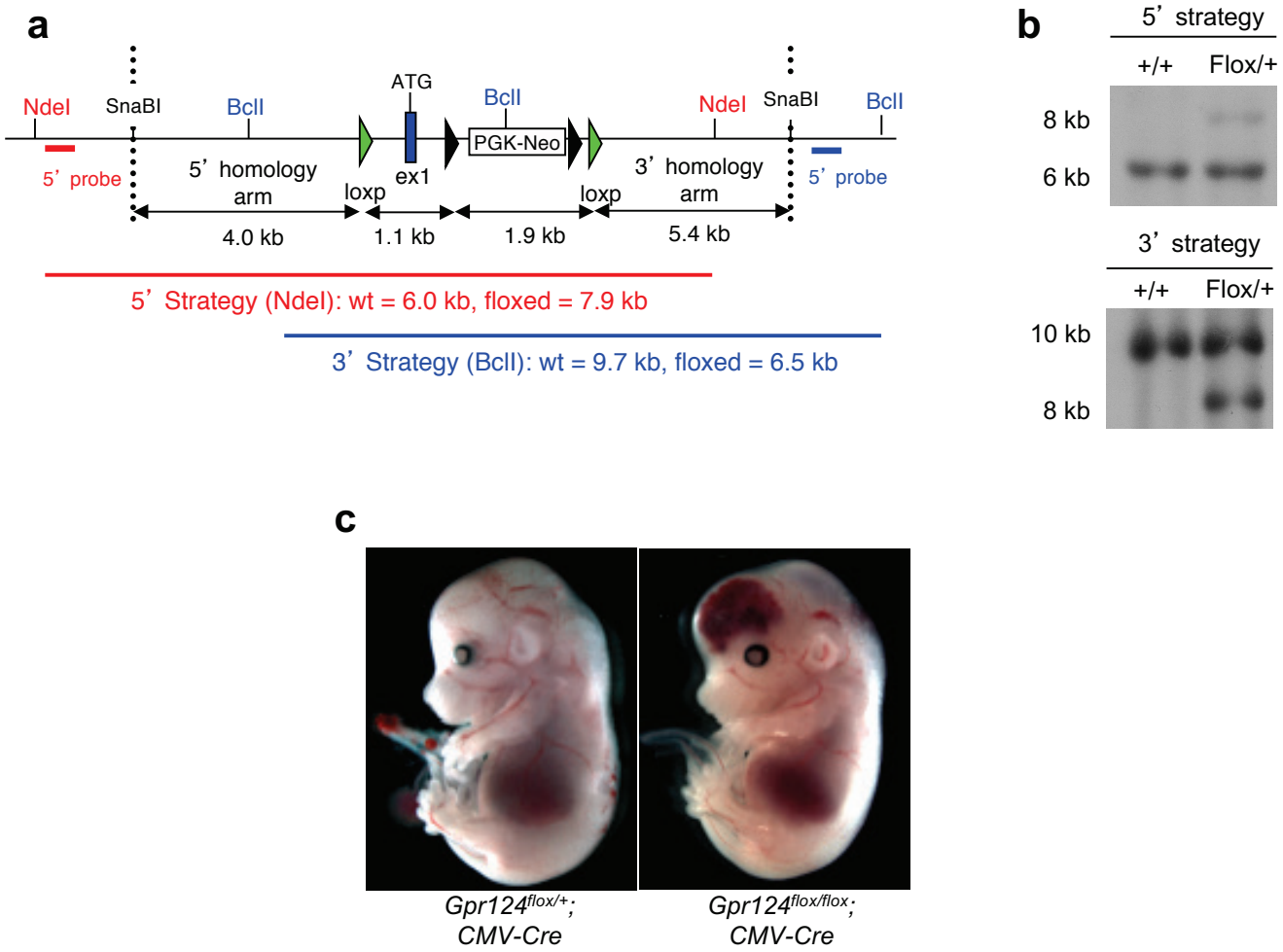
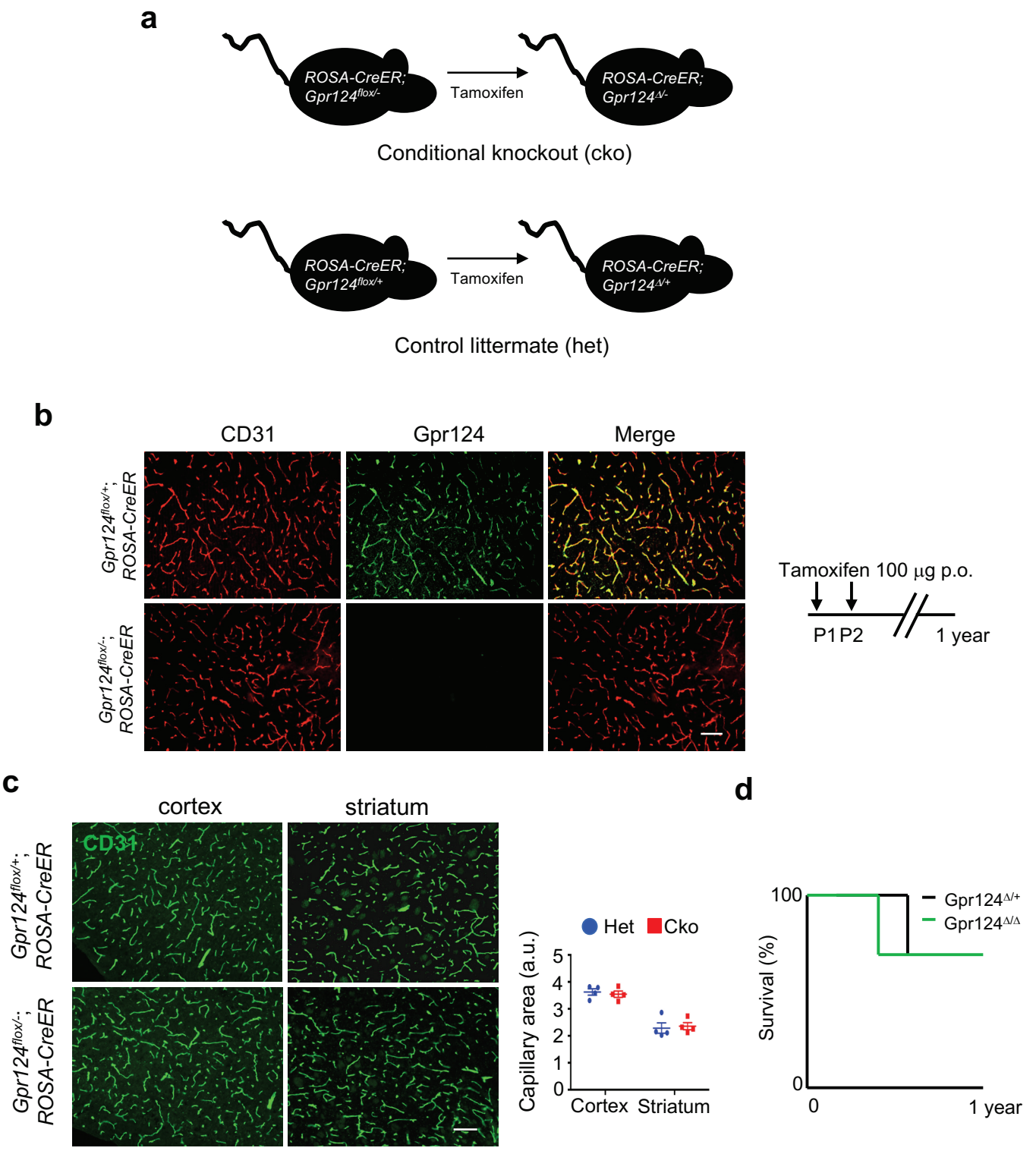
Supplementary Figure 1



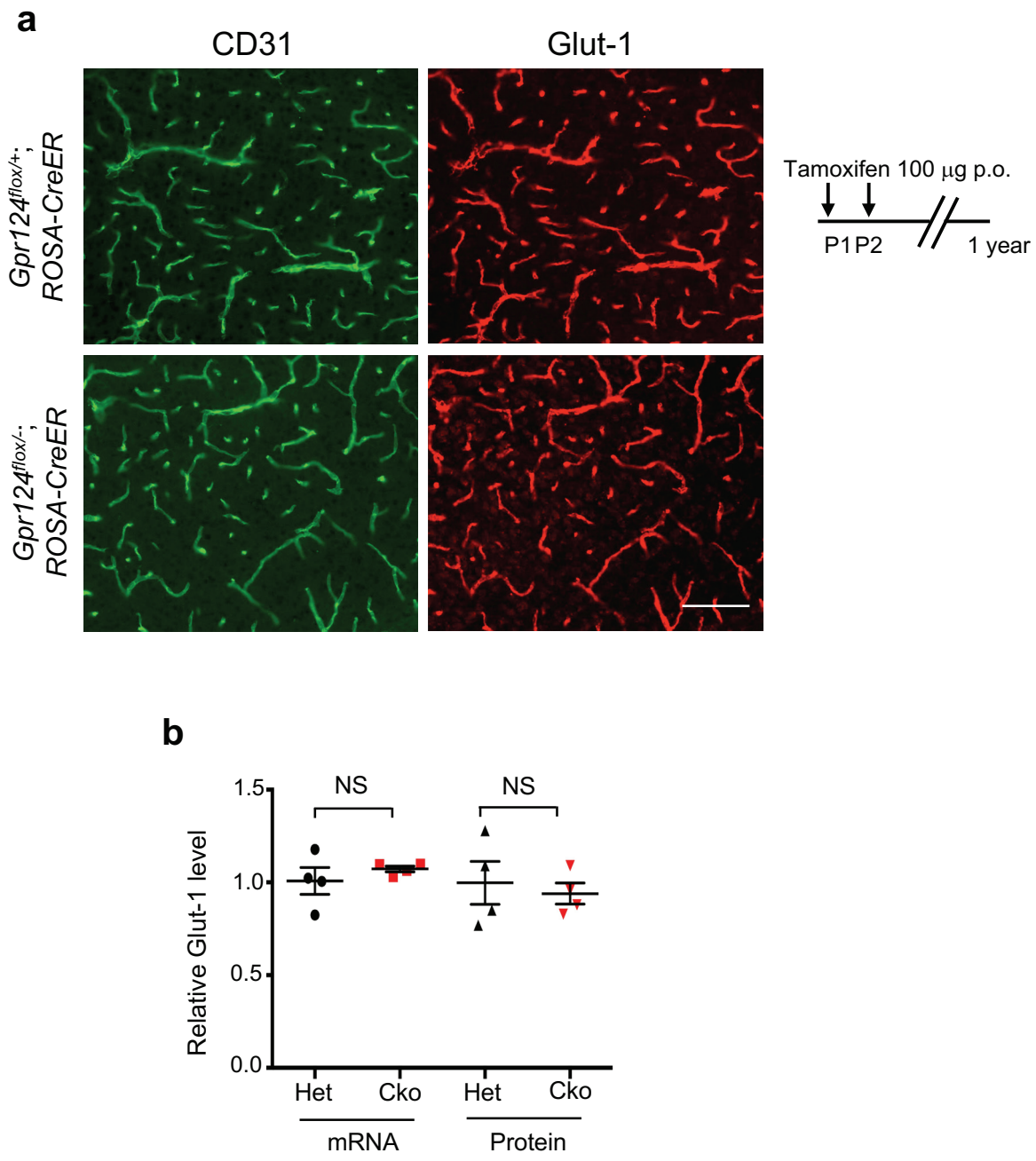
Supplementary Figure 1. Generation of the *Gpr124* conditional knock-out allele. (**a**) A targeting construct containing the first coding exon of *Gpr124* flanked with loxP sites ("floxed") was cloned and electroporated into mouse embryonic stem cells for targeting of the endogenous locus to generate the *Gpr124*^{flox} allele. (**b**) Southern blot demonstrated correctly targeted clones that were then injected into mouse blastocysts for derivation of the conditional knock-out lines, followed by confirmation of germline transmission of the *Gpr124* flox allele. (**c**) Ubiquitous deletion in *Gpr124*$^{flox/flox}$*; CMV-Cre* mice at E14.5 robustly phenocopied the forebrain hemorrhage and embryonic lethality of our previously published *Gpr124*^{lacZ} knock-in allele [Kuhnert F. et al., *Science* 2010].

Supplementary Figure 2



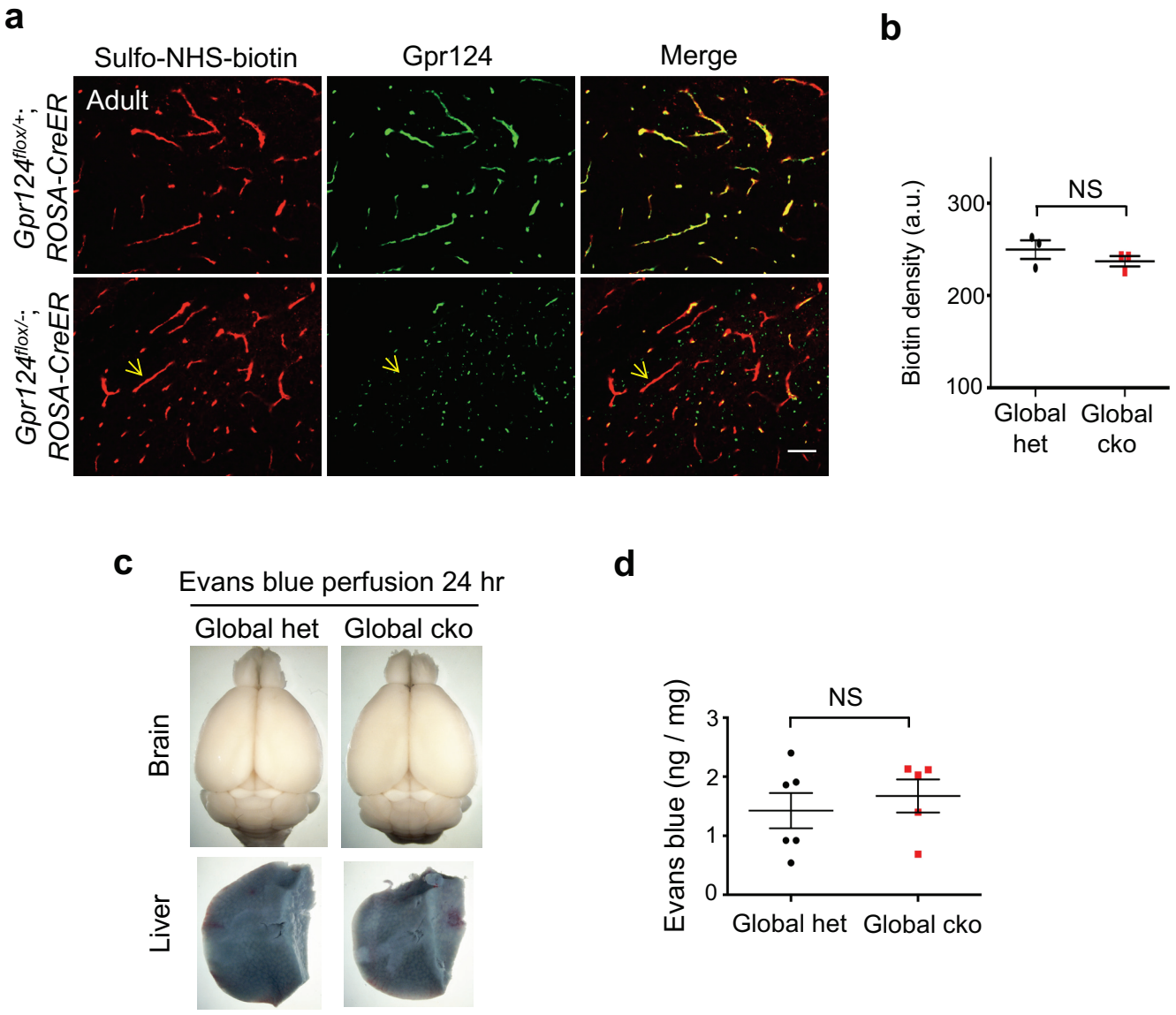
Supplementary Figure 2. Postnatal *Gpr124* deletion does not affect CNS angiogenesis and survival. (**a**) Breeding schema for global conditional *Gpr124* deletion by crossing to *ROSA-CreER* mice, deleting exon 1 and converting the $Gpr124^{flox}$ allele into a $Gpr124^{\Delta}$ allele in the setting of the *Gpr124* wild-type allele ($Gpr124^{+}$) or *Gpr124* lacZ knock-in allele ($Gpr124^{-}$). (**b**) *Gpr124* deletion at postnatal day 1 (P1) does not affect CNS vascular patterning in 1-year old $Gpr124^{flox/-}$; *ROSA-CreER* mice, as compared to $Gpr124^{flox/+}$; *ROSA-CreER* (termed as Het) mice. CD31 and Gpr124 immunofluorescence (IF), cerebral cortex. Scale bar, 100 μm. (**c**) Gpr124 deletion as early as postnatal day 1 (P1) does not affect CNS angiogenesis. a.u., arbitrary unit. 5-6 low power fields (LPFs) per mouse, *n* = 4 mice per group. Scale bar, 100 μm. (**d**) Survival was not affected by early postnatal *Gpr124* deletion. *n* = 15-20 mice per group

Supplementary Figure 3



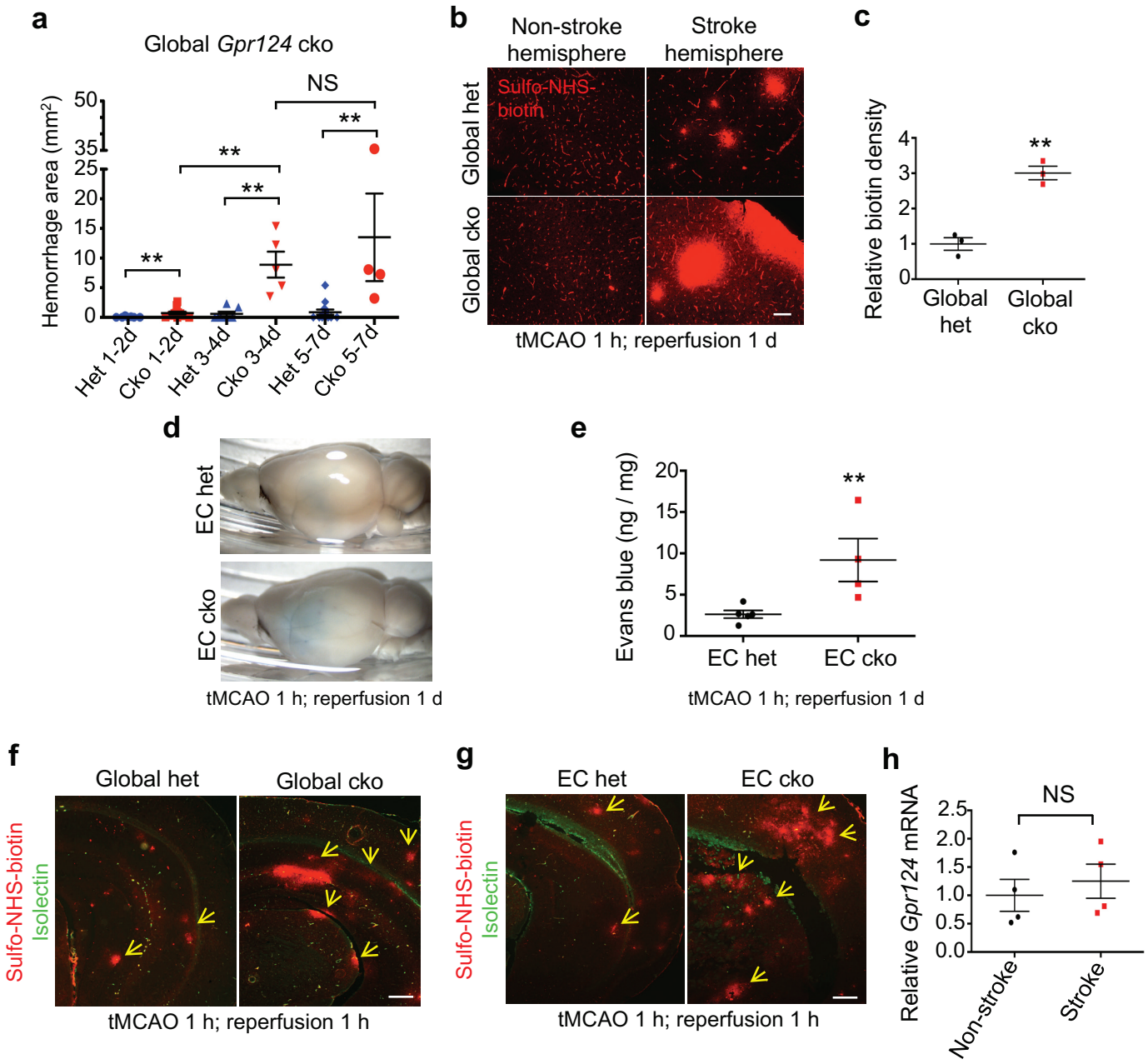
Supplementary Figure 3. Postnatal *Gpr124* deletion does not affect cerebrovascular *Glut-1* expression. (**a**) Gpr124 deletion at postnatal day 1 (P1) does not affect CNS Glut-1 expression in tamoxifen-treated 1-year old *Gpr124$^{flox/-}$; ROSA-CreER* mice. CD31 and Glut-1 co-IF, cerebral cortex. Scale bar, 100 μm. (**b**) Quantification of Glut-1 mRNA level by FACS sort/qRT-PCR of brain ECs. Glut-1 protein level was quantified by Glut-1 IF signal density normalized by CD31 signal area. 5-6 low power fields per mouse, *n* = 4 mice per group. NS, not significant, unpaired Student's *t*-test.

Supplementary Figure 4



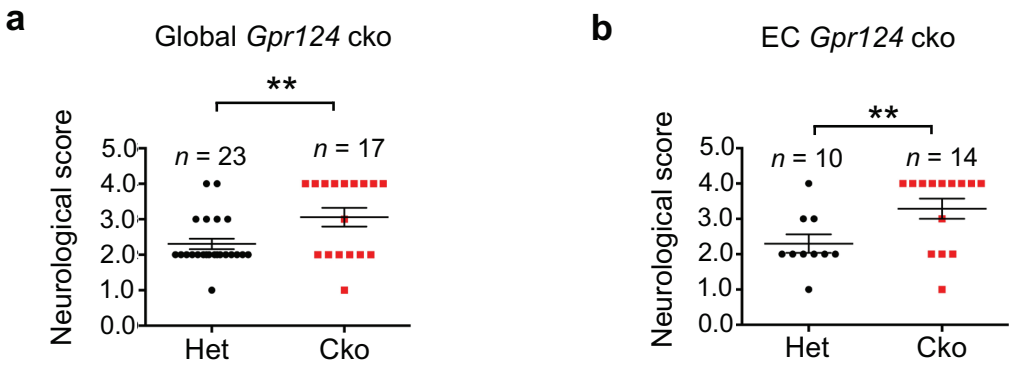
Supplementary Figure 4. Adult *Gpr124* deletion does not affect BBB integrity. Adult *Gpr124* deletion does not impact BBB function, as evaluated by Sulfo-NHS-biotin tracer perfusion (**a** and **b**) and Evans Blue perfusion (**c** and **d**). Arrows in (**a**) indicates a vessel deficient in Gpr124 with intact BBB. Scale bar, 50 μm. (**b**) Total biotin signal density was measured and normalized against vessel area (CD31 signal surface area). a.u., arbitrary unit. 5-6 low power fields per mouse, *n* = 3 mice per group. (**c** and **d**) Evans blue (2%, 100 μl/mouse) was injected IV 24 h prior to perfusion with 30 ml cold PBS. Livers were used to show successful perfusion of Evans blue. Whole brains were homogenized and Evans blue was extracted with 50% trichloroacetic acid and concentrations were measured with a fluorescent reader (620 nm excitation, 680 nm emission) and presented as ng per mg brain tissue. Tamoxifen was administered at 7-8 weeks of age. *n* = 6 for global het, *n* = 5 for global cko. Data are mean ± s.e.m. NS, not significant, unpaired Student's *t*-test.

Supplementary Figure 5



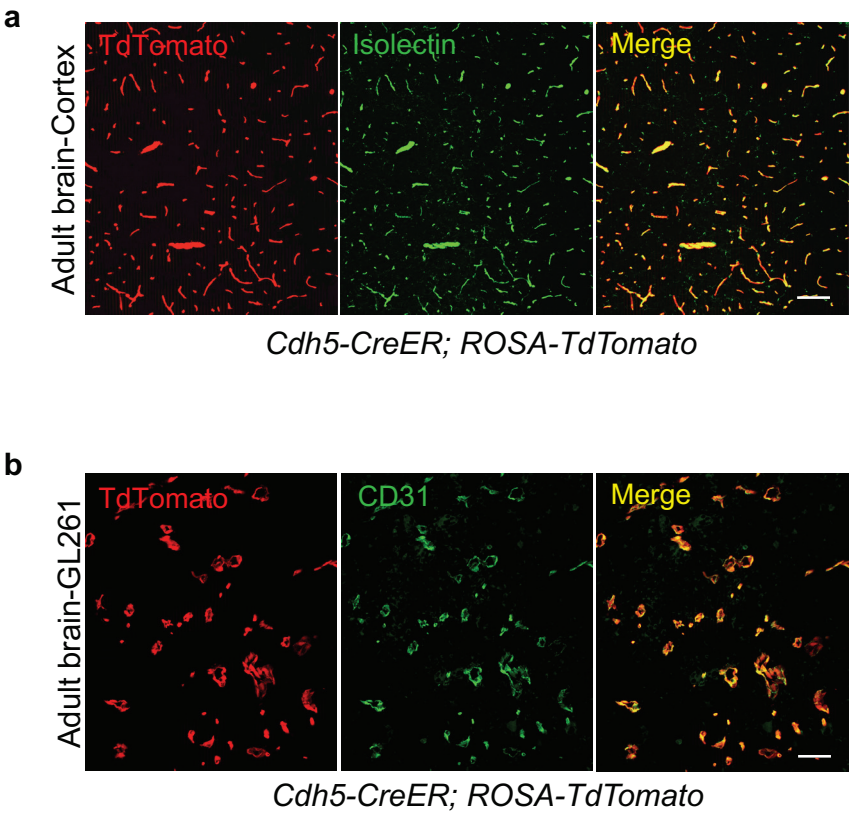
Supplementary Figure 5. *Gpr124* deletion increases hemorrhage and BBB leak following stroke. (**a**) Quantification of hemorrhage area on cortex surface and inside infarcted tissue of global *Gpr124* cko mice vs. het mice after 1 h tMCAO and 1-7 d reperfusion. Het 1-2 d, *n* = 7; Cko 1-2 d, *n* = 12; Het 3-4 d, *n* = 7; Cko 3-4 d, *n* = 5; Het 5-7 d, *n* =13; Cko 5-7 d, *n* = 4. NS, not significant, $^{**}P < 0.01$, unpaired Student's *t*-test. (**b**) Sulfo-NHS-biotin extravasation assay to evaluate BBB integrity in male global *Gpr124* cko mice and het controls after 1 h tMCAO and 1 d reperfusion. Representative images of regions in cerebral cortex were shown. Scale bar, 50 μm. (**c**) Quantification of Sulfo-NHS-biotin signal in (**b**). 3 low power fields per mouse, *n* = 3 mice per group. $^{**}P < 0.01$, unpaired Student's *t*-test. (**d, e**) Endothelial *Gpr124* het and cko mice (males) were subjected to 1 h tMCAO and Evans Blue (2%, 100 μl/mouse) was injected IV immediately. After 24 h reperfusion, mice were perfused with 30 ml cold PBS to remove blood Evans blue. Stroke hemispheres were homogenized, Evans Blue extracted with 50% TCA and concentrations were measured with a fluorescent reader (620 nm excitation, 680 nm emission) and presented as ng per mg brain tissue. *n* = 5 mice per group. $^{**}P < 0.01$, unpaired Student's *t*-test. (**f, g**) Sulfo-NHS-biotin tracer extravasation assay to assess BBB integrity after 1 h tMCAO and 1 h reperfusion. Arrows indicate biotin leaks. Representative images of both cortex and striatum from 3-4 mice per group. Scale bar, 400 μm. **(h).** *Gpr124* mRNA expression levels were not significantly changed 24 h after stroke as determined by RNA-Seq of FACS sorted brain ECs from stroke hemispheres and non-stroke hemispheres of Gpr124 het mice. NS, not significant, unpaired Student's t-test. *n* = 4 mice per group.

Supplementary Figure 6



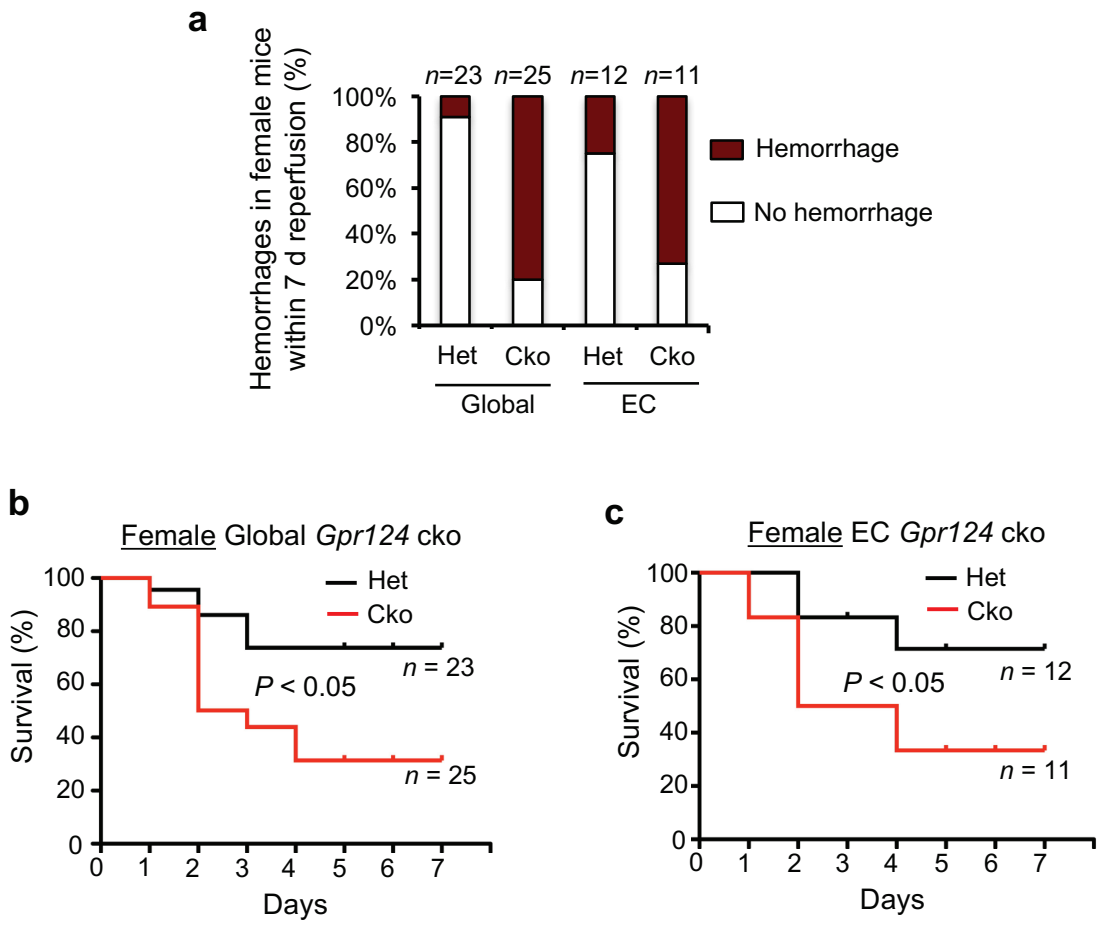
Supplementary Figure 6. *Gpr124* deletion increases neurological scores following stroke (**a**) Neurological score of male *global Gpr124 cko* mice and het controls 1 day after 1 h tMCAO. ***P* < 0.01, unpaired Student's *t*-test. (**b**) Neurological score of male endothelial *Gpr124* cko mice and het controls 1 day after 1 h tMCAO. ***P* < 0.01, unpaired Student's *t*-test.

Supplementary Figure 7



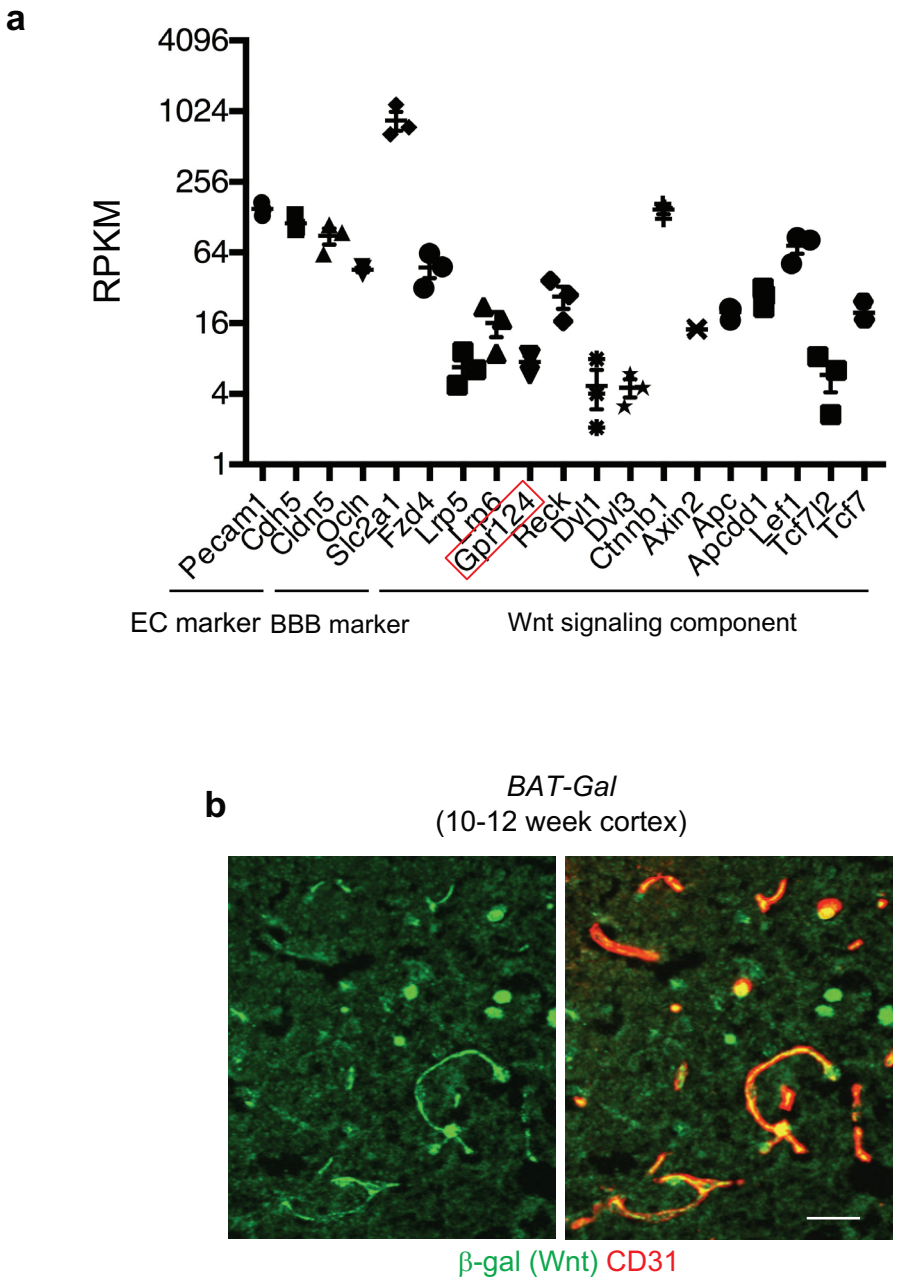
Supplementary Figure 7. Evaluation of the efficiency and specificity of the *Cdh5-CreER* driver using a *ROSA-LSL-TdTomato* reporter allele. Adult *Cdh5-CreER; ROSA-TdTomato* backcrossed to C57Bl/6J without (**a**) or with implanted GL261 glioblastoma cells (**b**) were treated by Tamoxifen (p.o. X4, every other day, 2 mg/10 g BW). TdTomato signal was determined 1 week after the last treatment by direct fluorescence. Scale bar, 100 μm. Note essentially complete overlap between TdTomato and isolectin/CD31 immunofluorescence. (**c**) Brains of adult *Cdh5-CreER; ROSA-TdTomato* mice were dissociated for FACS analysis of the TdTomato signal in the CD31$^+$ ECs and Pdgfrβ$^+$ pericytes (PC), respectively. (**d**) qRT-PCR of *Gpr124* mRNA levels in brain endothelial cells (EC) and pericytes (EC) of *Gpr124$^{flox/-}$; Cdh5-CreER* (Cko) and *Gpr124$^{flox/+}$; Cdh5-CreER* (Het) mice. Gpr124 expression levels were normalized to the corresponding cellular compartment of Het controls. Data shown as mean ± s.e.m., *n* = 4 mice per group.

Supplementary Figure 8



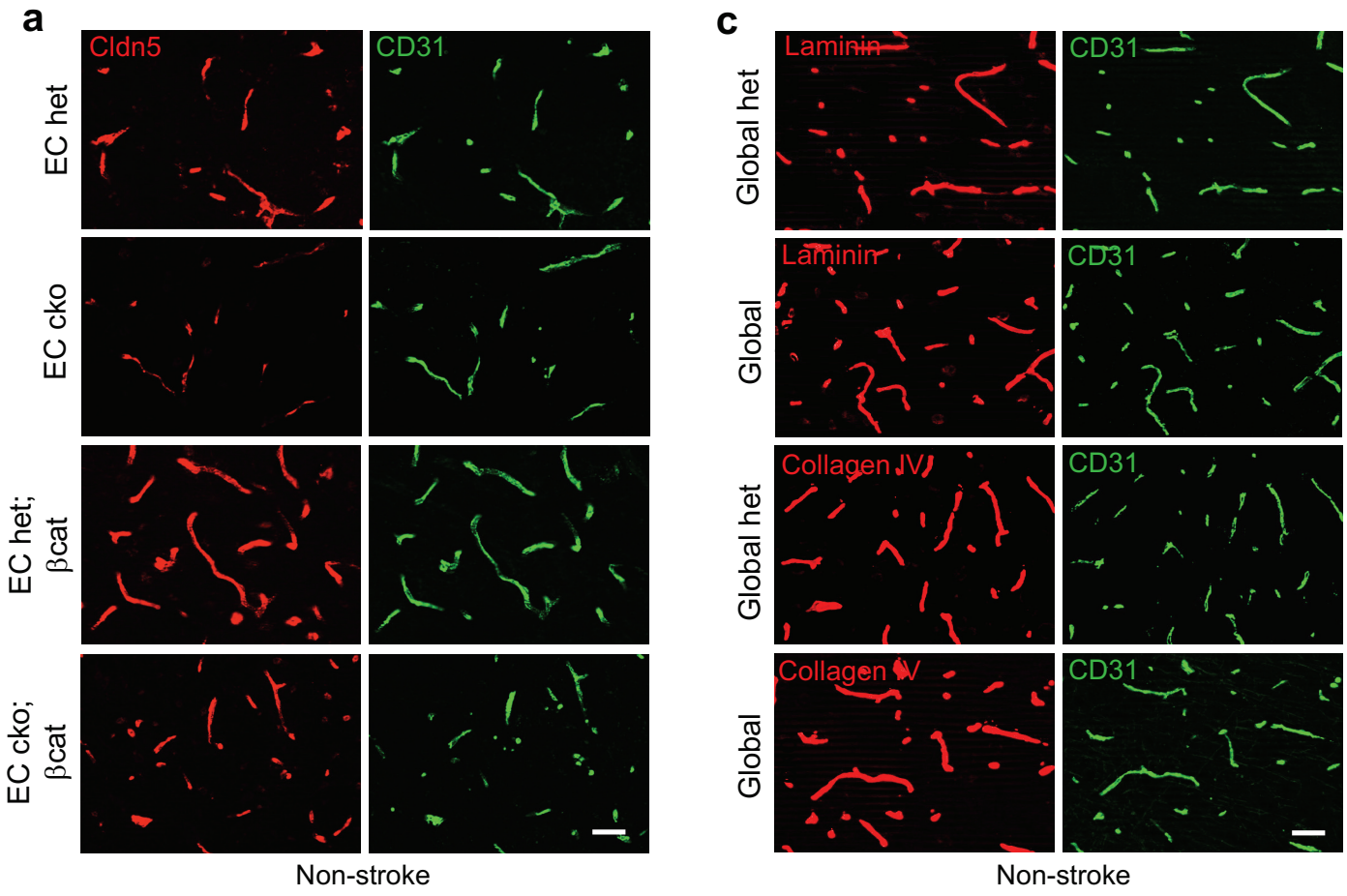
Supplementary Figure 8. *Gpr124* deletion induces hemorrhage and decreases survival in female mice after stroke. (**a**) Quantification of numbers of female mice with hemorrhage post-stroke within 7-d reperfusion, which is similar to males. Presence of hemorrhage was recorded at the time of premature death, morbidity, or sacrifice at day 7. (**b** and **c**) Survival of female *global Gpr124 cko* mice (**b**) or endothelial *Gp124* cko mice (**c**), together with het controls after 1 h tMCAO and 7 d reperfusion. $P < 0.05$, Log-rank test.

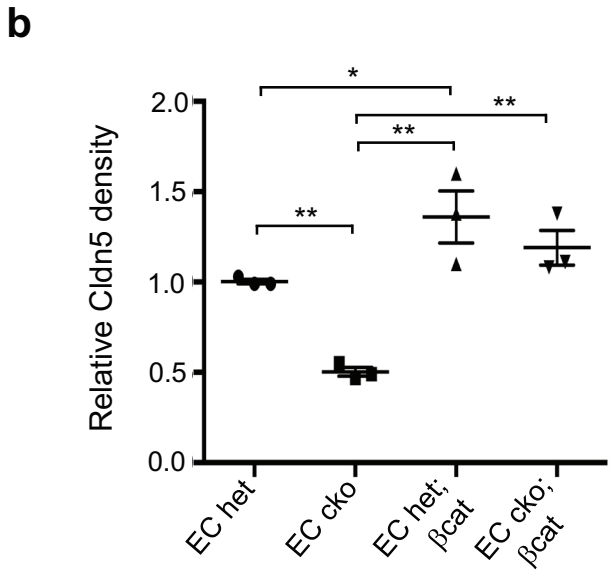
Supplementary Figure 9

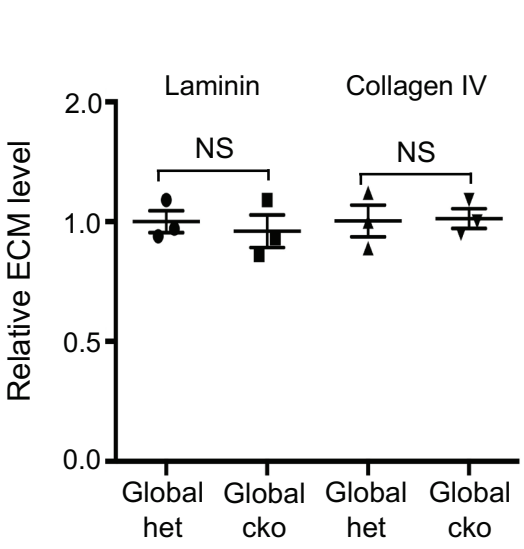


Supplementary Figure 9. Characterization of Wnt pathway status in adult cerebrovasculature. (a). Abundant expression of essential canonical Wnt signaling components in adult brain endothelium as determined by RNA-Seq of FACS-sorted CD31⁺ mouse brain ECs. RPKM expression levels of essential components of the Wnt/β-catenin signaling pathway were listed, in comparison with pan-endothelial markers and BBB markers. *Gpr124* is highlighted as a reference. *n* = 3 mice per group. Data were shown at average ± s.e.m. **(b)** β-galactosidase/CD31 co-IF staining of cerebral cortex of adult *BAT-Gal* mice. Scale bar, 25 μm.

Supplementary Figure 10

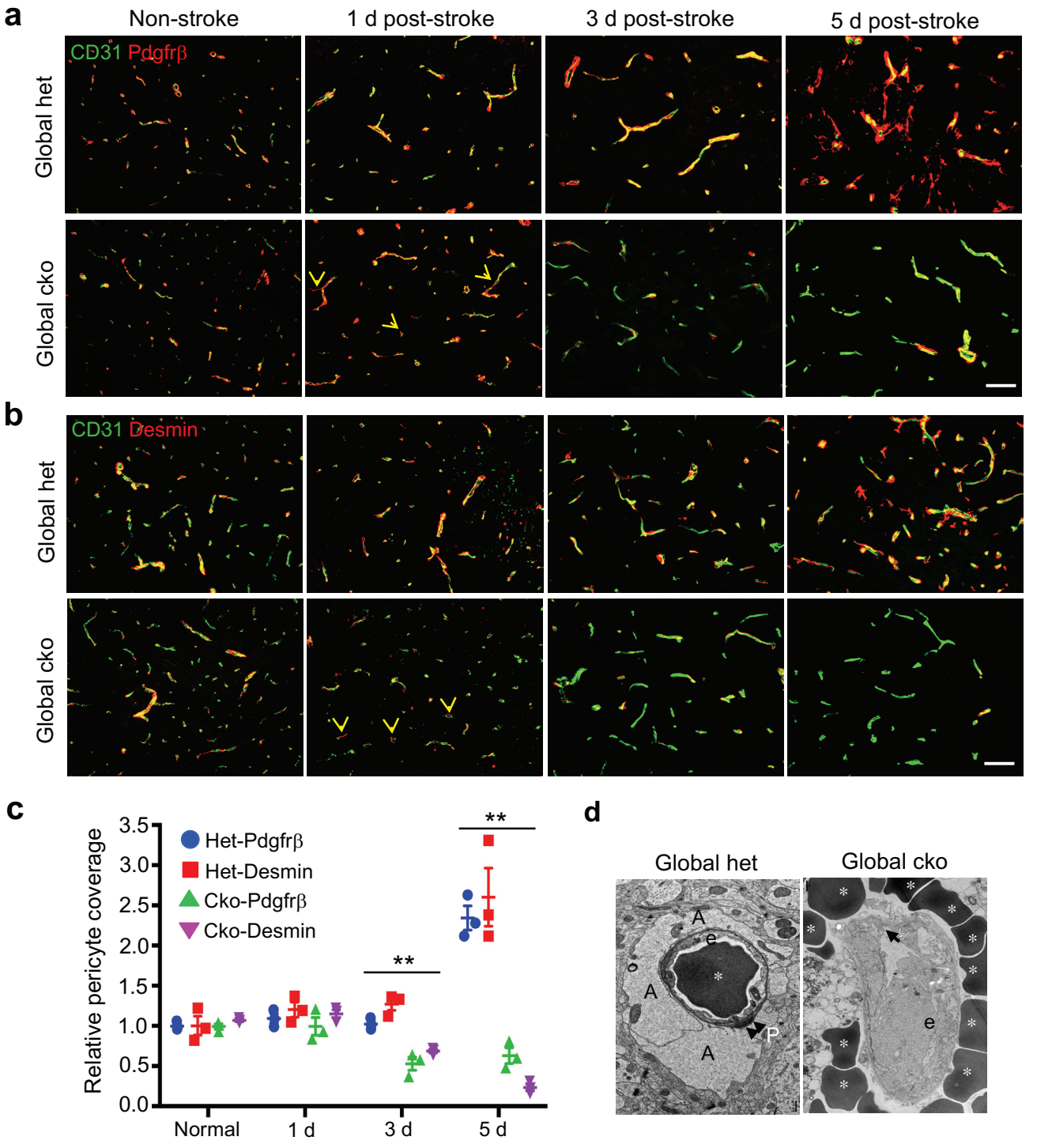






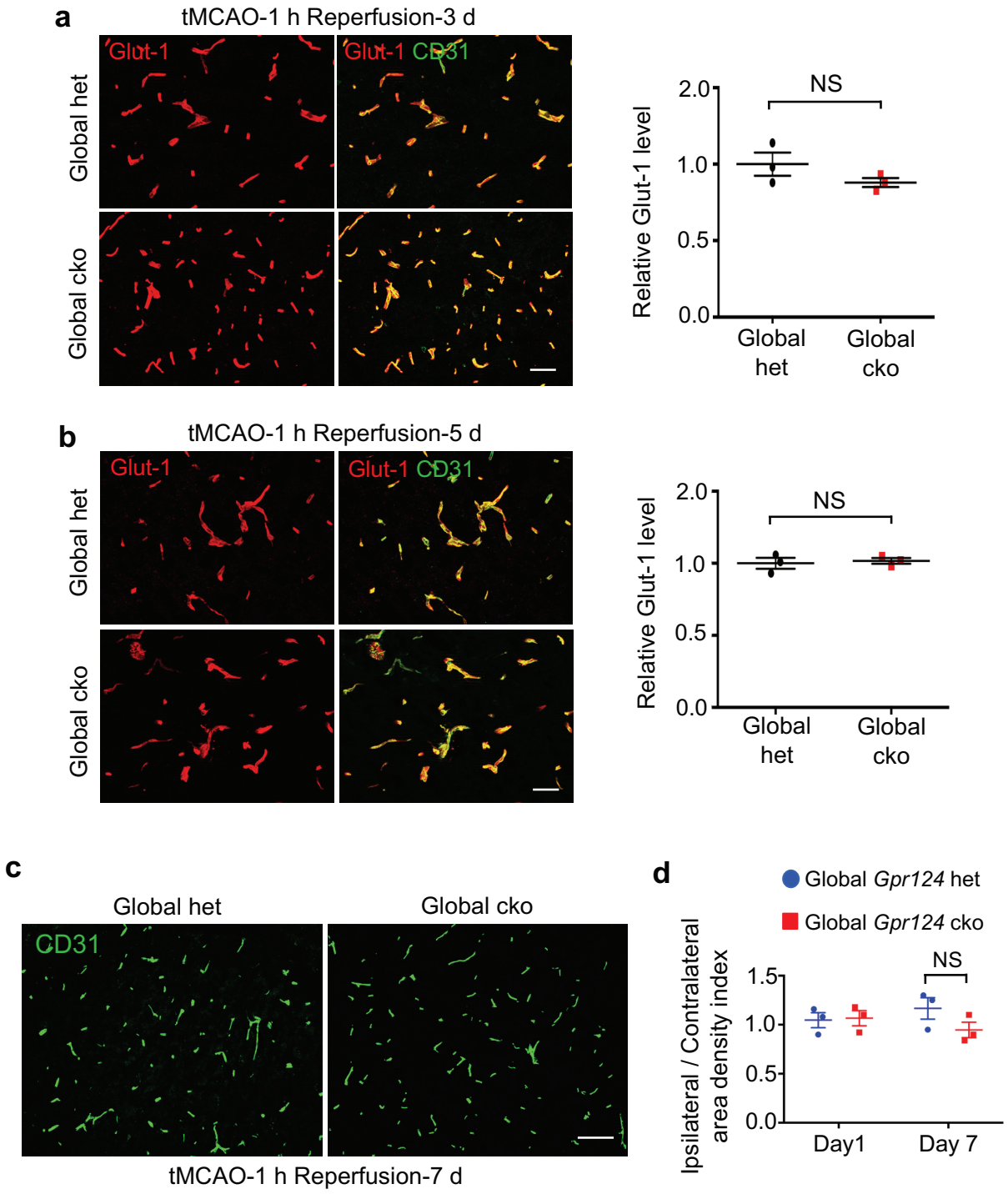
Supplementary Figure 10. Gpr124 regulation of Cldn5 and ECM proteins. (**a** and **b**) Cldn5 protein levels were determined by IF (**a**), and quantified by measuring Cldn5 IF signal density normalized by CD31 signal area. (**c** and **d**) Perivascular ECM (laminin, collagen IV) levels were determined by IF (**c**), and quantified by measuring IF signal density normalized by CD31 signal area. 5-6 low power fields per mouse, n = 3 mice per group. Data shown as mean ± s.e.m. NS, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, unpaired Student's t-test. Scale bar, 50 μm.

Supplementary Figure 11



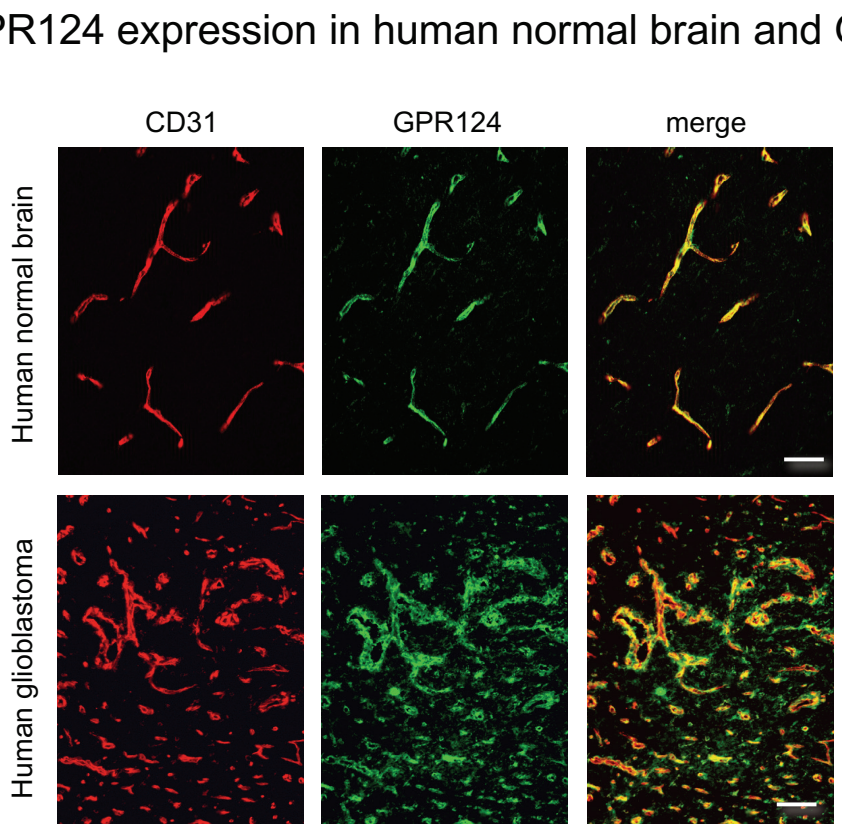
Supplementary Figure 11. Progressive pericyte loss in global *Gpr124* cko mice following stroke. (**a**) Pdgfrβ and CD31 co-IF staining of global *Gpr124* cko and het brains before and after 1 h tMCAO and 1-5 d reperfusion. (**b**) Desmin and CD31 co-IF staining of global *Gpr124* cko and het brains before and after 1 h tMCAO and 1-5 d reperfusion. Arrowheads in (**a**) and (**b**) show detached pericytes. (**c**) Quantification of Pdgfrβ^{+} pericyte coverage shown in (**a** and **b**). 5-6 low power fields were randomly selected from 3 mice per group and length of Pdgfrβ or Desmin signal profile was normalized to length of CD31 signal profile. Data shown as mean ± s.e.m. $^{**}P < 0.01$ vs. het controls, unpaired Student's *t*-test. (**d**) Electron microscopy analysis of the neurovascular unit in global *Gpr124* cko mice 3 d post-stroke vs. het controls. A, astrocyte; e, endothelium; P, pericyte; asterisk, red blood cell. Arrowheads show a pericyte tightly associated with the endothelium in het mice but not in global *Gpr124* cko mice. The arrow shows fragmented endothelium and basal lamina.

Supplementary Figure 12



Supplementary Figure 12. Cerebrovascular *Glut-1* expression and angiogenesis was not affected by *Gpr124* deletion following stroke. (**a** and **b**) Glut-1 protein levels were determined by IF after 1 h tMCAO and 3 d (**a**) or 5 d (**b**) reperfusion, and quantified by measuring Glut-1 IF signal density normalized by CD31 signal area. 5-6 low power fields per mouse, *n* = 3 mice per group. Data shown as mean ± s.e.m. NS, not significant, unpaired Student's *t*-test. Scale bar, 50 μm. (**c**) Representative CD31 IF staining of infarcted cortex after 1 h tMCAO and 7 d reperfusion. (**d**) Microvessel density was quantified by measuring CD31 signal area (6-8 low power fields per mouse, *n* = 3 mice per group) and corrected for edema. Data shown as mean ± s.e.m. NS, not significant, unpaired Student's *t*-test. Scale bar, 100 μm.

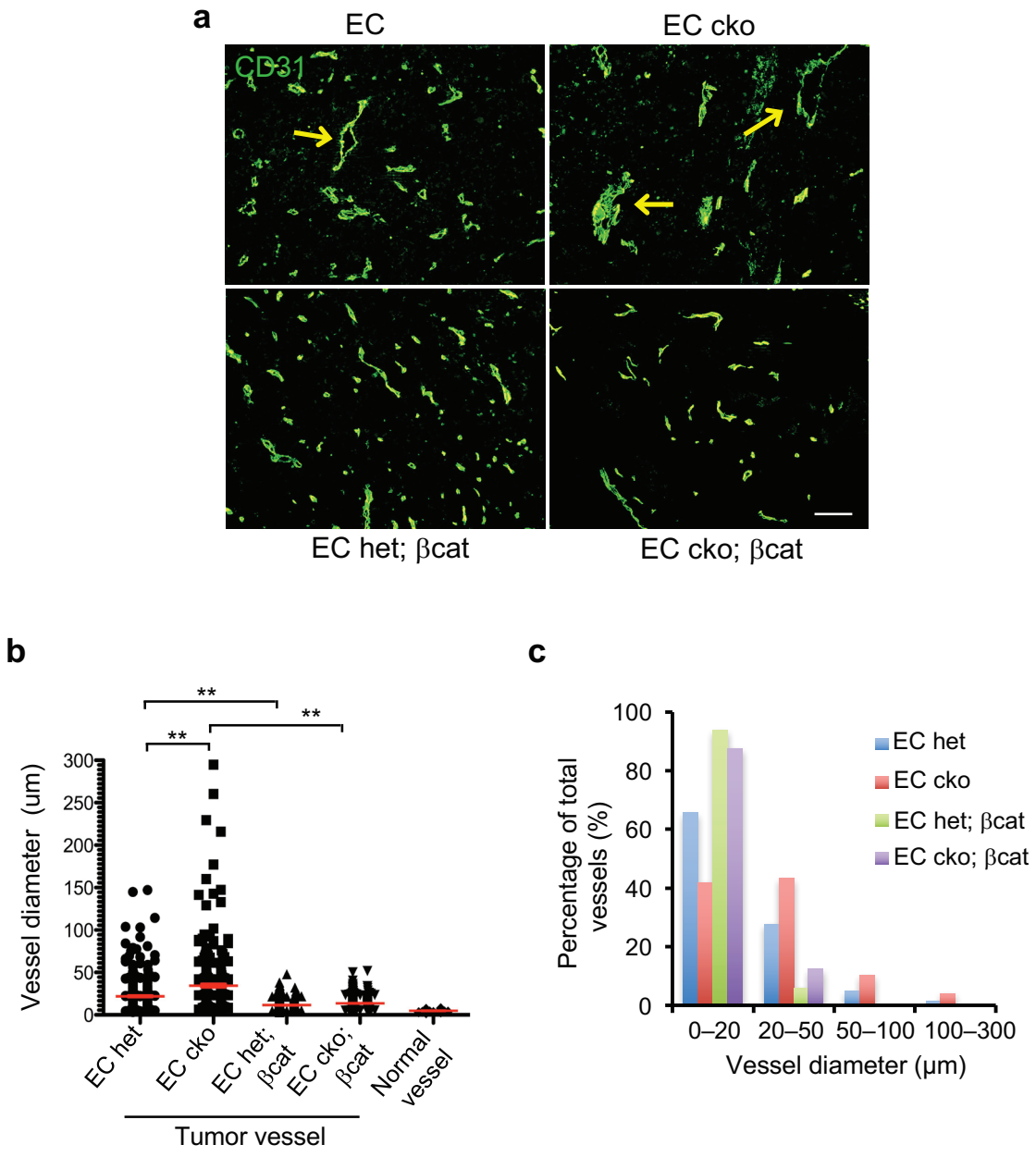
Supplementary Figure 13



Supplementary Figure 13. GPR124 expression in human normal brain and GBM. (**a**) CD31 and GPR124 immunofluorescence staining demonstrates abundant vascular expression of GPR124 in both normal human brain tissue and human glioblastoma. Scale bar, 100 μm.

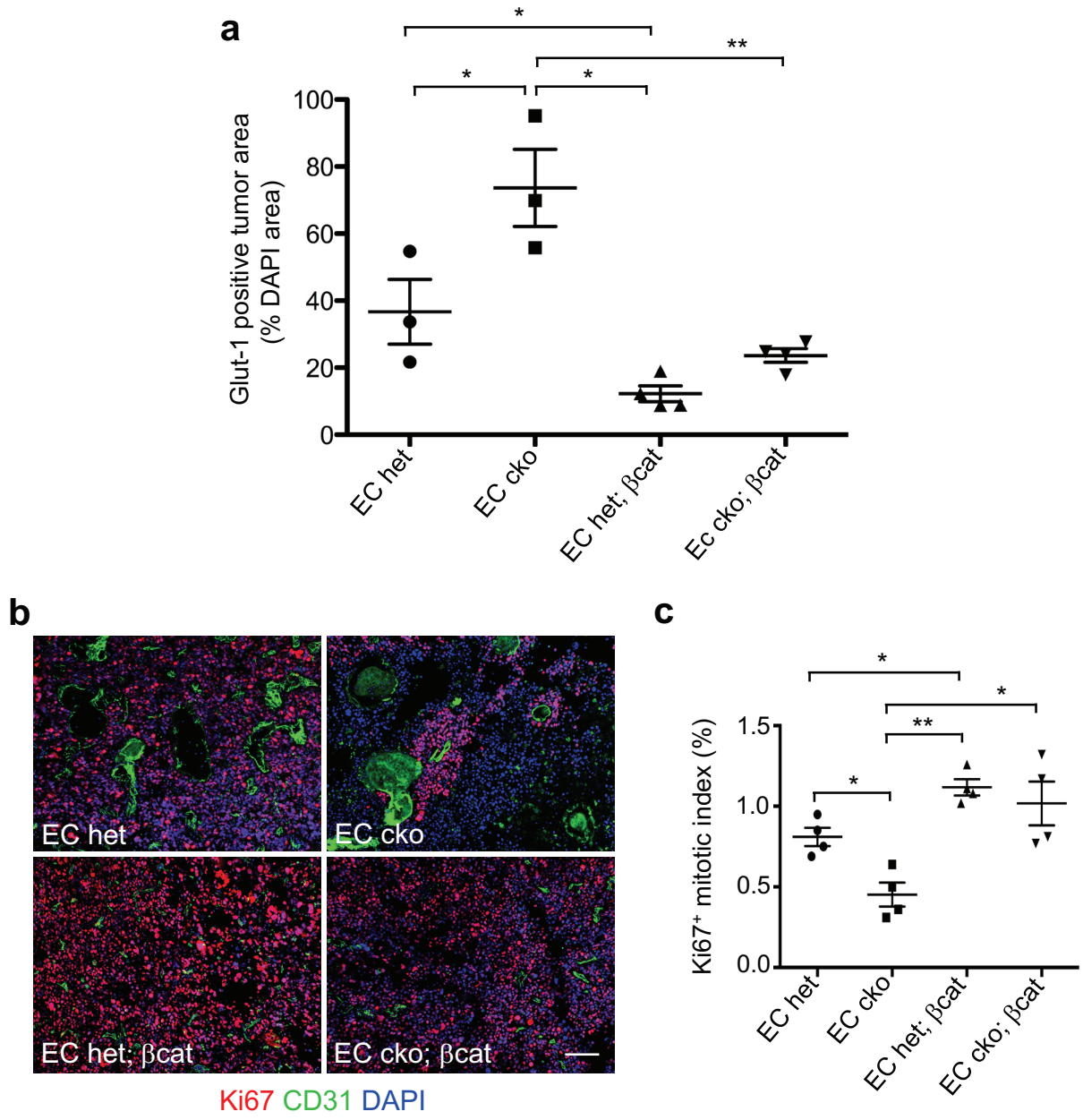
Nature Medicine: doi:10.1038/nm.4309

Supplementary Figure 14



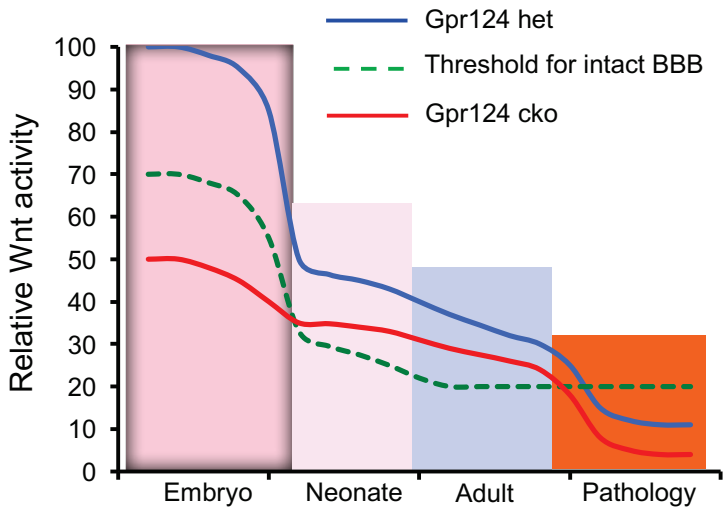
Supplementary Figure 14. Regulation of vascular normalization by Gpr124-Wnt signaling in GL261 glioblastoma. (**a**) CD31 IF staining of tumor vessels. Scale bar, 100 μm. Arrows show vessels with large diameter. (**b**) Vessel diameter was measured using ImageJ. 300-400 vessels from 4-5 tumors per group were measured. Vessel diameters of normal brain tissues were measured and presented as a control. (**c**) Vessel diameter grouped in categories. *$P < 0.01$, **$P < 0.01$, unpaired Student's *t*-test.

Supplementary Figure 15



Supplementary Figure 15. Regulation of tumor hypoxia and proliferation by Gpr124-Wnt signaling in GL261 glioblastoma. (**a**) As an indication of tumor hypoxia, Glut-1 expression in tumor cells was quantified by calculating the ratio of the Glut-1 IF staining positive area to DAPI area. 6-8 low power fields per mouse, *n* = 3-4 tumors per group. (**b**) Ki67/CD31 co-IF staining of tumors. (**c**) Quantification of Ki67 positive cells by calculating the ratio of the Ki67 IF staining positive area to DAPI area. 5-6 low power fields per mouse, *n* = 4 tumors per group. Data shown as mean ± s.e.m. **P* < 0.01, ***P* < 0.01, unpaired Student's *t*-test. Scale bar, 100 μm.

Supplementary Figure 16



Supplementary Figure 16. Schematic representation of the relationship between endothelial Wnt signaling activity and BBB integrity. In Gpr124 het mice, Wnt signaling in brain endothelium is elevated during embryogenesis to prompt CNS angiogenesis and BBB formation. During postnatal and adult life, Wnt signaling is reduced to much lower levels but still enough to maintain an intact BBB. During pathologies, Wnt signaling is further reduced to levels lower than those required to maintain an intact BBB ("threshold levels") so BBB leak occurs. Gpr124 cko decreases the endothelial Wnt signaling to levels below the threshold levels in embryos or adults with pathologies, resulting in extensive BBB breakdown and hemorrhage. Gpr124 cko does not reduces Wnt signaling below the threshold levels in neonates or adults without pathologies, so the BBB remains intact. Based on our data and a previous study [Liebner S. et al., *J Cell Biol* 2008].

Supplementary Table 1

Gene name	Mean RPKM	S.E.M.
Gpr124	**7.5**	0.8
Slc2a1 (Glut-1)	**854.7**	157.6
Cldn5	**88.9**	14.2
Plvap	**1.5**	0.7
Pdgfb	**28.2**	2.5
Axin2	**14.2**	0.2
Apcdd1	**27.1**	2.9
Prkcb	**4.9**	1.2
Nkd1	**4.4**	1.0
Cldn1	**0.9**	0.9
Cldn3	**Not detected**	

Supplementary Table 1. Expression levels of genes detected in this manuscript by qRT-PCR. RPKM values of RNA-Seq of FACS sorted brain ECs from WT mice. *n* = 3 mice.

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Supplementary Table 2

Up-Regulated Pathways	P value	Genes
ECM-receptor interaction	1.11E-03	*VWF, COL4A2, ITGB8, LAMA5, NPNT, COL3A1, COL1A2, THBS1, THBS2, FN1*
Cell-substrate adhesion	1.76E-03	*VWF, ITGB8, CTGF, TRPM7, NPNT, SIRPA, BCL2L11, FN1*
Cell-matrix adhesion	4.18E-03	*ITGB8, CTGF, TRPM7, NPNT, SIRPA, BCL2L11, FN1*
Vascular smooth muscle contraction	1.31E-02	*KCNMA1, ACTA2, MRVI1, MYH11, NPR1, PRKACA, CACNA1C, CACNA1D, KCNMB1, PRKCB*
Cell adhesion	1.51E-02	*NPNT, CDH1, NEO1, ALCAM, VCAM1, RGMB, ITGB8, CTGF, COL12A1, CNTNAP1, THBS1, THBS2, FN1, COL18A1, PCDHGA11, TRPM7, LPP, CPXM2, FBLIM1, NLGN3, SIRPA, BCL2L11, CTNNA3, VWF, LAMA5, PARVB, ROM1, CDH11*
Focal adhesion	2.20E-02	*COL4A2, COL3A1, PRKCB, VWF, LAMA5, ITGB8, CCND2, COL1A2, PdgfrB, THBS1, THBS2, PARVB, FN1*
Regulation of angiogenesis	4.92E-02	*GATA2, COL4A2, RUNX1, THBS1, ANGPT2*
Angiogenesis	5.36E-02	*COL18A1, SEMA5A, CXCR4, CTGF, ENPEP, ANGPT2, SRF, POFUT1, C1GALT1*
Regulation of growth	2.76E-02	*NPY1R, STAT3, BCL2L11, NTRK3, AGPAT6, RNF6, CTGF, MFSD7B, CACNA1C, BRMS1L, IGFBP4, CRIM1, IGFBP5, GHR, BRD8*

Down-Regulated Pathways	P value	Genes
Regulation of cell adhesion	5.13E-02	*EMCN, TNXB, PRLR, FBLN2, ECM2, ALOX12*
Death	5.48E-02	*PHLPP1, RHOX5, ADAMTSL4, LGALS12, NR4A2, PRKDC, TRIB3, BCAP31, ASAH2, BAK1, P2RX7, RABEP1, RIPK3, BUB1B, FGL2, SPR, TRAF5, TCF15*

Supplementary Table 2. DAVID biological process and signaling pathway analysis of RNA-Seq data for β-catenin activation. RNA-Seq was performed with FACS sorted brain ECs from βcat mice vs. WT mice (*n* = 3 mice per group).

Supplementary Table 3

Down-Regulated Pathways	P value	Genes
Cell adhesion	4.16E-04	*CTNNAL1, NRP1, NPNT, CTNND1, VCL, PNN, TGFB2, ALCAM, CDH20, PCDHB16, PCDHB9, COL4A3, HAPLN1, PTPRF, PIK3CB, FBLIM1, PCDH15, VEZT, NID1, ITGA4, PCDH7, COL16A1, KITL, PCDH18, HSPB11, CNTN4, ITGAD, ANTXR1, BMPR1B, ADAM12, SELE, OMG*
Cell-cell adhesion	1.96E-02	*PCDHB9, PTPRF, PIK3CB, CTNND1, VEZT, PCDH15, ITGA4, PCDH7, PCDH18, TGFB2, PNN, CDH20, PCDHB16, BMPR1B*
Regulation of cell-substrate adhesion	2.75E-02	*EGFLAM, PIK3CB, NPNT, NF1, NID1*
VEGF signaling pathway	1.32E-02	*MAPK1, PIK3CB, JMJD7, SHC2, AKT3, PRKCB, NFATC1*
Blood vessel development	5.07E-02	*FGFR2, MAPK1, NRP1, MYOCD, DHCR7, HMOX1, NF1, GBX2, AMOT, ITGA4, PPAP2B, QK, TGFB2*
Vasculature development	5.87E-02	*FGFR2, MAPK1, NRP1, MYOCD, DHCR7, HMOX1, NF1, GBX2, AMOT, ITGA4, PPAP2B, QK, TGFB2*
Regulation of growth	1.67E-02	*FGFR2, AR, NRP1, NDNL2, WRN, ESM1, TGFB2, UBE2E3, MYOCD, HTRA1, ULK2, EAF2, SEMA3A, GPAM, BRD8*
Regulation of cell growth	1.69E-02	*NRP1, MYOCD, HTRA1, ULK2, SEMA3A, EAF2, ESM1, TGFB2*

Up-Regulated Pathways	P value	Genes
Programmed cell death	2.85E-02	*SIVA1, MRPL41, TNFRSF12A, PGLYRP1, KIT, SHF, KCNIP3, AKT1, TNFRSF1A, FIS1, BAK1, SQSTM1, AEN, BCL2, NAIP5, BCAP29, DAP, DYRK2, FAS, IL6, HTT, SRA1, NFKBIL1, TNFRSF10B, ZDHHC16, MAPK7, PUF60, NEK6*
Cell death	5.88E-02	*SIVA1, MRPL41, TNFRSF12A, PGLYRP1, KIT, SHF, KCNIP3, AKT1, TNFRSF1A, FIS1, BAK1, SQSTM1, AEN, BCL2, NAIP5, BCAP29, DAP, DYRK2, FAS, IL6, HTT, SRA1, NFKBIL1, TNFRSF10B, ZDHHC16, MAPK7, PUF60, NEK6*

Supplementary Table 3. DAVID biological process and signaling pathway analysis of RNA-Seq data for *Gpr124* deletion. RNA-Seq was performed with FACS sorted brain ECs from global *Gpr124* cko mice vs. global *Gpr124* het mice (n = 4 mice per group).