SUPPLEMENTAL INFORMATION

Immunothrombosis and vascular heterogeneity in cerebral cavernous malformation

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Supplemental Methods

In vivo experiments

Ethics approval

All experiments involving animal studies were conducted according to the principles in the Swedish National Board for Laboratory Animals and European Convention for Animal Care. Animal experiments were approved by the regional ethics committees in Uppsala, Sweden (C145/15, 5.8.18-16224-2020).

RNA extraction and library preparation

RNA was extracted from isolated endothelial cells (CD31+, CD45-) with the RNeasy Micro Kit (Qiagen) in the automated QIACube (Qiagen) platform. RNA libraries were prepared with the SMARTer Stranded Total RNA Sample Prep Mammalian Kit (Clontech). The quality and concentration of the RNA libraries was checked with the High Sensitivity DNA Kit in the Agilent 2100 Bioanalyzer. Each sample was run on a single lane (paired-end sequencing, 125bp reads) and sequenced on a HiSeq2500 sequencing system with v4 chemistry from Illumina at the Swedish National Genomics Infrastructure platform (Science for Life Laboratory, Uppsala). About 30 million reads were received per sample.

Lectin perfusions

At P8 wild-type and *Ccm3-iECKO* mice were anesthetized with Avertin and given a cardiac injection (25 ul) of Biotinylated Lycopersicon Esculentum (Tomato) Lectin (LEL, TL) from Vector Labs B-1175 (2 mg/mL) into the left ventricle over a period of 30 seconds. The lectin was allowed to circulate for one minute and then the mice were perfused with PBS for 2 min and then with 4% PFA for 3 minutes (min). The brains were processed as described below and for immunofluorescence staining, streptavidin AF488 was used to detect the Biotinylated Lectin.

Tissue processing for immunofluorescence

At P6, 7, and 8 wild-type and *Ccm3-iECKO* mice were given an intraperitoneal injection of pimonidazole-hydrochloride (from the Hypoxyprobe™ Red549 Kit, Cat# HP7-100, 60 μg/g) 1 hr prior to sacrification. The pups were anesthetized with Avertin, and perfused through the heart with Dulbecco's phosphate buffered saline (DPBS, Gibco; to ensure the removal of blood) for 2 min and then 1% paraformaldehyde (PFA) in DPBS for 2 min. The brains were dissected and fixed by immersion in 4% PFA diluted in phosphate buffered saline (PBS) overnight at 4 °C. The brains were washed three times with PBS, treated with 30% sucrose/PBS for 48 hours (hrs) and then cut in two sagittal halves. One half was processed for vibratome sectioning and the second half was processed for cryosectioning.

Wildtype and *Ccm3-iECKO* samples that were used to detect CD42b and ANXA IV were perfused through the heart with HBSS, dissected, and fixed by immersion in methanol overnight at 4 °C. The brains were washed with PBS for 24 hrs and then treated with 30% sucrose/PBS for 48 hrs.

Vibratome sectioning

Fixed and cryoprotected brains were embedded in 4% low melting-point agarose diluted in PBS and then fully sectioned (80 μm) with a vibratome. The brain slices were mounted on SuperFrost Plus Gold slides (ThermoFisher) and then stored at -80°C until ready to stain.

Cryosectioning

Fixed and cryoprotected brains were embedded OCT, frozen, and then sectioned with a cryostat (7 μm). The brain slices were mounted on SuperFrost Plus slides (ThermoFisher) and then stored at -20°C until ready to stain.

Immunofluorescence of vibratome sections – for the detection of hypoxia

At least 5-6 brain slices of each sample (representing a different region of the brain) were processed for staining. Sections were permeabilized with PBS+ 0.05% Tween-20 (PBST), blocked for 2 hrs in blocking buffer (1X PBS, 0.05% Triton, 3% bovine serum albumin and 3% fetal calf serum), and then incubated with HP-Red549 (1:200, from the Hypoxyprobe™ Red549

Kit) and CD31 (1:200, R&D #AF3628) overnight at 4°C. The sections were rinsed with PBST and then incubated with the secondary antibodies (ThermoFisher) diluted in blocking buffer for 2 hrs at room temperature. The sections were rinsed with PBST, counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 10 µg/mL, Molecular Probes), and mounted with Fluoromount-G (ThermoFischer).

Immunofluorescence of cryosections

Sections were rehydrated and permeabilized with PBST and then, blocked and stained simultaneously with the primary antibodies diluted in blocking buffer (PBS with 0.3% Triton-X and 5% donkey serum) overnight at 4°C. The primary antibodies used for cryosections are listed in Suppl. Table 2. The sections were washed with PBST and incubated for 1 hr with the secondary antibodies diluted in PBS. Sections were washed with PBST, counterstained with DAPI, rinsed with PBS, and mounted with Fluoromount-G.

Signal amplification for Tissue Factor

The Alexa Fluor™ 555 Tyramide SuperBoost™ Kit (goat anti-rabbit IgG, ThermoFisher # B40923) was used to amplify the signal of Tissue Factor (Abcam #ab151748). Cryosections were rehydrated with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) and incubated at 95°C for 20 min in pre-heated citrate buffer (10mM citric acid, 0.05% Tween 20, pH 6). Sections were left in the citrate buffer to cool to room temperature for 1 hr. Endogenous peroxidase was blocked with 3% hydrogen peroxidase for 10 min at room temperature. Sections were rinsed with TBS, blocked for 1hr at room temperature with 10% goat serum in TBST and then incubated overnight at 4°C with primary antibodies diluted in blocking solution. The slides were washed with TBST and incubated for 1hr at room temperature with poly HRP-conjugated goat anti-rabbit secondary antibody. The slides were then washed with TBST, and the signal was amplified according the manufacturer's instructions of the Alexa Fluor™ 555 Tyramide SuperBoost™ Kit. The sections were counter stained with a vessel marker and DAPI and then mounted with Fluoromount-G.

Scanning electron microscopy (SEM)

Wild-type (n=3) and *Ccm3-iECKO* (n=3) P8 mice were anaesthetized by an intraperitoneal injection of Avertin and subject to cardiac perfusion with electron microscopy fixative (2% PFA, 2.5% glutaraldehyde (EMS, USA), 2% sucrose, 2 mmol $CaCl_2$, in PBS, pH 7.4). After perfusion, the mice were kept in a sealed plastic bag for 2 hrs at room temperature. The brains were dissected and post-fixed in electron microscopy fixative for 72 hrs at 4 °C. Afterwards, the samples were transferred to PBS and sectioned in a sagittal manner at 1 mm intervals using a mouse brain matrix (Zivic instruments BSMAS005-1 and 2) under a stereomicroscope (Wild Herrbrugg, Wild MB, Switzerland). Sections were then post-fixed in 1% unbuffered osmium tetroxide for 1 hr. After osmication, the sections were washed in the buffer three times, dehydrated through a series of graded ethanol, critical point dried in a critical point dryer (Agar E3000; Quorum Technologies Ltd., East Essex, UK) using liquid CO_2 as the drying agent, mounted on stubs, and coated with gold using a sputter device (K550X; Emitech Ltd., Kent, UK). Observations were made using a scanning electron microscope (XL 30 ESEM; Philips, Eindhoven, The Netherlands) operated at 10 kV accelerating voltage, with images recorded digitally.

Brain CLARITY and imaging

Wild-type (n=3) and *Ccm3-iECKO* (n=3) P8 mice were injected with the hypoxyprobe as described above. Brains were perfused, collected and fixed as described above. The cerebellum was embedded in agarose as described above and sectioned coronally with a vibratome (1 mm). Passive tissue clearing was carried out as previously described[1 2]. In brief, the sections were incubated overnight at 4 °C with a hydrogel solution (4% Acrylamide, 0.25% VA-044 in PBS). Sections were then transferred to cold microwave reaction vials and the hydrogel solution was replaced with fresh hydrogel solution. The tubes were sealed with a rubber septum cap and before initiatings the polymerization, the hydrogel solution was degassed by bubbling nitrogen through the septum for 2 min on ice. The polymerization was activated by incubating the samples at 37 °C for 2 hrs. Excess acrylamide was removed and sections were incubated overnight at 37 °C with a clearing solution (8% sodium dodecylsulfate (SDS), 0.01% sodium azide in water, pH 8.5). The clearing solution was replaced and passive clearing was

continued for 72 h at 37 °C. The cleared sections were then washed four times with PBS containing 0.1% Triton X100 and 0.01% sodium azide (washing buffer) for 48 h. To enhance both clearing and washing efficacy, the buffer-to-section volume ratio was always >50. Immunostaining was performed at room temperature as following: 1) sections were incubated with the indicated antibody in washing buffer supplemented with 5% donkey serum for 72 h. Samples were then washed for 30 hrs and incubated, for 72 hrs, with the appropriate secondary antibody diluted in washing buffer. Finally, samples were washed for 30 h. Sections were then incubated and, subsequently, mounted in a refractive index matching solution (RIMS, 88% w/v Hystodenz in water) for confocal imaging with a Leica SP8 microscope.

Bioinformatics analysis for RNA-sequencing data

Trimming and mapping of the raw reads

Each sample (n=3 wild-type and n=3 *Ccm3-iECKO*) received ~30 million paired-end reads. Trim Galore[3] was used for quality control and for processing the raw reads. In addition, 5 nucleotides from both the 5' and the 3' ends were clipped from the reads to remove unwanted bias. The trimmed reads were mapped to the mm10 mouse genome (~50% unique mapping rate) with STAR[4] (version 2.5.3a). The number of reads per gene were also counted by mapping with STAR.

Differential gene expression analysis

DESeq2[5], an R package on Bioconductor, was used to identify differentially expressed genes (DEGs) between wild-type and *Ccm3-iECKO* samples. The thresholds for identifying DEGs were: *adjusted p-value < 0.05* and *|Log2FC| > 0.5*. The gene expression read counts were normalized with the factor-based methods from R package DESeq2.

Annotations of differentially expressed genes

The gene set collections – "Gene Ontology" (GO) [6-8], "Hallmark" [9-11] and "KEGG" [12-14] were downloaded from the Molecular Signatures Database (MSigDB, version 5.2)[10 11] and DEGs were annotated with their associated MSigDB gene set ID's in those three collections.

Identification of coagulation and hypoxia-related genes

Coagulation and hypoxia-related genes were defined as differentially expressed genes that were associated with the following MSigDB gene sets:

- "KEGG_COMPLEMENT_AND_COAGULATION_CASCADES"
- "GO_BLOOD_COAGULATION_FIBRIN_CLOT_FORMATION"
- "GO_BLOOD_COAGULATION_INTRINSIC_PATHWAY"
- "GO_FIBRINOLYSIS"
- "GO_HEMOSTASIS"
- "GO_POSITIVE_REGULATION_OF_COAGULATION"
- "GO_REGULATION_OF_COAGULATION"
- "GO_REGULATION_OF_EXTRACELLULAR_MATRIX_DISASSEMBLY"
- "GO_REGULATION_OF_WOUND_HEALING"
- "HALLMARK_COAGULATION"
- "HALLMARK_COMPLEMENT"
- "HALLMARK_HYPOXIA"

Functional analysis

R package clusterProfiler[15] was used for the GO over-representation analysis. The cutoff was $p_{adj}<0.05$ and redundant enriched GO terms were removed. The gene set enrichment analysis was made with the Gene Set Enrichment Analysis (GSEA) software[9 16] with the gene sets from the Molecular Signatures Database (MsigDB v5.2). GSEA conducted 1000 permutations. The maximum and minimum sizes for gene sets were 1000 and 5, respectively. The cutoff for significant gene sets was false discovery rate < 25%.

Heatmap of the differentially expressed genes

To visualize different gene expression levels on a heatmap, normalized expression values were regularized logarithm-transformed (rlog). This method produces transformed data on a log2 scale. Z-scores were then calculated for each gene using the rlog-transformed value and used for plotting in the heatmap to avoid the overwhelming of expression values over patterns. The

formula used to calculate a z-score is $z = (x-\mu)/\sigma$, where x is the rlog-transformed value for each gene, μ is the rlog-transformed value mean, and σ is the rlog-transformed value standard deviation. The red lines listed next to each gene indicate that gene's location in the heat map and which GO term it appears in. Some genes only appear in one GO term (*Klf4*) and some genes appear in all four GO terms (*Ano6*).

Spatial transcriptomics data analysis

The spatial transcriptomics data was obtained from our previous study[17]. In summary, coronal brain sections from two wild-type and two acute *Ccm3-iECKO* P8 mice were sequenced with the 10x Visium Spatial Transcriptomics platform. The sequencing data and histological images were first processed with the software Space Ranger (v1.0, 10× Genomics) and then the pipeline from Seurat (v.3.9) R package was used to perform the integrated analysis. The spots were color-coded by cluster and overlaid on the hematoxylin and eosin-stained tissue images to identify the spots/clusters that belong to the cerebellum region. Then spots in the cerebellum region were analyzed for their expression levels of *F3*, *Serpine1*, and *Vegfa*.

Bioinformatics analysis for F3

The violin plot of *F3* expression in cerebellar cells was generated with the Seurat package for R[18] and the publicly available mouse cerebellar single-nucleus RNA-seq (snRNA-seq) dataset generated by the Broad Institute of MIT and Harvard[19].

In vitro **experiments**

shCCM3 Lentivirus infection

Primary human microvascular endothelial cells (HBMEVECs) were purchased from iXCells (10HU-051) at passage 1(p1). Cells were seeded on Collagen I coated dishes and maintained in Endothelial Cell Growth Medium MV 2 (Lonza) without antibiotics. Lentivirus vectors were prepared by transfecting Lenti-X 293T cells (Takara #632180) with 3rd generation Lentiviral system plasmids. After 48 hrs, the supernatant was collected and centrifuged for 2 hrs at 20,000xg. The viral particles that remained in the pellet were resuspended in ice-cold sterile

1% BSA/PBS and then aliquoted and stored at -80°C. Semi-confluent HBMVECs were transduced with Lentivirus containing short hairpin RNA (shRNA) against human *CCM3* (Santa Cruz #62084) for 24 hrs. The viruses were removed by adding fresh medium, and the cells were cultured for an extra 48 hrs in complete medium. Transduced cells were selected with 2 µg/mL of Puromycin. After selection, cells were trypsinized and expanded for experiments (p3 to p7).

Microfluidics

Human brain endothelial cells (shScramble and *shCCM3*) were exposed to 10 dynes of flow for 48 hrs with the 0.4 Ibidi *µ*-Slide I Luer (ibidi #80176) with the Ibidi pump system (ibidi #10902) at 37°C.

Western blot

After transfection, shScramble and *shCCM3* HBMEVECs were rinsed twice with cold DPBS and lysed with ice cold RIPA buffer (ThermoFisher #89901) supplemented with protease and phosphatase inhibitors (ThermoFisher #87785). The protein concentration of each cell lysate was determined with the Pierce BCA Protein Assay Kit (ThermoFischer #23225). Samples were prepared for western blot with a homemade 6X SDS sample buffer[20]. The samples were boiled for 5 min at 95°C and then loaded onto a 1.5 mm 4-12% BisTris precast gel (NuPAGE) and run with a MOPS running buffer (ThermoFisher) for 15 min at 80V and then for 1 hr at 130V. The Page Ruler Prestained Protein Ladder (ThermoFisher #26616) was used as molecular weight marker. Proteins were dry-transferred onto a PVDF membrane with the iBLOT 2 Gel Transfer Device (ThermoFisher). The membrane was blocked for 1 hr at room temperature with 3% BSA/TBS-T, incubated with the primary antibodies diluted in 3% BSA/TBS-T overnight on a rocker at 4°C, washed with TBS-T, and then incubated with the secondary antibodies diluted in 3% BSA/TBS-T for 1 hr at room temperature. The membrane was washed with TBS-T, then TBS, and then incubated with the ECL Prime Western Blotting Detection Reagents (GE Healthcare #RPN2232) and then exposed under a transilluminator (iBright CL1500, ThermoFisher). The iBright software was used to assemble the images.

qPCR

Quantitative PCR (qPCR) was carried out with the TaqMan system (Applied Biosystems). The TaqMan probes Hs003600439_g1 and Hs00264920_s1 were used to determine transcript levels of *KLF2* and *THBD*, respectively. Transcript levels of *KLF2* and *THBD* were normalised to *GAPDH* (Hs02786624_g1).

Immunofluorescence for HBMEVECs

After transfection, confluent shScramble and *shCCM3* HBMEVECs were fixed with 4% PFA for 20 min at room temperature and then washed twice with PBS. Fixed cells were permeabilized with 0.05% Triton-X100 in PBS for 5 min and then rinsed twice with PBS. The cells were incubated for 30 min with blocking buffer (3% BSA diluted in PBS). The primary antibodies (listed on Table 2) were diluted in fresh blocking buffer and incubated overnight at 4°C in a humid chamber. The morning after, the fixed cells were washed three times with PBS. The secondary antibodies (ThermoFisher) were diluted in blocking buffer and incubated for hr at room temperature. The cells were washed three times with PBS, and counterstained with DAPI (Molecular Probes, 2ug/mL) for 5 min. The cells were mounted with Fluoromount-G, and the slides were allowed to dry overnight at room temperature before imaging.

Analysis of human CCM brain biopsies

Ethics approval

The use of human biopsies in this study was approved by the Organizational and Ethical Committees of the Swedish Ethical Review Authority, under permit numbers 2019-04715 and 2019-06374, by Helsinki University Hospital (HUS/125/2018), and by the Committee on Research Ethics of Helsinki University Hospital (HUS/3648/2017).

Collection of human CCM brain biopsies

Biopsies from three patients with sporadic CCM were surgically removed at the Department of Neurosurgery at Helsinki University Hospital in Finland. The decision to surgically remove the CCM lesions from the patients was solely based on the patient's clinical needs. After

surgical resection, the biopsies were embedded in OCT and snap frozen on an isopentane-dry ice slurry. The snap frozen biopsies were delivered to Uppsala University for further processing. Paraffin-embedded biopsies from three patients with familial CCM were obtained from the Angioma Alliance's DNA/Tissue Bank as defined through a Material Transfer Agreement.

Immunofluorescence of human CCM brain biopsies

Snap frozen biopsies from three sporadic CCM patients were sectioned (7 µm) and mounted onto positively charged glass slides (ThermoFisher). The samples were fixed with ice cold methanol for 10 min and then rehydrated, permeabilized, blocked and stained as mentioned above for cryosections. The samples were counterstained with DAPI and mounted with Fluoromount-G.

Immunohistochemistry of human CCM brain biopsies

Formalin fixed and paraffin embedded (FFPE) biopsies from patients with familial CCM were sectioned and stained for CD34 (Dako # IR632, mouse monoclonal, clone QBEnd10), thrombomodulin (Dako #M0617, mouse monoclonal, clone 1), and Fraser-Lendrum according to standard routine protocols in the clinical pathology lab

Image acquisition and analysis

Images were acquired with a wide-field DMI8 microscope (Leica) or with an SP8 confocal microscope (Leica). For comparison purposes, different sample images of the same antigen were acquired under constant acquisition settings. The imaging processing package Fiji[21] was used for image analysis. In brief, immunofluorescent images were analyzed as following: the area of the antigen of interest was measured by selecting the positive signal with an algorithm threshold. The positive area was then divided by the total area of the cerebellum. The values were then converted to percentages unless otherwise indicated. Antigens that were only present in the vasculature were normalized to the respective vascular marker. Arivis Vision4D was used to make the supplemental movies for the CLARITY experiments.

Fraser-Lendrum, thrombomodulin and CD34 stained CCM biopsies (n=3) were imaged using AxioScan (Zeiss) and analysed with ZEN (blue edition; Zeiss) and QuPath v0.2.3[22]. Images from randomly selected regions (40 in total) of all three biopsies were selected and blindly scored by five investigators for activated erythrocytes (based on their color) and the presence or absence of thrombomodulin, as illustrated in Suppl. Fig. 5. The results were transformed to percentages and then plotted on a pie chart.

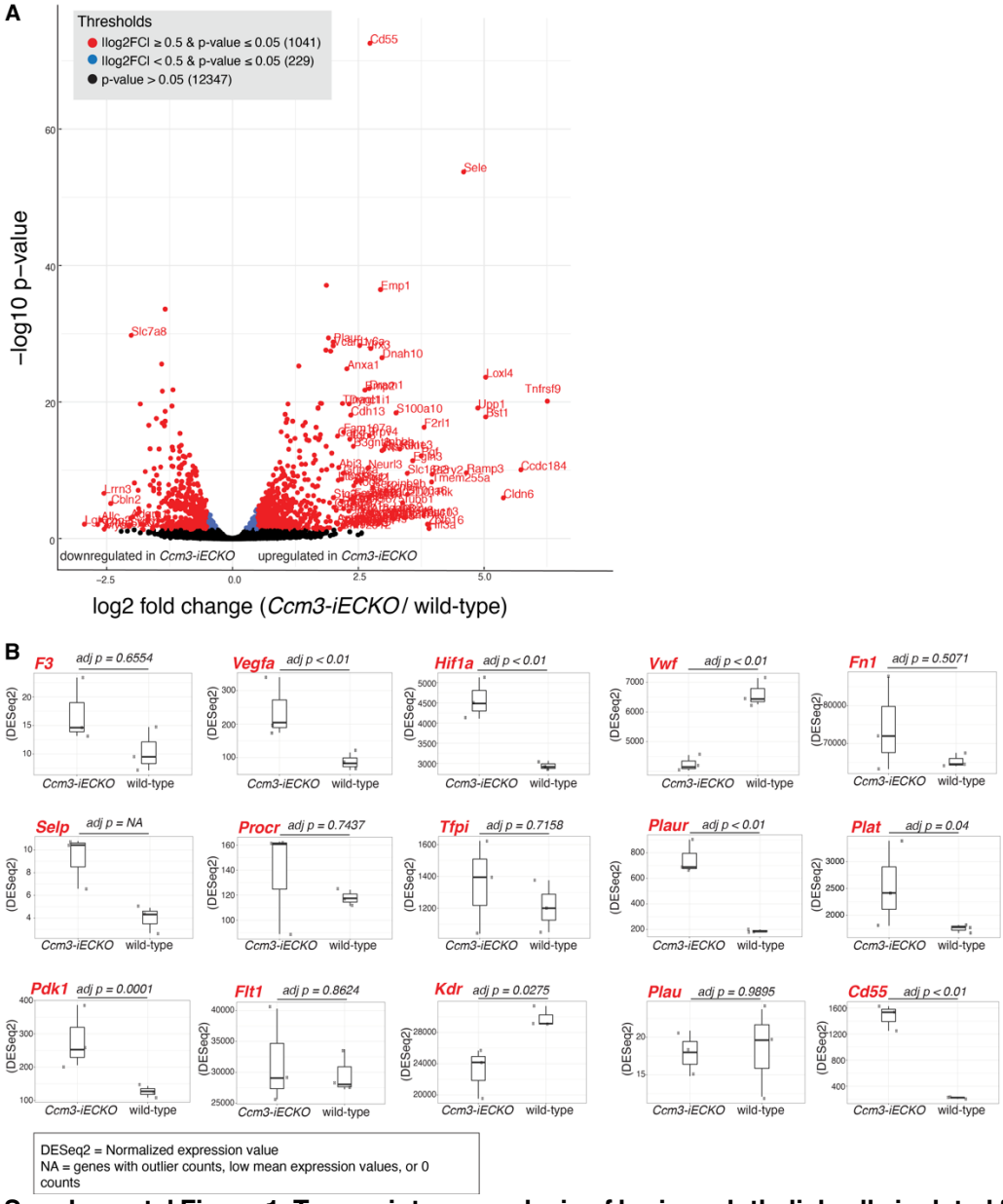
Statistical analysis

For all experiments the D'Agostino & Pearson test was used to assess whether or not the data was normally distributed. When the data was normally distributed, an unpaired t-test was used to determine statistical differences. When the data was not normally distributed, a Mann-Whitney U tests was used to determine statistical differences. When $p < 0.05$, the difference was considered statistically significant. All graphs were made with GraphPad Prism 7, except for the graphs in Suppl.Fig 2B which were made with RStudio[23].

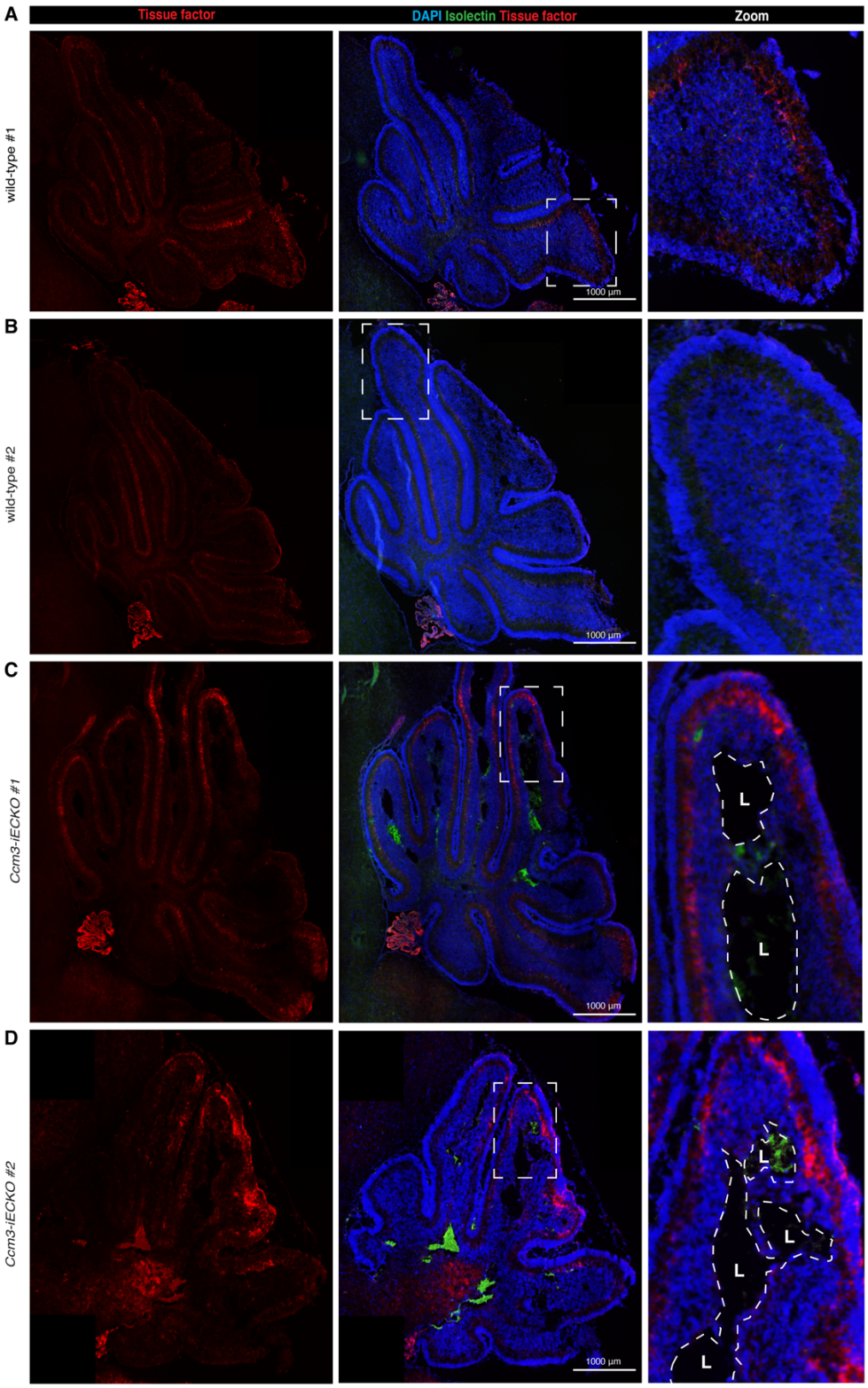
Figure assembly

All figures were assembled with Adobe Illustrator. The visual abstract and Figure 1A were assembled with Adobe Illustrator, Smart Servier Medical Art[24] and BioRender (Agreement number OP2499CY40). The Annexin IV cartoon in the visual abstract was adapted from van Genderen and colleagues[25].

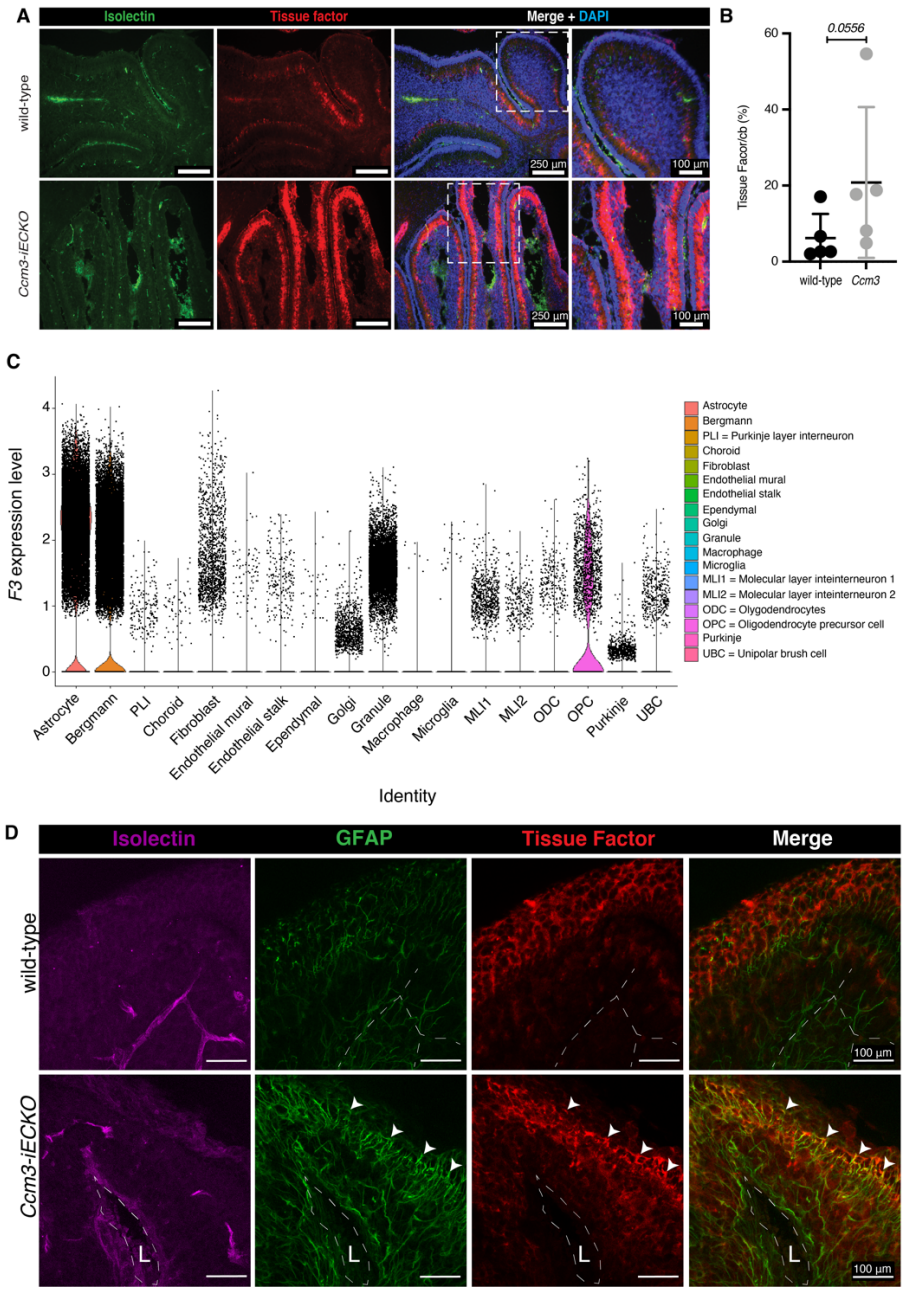
Supplemental Figures



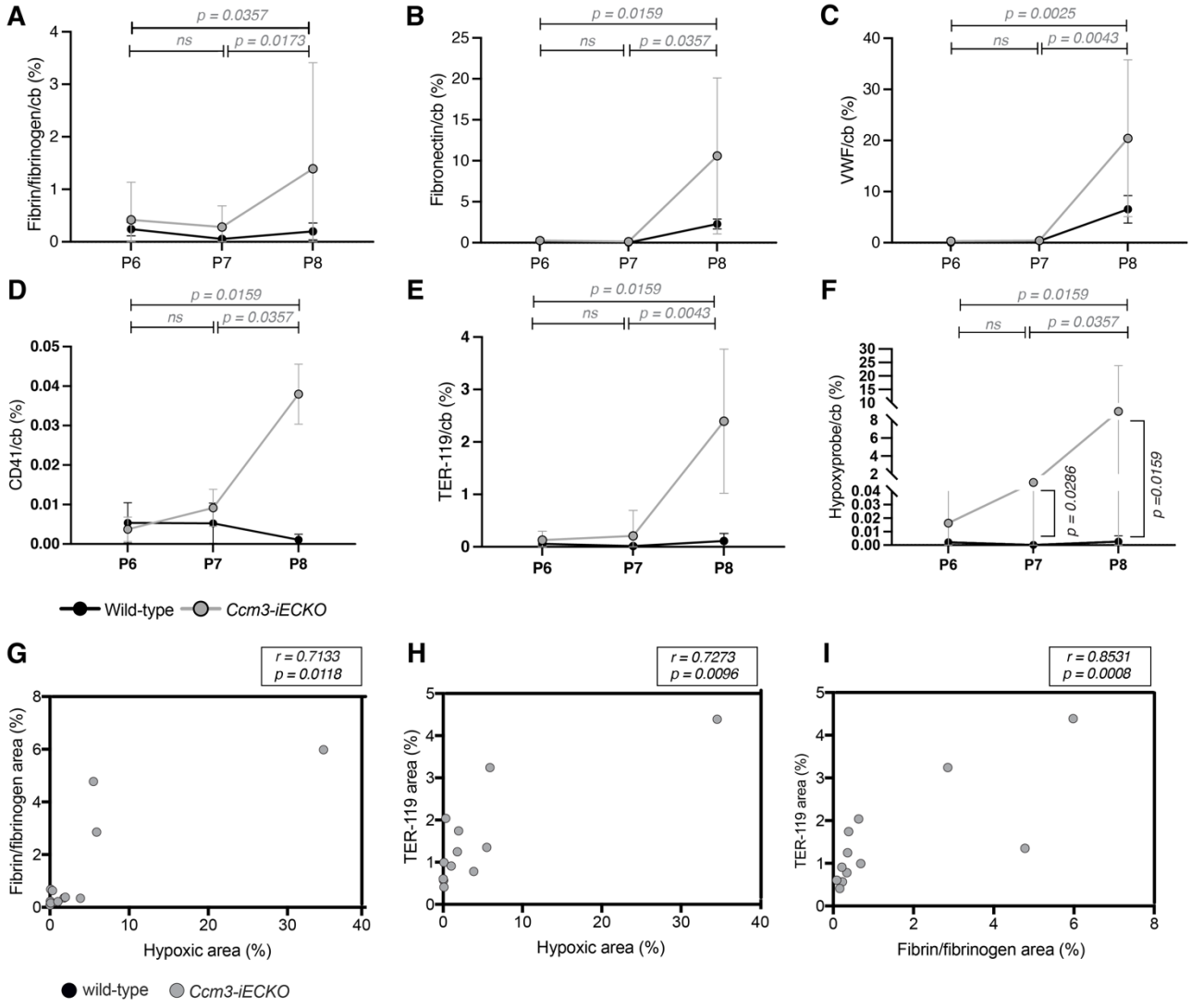
Supplemental Figure 1. Transcriptome analysis of brain endothelial cells isolated from wild-type and *Ccm3-iECKO* mice identified genes related to coagulation and hypoxia.
(A) Volcano plot illustrating the differentially expressed genes (DEGs) identified between wild-type and *Ccm3-iECKO* mice at P9. A total of 1041 genes were significantly upregulated (red) and 229 were significantly downregulated in *Ccm3-iECKO* mice. **(B)** Box plots of selected genes involved in coagulation and hypoxia. The gene names are listed in red on the top left corner of each graph and the p-values are written above the two groups. In the boxplot datapoints represent biological replicates (n=3 per group), the bar indicates the median, the whiskers indicate the minimum and maximum values and the box represents the first quartile (Q_1) and third quartile (Q_3). The adjusted (adj) p-value is written on all graphs.



Supplemental Figure 2. Representative images of tissue factor staining in P8 wild-type and *Ccm3-iECKO* murine cerebellums.
(A) Wild-type #1 **(B)** Wild-type #2 **(C)** *Ccm3-iECKO* #1 and **(D)** *Ccm3-iECKO* #2 stained with tissue factor (red), isolectin (green) and DAPI (blue). Dashed white rectangles mark magnified regions to the right. L = lesion

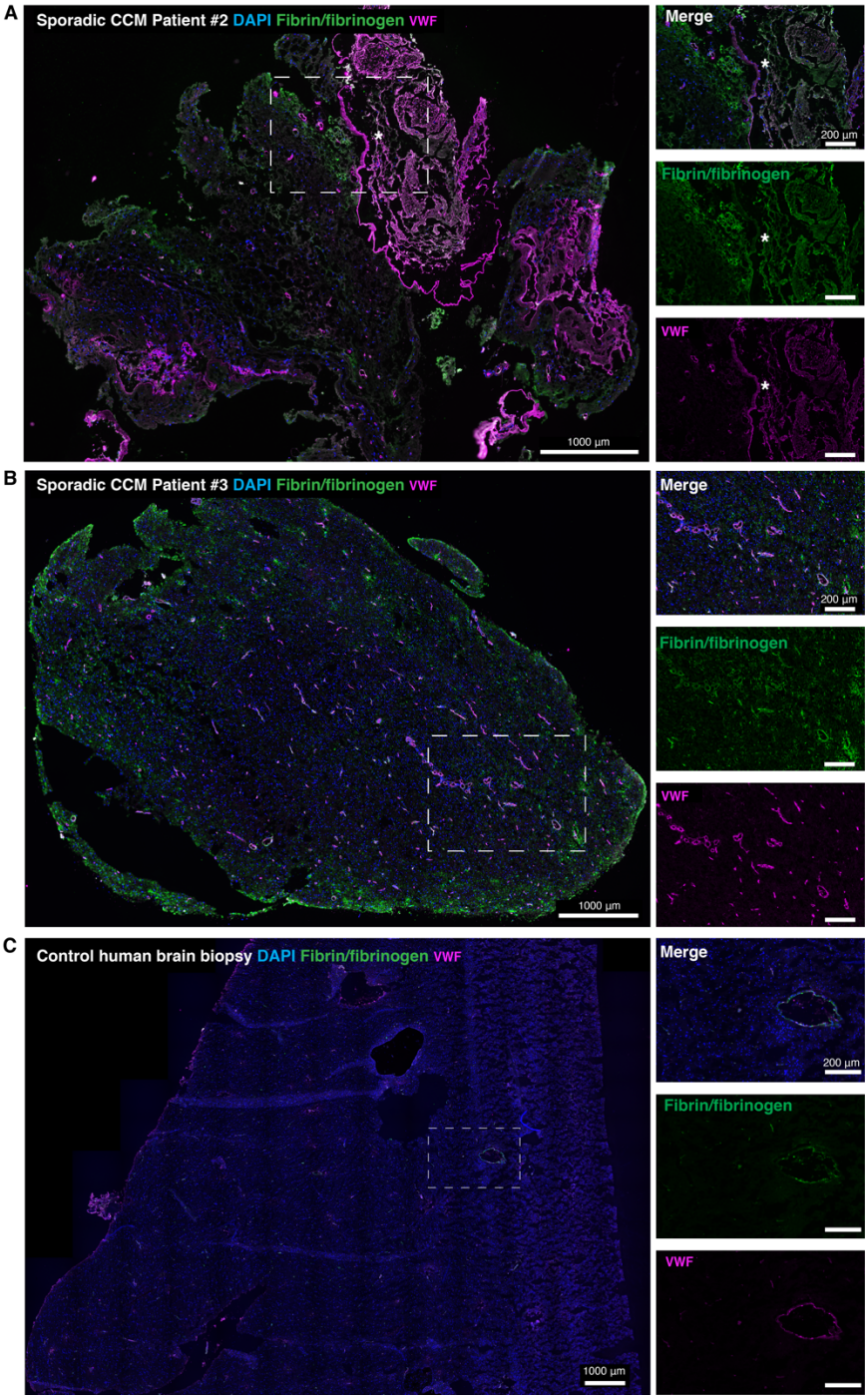


Supplemental Figure 3. Tissue factor expression in the mouse cerebellum.
(A) Representative images of the cerebellum of wild-type (upper panel) and *Ccm3-iECKO* (lower panel) P8 mice stained with isolectin (green), tissue factor (red) and DAPI (blue). A magnified image is shown in the right panel. **(B)** Quantification of tissue factor in the cerebellum (cb) of wild-type and *Ccm3-iECKO* mice (p=0.0556). Each data point represents one biological replicate (n=5 per group), the bar indicates the mean of each group and the error bars represent the standard deviation. A Mann-Whitney *U* test was used to compare wild-type mice with *Ccm3-iECKO* mice. **(C)** Tissue factor transcript (*F3*) expression in adult mouse cerebellar cells generated with the publicly available mouse cerebellar single-nucleus RNA-seq (snRNA-seq) dataset generated by the Broad Institute of MIT and Harvard. Each dot represents a single cell and the violin plot describes the distribution of *F3* expression level. **(D)** Tissue factor (red) expression in glial fibrillary acidic protein (GFAP) positive astrocytes (green) in P8 wild-type (upper panel) and *Ccm3-iECKO* (lower panel) mice. Vasculature is detected by isolectin (magenta). Arrowheads showing the co-localization of tissue factor and GFAP expression in astrocytes. L = lesion.

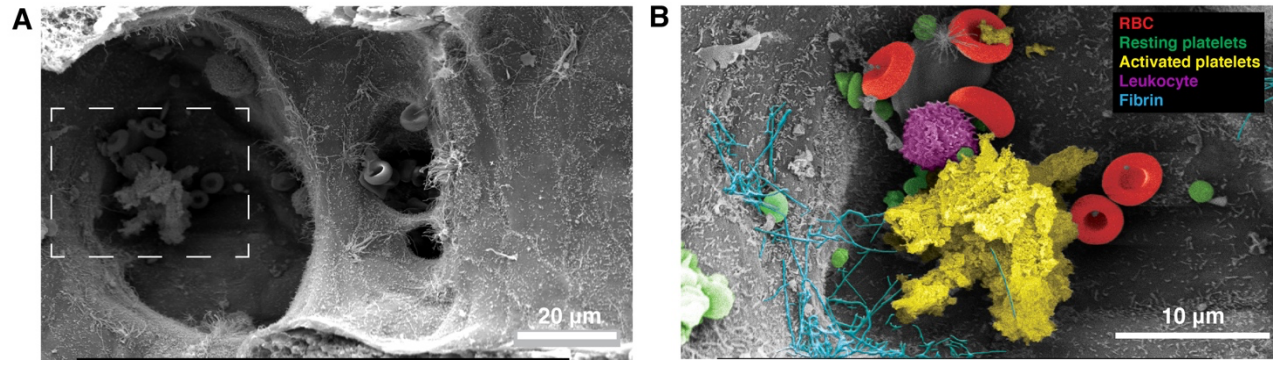


Supplemental Figure 4. Kinetic analysis of coagulation factors, erythrocytes, and hypoxia in wild-type and *Ccm3-iECKO* mice.

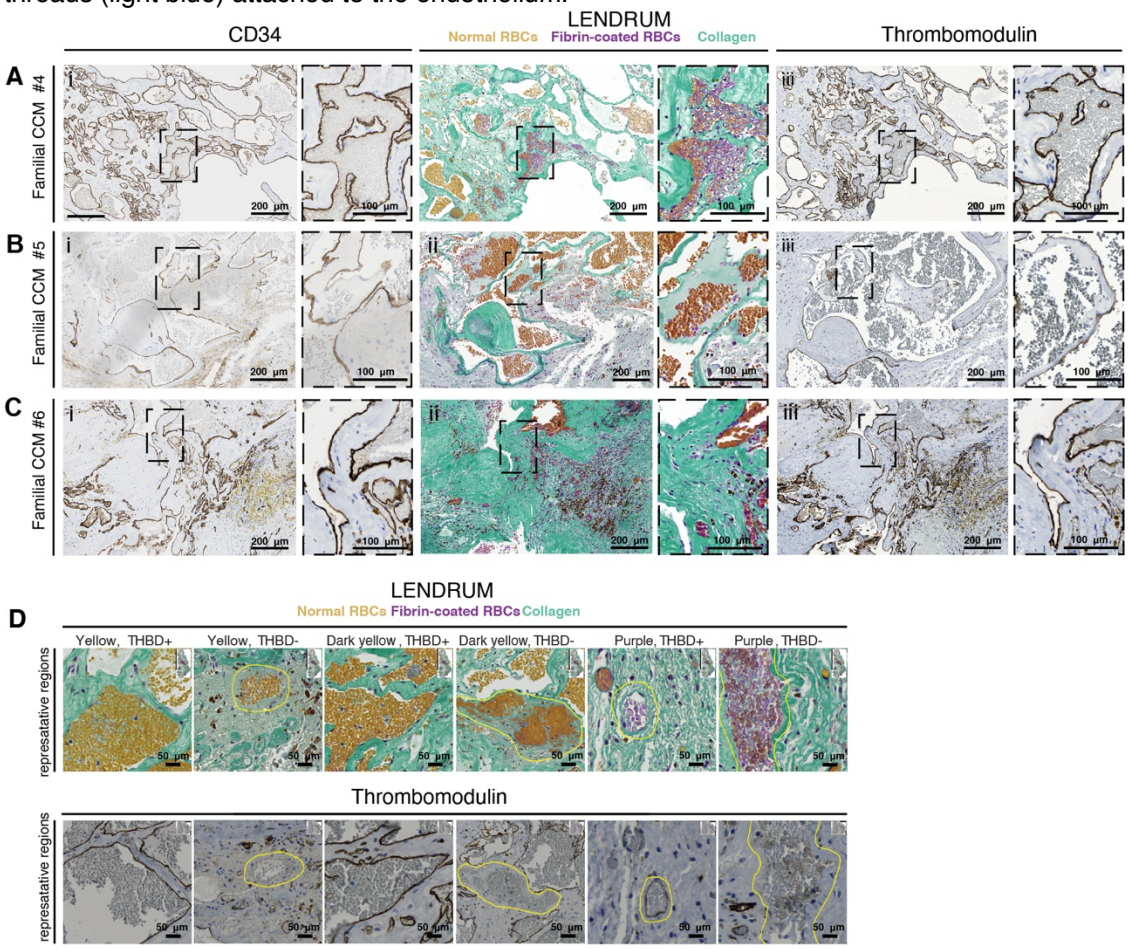
A kinetic analysis of **(A)** Fibrin-fibrinogen **(B)** Fibronectin **(C)** VWF **(D)** CD41 **(E)** TER-119 and **(F)** hypoxia (hypoxyprobe) in P6-P8 in cerebellum (cb) of wild-type (black circles) and *Ccm3-iECKO* (gray circles) mice. Gray p-values compare different timepoints between *Ccm3-iECKO* mice while black p-values compare wild-type mice to *Ccm3-iECKO* mice. Each data point represents the mean of biological replicates (n= 4-12 mice per group) and error bars represent the standard deviation in each group. A Mann-Whitney *U* test was used to compare the difference between groups. **(G-H)** Spearman correlation analysis of **(G)** Fibrin-fibrinogen vs hypoxyprobe area **(H)** erythrocytes (TER-119) vs hypoxyprobe area and **(I)** erythrocytes (TER-119) vs fibrin-fibrinogen area. The p-values (p) and correlation values are written in each graph.



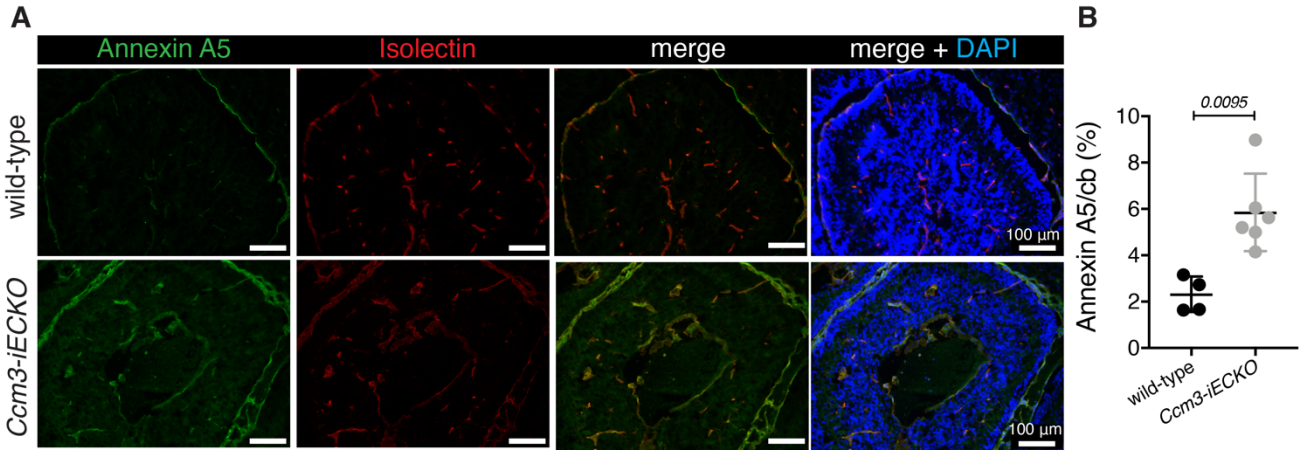
Supplemental Figure 5. Coagulation factors in human sporadic CCM biopsies.
(A) Scanned section of the biopsy from patient number 2 stained with small images to the right show color separation of fibrin and VWF within the dashed insert in the larger image. A representative clot is highlighted by an asterisk. **(B)** Scanned section of patient number 3 biopsy stained with fibrin-fibrinogen (green), VWF (magenta) and nuclei stained with DAPI (blue), but no obvious clot in this biopsy. Small images to the right show color separation of fibrin and VWF staining of slightly enlarged vessels within the dashed insert in the larger image. **(C)** A biopsy from a control human with no known neurological symptoms stained with fibrin-fibrinogen (green), VWF (magenta), and DAPI (blue).



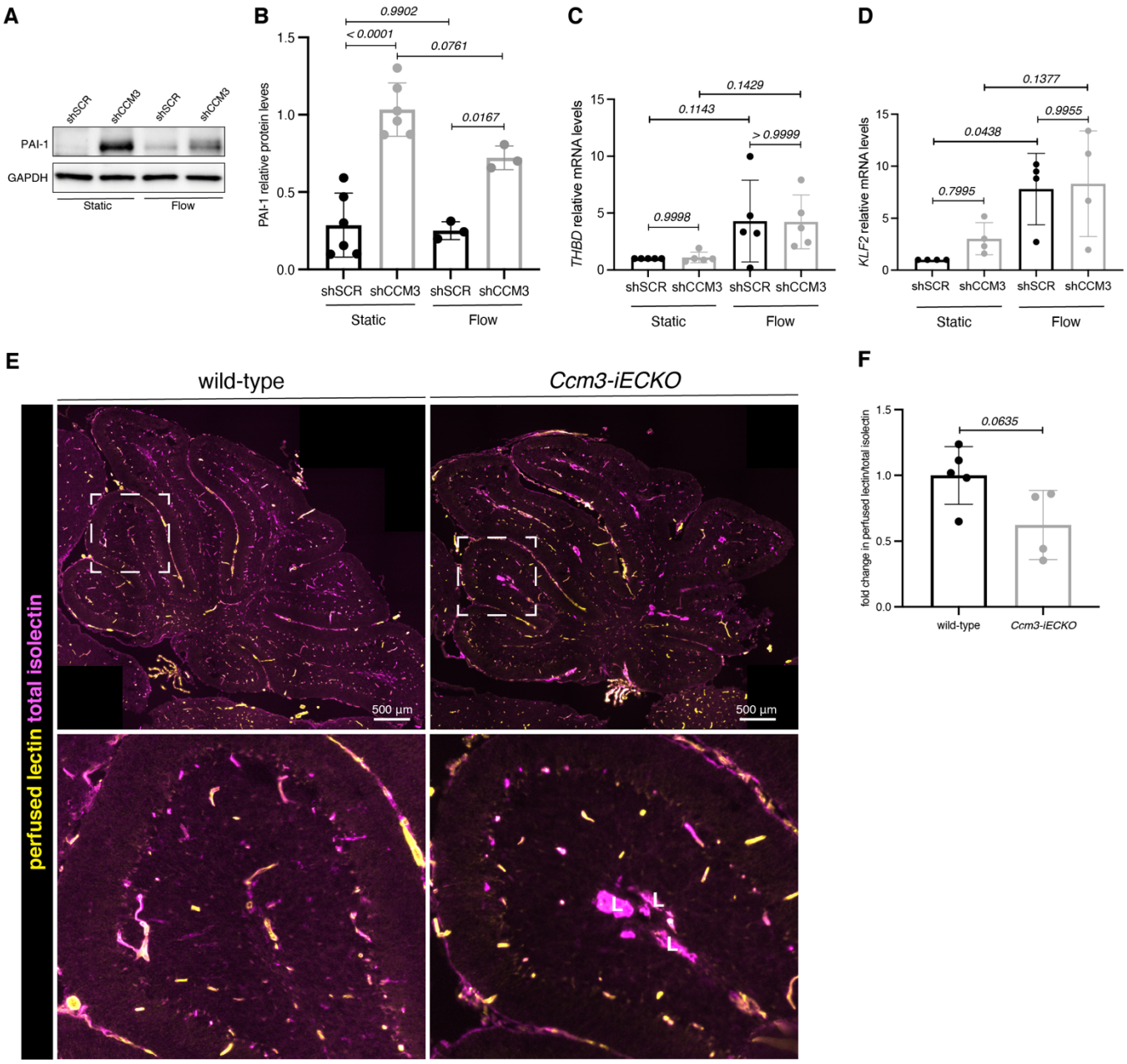
Supplemental Figure 6. Scanning electron microscopy images of *Ccm3-iECKO* lesions.
(A) Representative image of a CCM lesion devoid of clots. **(B)** Magnification of the dashed area in (A) showing a cluster of activated platelets (yellow), naïve platelets (green), erythrocytes (red), a lymphocyte (magenta) and fibrin threads (light blue) attached to the endothelium.



Supplemental Figure 7. Human familial CCM biopsies show coagulant and anticoagulant domains.
(A-C) Biopsies of **(A)** CCM patient #4 **(B)** CCM patient #5, and **(C)** CCM patient #6 stained for CD34, Fraser-Lendrum, and thrombomodulin. **(D)** Representative regions of the different levels of erythrocyte activation identified in all CCM patients (from non-activated yellow to activated and fibrin coated purple) in the presence of thrombomodulin (THBD+) or in the absence of thrombomodulin (THBD-).



Supplemental Figure 8. Annexin A5, an anticoagulant marker, is higher in *Ccm3-iECKO* mice.
(A) Annexin A5 (green), isolectin (red) and DAPI (blue) in wild-type and *Ccm3-iECKO* cerebellum section at P8. **(B)** Image quantification of Annexin A5 (p=0.0095) in the cerebellum (cb). Each data point represents one biological replicate (n=4-6 per group), the bar indicates the mean of each group and the error bars represent the standard deviation. A Mann-Whitney *U* test was used to compare wild-type mice to *Ccm3-iECKO* mice.



Supplemental Figure 9. Human brain endothelial cells transduced with shRNA (scramble or *CCM3*) exposed to flow.

(A) Western blot of PAI-1 and GAPDH in HBEC exposed to either static or flow (10 dynes) conditions. **(B)** Quantification of PAI-1 protein, **(C)** thrombomodulin transcript (*THBD*), **(D)** *KLF2* transcript in static or flow conditions of shScramble (shSCR) and *shCCM3*. An ordinary one-way ANNOVA with Tukey's multiple comparison test was used to determine the difference between the groups. Respective p-values are indicated in each graph. **(E)** Representative images of wild-type and *Ccm3-iECKO* sectioned brains post-lectin perfusion. **(F)** Quantification of perfused vessels. A Mann-Whitney *U* test was used to compare wild-type mice with *Ccm3-iECKO* mice (p=0.0635).

Supplemental Tables

Supplemental Table 1. Antibodies and reagents used for immunofluorescence

Reagent	*Company and catalog number*	*Dilution*
Annexin V	ProteinTech #11060-1-AP	1:100
CD31 (PECAM1)	R&D #AF3628	1:200
CD41-PE	BD Pharmingen #558040	1:200
CD62P-FITC	BD Pharmingen #553744	1:200
CD42b	Abcam #ab183345	1:100
Collagen IV	AbD Serotec #2150-1470	1:100
Isolectin B4	Vector Laboratories #B-1205	1:200
Fibrin-fibrinogen	Nordic MU Bio #GAM/Fbg/7S	1:500
Fibronectin	Abcam #ab2413	1:300
TER-119	BD Pharmingen #553671	1:500
Tissue factor	Abcam #ab151748	1:300
Thrombomodulin	R&D #AF3894	1:200
VE-cadherin	Cell Signaling #2500	1:200
VWF	Dako #A0082	1:500

Supplemental Table 2. Antibodies used for western blot

Reagent	*Company and catalog number*	*Dilution*
CCM3	ProteinTech #10294-2-AP	1:1000
β-actin	Abcam #ab8227	1:1000
GAPDH	Abcam #ab181602	1:1000
PAI-1	Abcam #ab66705	1:1000
Tissue factor	Abcam #ab228968	1:1000
VEGF-A	Abcam #ab46154	1:1000

Supplemental Table 3. Number of mice used in animal experiments

Methods	*No. of wild-type mice/ timepoint*	*No. of Ccm3-iECKO mice/ timepoint*
RNA-seq	3	3
scRNA-seq	2	2
Visium	2	2
RNAscope	4	5
Immunofluorescence	4-12	4-12
CLARITY	3	3
Lectin perfusions	5	4

Supplemental Movies

Supplemental movie 1. Wild-type mice do not exhibit fibrin clots or hypoxia. The brains of wild-type mice were cleared and imaged with a confocal microscope (1 mm sections). A 3-dimensional reconstruction of a z-stack was made into a movie to show that wild-type mice did not exhibit any fibrin clots or hypoxia. Fibrin-fibrinogen is shown in green; hypoxia is shown in red, and Collagen IV marks the vasculature in white.

Supplemental movie 2. *Ccm3-iECKO* mice have fibrin clots and hypoxia. The brains of *Ccm3-iECKO* mice were cleared and imaged with a confocal microscope (1 mm sections). A 3-dimensional reconstruction of a z-stack was made into a movie to show that *Ccm3-iECKO* mice had fibrin clots in the lesions and that hypoxia surrounded large lesions. Fibrin-fibrinogen is shown in green; hypoxia is shown in red, and Collagen IV marks the vasculature in white.

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