Cavin1 Deficiency Causes Disorder of Hepatic Glycogen Metabolism and Neonatal Death by Impacting Fenestrations in Liver Sinusoidal Endothelial Cells

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It has been reported that Cavin1 deficiency causes lipodystrophy in both humans and mice by affecting lipid metabolism. The ablation of Cavin1 in rodents also causes a significant deviation from Mendelian ratio at weaning in a background-dependent manner, suggesting the presence of undiscovered functions of Cavin1. In the current study, the results show that Cavin1 deficiency causes neonatal death in C57BL/6J mice by dampening the storage and mobilization of glycogen in the liver, which leads to lethal neonatal hypoglycemia. Further investigation by electron microscopy reveals that Cavin1 deficiency impairs the fenestration in liver sinusoidal endothelial cells (LSECs) and impacts the permeability of endothelial barrier in the liver. Mechanistically, Cavin1 deficiency inhibits the RhoA-Rho-associated protein kinase 2-LIM domain kinase-Cofilin signaling pathway and suppresses the dynamics of the cytoskeleton, and eventually causes the reduction of fenestrae in LSECs. In addition, the defect of fenestration in LSECs caused by Cavin1 deficiency can be rescued by treatment with the F-actin depolymerization reagent latrunculin A. In summary, the current study reveals a novel function of Cavin1 on fenestrae formation in LSECs and liver glycogen metabolism, which provide an explanation for the neonatal death of Cavin1 null mice and a potential mechanism for metabolic disorders in patients with Cavin1 mutation.

Congenital generalized lipodystrophy type 4 (CGL4) is a rare disease caused by mutations in the gene CAVIN1, also known as polymerase I and transcript release factor, with systemic loss of body fat and predisposition for diabetes mellitus, hypertriglyceridemia, and hepatic steatosis.[1,2]

Cavin1 is the first found member of Cavins family, a family of proteins play important roles in the formation and maintenance of caveolae.[3] The other three members in Cavins family: Cavin2 (serum-deprivation response protein, SDPR), Cavin3 (SDR related gene product that binds to c-kinase), and Cavin4 (muscle-restricted coiled-coil protein, MURC) all share homology with Cavin1 and regulate the dynamics of Caveolae.[4,5] Cavin1 and Caveolin1 are two important component proteins for caveolae. Lacking either one ablates caveolae completely.[6] The defective caveolae will lead to diverse diseases including cancer, lipodystrophy, cardiomyopathy, and muscular dystrophies.[6]

Most knowledge about the functions of caveolae comes from the studies of caveolins and cavins, especially the studies of Caveolin1 or Cavin1 deficient mice.[4,6,7] Most phenotypes of Caveolin1 or Cavin1 deficient mice are centered on metabolism, such as reduced adipose tissue, hyperlipidemia, glucose intolerance, hyperinsulinemia, and less physical activity.[4,6,7]

Cavin1 deficient mice show similar phenotypes as humans with severe lipodystrophy and metabolic disorders.[8] Thus,
Cavin1-deficient mice are a viable representative model for studying CGL4 disease. Recent studies reported a significant deviation from Mendelian inheritance for Cavin1 knockout pups in certain genetic background at weaning,\[^{9-11}\] but the cause of this deviation remains unclear. Studying the underlying mechanisms for this phenomenon may reveal novel functions of Cavin1 and provide better care for CGL4 patients.

The survival rate of the Cavin1 knockout pups at weaning (3 weeks of age) is much lower than expected and varies from 5.7% to 13% in previous reports.\[^{9-11}\] A possible explanation for the variation is the mixed genomic background of the mice used in different laboratories. In order to study the cause of Cavin1 knockout pup mortality in a pure genetic background, we backcrossed the Cavin1\(^{-/-}\) mice with C57BL/6J for 20 generations. Then, we crossed the backcrossed Cavin1\(^{-/-}\) heterozygous parents to produce Cavin1\(^{+/+}\) homozygous progenies. Among 72 offspring (from 15 litters) genotyped at weaning, only one was identified as Cavin1\(^{-/-}\) (Figure 1A and Table S1, Supporting Information). To ascertain the cause of missing adult Cavin1\(^{-/-}\) mice, we analyzed 107 (from 12 litters) newborn pups from heterozygous parents. The ratio of Cavin1\(^{+/+}\)/Cavin1\(^{+/−}\)/Cavin1\(^{-/-}\) at birth followed the Mendelian inheritance (Table S2, Supporting Information), suggesting that the death of Cavin1\(^{-/-}\) mice happened after birth rather than at the embryonic stage. The survival survey concluded that most of the death occurred at around 10 h after birth (Figure 1B). We also observed that all the dying Cavin1\(^{-/-}\) mice exhibited cyanosis (Figure 1C,D). Cyanosis is a blue color throughout the skin and mucous membranes due to reduced hemoglobin in the blood, which could be caused by multiple conditions including heart disease, respiration failure, and extreme hypoglycemia.\[^{12}\] Generally, cyanosis and death caused by respiration failure would occur within 2 h after birth,\[^{13}\] but this timeline did not match our observations where Cavin1\(^{-/-}\) mice survived ≈10 h after birth (Figure 1B). Moreover, the morphology of lung and heart in Cavin1\(^{-/-}\) mice was normal (Figure S1A,B, Supporting Information). These results suggested that it might not be the respiration failure or heart diseases, but the disorder of glucose homeostasis caused the neonatal death of Cavin1\(^{-/-}\) mice. To test this hypothesis, we measured the blood glucose level at 9 h after birth in the pups that survived and found that the blood glucose levels in the Cavin1\(^{-/-}\) pups were extremely low, while the glucose levels in other littersmates remained normal (Figure 1E). To determine whether the lethal hypoglycemia in Cavin1\(^{-/-}\) pups was prenatal or postnatal, we measured fetal blood glucose level at 24 h before birth. Our data showed that the Cavin1\(^{+/+}\) and Cavin1\(^{-/-}\) fetus had a similar level of blood glucose before birth (Figure 1F). In contrast, in the same cohort, the blood glucose level in Cavin1\(^{-/-}\) pups was significantly lower than their wild-type littersmates at 9 h after birth (Figure 1F). These results indicated hypoglycemia in Cavin1\(^{-/-}\) pups occurred at the postnatal stage. To test if this severe hypoglycemia led to neonatal death, we performed a rescue experiment. We found that two intraperitoneal injections of glucose at 1 and 5 h after birth rescued Cavin1\(^{-/-}\) neonates (Figure 1G,H). Without any further intervention, these glucose-treated mice survived the weaning stage like their wild-type littersmates except a leaner body (Figure 1H,I). In summary, our data demonstrated that Cavin1\(^{-/-}\) mice backcrossed to the C57BL/6J background suffered severe hypoglycemia leading to neonatal death.

Before suckling, the blood glucose level in newborn pups is maintained by the mobilization of liver glycogen,\[^{14}\] thus deficits in glucose release from liver glycogen would cause hypoglycemia.\[^{15}\] We assessed the liver glycogen level in Cavin1\(^{-/-}\) mice and their wild-type littersmates at 9 h after birth while they are still alive. In wild-type newborn pups, the fetal liver was full of glycogen before birth and depleted of glycogen within 9 h after birth (Figure 2A,B). However, wild-type pups maintain healthy blood glucose level (Figure 1F) because the milk intake would catch up with the glucose supply. In contrast, the liver of Cavin1\(^{-/-}\) fetus contained much less glycogen before birth (Figure 2A,B). Additionally, this stored glycogen could not be efficiently mobilized and 9 h after birth there was still substantial amount of residual glycogen in the liver (Figure 2A,B). Without the glucose supply from liver glycogen, the blood glucose level in Cavin1\(^{-/-}\) pups dropped dramatically and the pups developed lethal hypoglycemia (Figure 1F). The adult Cavin1\(^{-/-}\) mice that survived after glucose injection could maintain a healthy blood glucose level similar to wild-type littersmates (Figure 2C). However, they still possess abnormal storage and mobilization of liver glycogen (Figure 2D,E). The liver glycogen in wild-type mice was synthesized and degraded according to the change of food supply, while the liver glycogen in Cavin1\(^{-/-}\) mice was almost fixed at a low level regardless of the food supply (Figure 2D,E). The liver of homozygous mice could neither store sufficient amount of glycogen during feeding nor efficiently mobilize glycogen during fasting (Figure 2D,E). The liver, skeletal muscle, and heart are the major organs to store glycogen. Glycogen from the liver is degraded and released to regulate blood glucose, while glycogen from skeletal muscle and heart is mostly used by themselves. Cavin1 deficiency only decreased the storage of liver glycogen, but not skeletal muscle or heart glycogen (Figure 2B,E and Figure S2A, Supporting Information). Of these three glycogen storage organs, Cavin1 deficiency only targeted the liver, which could...
not function as the reservoir for blood glucose regulation. The reduction of glycogen storage and the inhibition of glycogen mobilization indicated that the bilateral flow between liver glycogen and blood glucose was blocked in Cavin1-/- mice.

Liver glycogen is synthesized and degraded according to food intake. It is regulated by several cellular signal pathways to balance the activities of glycogen synthesis and glycogen phosphorylase. The mitogen-activated protein (MAP) kinases in the liver are regulated according to the feeding status, i.e., the liver responds to the influx of nutrients following feeding by activating the MAP kinase signal (Figure 3A). However, Cavin1 deficiency specifically inhibited this feeding-induced MAP kinase in the liver without affecting the same signal cascade in skeletal muscle or heart (Figure 3A). The fixed MAP kinase signal in Cavin1-deficient mice suggested that some of the metabolic activities in the liver will also be fixed in certain state regardless of the nutritional status. The balance of glycogen synthase and phosphorylase regulates the liver glycogen level. Glycogen phosphorylase is the major enzyme involved in the mobilization of glycogen, catalyzing the removal of a glucose residue from the nonreducing end of a glycogen chain. We checked the activity of glycogen phosphorylase in the livers of Cavin1-/- mice at 9 h after birth and found that the activity of glycogen phosphorylase was significantly lower in Cavin1-/- livers (Figure 3B), which supported the idea that the absence of Cavin1 would inhibit the glycogen mobilization. Since glycogen mobilization is stimulated mainly by glucagon secreted by pancreatic islets, we considered the possibility that impaired pancreatic function contributed to the deficits in glycogen mobilization in Cavin1-/- pups. Neither α nor β cells expressed Cavin1 (Figure S3A, Supporting Information).
Figure 2. Cavin1 deficiency affected the storage and mobilization of glycogen in the liver. A,B) Periodic acid-Schiff (PAS) staining and quantitative analysis of hepatic glycogen in wild-type and Cavin1 deficient newborn pups at 24 h before birth and 9 h after birth. **p < 0.01; n = 3 mice each group. C) Blood glucose in adult wild-type or Cavin1-/- homozygous mice when fed or fasted. ns, not significant; n = 3–5 mice each group. D,E) PAS staining and quantitative analysis of hepatic glycogen in wild-type and Cavin1 deficient adult mice when fed and fasted. **p < 0.01; n = 3–5 mice each group.
Primary hepatocytes from Cavin1-deficient mice and treated with insulin and glucagon. We found that the hepatocytes from Cavin1+/− mice had no problem to store or mobilize glycogen upon the treatment of insulin or glucagon (Figure 3I,J). Thus, the lower rate of glucose uptake in Cavin1 null mice is because of insufficient amount of glucose in disse space, rather than the inability of the hepatocytes to transport glucose. In summary, our data demonstrated that the Cavin1 deficiency blocked the bilateral flow between liver glycogen and blood glucose and this led to abnormal storage and mobilization of glycogen in mouse liver and the eventual hypoglycemia in Cavin1+/− mice.

In order to understand how Cavin1 deficiency impacts liver function, we investigated in which cell populations the Cavin1 expresses. Immunostaining in liver samples revealed that Cavin1 predominantly expressed in the endothelial cells of sinusoids, portal venule, and central veins (Figure 4A and Figure S4A,B, Supporting Information). A recent publication provided single-cell RNA sequencing data on nonparenchymal cells isolated from the livers of wild-type C57BL/6 mice. We reanalyzed these data and clustered all cells by the Louvain algorithm. We then performed dimensionality reduction for presentation using t-distributed stochastic neighbor embedding and uniform manifold approximation and projection (Figure 4B). All clusters were marked by known markers of different cell populations existing in the liver. Our data showed that Cavin1 only expressed in cluster 0 and cluster 9, which were marked as endothelial cells and hepatic stellate cells, respectively (Figure 4C). These results implied that the loss of Cavin1 might affect the endothelial cells or hepatic stellate cells instead of hepatic parenchymal cells. Thus, we evaluated hepatic tissue morphology (Figure 4D), the expression of endothelial markers (CD36, VEGFR2, and Tie2) (Figure 4E,F), and calculated the vascular network distribution (Figure 4G). Taken together, our results showed that Cavin1 predominantly expressed in the endothelial cells, but there is no differences in the vascular network between wild-type and Cavin1-deficient mice (Figure 4A–G).

The permeability of the sinusoidal endothelial wall determined the amount of glucose, lipids, proteins, and other molecules brought in and out of the liver. The unchanged vascular network suggested that the permeability of the sinusoidal endothelial wall might be affected by Cavin1 deficiency. It was believed that the permeability of the sinusoidal endothelial wall is reg-

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**Figure 3.** Cavin1 deficiency impaired the metabolism of liver glycogen. A) Specific inhibition of MAP kinase signal in liver by Cavin1 deficiency. Tissues (liver, fat, heart, muscle, and lung) of normally fed wild-type (+/+ ) and Cavin1−/− mice (−/−) were obtained for protein analysis. Phosphorylated extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase kinase (MEK) and total ERK and MEK were analyzed by western blot. B,C) Glycogen phosphorylase activity in liver and serum glucagon level in Cavin1+/+ and Cavin1−/− pups at 9 h after birth. ** p < 0.01; n = 6–9 mice each group. D) Quantitative analysis of 3H-labeled glucose uptake to liver in Cavin1+/+ and Cavin1−/− pups. ** p < 0.01; n = 7–10 mice each group. E) Measurement of glycogen synthase activity in Cavin1+/+ and Cavin1−/− fetuses. ** p < 0.01; n = 9–10 mice each group. F) Western blot analysis for related glycogen synthesis enzymes including glycogen synthase (GS), phosphorylated glycogen synthase (pGS(S641)), glycogen synthase kinase 3 β (GSK3β), and its inactive form (pGSK3β(Ser9)) in Cavin1+/+ and Cavin1−/− fetuses. G) Analysis of Cavin1−/−/− mice. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as loading control. The ratio of the bands were quantified and normalized with GAPDH. The ratio between pGSK3β and GSK3β in Cavin1+/+ mice (marked as +/+ ) and Cavin1−/− mice (−/−) were calculated. The fold change between two groups was plotted. G) The expression and H) distribution of Glut1 and Glut2 in the livers of Cavin1-deficient mice (Figure 3G,H).

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In contrast, the livers of Cavin1−/− fetus had a slightly higher activity of glycogen synthase than the ones of the control fetus (Figure 3E). One explanation for this inconsistency is that the higher activity of glycogen synthase in the liver of Cavin1−/− fetus could be a mechanism for the liver to compensate for the shortage of glycogen due to the limited glucose uptake. The glucose in blood crosses the sinusoidal endothelial wall and enters the disse space where it is uptaken by hepatocytes with the help of glucose transporters. Western blot analysis revealed no change in the expression and distribution of glucokinase transporter (Glut1 and Glut2) in the liver of Cavin1-deficient mice (Figure 3G,H). In order to rule out the possibility that any defects in hepatocyte cause the abnormal glycogen metabolism, we isolated
Figure 4. Cavin1 deficiency did not affect liver vascular network. A) The expression of Cavin1 in endothelial cells but not in hepatocytes. Liver of wild-type mouse was stained for Cavin1 and CD31. B) Nonparenchymal cells from the livers were clustered into ten populations and each population was marked by known cell marker: Endo: Endothelial cells; TC: T cells; BC: B cells; Mac: macrophages; Chol: cholangiocytes; PlaB: plasma B cell; DC: dendritic cells, and SC: hepatic stellate cells. C) Cavin1 labeled cells were in cluster 0 and cluster 9, identified as endothelial cells and hepatic stellate cells, respectively. D) Hematoxylin-eosin staining to reveal the central veins/portal venule and sinusoids in Cavin1+/+ and Cavin1−/− liver. E,F) The expression and distribution of vascular markers: CD36, Tie2, and VEGFR2 in Cavin1+/+ and Cavin1−/− liver. GAPDH served as loading control. G) Quantitative analysis of blood vessels area fraction in Cavin1+/+ and Cavin1−/− liver. ns, not significant; n = 3 mice each group.
Figure 5. The neonatal death of Cavin1 null mice is independent of Caveolae or Caveolin1. A) The survival rate of newborn pups of Cavin1+/−, Cav1+/−, Cav1+/−::Cavin1−/− heterozygous parents. The numbers of pups in each group were listed in the figure. B) Periodic acid-Schiff staining for liver glycogen. Liver from normally fed adult wild-type or Caveolin1-deficient mice was stained for glycogen. Hepatic glycogen was calculated by the PAS positive area and relative hepatic glycogen was plotted. ns, not significant; n = 3 mice each group. C) Western blot analysis of Cavin1–4 and Caveolin1-3 expressed in Cavin1 or Caveolin1-deficient mice. Hsp90 served as loading control. The relative expression level of each protein was calculated based on western blot results. ns, not significant. All other comparisons between Cavin1−/− and wild-type (WT) or between Cav1+/- and WT groups reach significant.

ulated by different endothelial subcellular structures: caveolae, fenestrae, and transendothelial channels (TECs).[21] There is little knowledge of the function of TECs, which are thought to be fenestrae precursors.[22] Thus, we checked if the deficiency of Cavin1 impaired the endothelial permeability and caused the disorder of glycogen metabolism by affecting the formation of caveolae and/or fenestrae. Caveolae have two essential component proteins, Caveolin1 and Cavin1, and both proteins are equally necessary for caveolae formation. Lacking either one ablates caveolae completely.[8,23] We backcrossed Cav1+/− mice over 20 generations to pure C57BL/6J background and then crossed Cav1+/− to generate Caveolin1 null mice. Surprisingly, no neonatal death occurred in Cav1+/− newborn pups, suggesting that ablation of caveolae did not mimic the phenotype in Cavin1 null mice (Figure 5A). We also bred some Cavin1+/−::Cavin1−/− double knockout mice. These double knockout died at the neonatal stage, similar to the Cavin1−/− mice (Figure 5A). The ablation of caveolae in Cav1+/− did not alter the liver glycogen as in the Cavin1−/− mice (Figure 5B). These results indicated that the deficiency of Cavin1 caused neonatal death through a caveolae-independent mechanism. Although the depletion of caveolin1 dramatically decreased the expression level of Cavin1 and other members of the Cavin family in mouse liver (Figure 5C), it seems that the residual Cavin1 is enough for the survival of these Cav1 knockout mice.

Next, we tested if Cavin1 deficiency impaired endothelial permeability and glycogen metabolism by disrupting fenestrae formation. It has been reported that prominently increased number of fenestration in liver sinusoidal endothelial cells (LSECs) can promote glucose uptake in liver,[24] while a decreased number of fenestration of LSECs can significantly impair the absorption of glucose in the liver, thereby affecting hepatic glycogen synthesis.[25,26] First, we compared the morphology of fenestrae in the liver of wild-type and Cav1−/− mice by transmission electron microscope (TEM) and scanning electron microscope (SEM). Our results confirmed that the density of fenestrae in the liver of Cav1−/− mice was less than in wild-type mice (Figure 6A). The results from SEM examination on primary cultured LSECs also demonstrated that primary LSECs isolated from Cav1−/− mice contained fewer fenestrae, compared with that from wild-type mice (Figure 6B). Quantitatively, in Cav1−/− mice, the number of fenestrae in LSECs was reduced by half (Figure 6C). Combined, our data verified that the depletion of Cavin1 resulted in a decreased amount of fenestrae in the LSECs. Next, we tested if the reduction of fenestrae in LSECs would lead to glycogen disorder, mimicking the effect of Cavin1 deficiency. Here we used two known fenestrae inhibitors, histamine and nicotine[27,28] to treat adult wild-type C57BL/6J mice and measured how much residual glycogen was left after fasting for 6 h. Our results showed that the mice treated with the inhibitors had
Figure 6. Cavin1 deficiency dampened the fenestration in LSECs. A) Transmission (TEM) and scanning electron microscopy (SEM) showed fenestrae in the livers. Red arrows indicated the fenestrae; Hep, hepatocyte; L, lumen of the sinusoid. B) SEM showed fenestrae in primary cultured LSECs from Cavin1+/+ and Cavin1−/− mice. C) Quantitative analysis of fenestrae in the LSECs. ***p < 0.001; each dot stands for one SEM picture of LSECs; LSECs are from three to five mice each group. D,E) The hepatic glycogen content in adult wild-type mice at fed and 6 h after fasting. Treatments with D) histamine or E) nicotine inhibited glycogen mobilization upon fasting. **p < 0.01; n = 2–5 mice each group. F) The hepatic glycogen content in adult wild-type mice after 6 h of refeeding. Treatments with histamine or nicotine inhibited glycogen storage upon refeeding.

more leftover glycogen in their liver (Figure 6D,E), suggesting that the reduction of the fenestrae decreased the mobilization of liver glycogen to the bloodstream. Reversely, we found that histamine and nicotine also blocked glycogen formation during fed state (Figure 6F). In summary, our results indicated that the loss of Cavin1 resulted in fewer fenestrae in LSECs and consequently blocked bilateral flow between liver glycogen and blood glucose.

The fenestrae in LSECs are formed after cell differentiation.[17,18] Initially, the lining of a liver sinusoid is a continuous endothelial wall with an outer basement membrane. After differentiation the endothelial lining becomes fenestrated and the outside basement membrane disappears. The marker protein of CD31 expressed in undifferentiated LSECs also becomes the differentiated cell expressed CD4.[17,18] Cavin1 was expressed in LSECs since early developmental stage of LSECs (Figure S5A, Supporting Information). Cavin1 deficiency did not alter the endothelial nature of LSECs before birth (Figure 7A) or at weaning (Figure 7B). What was affected by Cavin1 deficiency was the differentiation of LSECs. In Cavin1-deficient mice the expression of undifferentiated LSEC marker CD31 was high and the expression of differentiated marker CD4 was low (Figure 7C). There were also more basement membrane
proteins, laminin β1 (LAMB1) and collagen α1 (IV) (COL4A1) (Figure 7D). The reduced fenestration plus incomplete loss of outside basement membrane indicated that the impairment of LSEC differentiation was due to the absence of Cavin1.

Fenestrae are sensitive to environmental conditions and change in number and diameter in response to external stimuli such as hormones, drugs, and toxins. The molecular mechanisms regulating their structure are not fully understood, but previous studies have shown that the Rho signaling pathway regulates fenestrae formation by promoting the dynamics of the actin cytoskeleton. Active Rho (guanosine triphosphate (GTP)–bound Rho) regulates Cofilin via Rho-associated protein kinase (ROCK) and LIM domain kinase (LIMK). Cofilin is an actin-binding protein and is essential for the rapid turnover of the actin filament. By inducing Cofilin phosphorylation, Rho abolishes the actin-binding activity of Cofilin, thereby en-
hancing the dynamics of actin filaments.\textsuperscript{[37]} In addition, it has been reported that Cavin4/MURC, one of the Cavin1 homologs, can activate RhoA-ROCK in cardiomyocytes.\textsuperscript{[38]} To test whether the knockout of Cavin1 affected the fenestrae through the RhoA-ROCK signal pathway in LSECs, we investigated the level of active RhoA and its downstream ROCK2-LIMK-Cofilin signaling pathway in our newborn wild-type and Cavin1 knockout mice. We used anti-RhoA-GTP antibodies to immunoprecipitate the active RhoA (RhoA-GTP) and found that the level of active RhoA was significantly lower in the liver of Cavin\textsuperscript{1+/−} mice when compared to wild-type mice (Figure 8A). We also assessed the amount of ROCK2 associated with active RhoA by immuno-blottting the anti-RhoA-GTP co-immunoprecipitated mixture with the ROCK2 antibody. Our data showed that the level of ROCK2 associated with active RhoA was lower in Cavin\textsuperscript{1+/−} mice (Figure 8A). Furthermore, our analysis revealed that the phosphorylated level of LIMK1 at Thr508 and Cofilin at Ser3 were also decreased in the liver of Cavin1-deficient mice, which is consistent with the lower activity of RhoA (Figure 8B). Subsequently, we evaluated the level of actin associated with Cofilin by co-immunoprecipitation with Cofilin antibody. Our results showed that the level of actin associated with Cofilin was increased in the liver of Cavin\textsuperscript{1+/−} mice, suggesting fewer dynamics of actin cytoskeleton than that in Cavin\textsuperscript{1+/−} mice (Figure 8C). In order to prove that the observed inhibition of RhoA-ROCK2-LIMK-Cofilin signaling pathway is not coming from other liver cell types, we performed similar experiments in an endothelial cell line, human umbilical vein endothelial cells (HUVEC). Using siRNAs, we successfully knocked down the expression of Cavin1 in HUVECs (Figure S6A, Supporting Information) and confirmed that depletion of Cavin1 inhibited RhoA-ROCK2-LIMK-Cofilin signaling pathway in HUVECs as well (Figure S6B–D, Supporting Information). Combined, our data demonstrated that the LSECs from Cavin\textsuperscript{1−/−} mice had inhibited RhoA-ROCK2-LIMK-Cofilin signaling pathway and impaired dynamics of cytoskeleton, which would eventually result in a reduction of fenestrae. Furthermore, it had been reported that latrunculin A could promote the dynamic cytoskeleton remodeling and lead to the formation of fenestrae in the LSECs.\textsuperscript{[19]} Thus, we tested if the treatment with latrunculin A would be able to rescue the reduction of fenestrae in the LSECs caused by Cavin1 deficiency. Indeed, the treatment of latrunculin A caused massive depolymerization of F-actin (Figure 8D) and induced substantial fenestration in the primary cultured LSECs from Cavin\textsuperscript{1−/−} mice (Figure 8E). We then went further to test if latrunculin A treatment would rescue Cavin\textsuperscript{1−/−} mice from neonatal death. Indeed, two injections of 1.25 mg kg\textsuperscript{−1} of latrunculin A, at 1 and 5 h after birth, rescued Cavin\textsuperscript{1−/−} mice (Figure 8F) and the rescued mice presented a healthy blood glucose level at 9 h after birth (Figure 8G). Collectively, our data suggested that the loss of Cavin1 impaired the dynamics of the actin cytoskeleton through downregulation of RhoA signaling pathway and resulted in a reduction of fenestrae in the LSECs.

In summary, our data demonstrated that the depletion of Cavin1 impaired the permeability of hepatic sinusoidal capillaries by decreasing the number of fenestrae in LSECs and affected the homeostasis of glycogen, which led to the neonatal death of Cavin\textsuperscript{1−/−} mice in C57BL/6 background. So far, there is no recorded neonatal death of CGL4 patients, but this may be because CGL4 patients are rare and often living in rural areas. One clinical report mentioned that a sister of their CGL4 patient died 6 h after birth,\textsuperscript{[40]} but her genotype and the cause of death were not recorded. Thus, it is not clear if her death was caused by a CAVIN1 mutation. Nevertheless, our results raised the concern that Cavin1 deficiency may cause neonatal mortality in CGL4 patients. It is worth pointing out that the neonatal death of Cavin1 null mice only occurs in certain genetic background, such as C57BL/6J. Once we cross these mice with Friend Virus B (FVB), the Cavin\textsuperscript{1−/−} mice with 50% FVB background can improve survival with mild neonatal hypoglycemia (Figure S7, Supporting Information). This points to the impact of genetic background on susceptibility to the disorder of glucose homeostasis.\textsuperscript{[41–43]} A more recent study on PV1, an essential protein for endothelial permeability, also showed that the deletion of PV1 is 100% lethal in pure C57BL/6J background but causes a much milder defect in ones with other backgrounds.\textsuperscript{[22]} Given the complexity of human genetic background, neonatal death may only occur in few CGL4 patients with a certain background. Nevertheless, our finding suggests close monitoring on glucose level for newborn CGL4 patients.

The roles of fenestration and permeability of LSECs in liver diseases have drawn more attention in recent years.\textsuperscript{[22,44]} The disorder of substance exchange between blood and hepatic parenchyma leads to the development of liver-related metabolic diseases.\textsuperscript{[17]} In fact, some previously reported phenotypes of Cavin\textsuperscript{1−/−} mice, such as glucose intolerance and hyperinsulinemia, are metabolically related to liver functions.\textsuperscript{[45]} The novel function of Cavin1 in regulating the fenestration of LSECs may partially contribute to the phenotypes in Cavin1 knockout mice. A mouse model with an endothelial specific knockout of Cavin1 is needed for further study on the roles of endothelial Cavin1 in liver diseases.

Fenestrae are dynamic structures. Their numbers and diameters are affected by physiological and pathological factors such as aging, nutritional status (feeding or fasting) or diseases (cirrhosis, stress-related disorders, cancer, etc.).\textsuperscript{[29,32,46]} but the molecular mechanisms regulating their structure remain largely elusive. A decade ago, LSECs fenestrations were thought to be sort of caveolae.\textsuperscript{[47]} However, Caveolin1 null mice have normal fenestrations,\textsuperscript{[48]} suggesting that normal structure of fenestrations in the LSECs is not dependent upon Caveolin1 or caveolae. Indeed, our results showed that neonatal Caveolin1 null mice had normal survival rates (Figure 5A), while both Cavin1 single knockout and Cavin1/Cav1 double knockout resulted in neonatal death. Taken together, we ruled out the possibility that Cavin1 regulates the fenestration of LSECs in a caveola-dependent manner. Instead, our data suggested that the loss of Cavin1 inhibited the differentiation of LSECs (Figure 7A–D) and impaired the dynamic of actin cytoskeleton through the downregulation of the RhoA signaling pathway (Figure 8A–E). The activity of RhoA is regulated by three kinds of regulators: guanine nucleotide exchange factors, GTPase-activating proteins, and Rho guanosine diphosphate dissociation inhibitors. Cavin1 has no typical motif as any of these three regulators. Thus, Cavin1 probably interacts with molecules that directly or indirectly regulate the activity of upstream regulators for RhoA, which subsequently promotes the dynamics of the actin skeleton. Our immunostaining results showed that Cavin1 is not colocalizing with F-actin (Figure S8A, Supporting Information). Instead, they
Figure 8. Cavin1 deficiency inhibited the dynamics of the actin cytoskeleton and reduced the fenestrae in LSECs. A) Western blot analysis of active Rho A and its binding with ROCK2 in the liver of Cavin1+/+ and Cavin1−/− pups. Input Rho A, input ROCK2 and HSP90 are shown as control. B) Western blot analysis of phosphorylated LIMK1 at Thr508 and phosphorylated cofilin at Ser3 in the liver lysate of Cavin1+/+ and Cavin1−/− pups. C) Western blot analysis of cofilin co-immunoprecipitation with actin in the liver lysate of Cavin1+/+ and Cavin1−/− pups. D) Immunofluorescent staining of F-actin in LSECs treated with or without latrunculin A (1 µg mL−1). E) Scanning electron microscopy analysis of fenestrae in the LSECs with or without latrunculin A treatment. Red arrows indicate fenestrae in the LSECs. Zoom shows the details of the enlarged area (black squares). F) The survival rate of Cavin1−/− pups when treated with Latrunculin A (LA). * p < 0.05; n = 6–7 mice per group. G) The blood glucose in LA treated mice. ns, not significant; n = 4 mice each group.
are locating adjacent to or right in the empty rings of the F-actin (Figure S8A, Supporting Information), suggesting that the Cavin1 may cause the depolymerization of F-actin through an effector locally. One of the future directions of this study will be to find the effector of Cavin1 and we will test if Cavin1-associated proteins are such potential targets. The analysis on single-cell RNA sequencing data suggested that Cavin1 also expresses in hepatic stellate cells. How does the Cavin1 deficiency in hepatic stellate cells contribute to the abnormalities of Cavin1−/− mice will be an interesting question to ask. It is also intriguing to know how Cavin1 is regulated according to extracellular stimuli. A recent study showed that, upon insulin stimulation, Cavin1 is acutely translocated to focal complex compartments and regulates focal complex formation in adipocytes.[23] Whether similar mechanism also exists in LSECs will need further studies.

**Experimental Section**

Materials and methods are described in detail in the Supporting Information. All experiments were performed with approval under the ethical guidelines of the Institute of Biochemistry and Cell Biology.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

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[1] N. Patni, A. Garg, Nat. Rev. Endocrinol. 2015, 11, 522.
[2] G. Nilay, T. Kutlu, G. T. Tekant, A. G. Eroglu, N. C. Ustundag, B. Oz- turk, H. Onay, B. Tuysuz, Eur. J. Med. Genet. 2020, 63, 103819.
[3] M. M. Hill, M. Bastiani, R. Lutterforst, M. Kirkham, A. Kirkham, S. J. Nixon, P. Walser, D. Abankwa, V. M. Oorschot, S. Martin, J. F. Hancock, R. G. Parton, Cell 2008, 132, 113.
[4] L. Liu, Biochem. Soc. Trans. 2020, 48, 147.
[5] N. Briand, I. Dugail, S. Le Lay, Biochimie 2011, 93, 71.
[6] R. G. Parton, M. A. del Pozo, Nat. Rev. Mol. Cell Biol. 2013, 14, 98.
[7] R. Gupta, C. Toufaily, B. Annabi, Biochimie 2014, 107, 188.
[8] L. Liu, D. Brown, M. McKee, N. K. Lebrasseur, D. Yang, K. H. Albrecht, K. Ravid, P. F. Pilch, Cell Metab. 2008, 8, 310.
[9] M. S. Karbalaei, C. Rippe, S. Albinsson, M. Ekman, A. Mansten, B. Uvelius, K. Sward, Eur. J. Pharmacol. 2012, 689, 179.
[10] C. G. Hansen, E. Shvets, G. Howard, K. Riento, B. J. Nichols, Nat. Commun. 2013, 4, 1831.
[11] T. Taniguchi, N. Maruyama, O. Ogata, T. Kasahara, N. Nakanishi, K. Miyagawa, D. Naito, T. Hamaoka, M. Nishi, S. Matoba, T. Ueyama, PLoS One 2016, 11, e0162513.
[12] M. H. Lees, J. Pediatr. 1970, 77, 484.
[13] B. Turgeon, S. Meloche, Physiol. Rev. 2009, 89, 1.
[14] J. Girard, P. Ferre, J. P. Pegorier, P. H. Duee, Physiol. Rev. 1992, 72, 507.
[15] N. D. Wang, M. J. Finegold, A. Bradley, C. N. Ou, S. V. Abdelsayed, M. D. Wilde, L. R. Taylor, D. R. Wilson, G. J. Darlington, Science 1995, 269, 1108.
[16] H. S. Han, G. Kang, J. S. Kim, B. H. Choi, S. H. Koo, Exp. Mol. Med. 2016, 48, e218.
[17] J. Poisson, S. Lemoine, C. Boulanger, F. Durand, R. Moreau, D. Valla, P. E. Rautou, J. Hepatol. 2017, 66, 212.
[18] K. K. Sorensen, J. Simon-Santamaria, R. S. McCuskey, B. Smedsrod, Compr. Physiol. 2015, 5, 1751.
[19] X. Xiong, H. Kuang, S. Ansari, T. Liu, J. Gong, S. Wang, X. Y. Zhao, Y. Ji, C. Li, L. Guo, L. Zhou, Z. Chen, P. Leon-Mimila, M. T. Chung, K. Kurabayashi, J. Opp, F. Campos-Perez, H. Villamil-Ramirez, S. Canizales-Quinteros, R. Lyons, C. N. Lumeng, B. Zhou, L. Qi, A. Huertas-Vazquez, A. J. Luis, X. Z. Xu, S. Li, Y. Yu, J. Z. Li, J. D. Lin, Mol. Cell 2019, 75, 644.
[20] K. Auvinen, E. Lokka, E. Mokkala, N. Jappinen, S. Tystjarvi, H. Saine, M. Peurla, S. Shetty, K. Elima, P. Rantakari, M. Salmi, Sci. Rep. 2019, 9, 15698.
[21] E. Tkachenko, D. Tse, O. Sideleva, S. J. Deharvengt, M. R. Luciano, Y. Xu, C. L. McGarry, J. Chidlow, P. F. Pilch, W. C. Sessa, D. K. Toomre, R. V. Stan, PLoS One 2012, 7, e32655.
[22] R. V. Stan, D. Tse, S. J. Deharvengt, N. C. Smits, Y. Xu, M. R. Luciano, C. L. McGarry, M. Buitendijk, K. V. Nemani, R. Elgueta, T. Kobayashi, S. L. Shipman, K. L. Moodie, C. P. Daghlian, P. A. Ernst, H. K. Lee, A. A. Suriawinata, A. R. Schned, D. S. Longnecker, S. N. Fiering, R. J. Noelle, B. Gimi, N. W. Shworak, C. Carriere, Dev. Cell 2012, 23, 1203.
[23] M. Drab, P. Verkade, M. Elger, M. Kasper, M. Lohn, B. Lauterbach, J. Menne, C. Lindscha, F. Mende, F. C. Luft, A. Schedl, H. Hailer, T. V. Kurzchalia, Science 2001, 293, 2449.
[24] M. Mohamad, S. J. Mitchell, L. E. Wu, M. Y. White, S. J. Cordwell, J. Mach, S. M. Solon-Biet, D. Boyer, D. Nines, A. Das, S. Y. Catherine Li, A. Warren, S. N. Hilmer, R. Fraser, D. A. Sinclair, S. J. Simpson, R. de Cabo, D. G. Le Couteur, V. C. Cogger, Aging Cell 2016, 15, 706.
[25] S. M. Raines, O. C. Richards, L. R. Schneider, K. L. Schueler, M. E. Rabaglia, A. T. Oler, D. S. Stapleton, G. Genove, J. A. Dawson, C. Betscholtz, A. D. Attie, Am. J. Physiol.: Endocrinol. Metab. 2011, 301, E517.
[26] L. Herrnberger, R. Hennig, W. Kremer, C. Kellerbrand, A. Goepferich, H. R. Kalbitzer, E. R. Tamm, PLoS One 2014, 9, e115005.
[27] F. Braet, E. Wisse, *Comp. Hepatol.* 2002, 1, 1.

[28] J. N. O’Reilly, V. C. Cogger, D. G. Le Couteur, *J. Electron Microsc.* 2010, 59, 65.

[29] F. Braet, *Liver Int.* 2004, 24, 532.

[30] F. Braet, M. Shleper, M. Paizi, S. Brodsky, N. Kopeiko, N. Resnick, G. Spira, *Comp. Hepatol.* 2004, 3, 7.

[31] H. Yokomori, K. Yoshimura, S. Funakoshi, T. Nagai, K. Fujimaki, M. Nomura, H. Ishii, M. Oda, *Lab. Invest.* 2004, 84, 857.

[32] H. Yokomori, *Med. Mol. Morphol.* 2008, 41, 1.

[33] H. Yokomori, K. Yoshimura, T. Nagai, K. Fujimaki, M. Nomura, T. Hibi, H. Ishii, M. Oda, *Hepatol. Res.* 2004, 30, 169.

[34] C. Xu, X. Wu, B. K. Hack, L. Bao, P. N. Cunningham, *Physiol. Rep.* 2015, 3.

[35] S. T. Sit, E. Manser, *J. Cell Sci.* 2011, 124, 679.

[36] P. Lappalainen, D. G. Drubin, *Nature* 1997, 388, 78.

[37] J. R. Bamburg, A. McGough, S. Ono, *Trends Cell Biol.* 1999, 9, 364.

[38] T. Ogata, T. Ueyama, K. Isodono, M. Tagawa, N. Takehara, T. Kawashima, K. Harada, T. Takahashi, T. Shioi, H. Matsubara, H. Oh, *Mol. Cell. Biol.* 2008, 28, 3424.

[39] F. Braet, R. De Zanger, D. Jans, I. Spector, E. Wisse, *Hepatology* 1996, 24, 627.

[40] A. Ardissone, C. Bragato, L. Caffi, F. Blasevich, S. Maestrini, M. L. Bianchi, L. Morandi, I. Moroni, M. Mora, *BMC Med. Genet.* 2013, 14, 89.

[41] E. H. Leiter, D. L. Coleman, K. P. Hummel, *Diabetes* 1981, 30, 1029.

[42] S. Kooptiwut, S. Zraika, A. W. Thorburn, M. E. Dunlop, R. Darwiche, T. W. Kay, J. Proietto, S. Andrikopoulos, *Endocrinology* 2002, 143, 2085.

[43] R. N. Kulkarni, K. Almind, H. J. Goren, J. N. Winnay, K. Ueki, T. Okada, C. R. Kahn, *Diabetes* 2003, 52, 1528.

[44] A. Hammoutene, P. E. Rautou, *J. Hepatol.* 2019, 70, 1278.

[45] H. Wang, P. F. Pilch, L. Liu, *J. Biol. Chem.* 2019, 294, 10544.

[46] V. C. Cogger, M. Mohamad, S. M. Solon-Biet, A. M. Senior, A. Warren, J. N. O’Reilly, B. T. Tung, D. Svishtounov, A. C. McMahon, R. Fraser, D. Raubenheimer, A. J. Holmes, S. J. Simpson, D. G. Le Couteur, *Am J. Physiol.: Heart Circ. Physiol.* 2016, 310, H1064.

[47] H. Yokomori, M. Oda, M. Ogi, Y. Kamegaya, N. Tsukada, H. Ishii, *Liver* 2001, 21, 198.

[48] A. Warren, V. C. Cogger, I. M. Arias, R. S. McCuskey, D. G. Le Couteur, *Microcirculation* 2010, 17, 32.