presented. Data are mean ± s.e.m from three independent experiments. **P*<0.05 by one-way ANOVA.
c. Effects of CCM3 siRNA and Unc13B siRNA on ANGPT2 mRNA in HBMVECs. HBMVECs were transfected with 2 sets of siRNAs for CCM3 and UNC13B as indicated. Gene expression of CCM3, UNC13B and ANGPT2 were measured by qRT-PCR and was normalized with GAPDH, and presented as % changes by taking control siRNA as 100%. Data are mean ± s.e.m from three independent experiments.
d. MBMVECs were isolated from P5 $CCM3^{lox/lox}$:Cdh5-CreERT2 pups. MBMVECs were treated with DMSO/Ethanol (WT) or with 4-hydroxyl tamoxifen to induce CCM3 gene deletion in vitro (KO). CCM3-KO MBMVECs were then infected with lentivirus expressing CCM3-WT or CCM3-N (1-95aa). 72 h post-infection, cells were fed with fresh serum-free media and aliquots were collected at 2 h and 8 h. ANGPT2 levels released into the medium were determined by ELISA. Data are mean ± s.e.m, *n*=3, ***P* < 0.01 by two-way ANOVA.
e. HBMVECs were transfected with siRNAs as indicated for 72 hrs. Cells were replaced with fresh serum free media in the absence or presence of CHX (+CHX in G) for 2–8 h. ANGPT2, TIE2, CCM3 and VEGFR2 proteins were detected by Western blotting. Data are mean ± s.e.m from three independent experiments and represent fold changes by normalizing control siRNA to 1.0.
f. MBMVECs were transfected with control or CCM3 siRNAs. ECs were co-stained for VAMP3 and ANPGT2. VAMP3-positive ANGPT2 vesicles are indicated by arrows. Representative images are 1 of 10 microscope fields from three independent experiments. Scale bar: 20 μm.
g–h. CCM3-VAMP3 regulates ANGPT2 secretion in ECs. HBMVECs were transfected with siRNAs as indicated for 72 hrs. (**g**) Cells were replaced with fresh serum free media for 8 h. ANGPT2 levels released into the medium were determined by ELISA. Data are mean ± s.e.m, ***P* <0.01 by one-way ANOVA. (**h**) Intracellular proteins were subjected to Western blotting with indicated antibodies. Representative blots are 1 of 3 independent experiments. Data represent fold changes with WT levels normalized to 1.0.
i. CCM3-VAMP3 regulates vWF secretion in ECs. HBMVECs were transfected with siRNAs as indicated for 72 hrs. Cells were replaced with fresh serum free media for 8 h. vWF levels released into the medium were determined by ELISA. Data are mean ± s.e.m from three independent experiments, ***P* <0.01 by one-way ANOVA.
j. CCM3-VAMP3 regulates P-selectin surface expression in ECs. HBMVECs were transfected with siRNAs as indicated for 72 hrs. P-selectin surface expression was measured by FACS analyses. Data are mean ± s.e.m from three independent experiments and represent fold changes by normalizing control siRNA to 1.0. ***P* <0.01 by one-way ANOVA.



Supplementary Fig.5. CCM3-deficiency augments EC sprouting and lumen dilation.
a–b. Organotypic angiogenesis assay. EGFP^{+} (a) or EGFP^{-}(b) HBMVECs were transfected with control or CCM3 siRNAs. 24h after transfection, cells were seeded onto a confluent layer of fibroblasts and were co-cultured for 7–14 days. EC sprouts and lumens were visualized by EGFP at D10 (**a**) or VE-cadherin and collagen IV staining at D14 (**b**). Branches are indicated by arrows and lumens by asterisks. Scale bar: 100 μm.
c-f. 3D spheroid sprouting assay. siRNA-transfected HBMVECs were coated with microbeads, embedded in fibrin gels and grown in EGM2 medium for 2-13 days. A representative image of 10 beads for each sample is shown (**c**). Representative images for day 8 in high power are shown with sprouts and lumens indicated by arrowheads and asterisks (**d**). Day 8 samples were also immunostained for VE-cadherin (**e**). Scale bar: 100 μm. Quantifications of sprout number, sprout length and lumen areas are shown in **f**. All Data are means ± s.e.m, *n*=10; ***P* <0.01 by unpaired two-tailed Student's *t*-test. Additional three independent experiments were performed.

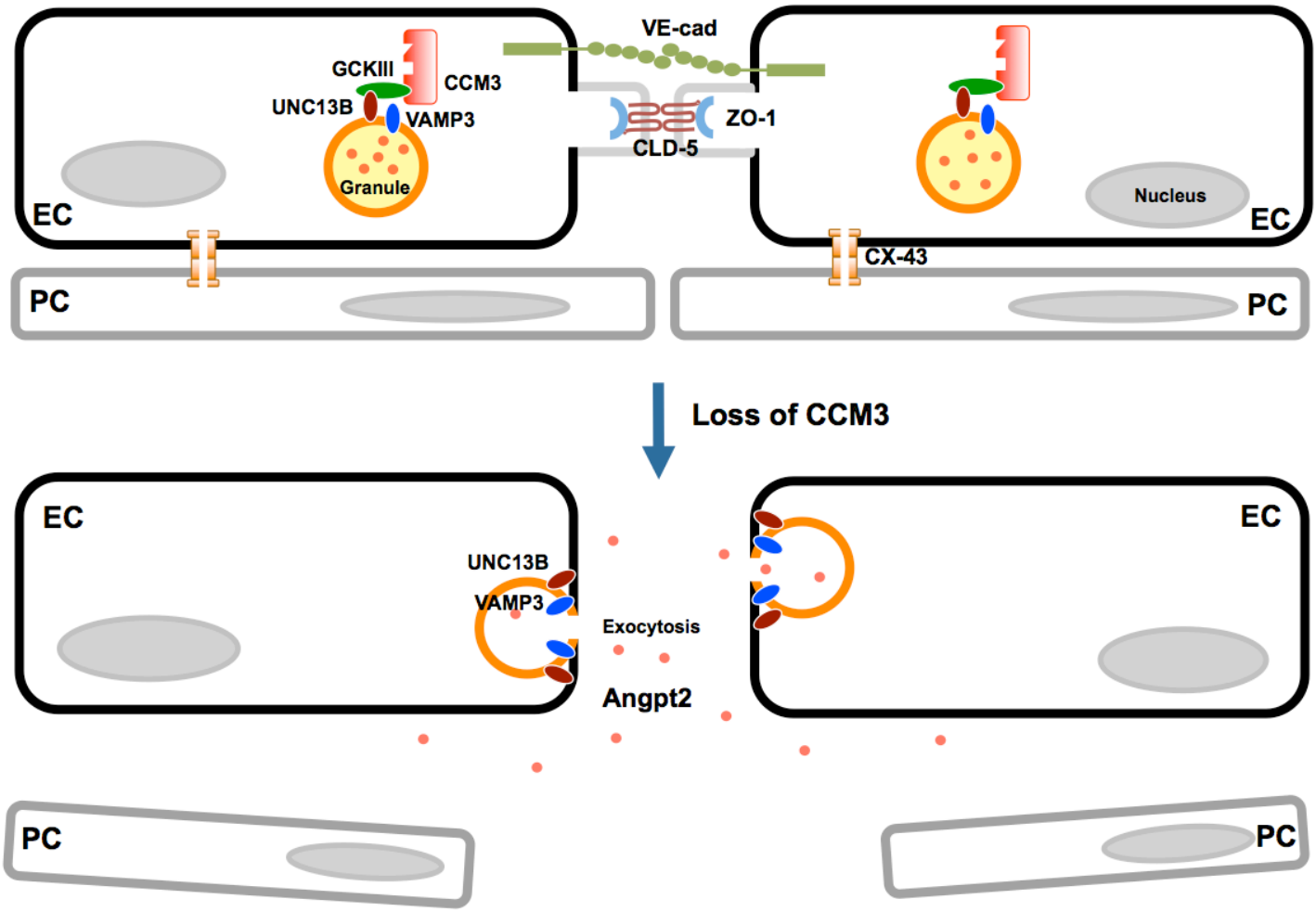


Supplementary Fig.6. Effects of ANGPT2 neutralization antibody on retinal and brain vascularization in newborn mice. WT (*Pdcd10*$^{fl/fl}$) pups were i.p. injected with control IgG or ANGPT2 neutralizing antibody (10 μg per g body weight) either once daily from P2 to P6 (P2–P6) or from P2 and every other day thereafter (P2,4,6). Retinas and cerebella were harvested at P10. **a**. Retinas were subjected to whole mount staining with isolectin B4 staining. Representative images and high power confocal images are shown. Asterisks indicate vascular tuft-like structures located at the edge of the advancing vessel network. **b**. Vascular areas are quantified as % of total retina. **c**. Images for fresh brain tissue. Scale bar: 2 mm. **d-e**. Cerebellum sections were stained with CD31 and vascular densities were quantified as % of IgG group. Scale bar: 100 μm. Data are means ± s.e.m, *n*=6, **P* <0.05, ***P* <0.01 by one-way ANOVA.



Supplementary Fig.7. ANGPT2 neutralization antibody reduces vascular lesion in retina of *Ccm3*^{ECKO} mice. WT and *Ccm3*^{ECKO} and *Ccm3*^{ECKO}:*Unc13b*$^{-/-}$ (DKO) pups were fed with tamoxifen from P1 to P3 to induce Ccm3 deletion. Pups were i.p. injected with control IgG or ANGPT2 neutralizing antibody (10 μg per g body weight) from P2 and every other day thereafter. Retinas were harvested at P10 and subjected for whole mount staining with isolectin B4 and CD31 staining.

a. Representative images are shown. Asterisks indicate CCM3 lesions. **b**. Representative confocal images are shown. Asterisks indicate CCM3 lesions. **c**. CCM lesion areas are quantified as % of total retina. All Data are means ± s.e.m, *n*=6; ***P* <0.01 by one-way ANOVA.



Supplementary Fig.8. A model for CCM3-restrained ANGPT2 release in CCM lesion formation. EC adherens and tight junctions, and EC-PC gap junctions are depicted. CCM3 via GCKIII (STK24/STK25) represses UNC13B and VAMP3-mediated exocytosis and ANGPT2 release; loss of CCM3 destabilizes GCKIII and releases its inhibitory effect on ANGPT2 secretion, which in turn induces disruption of EC-EC junctions and EC-PC interactions, followed by CCM lesion progression.