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The Rho/Rac guanine nucleotide exchange factor Vav1 regulates HIF-1α and GLUT-1 expression and glucose uptake in the brain

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Abstract.

Vav1 is a Rho/Rac guanine nucleotide exchange factor expressed in hematopoietic and endothelial cells that is involved in a wide range of cellular functions. It is also stabilized under hypoxic conditions when it regulates the accumulation of the transcription factor HIF-1α, which activates transcription of target genes to orchestrate a cellular response to low oxygen. One of the genes induced by HIF-1α is GLUT-1, which is the major glucose transporter expressed in vessels that supply energy to the brain. Here, we identify a role for Vav1 in supplying glucose to the brain. We found that Vav1 deficiency downregulates HIF-1α and GLUT-1 levels in endothelial cells, including blood-brain barrier cells. This downregulation of GLUT-1 in turn reduced glucose uptake to endothelial cells both in vitro and in vivo, and reduced glucose levels in the brain. Furthermore, endothelial cell-specific Vav1 knock-out in mice, which caused glucose uptake deficiency, also led to a learning delay in fear conditioning experiments. Our results suggest that Vav1 promotes learning by activating HIF-1α and GLUT-1 and thereby distributing glucose to the brain. They further demonstrate the importance of glucose transport by endothelial cells in brain functioning and reveal a potential new axis for targeting GLUT-1 deficiency syndromes and other related brain diseases.

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Introduction.

Energy sources and oxygen are delivered to organs and tissues throughout an organism via the vascular system, which acts as a critical lifeline for cellular metabolism. For delivery of these substrates to parenchymal cells, they must pass across the endothelium (1-4). Glucose is one of the main sources of cellular energy, and the endothelium plays an important role in tissue glucose metabolism. A key regulator of this endothelial metabolic function is the oxygen-sensing transcription factor, HIF-1α (5). HIF-1α maintains glucose homeostasis and is a well-known regulator of GLUT-1, which is the primary transporter of glucose across the endothelial blood-brain barrier (6). It has been reported that endothelial cells from HIF-1α knock-out mice showed decreased GLUT-1 levels followed by reduced glucose uptake (5).

The endothelium regulates glucose metabolism in parenchymal cells via two major pathways: by transporting glucose from blood to tissue; and by presenting endothelial endocrine or paracrine factors to the parenchyma. The first pathway involves the uptake of primary glucose across the membrane, which occurs by facilitated diffusion in an energy-independent manner by a glucose transporter (3). Glucose transporters are a structurally homologous family of proteins that are expressed in a tissue-specific manner (7,8). Among the family members, GLUT-1 is the major glucose transporter in endothelial cells including the blood-brain barrier (7,9-11). Human GLUT-1 malfunctioning mutations are associated with a reduction of glucose uptake to the brain and the tendency of seizures (2,12). GLUT-1 mutation is also a potential cause of CNS syndromes, including learning disabilities (9). GLUT-1 is also expressed in glia but to a lesser extent than in endothelial cells (10). Although the major glucose transporter of Vav1 controls HIF-1α and GLUT-1 in the brain

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neurons is GLUT-3, defective regulation of the large influx of glucose from endothelial cells to the parenchyma, including neurons, is likely to be an underestimated cause of neurological dysfunctions.

Vav proteins are a family of Rho/Rac guanine nucleotide exchange factors (GEF) that mediate a wide variety of cellular functions. They consist of three highly conserved members in vertebrates. Vav1 is largely restricted to hematopoietic cells where it acts as an important signaling factor involved in hematopoietic cell activation, growth, and differentiation (13,14). Mice lacking Vav1 are viable and fertile, and develop apparently normally except for a partial defect in lymphocyte development (15-17). Vav1 is also expressed in endothelial cells where it activates Rac1 and Cdc42 in association with δ-catenin, which may be involved in regulating cell motility and vascular network formation (18). In our recent study, Vav1 was identified as a critical regulator of the vascular response to hypoxia: hypoxia blocks Vav1 degradation, whereas under normoxic conditions it is continually produced and degraded. This accumulation of Vav1 leads to hypoxia-induced HIF-1α accumulation. Indeed, in mice lacking Vav1 that were subjected to cardiac ischemic stress, the absence of HIF-1α activation led to death (19).

The brain is directly affected by energy regulation (20,21). It utilizes glucose at a high rate, and a lack of glucose has been linked to various brain-related diseases, including dementia (22-24). The dysregulation of glucose metabolism in brain damage has been a focus of a therapeutic strategies because of the possibility to administer soluble molecules that stimulate glucose metabolism, such as insulin or glucagon-like peptide 1 (GLP-1). These molecules improve cognitive responses in humans diagnosed with Alzheimer's disease, as well as in mouse models (25-31). These reports suggest that the dysregulation of glucose

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in the brain is crucial in neurologic diseases, as well as the progression of other metabolic diseases including diabetes.

Given that Vav1 increases the stability of HIF-1α, and HIF-1α is the critical transcription factor for GLUT-1 (19), we hypothesized that Vav1 regulates the expression and function of GLUT-1 via HIF-1α, thereby regulating glucose metabolism in the brain. Glucose deficiency in the brain is directly related to various neurologic dysfunctions including learning disability. In this study, we suggest the regulation of glucose distribution is Vav1 dependent and Vav1 is an important factor for the brain function.

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Results.

Vav1 regulates HIF-1α and GLUT-1 in endothelial cells

In order to verify whether Vav1 regulates HIF-1α in endothelial cells, we incubated human umbilical vein endothelial cells (HUVEC) after knocking down Vav1 in normoxic or 1% O_2 hypoxic conditions for 5 hours. HIF-1α was strongly upregulated in hypoxic conditions in control cells, whereas it remained largely undetectable in Vav1 knock-down cells (Figure 1A). Vav1 was also upregulated when cells were incubated in hypoxic conditions, consistent with our previous findings (19). Since HIF-1α induces the transcription of GLUT-1, only 5-hours of hypoxia did not show any increase in GLUT-1 expression, as expected. Therefore, we incubated Vav1 knock-down HUVEC in hypoxic conditions for 16 hours and measured GLUT-1 levels. In control cells, GLUT-1 levels increased compared to normoxic conditions, whereas there was no difference in the Vav1 deficient HUVEC (Figure 1B). Next, we performed a similar experiment in human blood brain barrier endothelial hCMEC/d3 cells to validate whether GLUT-1 is controlled by Vav1 and HIF-1α. As expected, there was no upregulation of HIF-1α or GLUT-1 under hypoxic conditions in the Vav1 knock-down cells (Figure 1C). We further verified that HIF-1α controls GLUT-1 at the mRNA rather than the protein level by overexpressing HIF-1α in HUVEC. We found that the mRNAs of HIF responsible element (HRE) genes, including GLUT1 and VEGF, were upregulated by HIF-1α overexpression in both normoxic and hypoxic conditions (Supplementary Figure 1). To further verify that GLUT-1 is controlled by Vav1, we overexpressed HIF-1α in control or Vav1 shRNA-introduced hCMEC/d3 cells. Consistent with our previous results, Vav1 shRNA transfected cells showed downregulated

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HIF-1α followed by GLUT-1 levels compared to control shRNA transfected cells (Figure 1D). However, overexpression of HIF-1α blocked this downregulation. This GLUT-1 rescue shows that Vav1 controls HIF-1α, which in turn regulates GLUT-1 transcription, in endothelial cells.

Vav1 regulates glucose uptake in endothelial cells

Next, we determined whether Vav1 affects the ability of GLUT-1 to take up glucose. Vav1 shRNA or control shRNA were introduced into HUVEC or hCMEC/d3 cells. We treated these cells with 2-NBDG, a fluorescent glucose analogue, after starvation in glucose-free medium for 2 hours. The Vav1-deficient groups of both HUVEC and hCMEC/d3 showed a decreased 2-NBDG signal (Figure 2A). To quantify this, we measured the mean fluorescent intensity of 2-NBDG from both HUVEC (Figure 2B) and hCMEC/d3 cells (Figure 2C). Compared to control cells, the Vav1 knock-down cells showed a significantly reduced 2-NBDG signal. This reduction was comparable to that seen upon treatment with the GLUT-1 inhibitor, apigenin. These results demonstrate that, under hypoxic conditions, Vav1 regulates glucose uptake through GLUT-1 in endothelial cells.

Vav1 deficiency delays glucose uptake in vivo

We next determined whether Vav1 regulates glucose uptake also *in vivo* by testing glucose uptake from the blood stream in endothelial-specific Vav1-deficient mice. VE-Cadherin-cre/ERT-Vav1$^{flox/flox}$ mice were treated with 4-OH tamoxifen for two weeks to generate endothelial-specific Vav1-deficient mice. First, we measured basal levels of blood glucose in wild type and Vav1-deficient mice. In the absence of endothelial Vav1, blood glucose

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levels were relatively upregulated, even without glucose administration (Figure 3A). Next, an intraperitoneal glucose tolerance test was performed to compare the uptake of administered glucose. Compared to the wild type group, Vav1-deficient mice showed significantly increased levels of blood glucose and delayed recovery back to basal levels (Figure 3B). Over 50% of the Vav1-deficient mice had basal blood glucose levels of 126 mg/dl or more, compared to less than 20% of the wild type mice (Figure 3C). Furthermore, Vav1-deficient animals were significantly heavier than wild type animals (Figure 4D). These characteristics are found in diabetic mice, although we could not detect any related histological structural changes in the tissues of Vav1-deficient mice (Supplementary Figure 2).

Vav1 deficiency downregulates glucose distribution in the brain

We focused on the brain of endothelial Vav1-deficient mice because the brain is one of the major organs that utilizes glucose. As expected, GLUT1 was expressed in the brain of wild type mice, but, notably, was largely undetectable in the endothelial Vav1-deficient mice (Figure 4A). Typically, Vav1 levels increase under stressful conditions such as hypoxia (Figure 1A-C) or starvation (Supplementary Figure 3). ^{18}F- Fludeoxyglucose (FDG) is widely used in positron emission tomography (PET) to measure inflammatory regions especially in cancer patients. FDG is also an analogue of glucose, which is taken up by glucose transporters. We compared the signal of injected FDG in wild type and endothelial Vav1-deficient mice using PET. The FDG signal was significantly lower in the brains of Vav1-deficient mice compared to wild type mice. The dilated heart also showed a high signal as it contains high amounts of blood, as well as the gall bladder and urinary bladder,

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which contain high levels of FDG metabolite. When the PET image was superimposed onto the CT image, the lower amount of FDG was more easily observed in the Vav1-deficient mouse (Figure 4B). Both the mean and total standardized uptake value (SUV) were significantly lower in Vav1-deficient mice (Figure 4C and D). Next, we evaluated the effect of endothelial Vav1-deficiency on brain function by subjecting wild type and endothelial Vav1-deficient mice to a fear conditioning test, see methods, which represents a form of learning (32). We found a significant decrease in the percentage of time spent freezing to context, i.e., in the same environment as the conditioning, in the endothelial Vav1-deficient mice compared to the wild type mice. This difference was lost when the mice were analyzed for freezing to cue, i.e., in a different environment but with the same stimulus (Figure 4E). This suggests that there is some impairment of learning when Vav1 is deficient. These results show that Vav1 in the endothelium regulates glucose distribution to the brain as well as the function of the brain, such as learning.

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Discussion

Since GLUT-1 is a component of HIF Responsible Elements (HRE) and highly expressed in endothelial cells, we focused on glucose uptake in endothelial cells. As we expected, Vav1 knockdown led to the downregulation of both HIF-1α and GLUT-1. In our experiments, HIF-1α was upregulated by hypoxia in 5 hours but GLUT-1 was not increased. Since HIF-1α controls the transcription of GLUT-1, the upregulation of proteins was not shown at the same time in HUVEC. We could observe upregulated GLUT-1 after 16-hour incubation of cells in hypoxia, unlike HIF-1α in HUVEC. Interestingly hCMEC/d3 showed a slightly different pattern than HUVEC. hCMEC/d3 showed increased HIF-1α and GLUT-1 together after 16-hour incubation in hypoxia. This difference may be dependent on the cell type. Notably, the upregulation of HIF-1α and GLUT-1 was abrogated by Vav1 knockdown in both types of cells. Additionally, the GLUT-1 decrease was rescued when HIF-1α was overexpressed in Vav1 knockdown cells, which demonstrates that Vav1 is upstream of both HIF-1α and GLUT-1.

The basal blood glucose level of Vav1 knock out mice was significantly lower than that of wild type mice, which may be because of a lower uptake of glucose from the blood stream to the endothelial cells. In the Vav1-deficient group, exogenously administered glucose was not taken up as quickly as wild type mice. Additionally, the increase in glucose levels after injection was higher than in the wild type mice. This in vivo data indicates that glucose uptake in Vav1-knockout mice is delayed. Furthermore, more than 50% of Vav1-knockout mice had over 125 mg/dl of blood glucose, while less than 20% of wild type mice had these high levels. Vav1-knockout mice also showed increased body weight compared to wild type mice. High blood glucose and body weight are characteristics of diabetic mice. Other Vav1 controls HIF-1α and GLUT-1 in the brain

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than this, the endothelial-specific Vav1-knockout mice were fertile and had no gross phenotypic abnormalities.

The brain requires a consistent supply of energy to maintain proper functioning, and it is the second most glucose-consuming organ after the liver. GLUT-1 is the main route to supply glucose from the blood stream to the brain. Thus, loss of GLUT-1 in endothelial cells of the brain is directly linked to brain damage and related to several diseases including GLUT-1 deficiency syndrome and Alzheimer's disease (33,34). Because Vav1 deficiency leads to the loss of GLUT-1, the distribution of glucose to the brain is remarkably decreased. In our studies, we observed that Vav1 deficiency not only downregulates glucose distribution to the brain but also memory deficiency observing the behavioral experiment. Considering these findings, we propose that endothelial Vav1 is a critical factor that controls brain function including memory.

GLUT-1 deficiency syndrome is a genetic disorder that varies in symptoms and severity between individuals. For example, some individuals with GLUT-1 deficiency syndrome do not develop epilepsy. Other possible symptoms include abnormal eye-head movements, body movement disorders, developmental delays, and varying degrees of cognitive impairment, slurred speech and language abnormalities (33). Although Vav1 has not been previously associated with conditions caused by the mutation of SLC2A1, which encodes GLUT-1, the phenotype of endothelial Vav1-deficient mice was very close to GLUT-1 deficiency syndrome, supporting further studies such as discovering the pathological mechanism and in vivo experiments. Glucose uptake deficiency in the brain is not only related to GLUT-1 deficiency syndrome but also to Alzheimer's disease associated with

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type 2 diabetes and various other brain disorders (18,22,23). In light of our results, it will be interesting to study the potential role for Vav1 in these disorders.

It is well-known that HIF-1α is a key transcription factor for GLUT-1. Our previous study revealed that Vav1 is continuously produced and degraded under normoxic conditions, whereas under hypoxic conditions it is stabilized and required for HIF-1α activation via activation of p38 MAPK (19). We now take the next step and show that Vav1-mediated HIF1 α activation is required for glucose uptake in the brain.

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Materials and Methods.

Cell culture and bioassays

Human umbilical vein endothelial cells (HUVECs) and the hCMEC/d3 human blood-brain barrier cell line were obtained from Lonza (Walkersville, MD) and Sigma (St. Louise, MO), respectively. The cells were cultured according to the manufacturer's protocols. For hypoxia, cells were incubated in 1% O_2 adjusted incubator (Thermo, Waltham, MA) for 5 hours or 16 hours. Control and Vav1 shRNA constructs were obtained from Sigma. HIF-1α construct was obtained from Addgene (Watertown, MA). Glucose uptake assay was performed with Glucos Uptake Cell-based Assay Kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's description.

Western blot and qRT-PCR

Cells were lysed with sample buffer and briefly ultrasonicated. Specific antibodies for HIF-1α, GLUT-1 and Vav1 were purchased from Cell Signaling Technology (Danvers, MA). Total RNA was isolated with RNeasy mini for qRT-PCR from cell lines. qRT-PCR was performed using 1-Step Quantitative Reverse Transcription PCR from RNA (Biorad, Hercules, CA) following the manufacturer's description. Relative mRNA level was measured with primers purchased against GLUT-1, VEGF and HIF-1α (Sigma).

Experimental animals

The mice were maintained in pathogen-free facilities in the National Cancer Institute (Frederick, MD). The study was approved by the NCI Animal Care and Use Committee, and in accordance with the ARRIVE guidelines. VE-Cadherin-cre/ERT2 and Vav1flox/flox mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and crossed to establish the colony and backcrossed to C57BL/6 mice. Age and sex matched mice were used for experiments. Blood glucose was measured with blood drops from tail tips of mice using a glucometer (Roche, Basel, Switzerland).

Fear Conditioning

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The conditioning apparatus consisted of a mouse shock chamber (Coulbourn Instruments) set up in a sound-attenuated box. On day 1, the conditioning day, after a 2 min acclimation period to the conditioning chamber, mice received three conditioning trials consisting of a 30 s presentation of a 5 kHz, 70-dB tone [conditioning stimulus (CS)] that coterminated with a 0.7 mA foot shock delivered through the grid floor during the last 1.0 s of the tone. Each conditioning trial was separated by a 30 s intertrial interval (ITI). After conditioning, mice were returned to their home cages. Mice were traced with infrared beam cue for subsequent quantification of behavior. Time spent "freezing" before and during the presentation of the CS tone was measured during the CS presentation as well as during a 30 s baseline period before the first tone trial. This latter measure served as an assay for unconditioned effects of the CS on general activity levels. Memory for the context and the tone was evaluated on days 2 and 3 respectively (~24 and 48 h after conditioning). For the contextual test, mice were placed in the conditioning chamber and allowed to explore for 2 min, after which freezing to the context was assessed for the remaining 4.5 min. For the tone test, mice were placed in a novel chamber with green walls. Mice could acclimate to the chamber for 2 min and were then presented with the CS (tone) on three consecutive trials (30 s, 5 kHz, 70 dB; ITI of 40 s). Freezing was evaluated during the 2 min acclimation period, during each presentation of the tone CS, and during the 40 s intertrial interval. After the memory tests, animals were returned to their home cage and colony. Memory for either the context or tone CS was quantified by the percentage of time engaged in a fear-related behavior (freezing) during context testing or CS presentation.

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PET/CT Scanning

PET/CT scanning was performed after mice were fasted for 12 h but had free access to water. Following determination of body weights (28.0±3 g on average) mice were anesthetized using vaporized isoflurane (4% for induction; 2.5% for maintenance). Sterile normal saline (0.1 ml) was injected subcutaneously to ensure adequate hydration. Following administration of ^{18}F-FDG (< 200 μCi) via lateral tail vein injection, mice were awakened and returned to individual holding cages. Precisely 60 min post FDG injection, anesthetized mice were placed prone in a heated (36 °C) multimodality chamber) in the PET scanner's gantry. PET list mode data were acquired for 15 min, using a small-animal PET/CT imaging system (GE Healthcare, Laurel, MD). In the same workflow, a CT image was acquired for attenuation correction purposes. Images were reconstructed using a three-dimensional OP-MAP algorithm (Siemens Medical Solutions USA, Inc., Malvern, PA, USA). Scanner reconstructions using this algorithm were calibrated back to a National Institute of Standards and Technology (NIST)-traceable positron-emitting dose calibrator source to assure quantitative accuracy. Standard Uptake Value (SUV) was normalized by injection activity (μCi) and animal body weight (g).

Histology

Brain, heart, skeletal muscle and pancreas were obtained from both wild type and endothelial Vav1-deficient mice after perfusion with PBS. For standard histology, formalin-fixed tissues were embedded in paraffin, sectioned, and stained (H&E) by the NCI-Frederick Histology Core.

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Figure Legends.

Figure 1. Vav1 regulates HIF-1α and GLUT-1 in endothelial cells

(A) HUVEC cells with control or Vav1 shRNA were incubated under normoxic or 1% O_2 conditions for 5 hours, or (B) 16 hours. Cells were lysed to measure HIF-1α, Vav1 and Glut1 levels by Western blotting. (C) hCMEC/d3 cells with control or Vav1 shRNA were incubated under normoxic or 1% O_2 conditions for 16 hours. Cells were lysed to measure HIF-1α, Vav1 and Glut1 levels by Western blotting. (D) Control or Vav1 shRNA expressing hCMEC/d3 cells were transfected with empty vector or HIF-1α overexpression vector. Cells were incubated under normoxic or 1% O_2 conditions for 16 hours and then subjected to Western blot.

Figure 2. Vav1 regulates glucose uptake in endothelial cells

(A) HUVEC and hCMEC/d3 cells with control or Vav1 shRNA were starved in serum-free RPMI for 1 hour before addition of 2-NBDG under hypoxic conditions. After 1 hour of incubation, cells were fixed with paraformaldehyde and observed by confocal microscopy (A). (B) HUVEC and (C) hCMEC/d3 cells with control or Vav1 shRNA in a 96-well plate were starved in serum-free RPMI for 1 hour and then treated with 2-NBDG with or without apigenin under hypoxic conditions for an hour. Mean fluorescence intensity (MFI) was measured to quantify 2-NBDG uptake levels (quadruplet mean ± SD). ***$p<0.001$

Figure 3. Vav1 deficiency delays glucose uptake in vivo

Wild type or endothelial Vav1-deficient mice were fasted for 12 hours in the presence of water. (A) Blood glucose levels were measured from the tail tip of each mouse (n=10/group). (B) After

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12 hours' fasting, mice were given with 2g/kg of glucose intraperitoneally. The blood glucose level was measured at each designated time point (n=10/group). (C) Ratio of animals with 126 mg/dl or higher of blood glucose in the basal state are displayed (n=12/group). (D) The weight of mice measured after 12-hour fasting (n=12/group). Mean ± SD , *p<0.05, **p<0.01, ***p<0.001

Figure 4. Vav1 deficiency downregulates glucose distribution in the brain

(A) The total lysate of mouse brain was subjected to Western blot to measure GLUT-1 protein expression levels (n=3/group). (B) Wild type or endothelial Vav1-deficient mice were subjected to PET/CT scan to measure the distribution of ^{18}F-FDG. Mice were fasted for 12 hours before intravenous injection of ^{18}F-FDG. 1 hour after ^{18}F-FDG injection, whole body of mice were scanned. The scanned signal in brains were normalized and then numerated as mean (C) and total (D) standardized uptake value (SUV) (n=10/group). (E) Wild type or endothelial Vav1-deficient mice were subjected to a fear conditioning test. Both contextual and cued conditions were given to measure the percentage of time in a frozen state. Mean ± SD , *p<0.05

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Supplementary Figure 1. HUVEC were transfected with empty vector (blank bar) or HIF-1α (filled bar). Cells were incubated in normoxia or hypoxia (1% O_2) for 16 hours. GLUT-1, VEGF and HIF-1α mRNA were measured by qRT-PCR. Mean ± SD , *$p<0.05$

Supplementary Figure 2. Brain, heart, skeletal muscle, and pancreas were obtained from wild type or endothelial Vav1-deficient mice after perfusion. Tissues were fixed with 4% paraformaldehyde and then embedded in paraffin. Slides were stained with hematoxylin and eosin to visualize the basal structure of organs. Representative figures are shown from three independent experiments.

Supplementary Figure 3. HUVEC were incubated in serum-free medium or complete medium for 4 hours and then subjected to Western blot to measure Vav1 protein.

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Figure 1

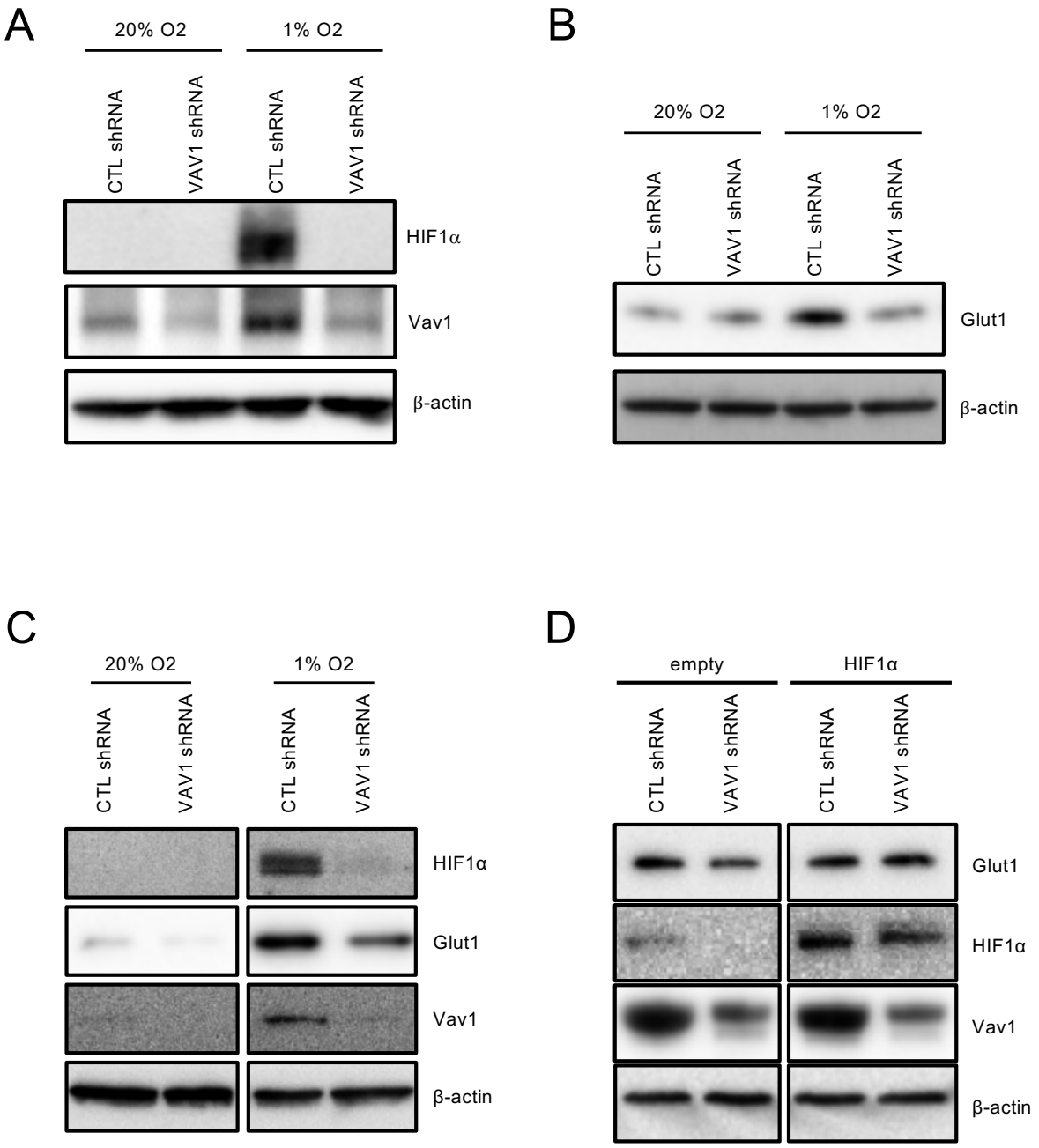

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Figure 2

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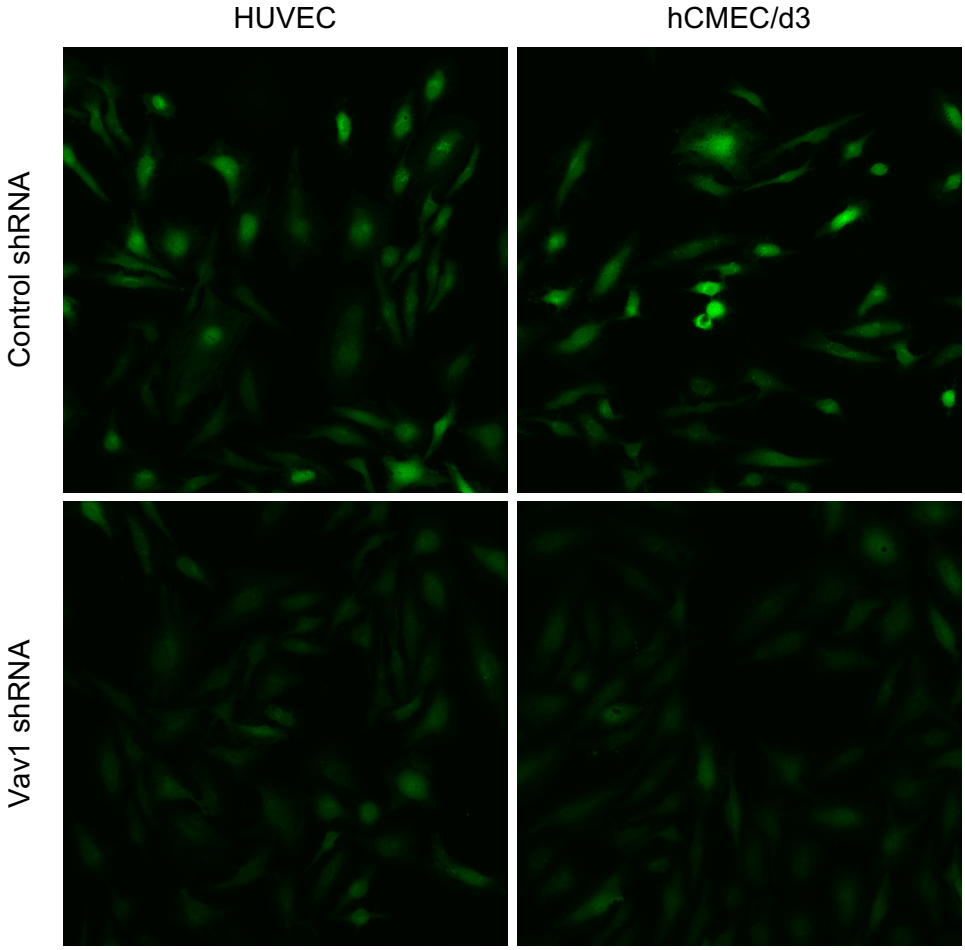

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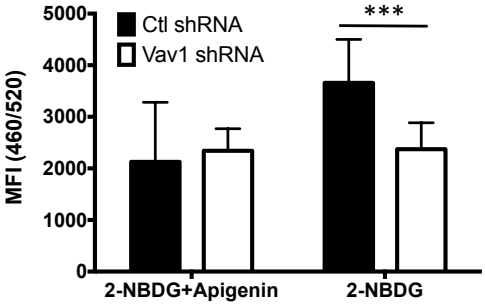

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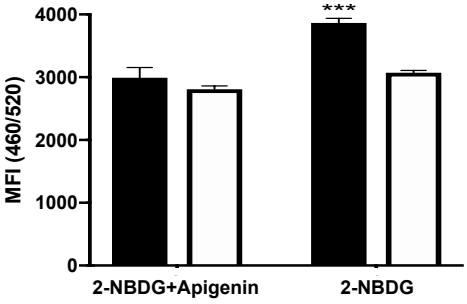

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Figure 3

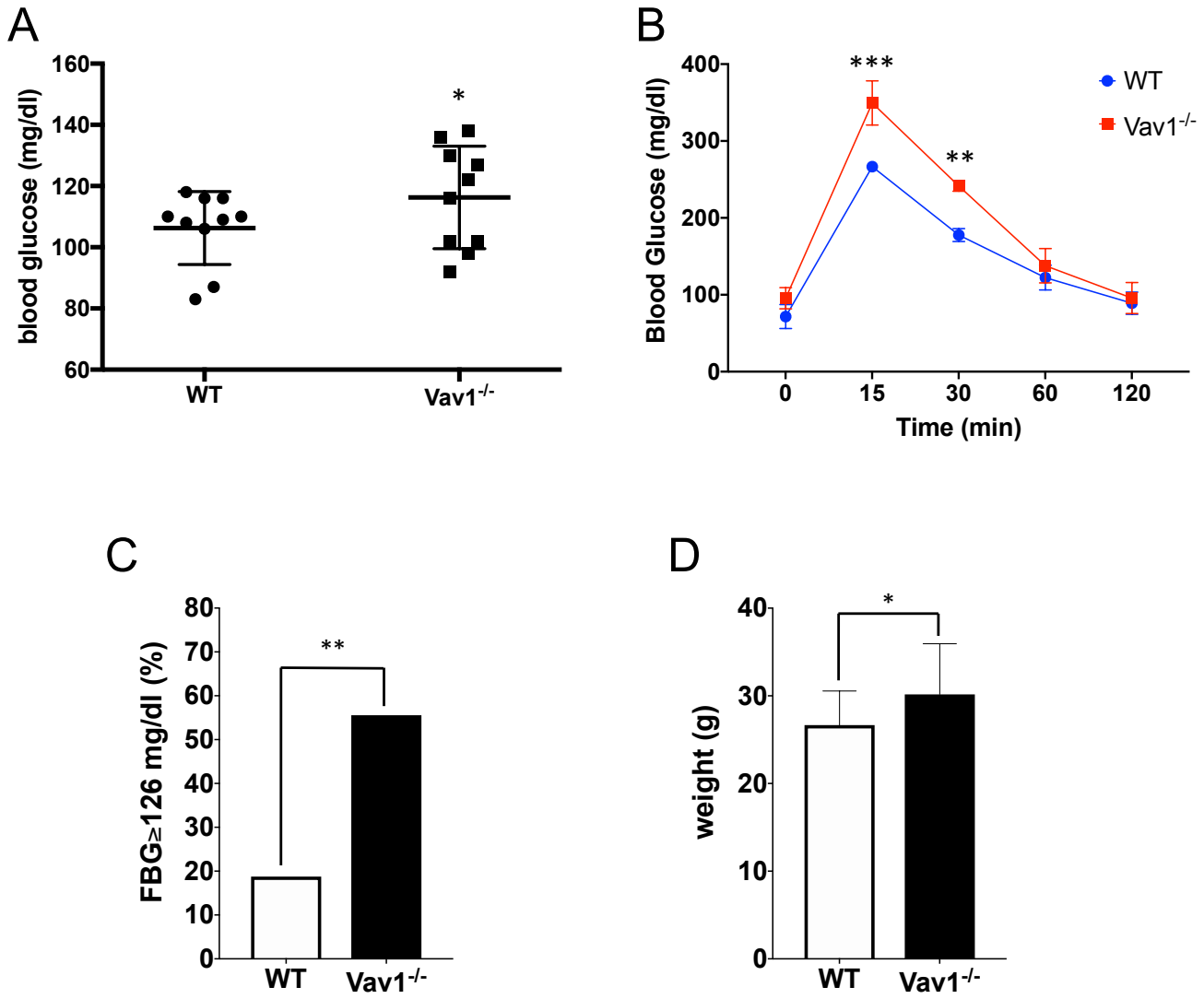

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Figure 4

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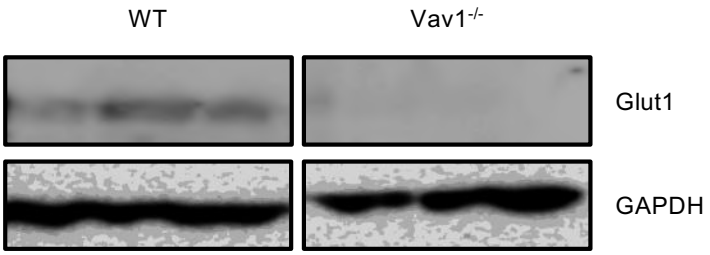

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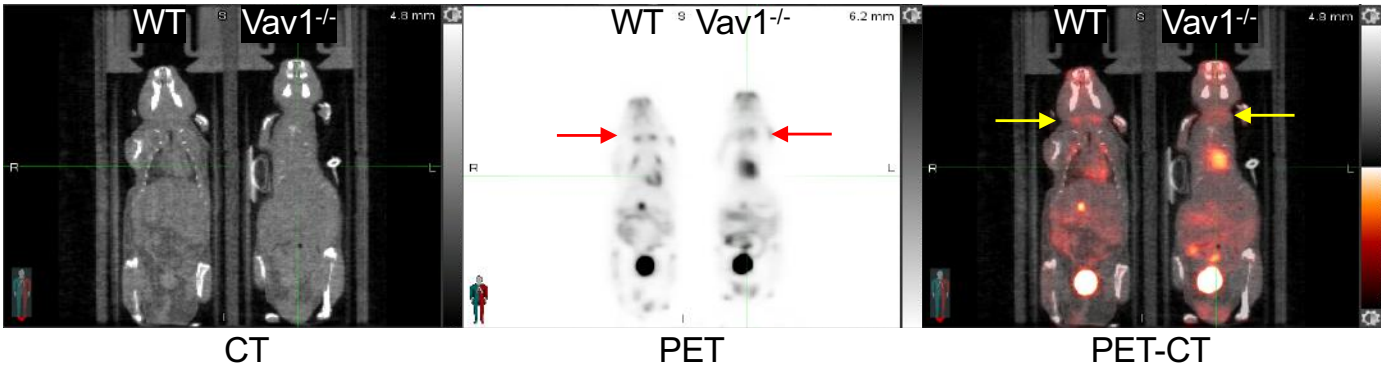

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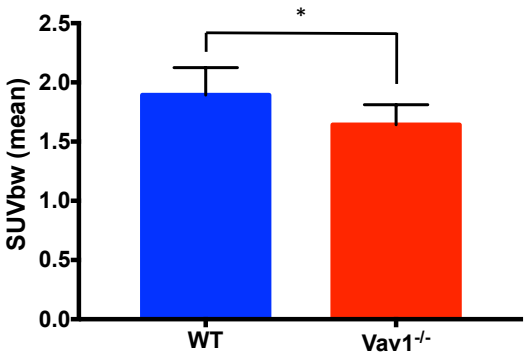

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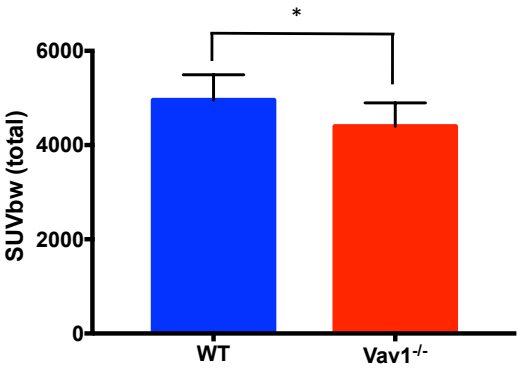

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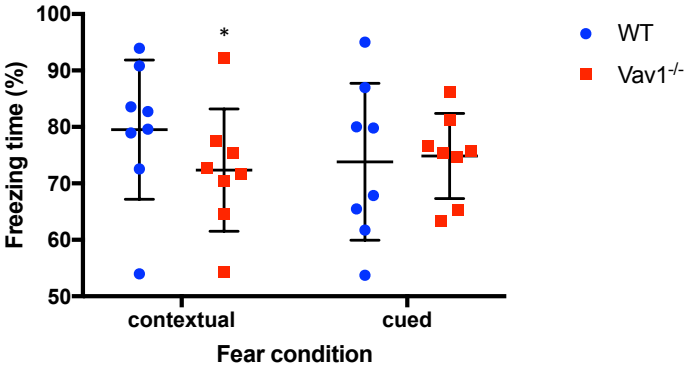

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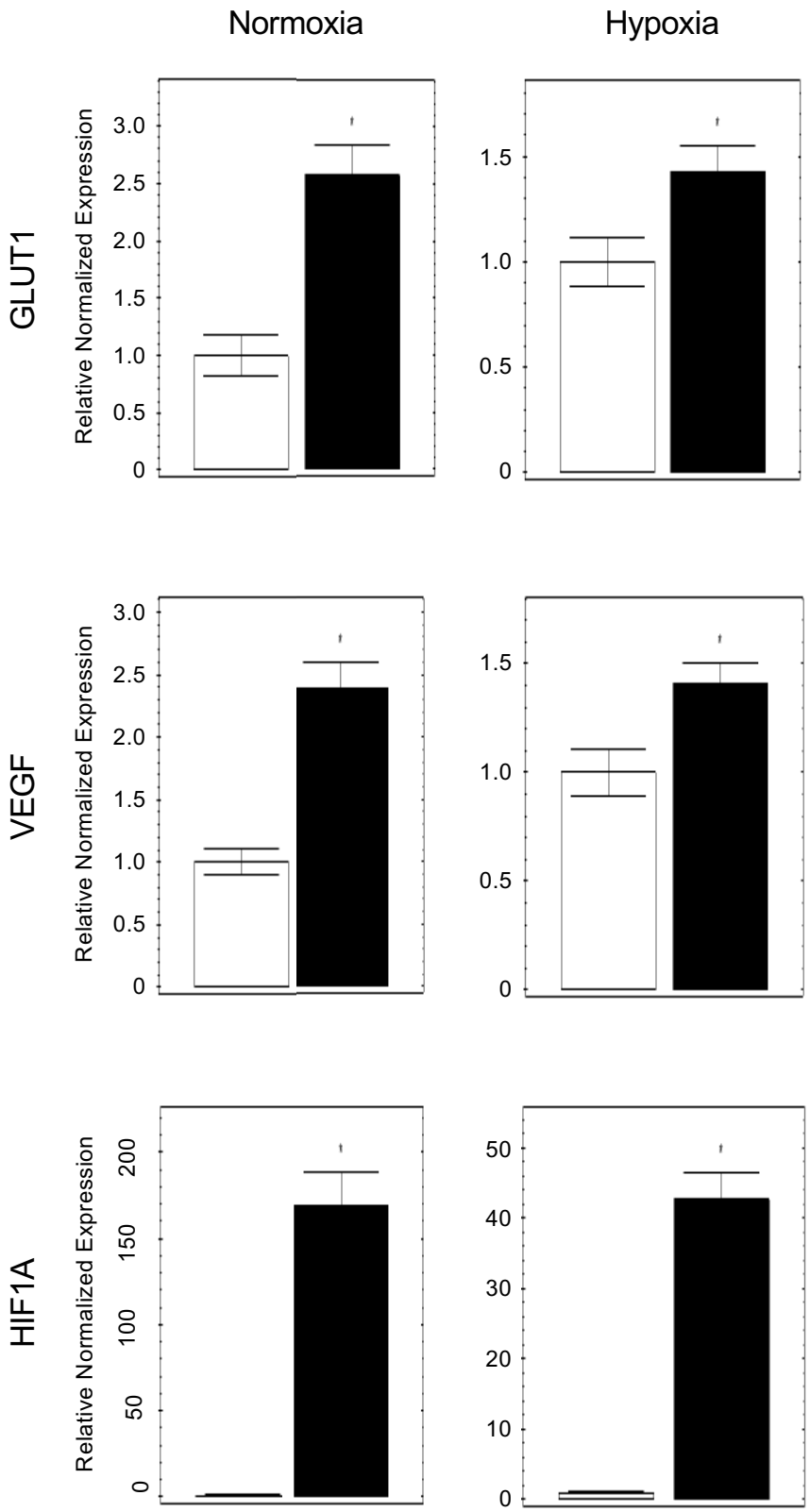
Sup Fig 1



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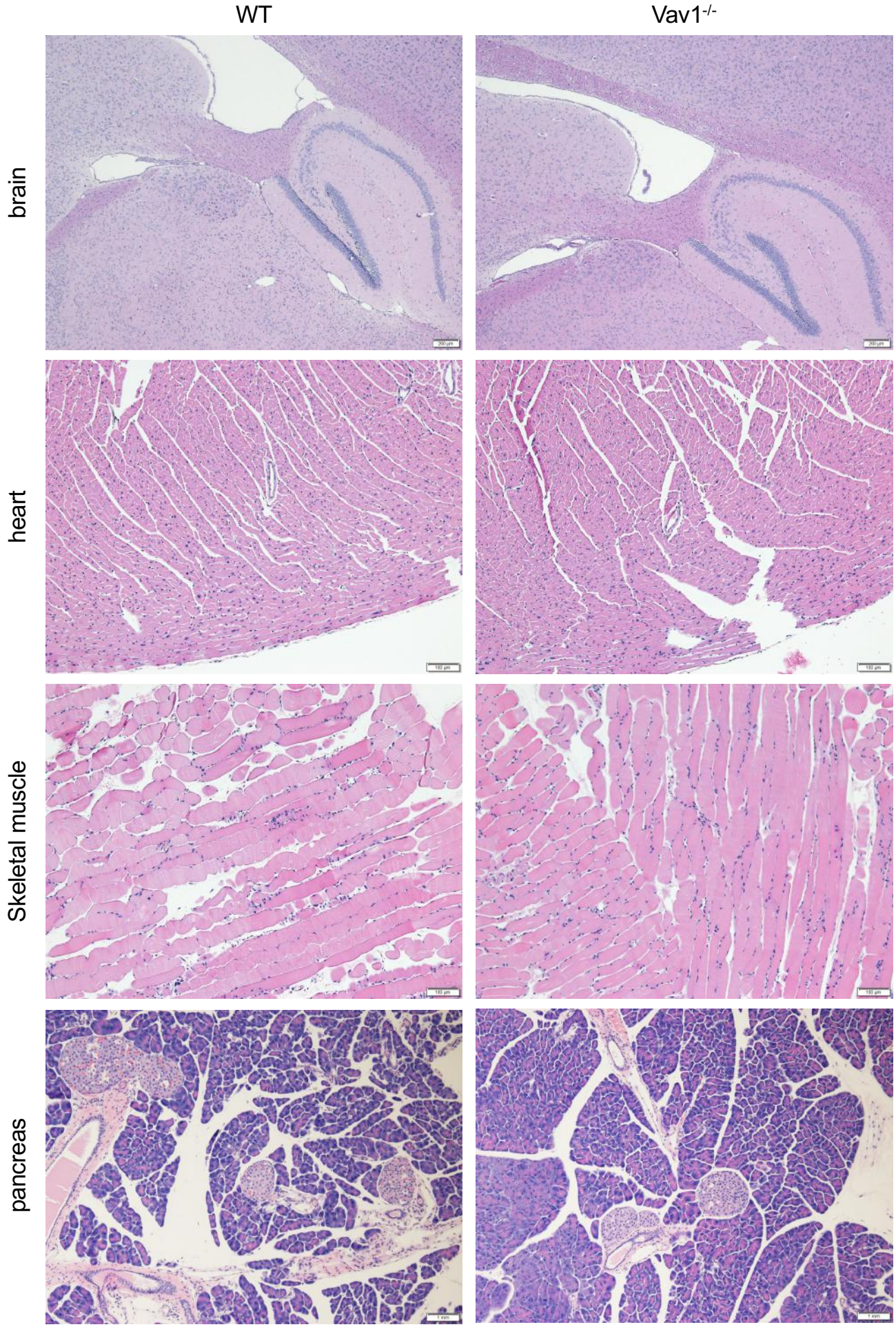
Sup Fig 2



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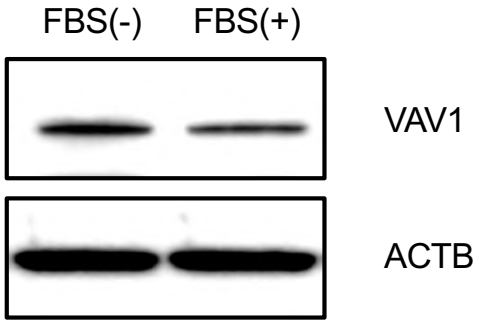
Sup Fig 3