

## Supplemental Materials and Methods

### Bone marrow transplantation

*Nemo<sup>beKO</sup>* and control mice aged 7-8 weeks received a total body irradiation of 10 Gy divided in 2 doses (1.38 Gy/min) with a 4 h break in between. The next day, we sacrificed *Actb-Egfp* mice as donors by decapitation under deep anesthesia. Hind limbs were removed and collected in PBS on ice. All following steps were carried out at a sterile workbench at 4 °C. Bone marrow was flushed out of tibia and femur. The cell suspension was centrifuged at 500 x *g* for 5 min at 4 °C, filtered through a 40- $\mu$ m cell strainer (BD), and washed again with PBS. We counted cells in a Fuchs-Rosenthal chamber and adjusted the concentration to 10<sup>8</sup> cells/ml. Then, anesthetized recipient mice received a retro-orbital injection of 5 x 10<sup>6</sup> cells. Six weeks after transplantation, mice received tamoxifen injections and were perfused after another 14-15 days. The GFP signal was detected by immunohistochemistry using anti-GFP antibodies (# ab13970, Abcam) and Alexa 488-labeled anti-chicken secondary antibodies (# ab150169, Abcam).

### Cell culture

Primary brain endothelial cells (PBECS) from *Nemo<sup>beKO</sup>* and *Nemo<sup>oFl</sup>* mice after 14 days of tamoxifen diet were isolated and cultured as described previously.<sup>1</sup>

### Western blotting

If not indicated otherwise, either the cerebellum or PBECS were homogenized in cell lysis buffer (Cell Signaling Technology) supplemented with 1 % PMSF. The supernatant was collected as protein samples after centrifugation (4 °C, 18,000 *g*, 5 min). Samples were mixed with SDS-PAGE buffer, incubated at 95 °C for 5 min and loaded on SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with the indicated primary antibodies overnight at 4 °C and, subsequently, with HRP-conjugated secondary antibodies for 1-2 h at room temperature. For detection, we applied enhanced chemiluminescence (SuperSignal West Femto Substrate; Thermo Fisher Scientific) and a digital detection system (GelDoc 2000; Bio-Rad Laboratories).

#### **Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay**

Brains were post-fixed in 4 % PFA in PBS for 24 h and cryoprotected in 30 % sucrose for another 24 h. Then, they were snap-frozen in isopentane on dry ice and kept at -80 °C. Cryosections (40 µm) underwent antigen retrieval with sodium citrate buffer (10 mM, 0.05 % TWEEN 20) at 95 °C for 20 min. After immunostaining as described above, the TUNEL assay was performed as described by the manufacturer (#11684795910, Roche).

#### **Image analysis**

Quantification of string vessels has been described previously.<sup>2</sup> Briefly, we determined string vessels or empty basement membrane tubes by measuring the length of ColIV<sup>+</sup> and CD31<sup>-</sup> vessels

with ImageJ software. Vessel length was measured in anti-CD31-stained sections. If not stated otherwise, all parameters were analyzed in two to four images in the cortex.

To analyze vessel intussusception, a 3D reconstruction was created by Imaris 9.3.0 (Bitplane, Belfast, UK). Vasculature from cortex of the *Nemo*<sup>beKO</sup> mice was immunostained for ColIV. A vessel fragment was 3D cropped from the vessel that underwent intussusception. A surface was created with the “Surface” function and smoothed under 0.4- $\mu\text{m}$  surface details.

To test for an association between proliferating and dying ECs, Ki67<sup>+</sup> ECs were counted in cortical sections and analyzed in comparison to the number of active caspase 3<sup>+</sup> ECs obtained in the same samples during a previous study.<sup>2</sup> All images were processed and analyzed using ImageJ software (National Institutes of Health) if not specified otherwise. A detailed description of the interaction analysis between string vessels and Ki67<sup>+</sup> ECs and between proliferating vessels and hypoxia is provided in the supplementary materials.

For the association analysis between string vessels and proliferating ECs, the confocal microscopy images of immunostainings for collagen IV (ColIV), CD31, and Ki67 were used. An image with a size of 775 x 775  $\mu\text{m}^2$  was evenly divided into 25 squares (155 x 155  $\mu\text{m}^2$ ) by using ImageJ (National Institutes of Health). Each small square was defined as one sample unit (SU). A string vessel (SV) was defined as a string-like structure which was ColIV<sup>+</sup>CD31<sup>-</sup>, whereas proliferating ECs (Ki67EC) were defined as cells which were Ki67<sup>+</sup>CD31<sup>+</sup>. All the SUs were sorted into four different groups, namely, SV<sup>+</sup>Ki67EC<sup>+</sup>, SV<sup>+</sup>Ki67EC<sup>-</sup>, SV<sup>-</sup>Ki67EC<sup>+</sup>, and SV<sup>-</sup>Ki67EC<sup>-</sup>. The number of these four different SUs was counted from different images of different mice. Therefore, a 2 x 2 contingency table (see below) was created. If there was no interaction between SV and Ki67EC, the number of SUs from those 4 groups would be evenly distributed, and vice versa.

		Ki67		SUM
		Present	Absent	
String vessel	Present	a	b	m=a+b
	Absent	c	d	n=c+d
SUM		r=a+c	s=b+d	N=a+b+c+d

A  $X^2$  test was used to test the spatial interaction between string vessels and proliferating ECs according to the following formula:

$$X^2 = \frac{N[|(ad) - (bc)| - (N/2)]^2}{mnr}$$

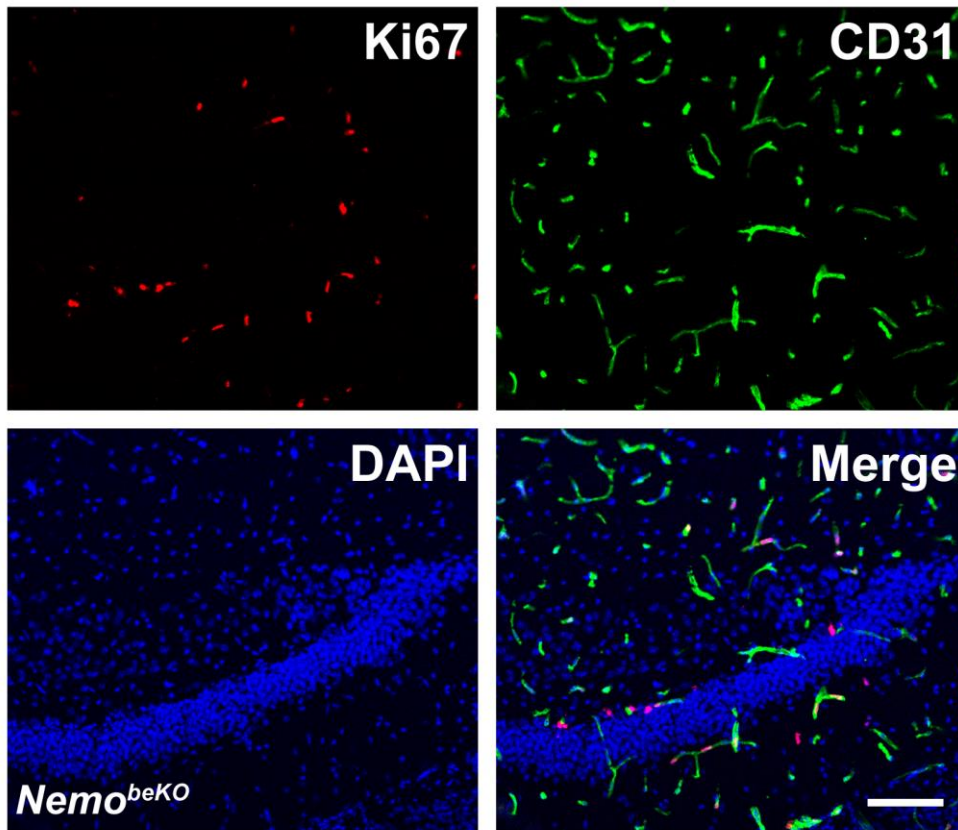
For the association analysis between proliferating vessels and hypoxic area, ImageJ software (National Institutes of Health) was used. COLIV<sup>+</sup> vessels with EdU<sup>+</sup> cells inside were identified as proliferating vessels (Online Figure IVA). The proliferating vessels were manually cropped and masked after setting the threshold in “Huang” mode (36, 255) (Online Figure IVB). Hypoxic areas were labeled by the hypoxia probe (HP) in the same section and masked by setting the threshold at (40,255) (Online Figure IVC). The image was then converted to a binary figure and the outliers were removed (radius=5, threshold =50, which=Bright). Thus, the hypoxic areas are shown as white areas (Online Figure IVD). The edges of the white areas were identified, selected automatically, and then enlarged for 150  $\mu$ m, which represented the area within 150  $\mu$ m to the nearest hypoxic staining (Online Figure IVE). Those selections were copied to the related proliferating vessel mask, showing the vessels within a certain distance to the nearest hypoxic area (Online Figure IVF). The proliferating vessel number per area within and outside were counted and calculated for the analysis of the spatial interaction between proliferating vessels and hypoxia.

To distinguish a successful recombination in different arteriolar cell types of *S/co1c1-Cre<sup>GFP→HA</sup>* mice a 3D-image was created with imaris 9.3.0 (Bitplane, Belfast, UK). Vasculature from the cortex of the mice was immunostained for SMA and HA. An arteriole with both GFP and HA signal was chosen. Three surfaces, one each for HA-, GFP- and SMA-signals, were created with the “Surface” function and smoothed under 0.481- $\mu$ m surface details.

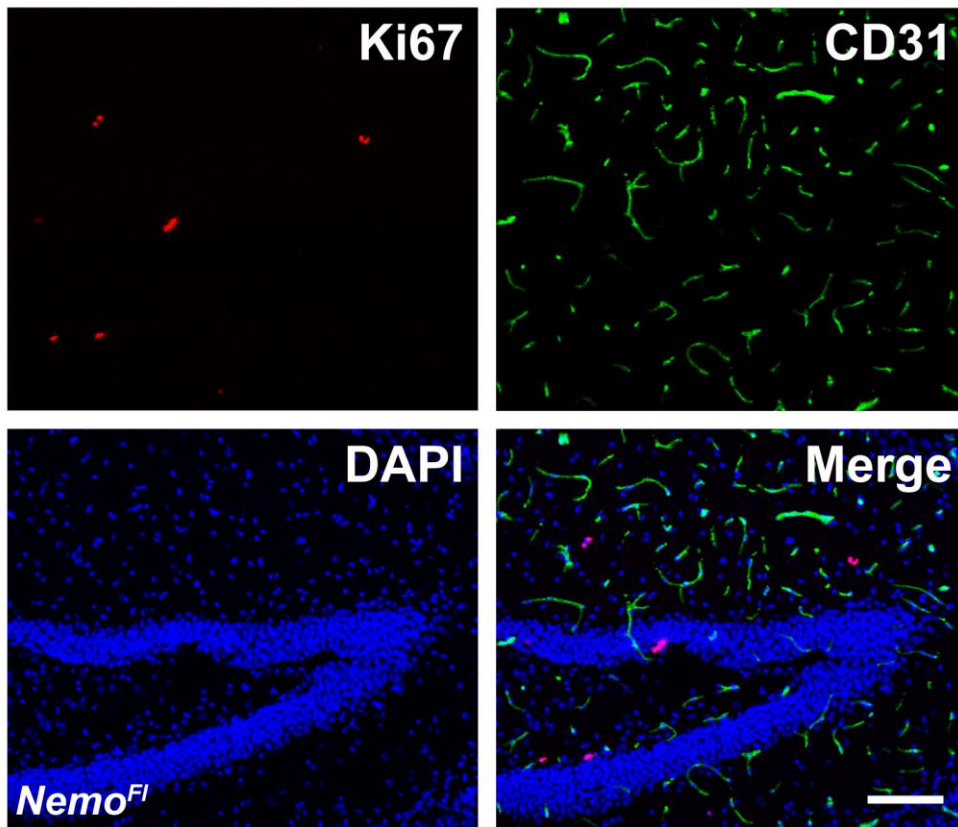
## Supplemental Figures and Figure Lengends

## Supplementary Figure 1

(a)



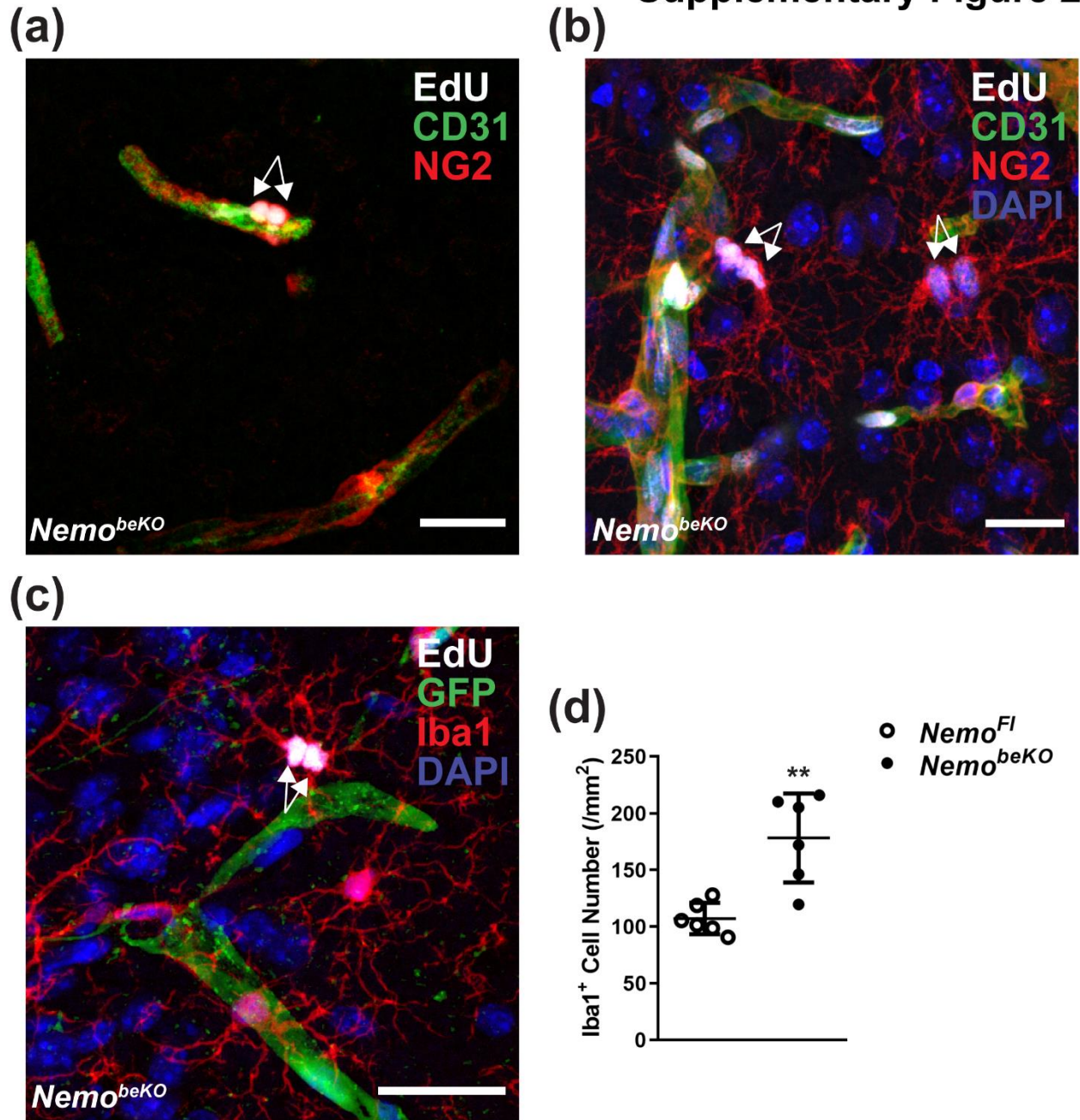
(b)



**Supplementary Figure 1.** Angiogenesis in the hippocampus of *Nemo<sup>beKO</sup>* mice. Representative immunostainings for Ki67 and CD31 showing proliferating cells along vessels in the hippocampus of *Nemo<sup>beKO</sup>* mice (a), but not *Nemo<sup>Fl</sup>* mice (b) at day 15 after starting tamoxifen injections. Nuclei were stained with DAPI. Scale bars, 100  $\mu$ m.



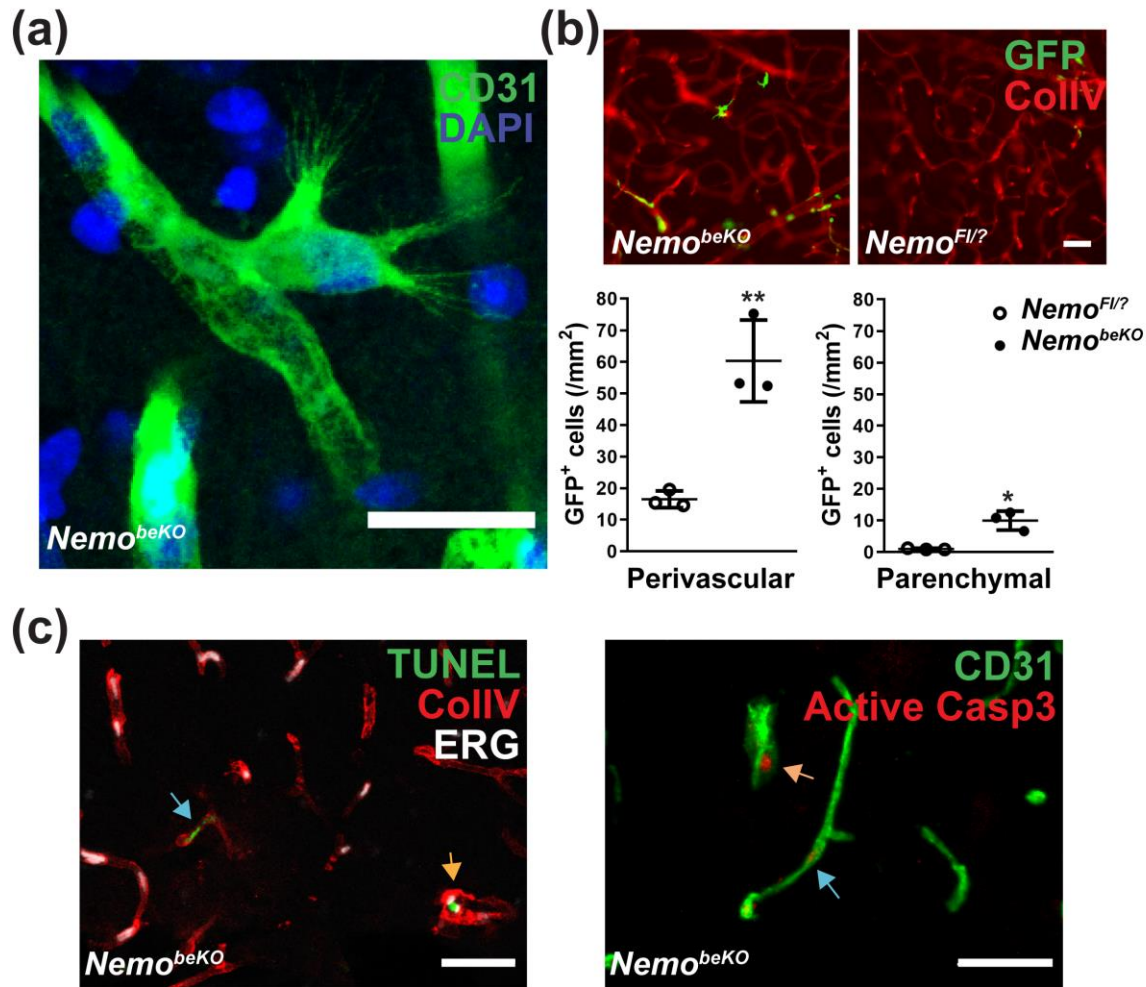
## Supplementary Figure 2



**Supplementary Figure 2.** Proliferation of non-ECs in *Nemo<sup>beKO</sup>* mice. (a) Representative stainings for EdU, CD31, and NG2 in cryosections of *Nemo<sup>beKO</sup>* mice showing proliferating pericytes (white arrows) in the cortex. (b) Representative stainings for EdU, CD31, and NG2 in free-floating sections of *Nemo<sup>beKO</sup>* mice showing proliferating oligodendrocyte precursor cells (OPCs) (white arrows) in the cortex. Nuclei were stained with DAPI. (c) Representative staining for EdU, Iba1, and GFP in the cortex. Nuclei were stained with DAPI. (d) Quantification of Iba1<sup>+</sup> cell number in *Nemo<sup>Flt1</sup>* (open circles) and *Nemo<sup>beKO</sup>* (filled circles) mice. \*\* indicates statistical significance (p < 0.01).

and GFP showing a proliferating microglial cell next to a vessel in the cortex of a *Nemo*<sup>beKO</sup> mouse. Microvessels here were labeled by injecting the mice with with the AAV-BR1-eGFP virus intravenously. Nuclei were stained with DAPI. (d) Quantification of Iba1<sup>+</sup> cells showing the increase of microglia population (n = 6 mice per genotype). Data are shown as mean  $\pm$  SD, \*\*P <0.01, determined by two-tailed unpaired t-test. Scale bars, 20  $\mu$ m.

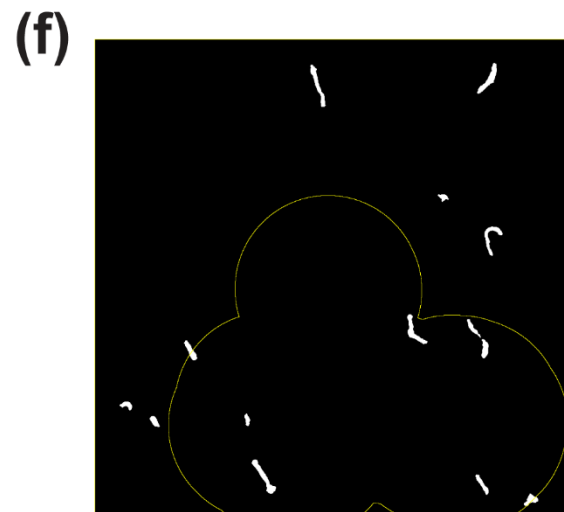
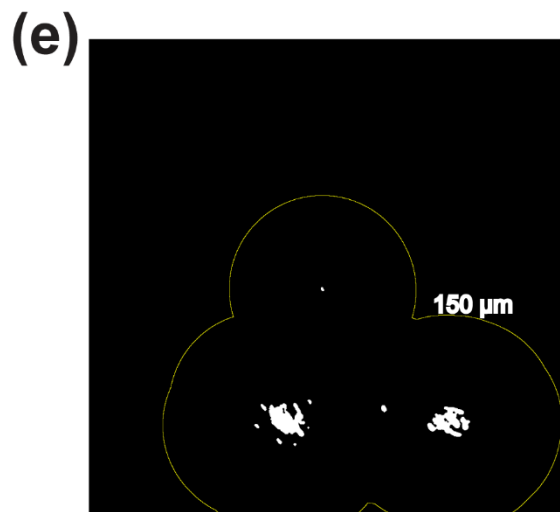
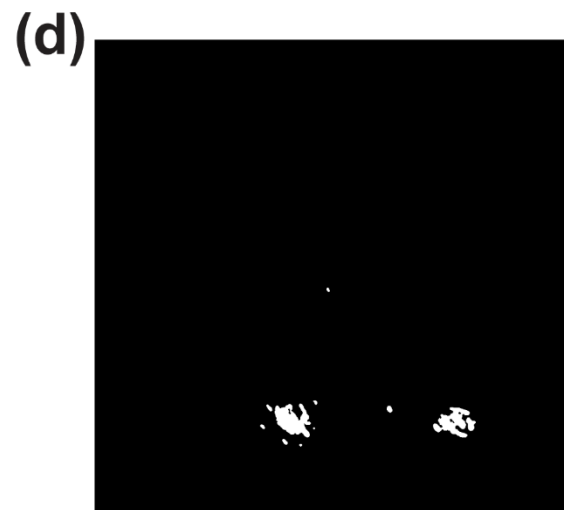
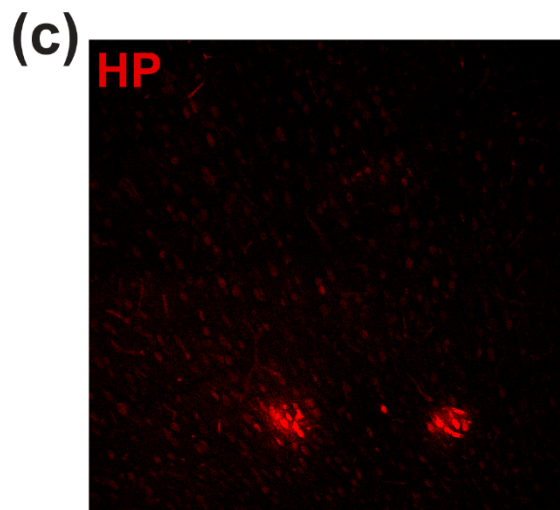
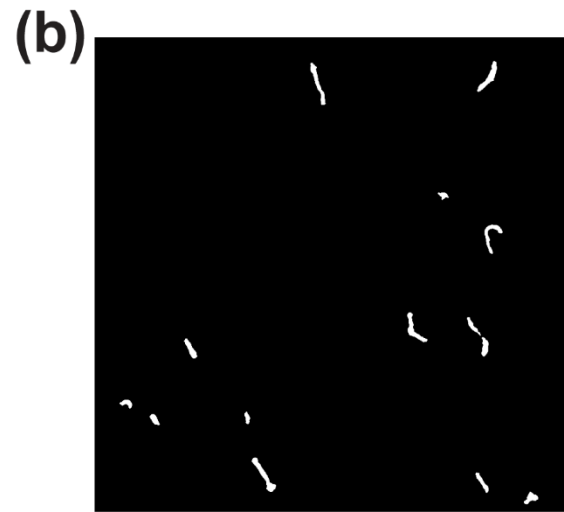
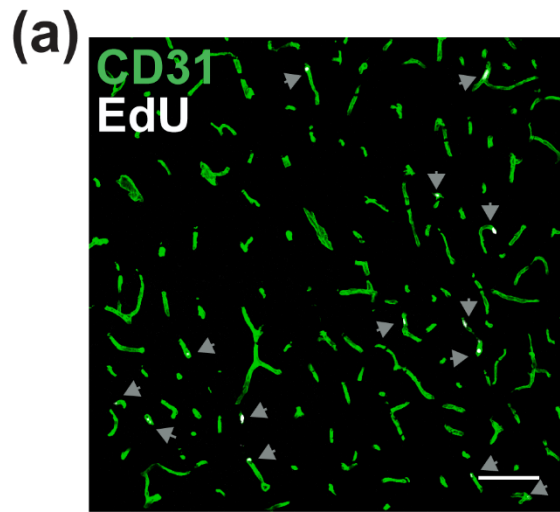
## Supplementary Figure 3



**Supplementary Figure 3.** Angiogenesis and vascular remodeling in *Nemo<sup>beKO</sup>* mice. (a) Representative immunostainings at day 16 after the start of tamoxifen injection for CD31 and DAPI, showing a tip cell with filopodia in the striatum of a *Nemo<sup>beKO</sup>* mouse. (b) Upper panels, representative immunostainings for GFP and ColIV in the cortex of *Nemo<sup>beKO</sup>* mice and littermate controls, which received a bone marrow transplantation from *Actb-Egfp* donors. Quantification showed an increased number of GFP<sup>+</sup> cells that were found within the perivascular space and in the parenchyma of *Nemo<sup>beKO</sup>* mice. (c) Representative stainings for TUNEL with ColIV and ERG (left), and CD31 with active caspase 3, showing dead ECs in both capillaries and bigger vessels. Data are depicted as means  $\pm$  SD. \*P < 0.05, \*\*P < 0.01, determined by two-tailed unpaired t-test

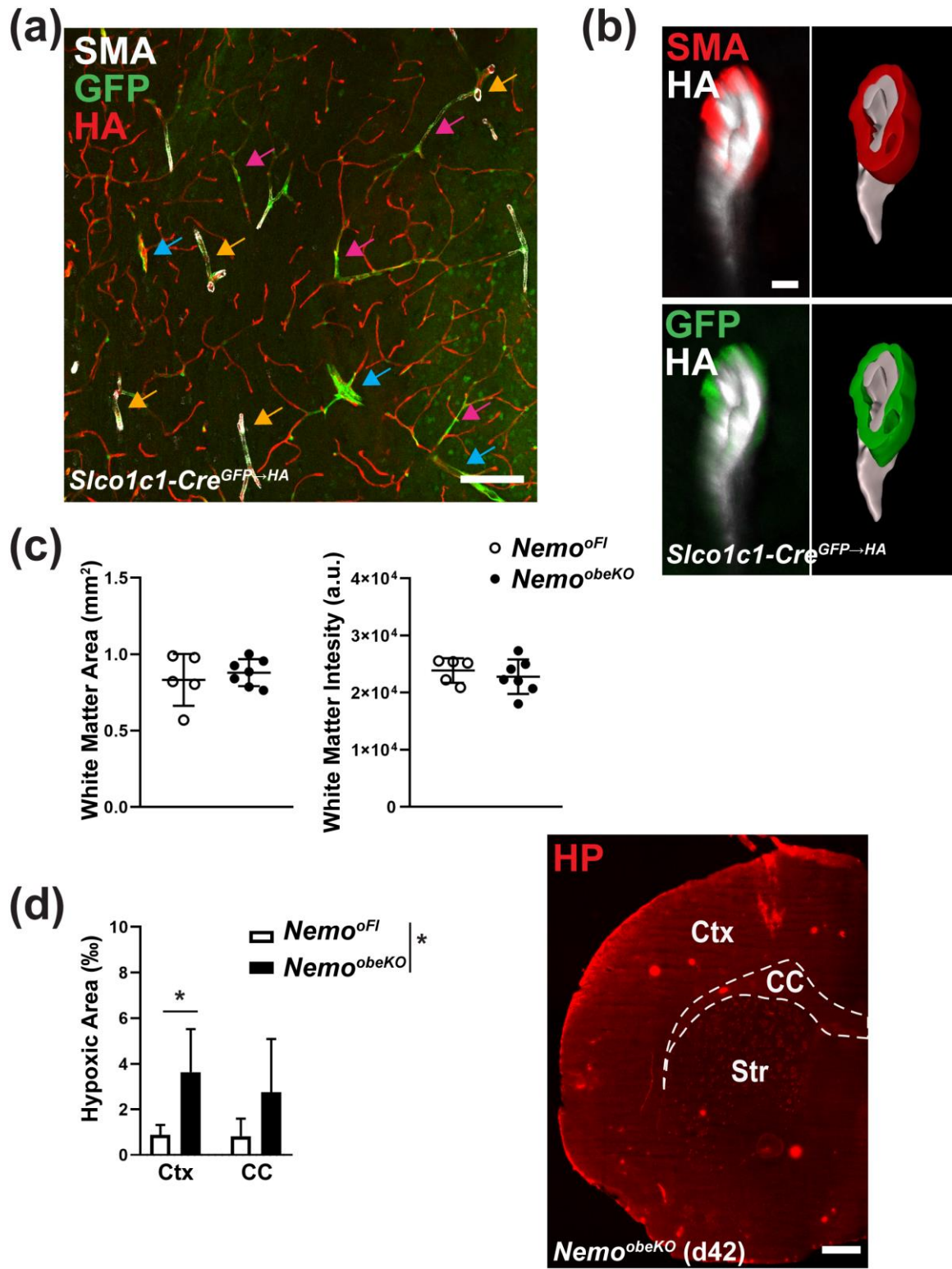
with (b down-left) without Welch's correction (b down-right). Scale bar 20  $\mu\text{m}$  (a), 100  $\mu\text{m}$  (b) and 50  $\mu\text{m}$  (c).

## Supplementary Figure 4



**Supplementary Figure 4.** Representative images showing the process of the interaction analysis between proliferating vessels and hypoxia. (a) Representative image showing the immunostaining for CD31 and EdU. Proliferating vessels (EdU<sup>+</sup>CD31<sup>+</sup>) are labeled by gray arrows. (b) A mask image of the cropped proliferating vessels. (c) Immunostaining of the hypoxia probe on the same section. (d) A mask image of the hypoxic area. (e) A hypoxia mask with yellow outlines covering the area which is 150  $\mu\text{m}$  to the nearest hypoxia area. These outlines were copied and pasted to the proliferating vessel mask (f). Scale bar, 100  $\mu\text{m}$ .

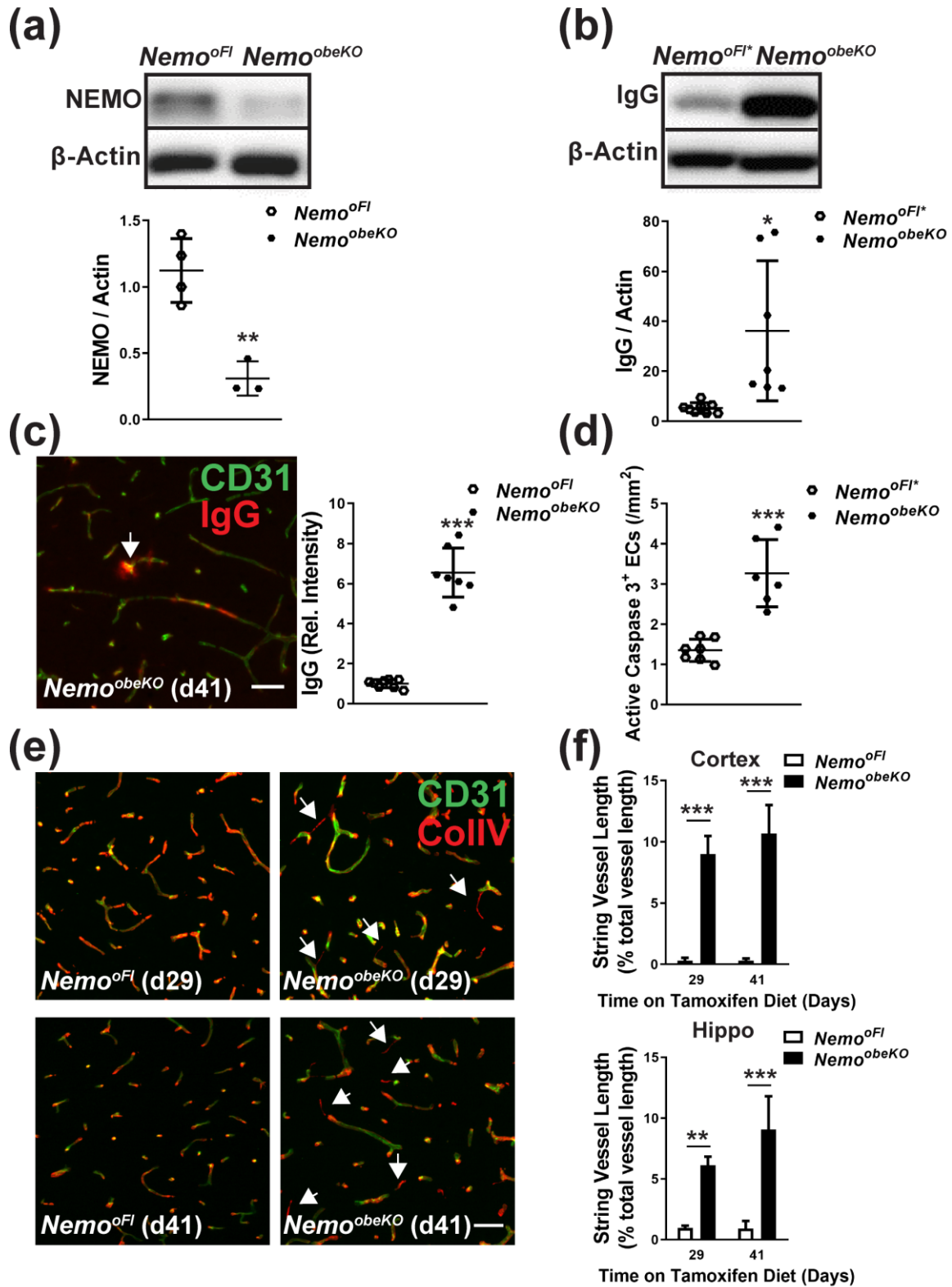
## Supplementary Figure 5



**Supplementary Figure 5.** Cre recombination and white matter properties in *Nemo*-deficient mice (a) Representative image for staining of SMA, GFP and HA in the *Slco1c1-Cre<sup>GFP→HA</sup>* mouse showing the incomplete recombination of the *Slco1c1*-cre recombinase in arterioles (orange arrows), venules (blue arrows) and capillaries (pink arrows) that leads to a genetic mosaicism in the brain vasculature. (b) Representative image of a stained arteriole for HA, SMA (top) and GFP (bottom) in high magnification showing that the cre recombination occurred rather in ECs but not in smooth muscle cells. This phenomenon most likely leads to the GFP signal in bigger vessels. The right panel of the images are the corresponding 3D reconstructions showing the same characteristics for GFP and SMA but not HA. (c) Quantification of size and intensity of FluoroMyelin-stained white matter of the corpus callosum showing no change in mice with long-term *Nemo* deficiency. (d) Representative staining for hypoxia probe (HP) (right) and its quantification (left) in different parts of the brain, showing an increased hypoxic area in the cortex (Ctx) and the corpus callosum (CC) of mice with long-term *Nemo* deficiency. Data are shown as means  $\pm$  SD, \*P < 0.05, determined by two-way ANOVA with Bonferroni's post-tests. Scale bar, 50  $\mu$ m ((a) and (d)), 5  $\mu$ m (b).



## Supplementary Figure 6



**Supplementary Figure 6.** Efficiency of oral tamoxifen treatment to induce brain endothelial *Nemo* knockout. (a) Quantification of NEMO protein levels in PBECs from *Nemo<sup>obeKO</sup>* and *Nemo<sup>oFl</sup>* mice by Western blotting (n=3-4). A representative blot is shown. (b) Quantification of IgG levels in the cerebellum of *Nemo<sup>obeKO</sup>* and *Nemo<sup>oFl\*</sup>* mice as a marker for IgG extravasation (n = 7-8). A representative blot is shown. (c) Representative immunostaining for IgG and CD31 (left) and quantification of IgG intensity (right) demonstrating increased IgG extravasation in *Nemo<sup>obeKO</sup>* mice after 41 days of tamoxifen diet (n = 7-8). (d) Quantification of active caspase 3<sup>+</sup> endothelial cells in the cortex of *Nemo<sup>obeKO</sup>* and *Nemo<sup>oFl\*</sup>* mice demonstrating increased apoptosis in *Nemo<sup>obeKO</sup>* mice (n = 6-7). (e) Representative immunostainings for CollIV and CD31 demonstrating a high string vessels level in *Nemo<sup>obeKO</sup>* mice after 29 days and 41 days of tamoxifen diet but not in the respective control mice. (f) Quantification of string vessels in the cortex (top) and hippocampus (bottom) of *Nemo<sup>obeKO</sup>* and control mice after 29 days and 41 days of tamoxifen diet as a percentage of total vessel length (n = 3-7). In this panel, *Slco1c1-CreER<sup>T2</sup>;Nemo<sup>Fl</sup>* mice were either fed with a tamoxifen diet (labeled as *Nemo<sup>obeKO</sup>*) or matched control diet (labeled as *Nemo<sup>oFl\*</sup>*) mice for 14-15 days, except for (a) and (e)-(f) where *Nemo<sup>Fl</sup>* mice were fed with tamoxifen diet as the control (labeled as *Nemo<sup>oFl</sup>*). Data are shown as means  $\pm$  SD. \*P < 0.05. \*\*P < 0.01, \*\*\*P < 0.001, determined by unpaired t-test (a, c and d) without Welch's correction (b), and two-way ANOVA with Bonferroni's post-test (f), Scale bars, 100  $\mu$ m.

**Supplementary Video.** Intussusceptive angiogenesis occurred in the cortex of *Nemo*<sup>beKO</sup> mice. The movie was rendered from confocal stacks. Vessels were labeled by anti-CollIV antibody (red) and proliferating cells were labeled by detecting EdU incorporation (white). In the movie, a cross-sectional view of an intussusceptive vessel from a *Nemo*<sup>beKO</sup> mouse is showed, followed by a 3D reconstruction of this vessel fragment that was obtained by creating the surface of CollIV staining. Scale bar, 5  $\mu\text{m}$ .

## Supplemental References

1. Assmann JC, Muller K, Wenzel J, et al. Isolation and Cultivation of Primary Brain Endothelial Cells from Adult Mice. *Bio Protoc* 2017; 7.
2. Ridder DA, Wenzel J, Müller K, et al. Brain endothelial TAK1 and NEMO safeguard the neurovascular unit. *J Exp Med* 2015; 212: 1529-1549.