Deficiency of MFSD7c results in microcephaly-associated vasculopathy in Fowler syndrome

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The authors have declared that no conflict of interest exists.

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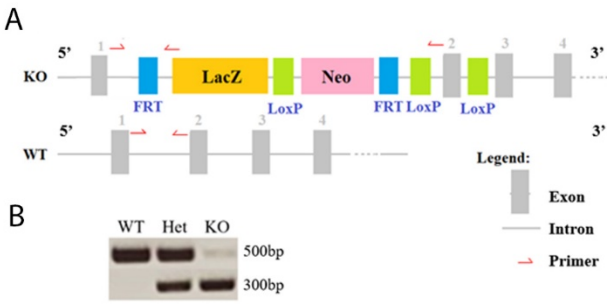
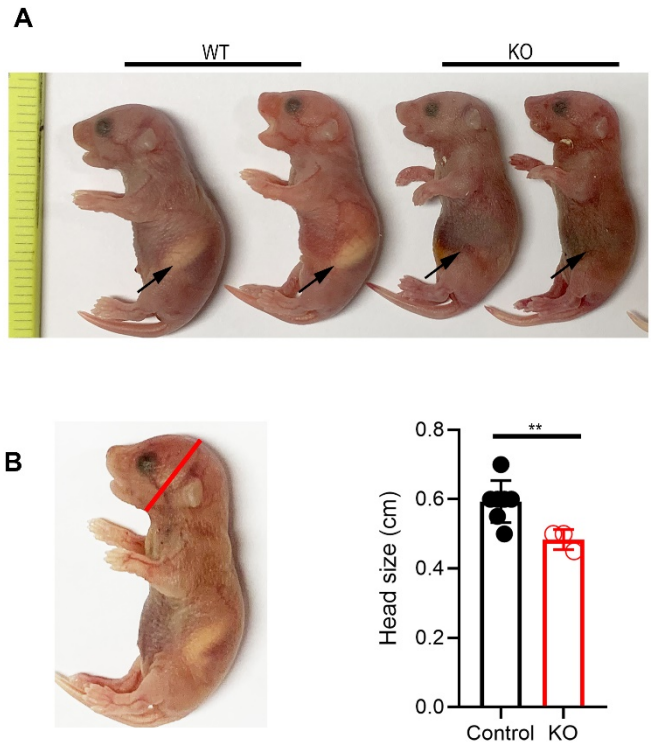
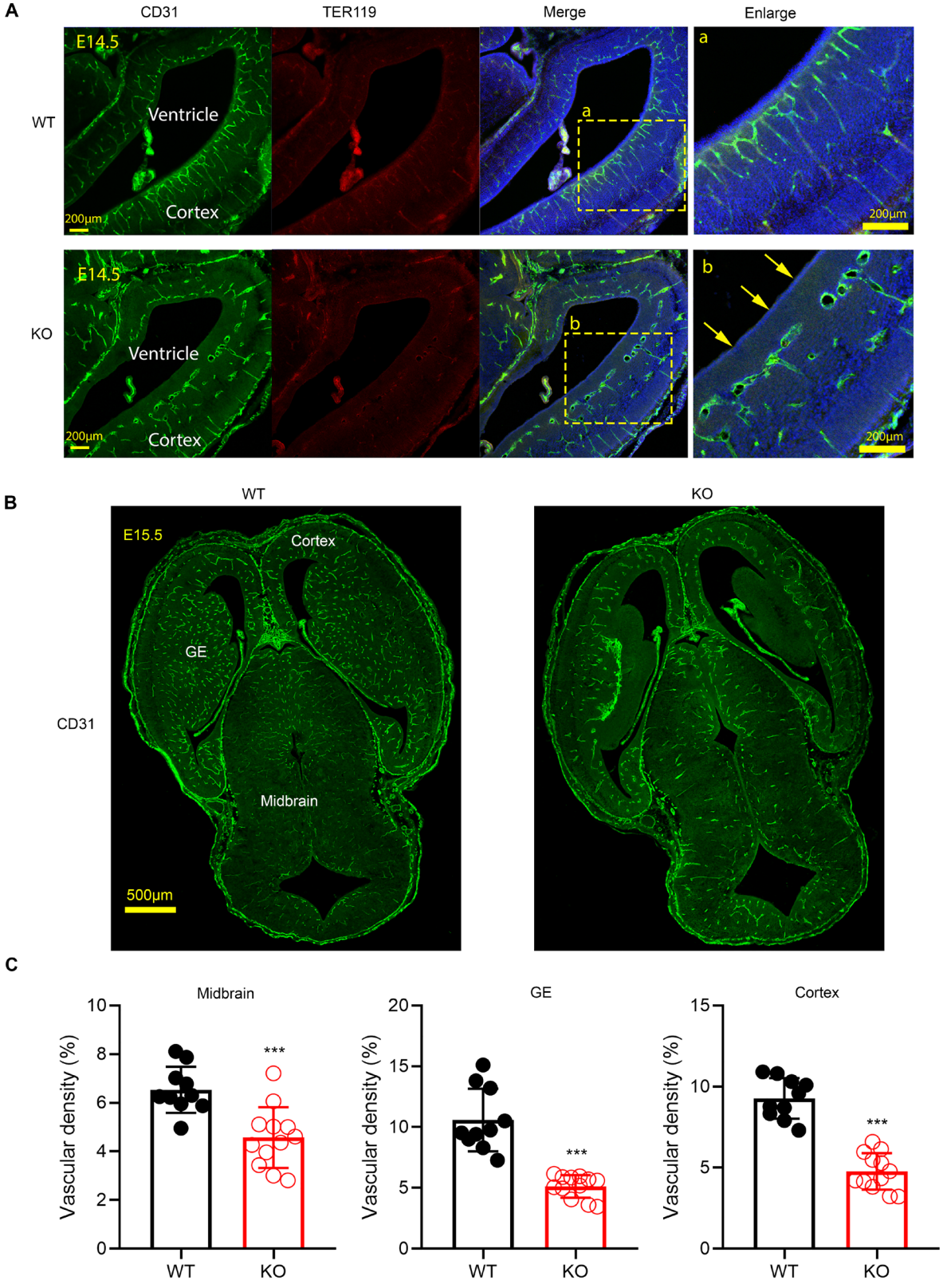
Supplemental figures



Supplemental Figure 1: Illustration of approach for generation of *Mfsd7c* knockout. A, Illustration of genomic locus of *Mfsd7c* global knockout (KO) allele and wild-type (WT) allele. *Mfsd7c* gene was disrupted by the insertion of LacZ-Neo cassette in the intron between exon 1 and 2. **B**, Representative genotyping results of WT, heterozygous (HET) and KO by PCR.

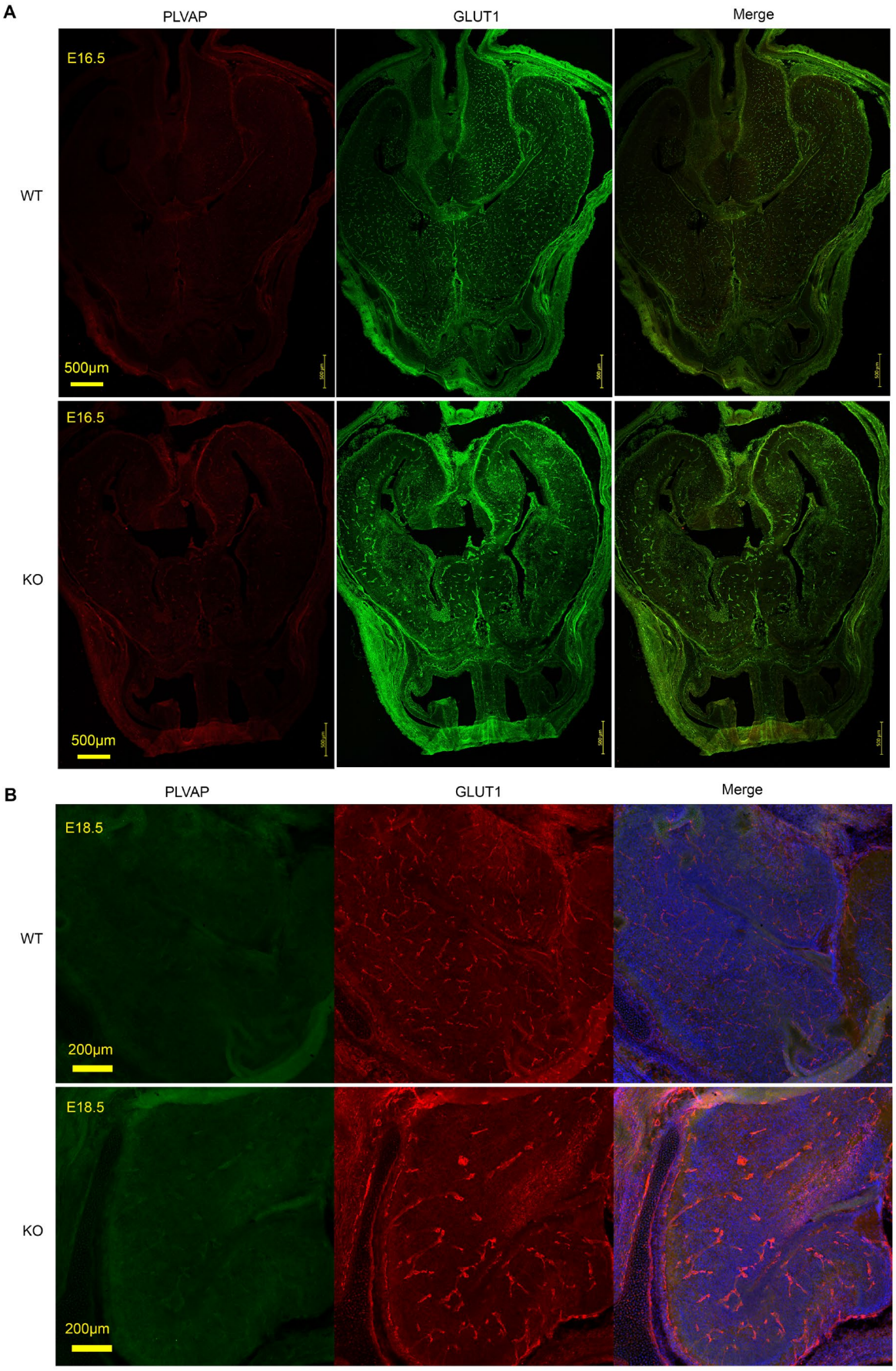


Supplemental Figure 2. Global knockout of *Mfsd7c* resulted in lethality. A, Representative of postnatal day 0 (PN0) of *Mfsd7c* knockout pups. The knockout pups were born alive but died few hours later. Their milk sac was empty (arrows). **B**, Head size measured as indicated by the red line. The head size of PN0 *Mfsd7c* knockout pups was significantly reduced compared with WT littermates. n=3-5 embryos per genotype. Data represent mean ± SD. **P<0.02. Two tailed *t*-test.

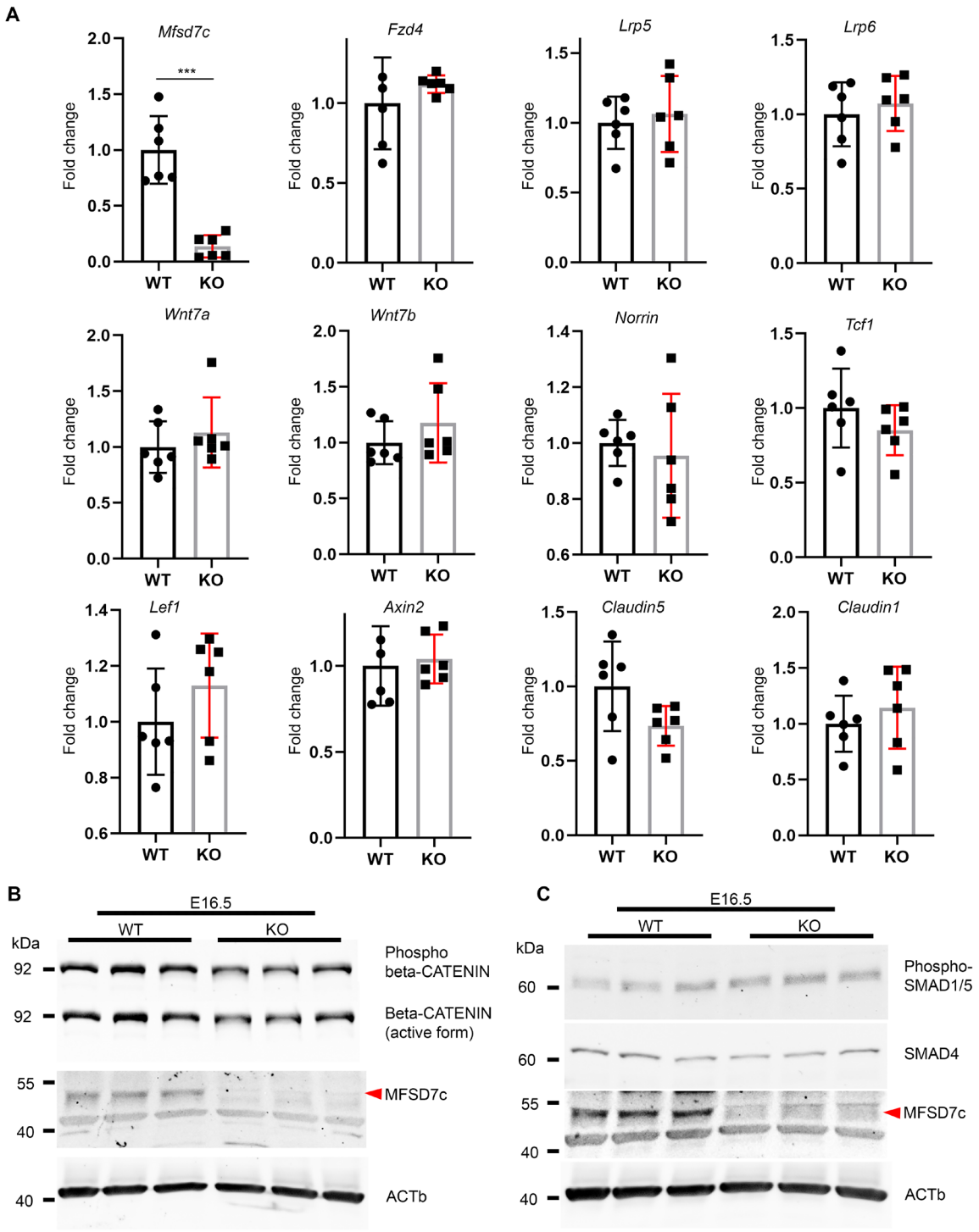


Supplemental Figure 3. Delayed vascular growth to the cortical ventricular zones and reduced vascular density in *Mfsd7c* knockout embryos. A, Representative images of brain sections from E14.5 WT and KO embryos that were stained with CD31. The growth of CNS blood vessels in *Mfsd7c* knockout was affected in ventricular zones of cerebral cortex. The sections were co-stained with

TER119 to visualize erythrocytes to detect haemorrhage. There was no bleeding in *Mfsd7c* knockout in cerebral cortices. a-b, enlarged images from WT and KO sections, respectively. Arrows indicate that *Mfsd7c* knockout blood vessels do not reach to the ventricular cell layers (n=3). **B,** representative images of whole coronal brain sections from E15.5 WT and KO embryos used for quantification of vascular density. **C**, Quantification of vascular density of E15.5 WT and KO embryos in mid-brain, ganglionic eminences (GE), and cortex regions. Data represent mean ± SD. ***P< 0.001; Two tailed *t*-test. Each dot represents a brain section. Data were quantified from 3-4 different WT and KO embryos.

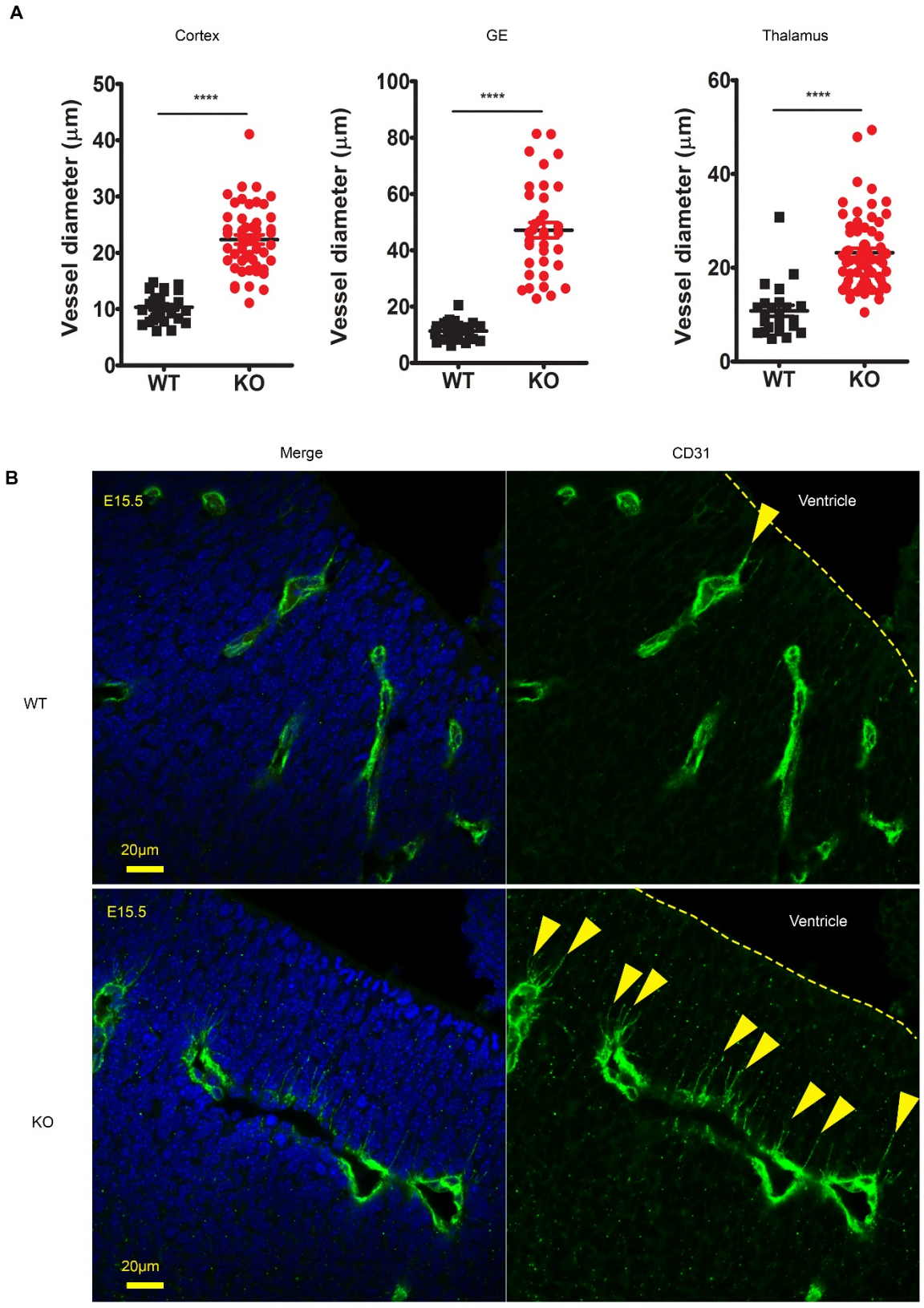

A
PLVAP
GLUT1
Merge
WT
KO
E16.5
E16.5
500μm
500μm
B
PLVAP
GLUT1
Merge
WT
KO
E18.5
E18.5
200μm
200μm

Supplemental Figure 4. Deletion of *Mfsd7c* did not result in increased permeability of CNS blood vessels. A-B, Representative images of whole coronal brain sections of E16.5 (n=5) (**A**) and hippocampal regions of E18.5 (n=5) (**B**) WT and KO embryos that were co-stained with PLVAP and GLUT1. PLVAP, which is a marker for fenestration of blood vessels, was used to detect the increased transcellular permeability of blood brain barrier. We examined at these time points as PLVAP is still expressed at E14.5-15.5 in mouse embryos (n=3). The expression of PLVAP was not increased in the CNS blood vessels from E16.5 or E18.5 knockout embryos compared with WT embryos. Experiments were performed three times, with n=3-5 WT and KO for each time point.



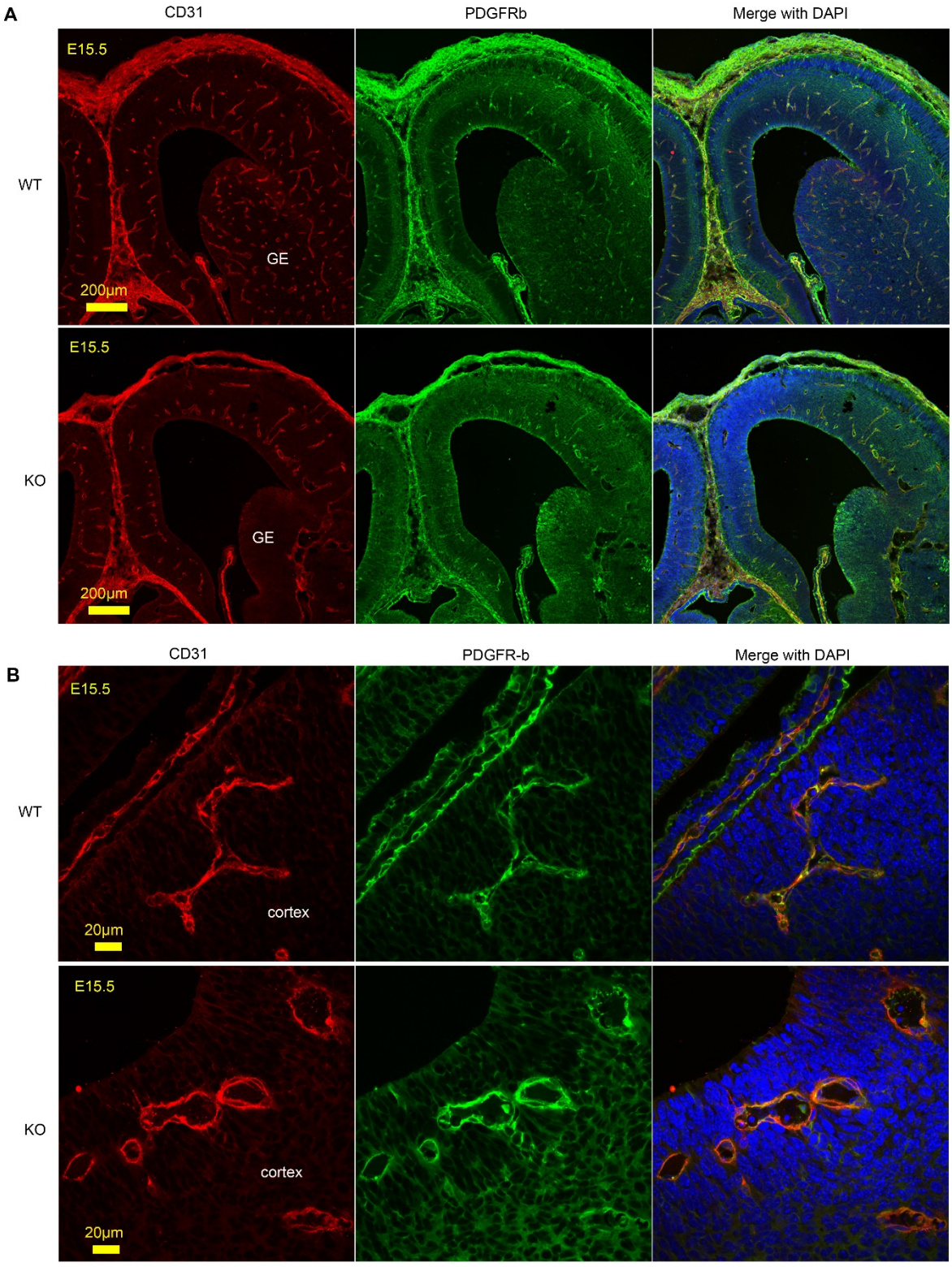
Supplemental Figure 5. Deletion of MFDS7c does not affect canonical WNT and TGFb signalling pathway. A, Quantitative PCR analysis of the expression of indicated genes involved in canonical WNT signalling in E14.5 WT and KO embryos. Expression of *MFSD7c* mRNA is significantly reduced. Experiments were repeated twice with n=6 per genotype. ***P< 0.001; Two tailed *t*-test. **B**, Western blot analysis of active (non-phosphorylated form) and phosphorylated form of beta-catenin. Whole brain lysate of E16.5 WT and KO was used. MFSD7c protein is absence in KO brain. ACTb was used as loading control. Experiment was repeated at least twice, n=3 per genotype. **C**, Western blot analysis of

TGFb signalling pathway. Phosphorylated Smad1/5 was used to determine whether TGFb signalling is altered in the KO brain. Whole brain lysate of E16.5 WT and KO was used. MFSD7c protein is absence in KO brain. ACTb was used as loading control. Experiment was repeated at least twice, n=3 per genotype.



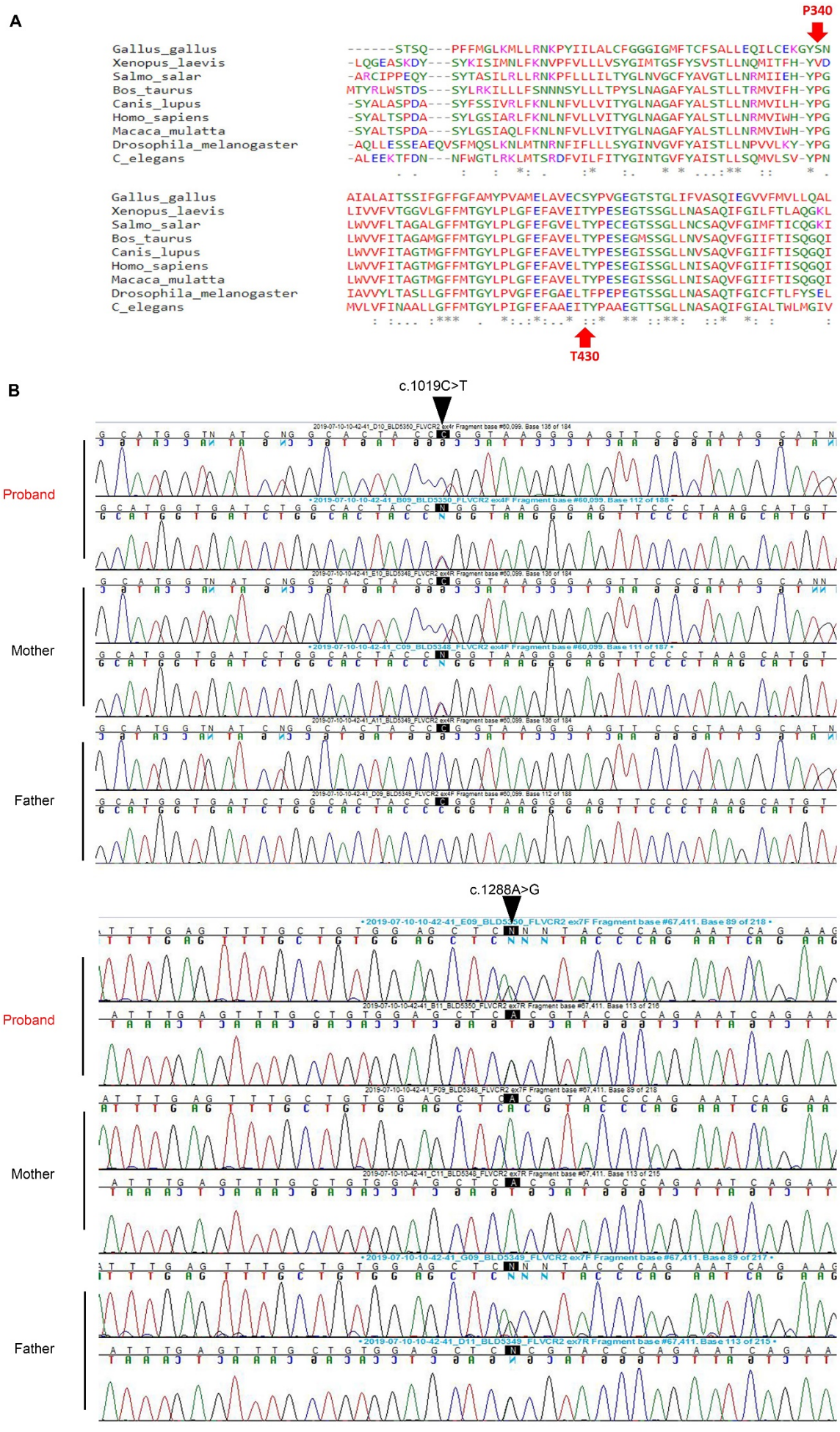
Supplemental Figure 6. Defects in CNS vasculature in *Mfsd7c* knockout embryos. A, Increased blood vessel diameters in the knockout embryos at different regions**.** The diameter of CNS blood vessels from E15.5 WT and KO embryos were measured. ****P<0.0001; Two tailed *t*-test. Each dot

represents one section. Measurement was performed with 3-4 WT and KO embryos. **B,** Representative images of the increased filopodia in endothelial cells from ventricular zones in *Mfsd7c* knockout embryos. Brain sections of E15.5 WT and *Mfsd7c* knockout embryos were stained with CD31 to visualize endothelial cells. Arrowheads indicate filopodia of endothelial cells. Experiment was repeated at least three times, n=4-6 per genotype.

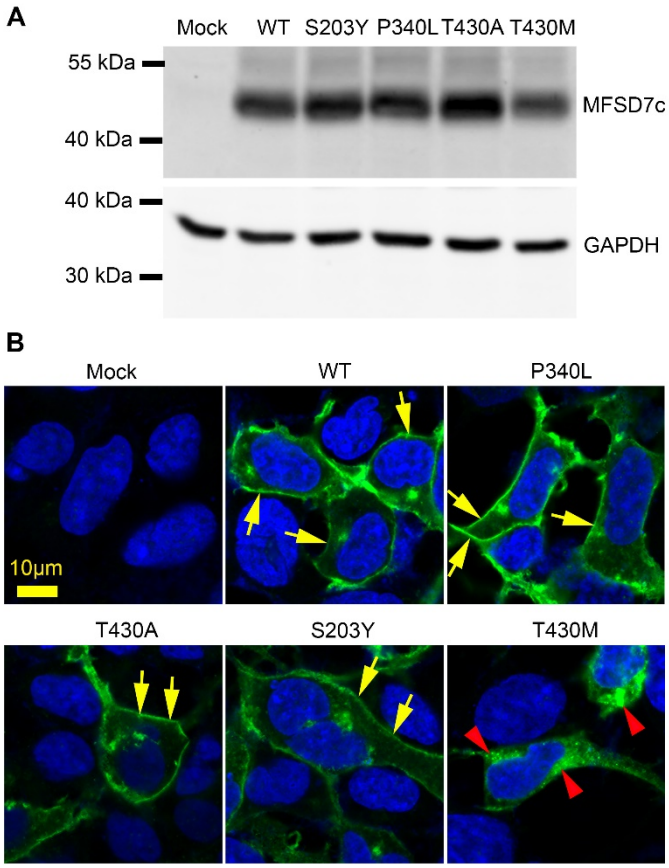


Supplemental Figure 7. CNS blood vessels from *Mfsd7c* knockout mice had normal pericyte coverage. A-B, Brain sections of E15.5 WT and KO embryos were stained with PDGFRb antibody and CD31 to visualize pericytes and endothelial cells. **A**, lower magnification images of lateral ganglionic eminence and cerebral cortex regions. **B**, high magnification images of ventricular regions. Expression

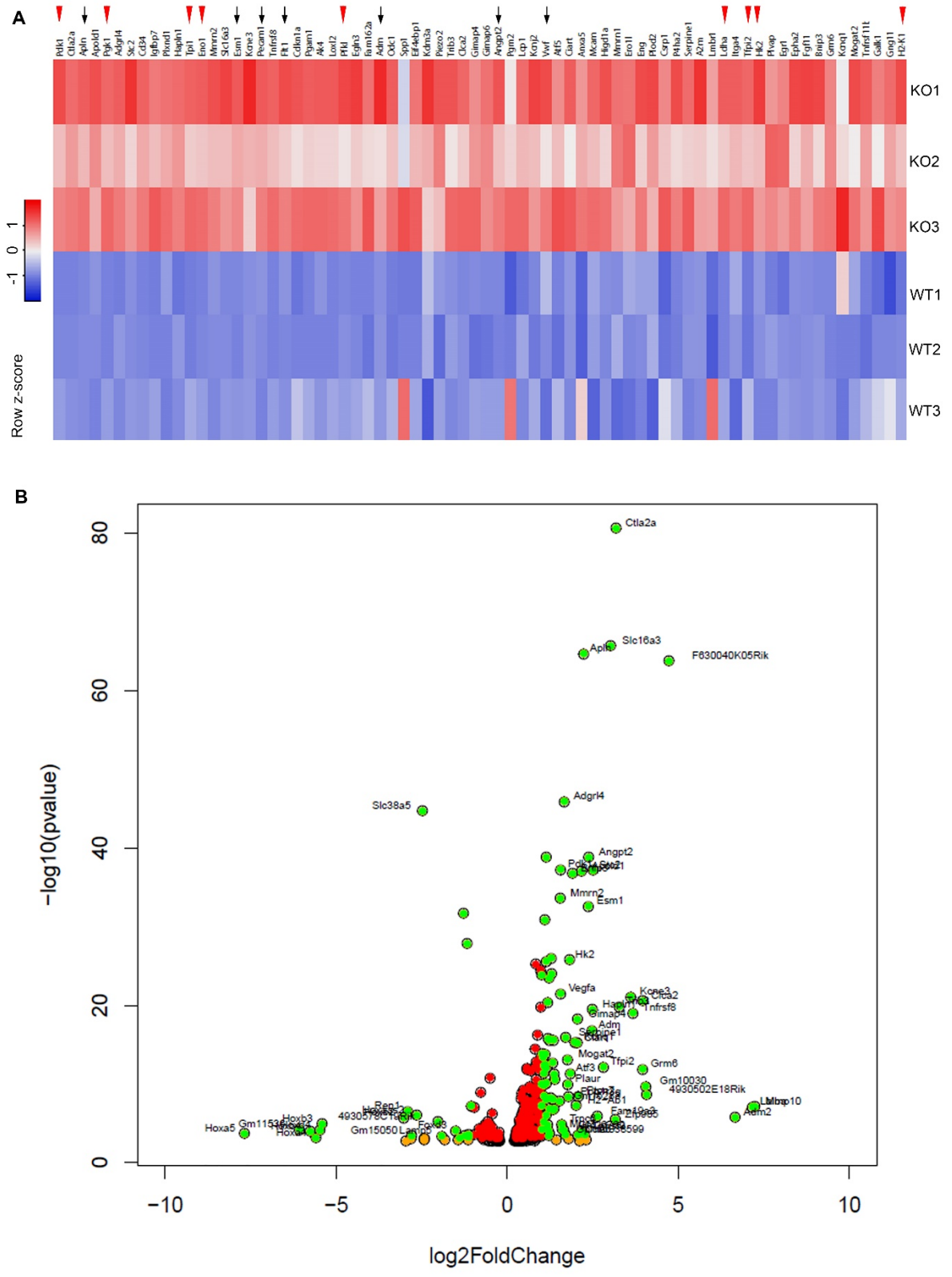
of PDGFRb is still found in the dilated CNS blood vessels in *Mfsd7c* knockout embryos. Experiment repeated at least three times, n=3-4 embryos per genotype.


A
P340
Gallus_gallus ------STSQ---PFFMGLKMLLRNKPYIILALCFGGGIGMFTCFSALLEQILCEKGYSN
Xenopus_laevis -LQGEASKDY---SYKISIMNLFKNVPFVLLLVSYGIMTGSFYSVSTLLNQMITFH-YVD
Salmo_salar -ARCIPPEQY---SYTASILRLLRNKPFLLLILTYGLNVGCFYAVGTLLNRMIIEH-YPG
Bos_taurus MTYRLWSTDS---SYLRKILLLFSNNNSYLLLTPYSLNAGAFYALSTLLTRMVIFH-YPG
Canis_lupus -SYALASPDA---SYFSSIVRLFKNLNFVLLVITYGLNAGAFYALSTLLNRMVILH-YPG
Homo_sapiens -SYALTSPDA---SYLGSIARLFKNLNFVLLVITYGLNAGAFYALSTLLNRMVIWH-YPG
Macaca_mulatta -SYALTSPDA---SYLGSIAQLFKNLNFVLLVITYGLNAGAFYALSTLLNRMVIWH-YPG
Drosophila_melanogaster -AQLLESSEAEQVSFMQSLKNLMTNRNFIFLLLSYGINVGVFYAISTLLNPVVLKY-YPG
C_elegans -ALEEKTFDN---NFWGTLRKLMTSRDFVILFITYGINTGVFYAISTLLSQMVLSV-YPN
Gallus_gallus AIALAITSSIFGFFGFAMYPVAMELAVECSYPVGEGTSTGLIFVASQIEGVVFMVLLQAL
Xenopus_laevis LIVVFVTGGVLGFFMTGYLPLGFEFAVEITYPESEGTSSGLLNASAQIFGILFTLAQGKL
Salmo_salar LWVVFLTAGALGFFMTGYLPLGFEFGVELTYPECEGTSSGLLNCSAQVFGIMFTICQGKI
Bos_taurus LWVVFITAGAMGFFMTGYLPLGFEFAVELTYPESEGMSSGLLNVSAQVFGIIFTISQGQI
Canis_lupus LWVVFITAGTMGFFMTGYLPLGFEFAVELTYPESEGISSGLLNVSAQVFGIIFTISQGQI
Homo_sapiens LWVVFITAGTMGFFMTGYLPLGFEFAVELTYPESEGISSGLLNISAQVFGIIFTISQGQI
Macaca_mulatta LWVVFITAGTMGFFMTGYLPLGFEFAVELTYPESEGISSGLLNISAQVFGIIFTISQGQI
Drosophila_melanogaster IAVVYLTASLLGFFMTGYLPVGFEFGAELTFPEPEGTSSGLLNASAQTFGICFTLFYSEL
C_elegans MVLVFINAALLGFFMTGYLPIGFEFAAEITYPAAEGTTSGLLNASAQIFGIALTWLMGIV
T430
B
c.1019C>T
Proband
Mother
Father
c.1288A>G
Proband
Mother
Father

Supplemental Figure 8. The variants of *MFSD7c* in the patient results in mutations in the conserved residues. A, comparison of amino acid sequences of MFSD7c from indicated organisms. Arrows indicate the amino acids that were mutated in the patient. **B,** Sanger sequencing results of the patient and the parent's DNA. The patient carries compound heterozygous mutations. The mother and father carries heterozygous mutation of P340L and T430A, respectively. Arrowheads show the missense mutations.

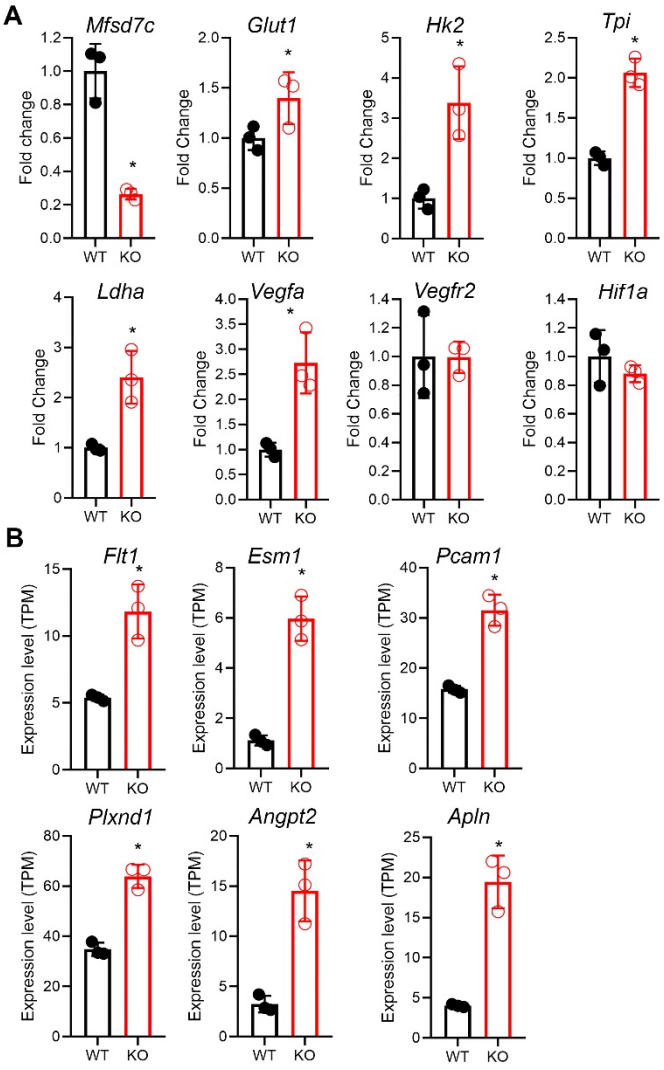


Supplemental Figure 9. Reduced transport activity of the mutants is likely responsible for the dysfunctions in the mutated MFSD7c proteins. A, expression of the indicated mutated protein in HEK293 cells. Expression pattern and levels of mutated proteins are similar to native **MFSD7c**. **B,** localization of the indicated mutated protein in HEK293 cells. The localization in the plasma membrane of the mutated proteins is similar to native **MFSD7c** in HEK293 cells, except for T430M mutant in which the localization was found mainly inside the cells. (n=3)

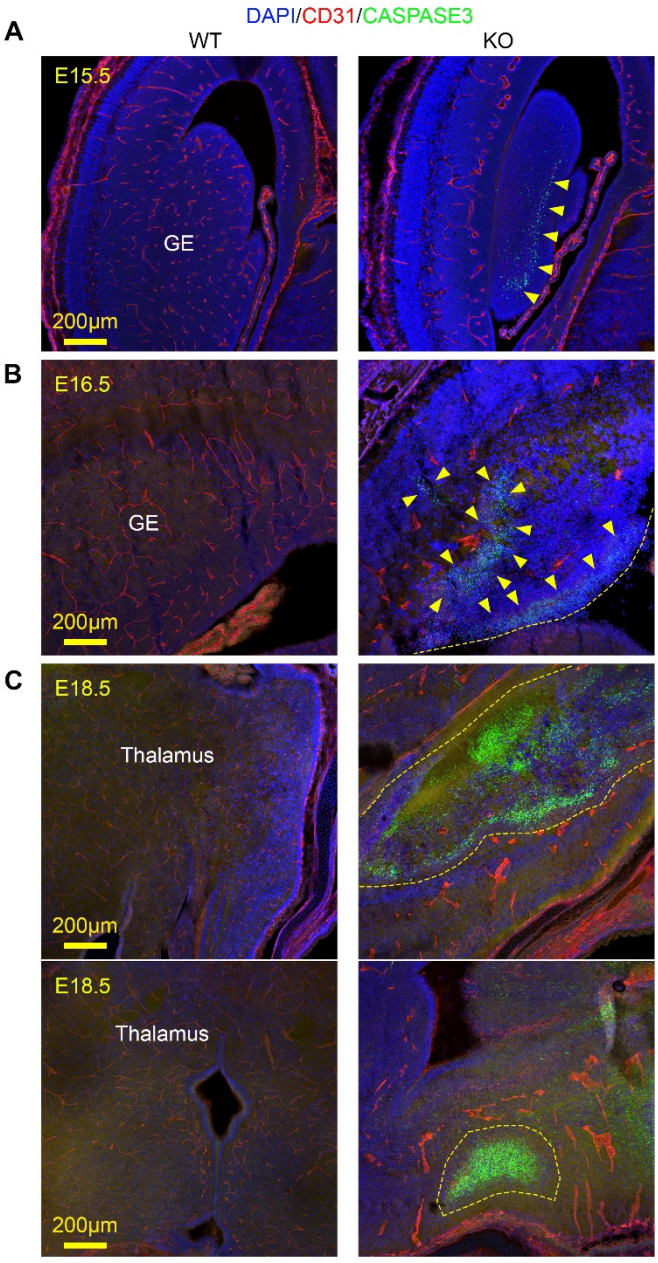


Supplemental Figure 10. Transcriptomic analysis of gene expression in *Mfsd7c* knockout embryos brain. A, Heatmap of top upregulated genes in the brain of E14.5 *Mfsd7c* knockout embryos. Arrowheads indicate glycolytic genes and arrows indicate angiogenic genes. n=3 for each genotype. **B**,

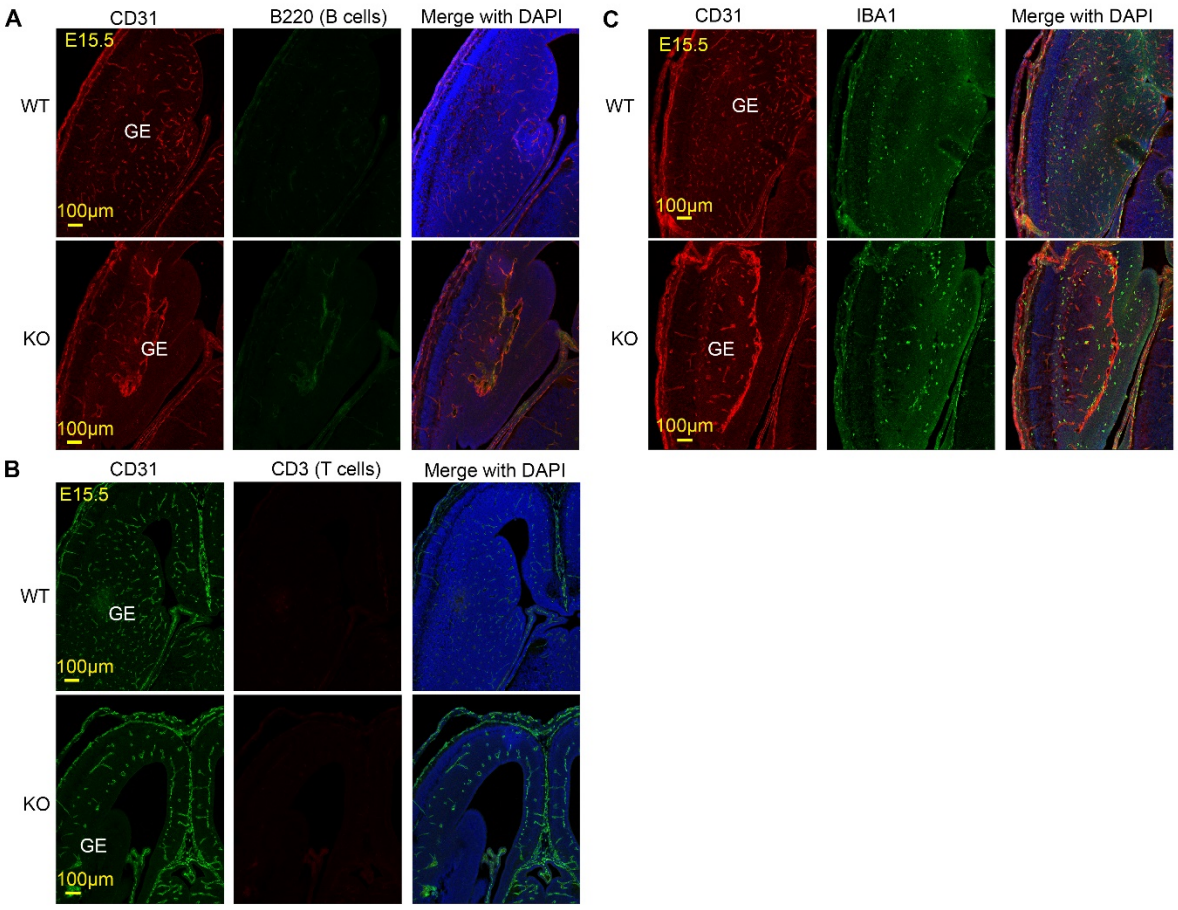
Vocano plot of differentially expressed genes in WT and KO embryos. The detailed gene expression levels can be found in the supplemental table 3.



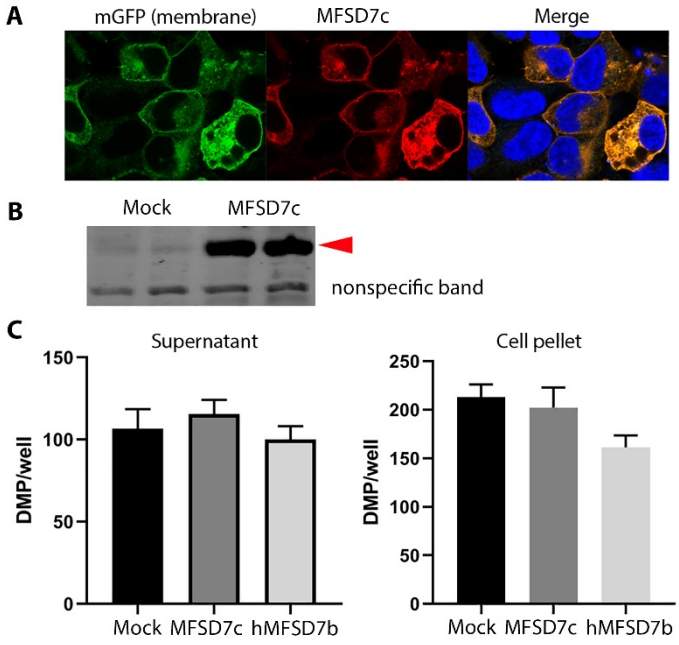
Supplemental Figure 11. Expression of hypoxia-related genes were upregulated in *Mfsd7c* knockout brain. A, Quantitative real-time PCR to measure the transcriptional expression level of hypoxia induced genes in E14.5 WT and KO brain. The expression level of indicated genes was shown as fold change to actin gene. The experiment was performed twice in triplicates. n=3 per genotype. **B**, Increased expression levels of angiogenic genes from RNA sequencing results. *P<0.05; Two tailed *t*-test.



Supplemental Figure 12. Increased cell death was detected in *Mfsd7c* KO embryos. A-C, Brain sections from E15.5 (n=3) (**A**), E16.5 (n=5) (**B**) and E18.5 (n=5) (**C**) embryos of WT and *Mfsd7c* knockout were stained with CD31 (red) and activated CASPASE3 (green). CASPASE3 positive cells were detected in the subventricular zone of the ganglionic eminence (GE) of E15.5 knockout embryos. We noted that there was an increased progress of cell death in the cortical cell layer in E16.5 knockout embryos and the cell death was also detected in the thalamus and hindbrain regions of E18.5 knockout embryos. Arrowheads show the CASPASE3 positive cells (green dots). Yellow dotted lines in **C** demarcate the cell death areas. Experiment was performed five times.



Supplemental Figure 13. Staining of immune cells in E15.5 WT and KO embryos. A-C, Brain sections of E15.5 WT and knockout embryos were stained with B220 (**A**), CD3 (**B**), and IBA1 (**C**) antibodies to detect the infiltration of B cells, T cells, and visualization of microglia cells, respectively. These sections were also co-stained with CD31 to visualize blood endothelial cells. There was insignificant increased amount of the infiltrated lymphocytes and microglia cells in the knockout brain. (n=1)



Supplemental Figure 14. Mfsd7c is dispensable for heme transport. A, MFSD7c is co-localized with plasma membrane GFP (mGFP) in HEK293 cells. **B,** Western blot analysis of human MFSD7C expression after 24h transfection in HEK293 cells. **C,** transport activity of mock (empty plasmid), MFSD7c, and MFSD7b with tritium labelled heme in HEK293 cells. There was a slight reduction of intracellular heme level in human MFSD7b (hMFSD7c) condition, whereas there was no difference in MFSD7c compared with mock for both supernatant and cell pellet. Experiments were repeated at least 3 times with 3 biological replicates.