

Liver specific Prox1 inactivation causes hepatic injury and glucose intolerance in mice

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Abstract

Previous reports have revealed that Prospero-related homeobox 1 (Prox1) is required for the migration and differentiation of hepatoblasts during embryonic liver formation. However, the role of Prox1 in adults remains to be elucidated. We created liver-specific Prox1 knockout mice to verify the role of Prox1 in adult hepatocytes. The mutant mice exhibited hepatic injury and non-obese, insulin-resistant diabetic phenotype *in vivo*. Hepatocyte injury was observed predominantly in the perivenous region and was characterized by the formation of vacuoles and emergence of round-shaped mitochondria, suggesting the effect of Prox1 on the maintenance of adult hepatocyte is region-dependent. Furthermore, glycolysis was suppressed, and both oxidative phosphorylation (OXPHOS) and autophagy were upregulated, indicating the role of Prox1 in regulating energy homeostasis in hepatocytes.

Keywords:

Prospero-related homeobox 1, glycolysis, type 2 diabetes

Abbreviations:

ALT, alanine transaminase; AST, aspartate transaminase; CK19, cytokeratin 19; D- or T-Bil, direct or total bilirubin; ECAR, extracellular acidification rate; GCK, glucokinase; G6Pase, glucose 6-phosphatase; IPGTT, intraperitoneal glucose tolerance test; LC3B, light chain 3B; LDH, lactate dehydrogenase; OCR, oxygen consumption

rate; OXPHOS, oxidative phosphorylation; PEPCK, phosphoenolpyruvate carboxykinase; PGC1a, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Prox1, Prospero-related homeobox 1; Prox1cKO mice, *Albumin-Cre;Prox1^{loxP/loxP}* mice; ROS, reactive oxygen species; SNP, single-nucleotide polymorphism

1. Introduction

Type 2 Diabetes is a disease caused by the insulin resistance of peripheral tissues/organs such as fat, muscle and liver. Recent advances in our understanding of the genetic susceptibility to type 2 diabetes include single nucleotide polymorphisms (SNPs) of several genes. Prox1 plays pivotal roles in the embryonic formation of several organs and tissues including liver, pancreas, eye, lymphatic vessel, nerve and cardiac muscle in mice [1-4] and a meta-analysis of a genome-wide association study revealed that a rs340874 SNP in the PROX1 gene is associated with type 2 diabetes [5]. Another report showed that people who carry this variance are more glucose intolerant and have more visceral fat than people without it [6]. Harvey et al. reported an animal model in which Prox1 heterozygous adult mice become obese and had higher serum insulin levels and hepatic lipid accumulation [7]. While the same group demonstrated that pancreas-specific Prox1 inactivation did not cause the diabetic phenotype in mice [8], obesity is likely the main mechanism of insulin resistance in both Prox1 heterozygous mice and patients who carry the rs340874 SNP in PROX1. Indeed, Prox1 is expressed in adipose tissues in human, with expression higher in omental adipose tissue than in subcutaneous adipose tissue [9], however, the involvement of hepatic Prox1 should not be neglected, since liver is the largest target organ of insulin effect.

The role of Prox1 in liver has been extensively analyzed during embryogenesis. During liver organogenesis, Prox1 is first expressed at embryonic day 8.5 (E8.5) in the endodermal cells of mice [10], and its global inactivation impairs the

migration of hepatoblasts to cause hypomorphic liver formation [11]. On the other hand, there is only limited information on the role of Prox1 in adult liver. Several *in vitro* experiments showed that Prox1 negatively regulates mitochondrial function through its physical association with peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1a) [12] and that Prox1 downregulation by siRNA upregulates phosphoenolpyruvate carboxykinase (PEPCK) [13], indicating its role in controlling the energy metabolism of hepatocytes. Despite these *in vitro* studies, the involvement of hepatic Prox1 in the pathogenesis of type 2 diabetes has not been clarified *in vivo*.

In this study, we used *Albumin-Cre* mice to inactivate Prox1 in a liver-specific manner and analyzed the liver phenotype and glucose homeostasis in the adult stage. As expected, the mutant mice exhibited insulin-resistant diabetic phenotype *in vivo* without obesity. Blood examination showed hepatic dysfunction characterized by elevated transaminase, bilirubin and lower albumin. Histologically, hepatocyte injury was characterized by the formation of vacuoles and the emergence of round-shaped mitochondria. Interestingly, the degree of hepatocyte injury was more severe in the perivenous region than periportal region, suggesting a region-specific dependency on Prox1 effects in adult liver. As for the energy metabolism in hepatocytes, glycolysis was suppressed and autophagy was stimulated, suggesting energy starvation in the perivenous region. We also provide evidence that upregulated oxidative phosphorylation in mitochondria and excess ROS generation resulted in hepatocyte injury and histological disorganization in the perivenous region. These results indicate a pivotal role of Prox1 in maintaining the energy homeostasis of adult hepatocytes and its

involvement in the pathogenesis of type 2 diabetes.

2. Materials and Methods

2.1. Mice

Prox1 floxed mice were generated by Unitech, Co. (Kashiwa, Japan), as reported previously. Mutant mice were produced on a C57BL/6J background. A neo-loxP cassette carrying the phosphoglycerate kinase promoter-driven neomycin-resistance gene flanked by FRT sequences was inserted into intron 2 of the Prox1 gene. A 3222-bp fragment containing exon 2 was replaced with a fragment carrying the corresponding sequence and a loxP sequence. The founder mouse (+/Flox [neo+]) was crossbred with FLPe transgenic mice to produce Prox1 floxed mice without neo cassette (+/Flox [neo-]). Deletion of this cassette was confirmed by PCR. To create liver-specific Prox1 knockout mice, we crossbred Prox1 floxed mice with Albumin-Cre-Tg mice (B6. Cg-Tg(Alb-Cre)21Mgn/J; The Jackson Laboratory). To verify the leaking effect, we crossbred *Albumin-Cre* mice and *Alubumin-Cre;Prox1^{loxP/loxP}* mice with *Rosa26R* mice [14] and performed X-gal staining. We analyzed *Alubumin-Cre;Prox1^{loxP/loxP}* (Prox1cKO) male mice from postnatal day 42 (P42) to P49. *Albumin-Cre* mice were analyzed as control mice. Samples were collected at around noon to exclude the influence of circadian rhythms. All animal experiments were performed in accordance with the Kyoto University guidelines for animal experiments and approved by the animal research committee of Kyoto university.

2.2. Genotyping

Genomic DNA from the tail tips of the mice was genotyped by PCR with the primer sets (*Prox1* forward) 5'-CTAGGTCTCCCAAGATACTCAGATG-3' and (*Prox1* reverse) 5'-TGAGATTAAAGAGAGAAGGCAAGCA-3'. The product sizes were 534 bp and 753 bp from WT and KO alleles, respectively.

2.3. Tissue preparation

Tissue preparation was performed as previously described with some modification [15]. Briefly, the fixative solution (PBS containing 4% PFA; for X-gal staining, 2% glutaraldehyde was further added) was perfused when the specimens were prepared for X-gal staining, hematoxylin and eosin staining, PAS staining or immunofluorescence. For the immunohistochemistry of 8-OHdG, fresh liver was cut into 3-mm thick pieces without perfusion and immersed in Bouin solution (Wako) overnight. Finally, the specimens were embedded in paraffin.

2.4. Immunohistochemistry and immunofluorescence

Paraffin sections were cut into 3- μ m thick slices. The immunohistochemistry of 8-OHdG was performed as previously described with some modification [16]. After deparaffinization and hydration, the antigen retrieval procedure was performed with Target Retrieval Solution pH 6.0 (Dako) in accordance with the manufacturer's instructions. Peroxidase blocking was done with 1% peroxidase diluted with methanol for 30 min. The sections were incubated for 30 min at room temperature with Protein

block (Dako), then incubated with primary antibody overnight at 4 °C. Sections were treated according to the protocol of the VECTASTAIN ABC kit (Vector Laboratories) and DAB Peroxidase Substrate (Vector Laboratories). Immunofluorescence and X-gal staining was performed as previously reported [15,17]. After the antigen retrieval procedure, the sections were incubated overnight at 4 °C with primary antibodies (Supplementary Table 1). The sections were then washed in PBS and incubated for 60 min with secondary antibodies (Supplementary Table 2). For the immunofluorescence of light chain 3B (LC3B), fresh frozen liver was cut into 6-μm thick slices and immersed in ice-cold 100% methanol for 10 min. The process after the antigen retrieval procedure was the same as the other immunofluorescence. Images were visualized using HS All-in-one Fluorescence Microscopy BZ-9000E (Keyence).

2.5. Electron microscopy

The fixation of liver samples was performed as previously reported with some modification [18]. Briefly, we used PBS containing 4% PFA and 2% glutaraldehyde as the fixative solution. Sections were observed by transmission electron microscopy (Hitachi H-7650).

2.6. RNA isolation and RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed using ReverTra Ace qPCR RT Master Mix (Toyobo). Quantitative RT-PCR was performed with TaqMan. The mouse primer and probe

sequences are listed in Supplementary Table 3 [19,20]. Independent experiments were performed three times and confirmed. Data were normalized in relation to the expression of beta-2 microglobulin. The mouse beta-2 microglobulin, human Prox1 and human beta-2 microglobulin probes used were TaqMan Gene Expression Assay no. Mm00437762, Hs00896294 and Hs00984230, respectively (Applied Biosystems). The analysis was performed using a standard curve (mouse primers) and comparative CT methods (primers for human).

2.7. Western blot

Liver tissues were lysed in RIPA buffer (Wako) in the presence of protease inhibitor (Roche). Obtained samples were electrophoresed in 10% SDS-PAGE gels and transferred to PVDF membrane (Millipore). Membranes were blocked with 5% skim milk in TBS-T and incubated with anti-Prox1 antibody (Proteintech 1:500), anti-LC3B antibody (CST 1:1000) or anti- β -actin antibody (Sigma 1:5000) overnight at 4 °C. After washing, the membranes were reacted with HRP-linked secondary antibodies (GE Healthcare) at 1:5000 dilution. Chemiluminescence was detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare) and visualized with ImageQuant LAS-4000 (GE Healthcare). The intensity of the bands was quantified with imaging analysis software (Image J) using β -actin as an internal control.

2.8. Blood examination

Blood was centrifuged, and serum was submitted to SRL Inc., Japan for

analysis.

2.9. Intraperitoneal glucose tolerance test and insulin ELISA

IPGTT and insulin ELISA were performed as previously described [21].

2.10. siRNA and Extracellular Flux Analysis

Knockdown experiments were performed as previously described with some modification [12]. We used the Hep3B cell line cultured in DMEM (Sigma) supplemented with 10% FBS and penicillin/streptomycin. Hep3B cells were transfected shortly after seeding with either Stealth RNAi[®] Negative Control Duplex (Invitrogen) or ON-TARGETplus Human PROX1 siRNA - SMARTpool (Dharmacon) using HiPerfect reagent (QIAGEN). 48 h after transfection, the cells were treated with Accutase (ICT), counted and seeded at 20000 cells/well in XFe96-well plates (Seahorse Bioscience). 24 h after re-seeding, the plates were washed and incubated for 1 h in a 37 °C incubator under air in 175 ml assay medium (Seahorse Bioscience) supplemented with 2 mM Glutamine and pH adjusted to 7.4. We used Glycolysis Stress Test Kit (Seahorse Bioscience) with glucose (final concentration 10 mM), oligomycin (final concentration 1 mM) and 2-Deoxy-D-glucose (final concentration 50 mM) added sequentially, and the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) at each time point were averaged from 6-10 wells. Experiments were performed in triplicate.

2.11. Statistical analysis

All indices were analyzed using an unpaired Student's t test (two-tailed). $P < 0.05$ was considered significant.

3. Results

3.1. Creation of liver-specific Prox1 knockout mice

To make liver-specific Prox1 knockout mice, we created Prox1-floxed mice, in which exon 2 is flanked by the loxP sequence (Figure 1A), and crossbred the mice with *Albumin-Cre* transgenic mice. The expression level of *Prox1* mRNA in the liver showed no significant difference among *Albumin-Cre* mice (control mice), Prox1 floxed mice and wild type mice (Figure 1D), and we observed no abnormal phenotype in these mice. Non-specific Cre recombination was not detected in other tissues/organs including pancreas, skeletal muscles, subcutaneous adipose tissues and omental adipose tissues, as judged by lineage tracing experiments in mice crossed with *Rosa26R* mice, confirming the specificity of *Albumin-Cre* (Supplementary Figure 1). Consistent with a previous report that used the same *Albumin-Cre* and a different Prox1-floxed strain [22], qPCR analyses showed 90% reduction of hepatic Prox1 expression without apparent histological phenotype at embryonic day 18.5 (E18.5) in our Prox1cKO mice (data not shown). At P42, Prox1 expression was detected in a very few hepatocytes and cholangiocytes in Prox1cKO mice (Figure 1B right panel). Quantitatively, its protein and mRNA levels were approximately 16% and 15% those of control mice, respectively (Figure 1C and 1D).

3.2. Non-obese glucose intolerance due to the hepatic insulin resistance in Prox1cKO mice

Mutant mice had normal appearance, body weight consistent with control mice during postnatal stages, and no abnormal subcutaneous or visceral fat accumulation (Figure 2A and 2B). Furthermore, their blood glucose level was comparable to control mice both in the feeding period and after starvation (Table 1). However, the intraperitoneal glucose tolerance test (IPGTT) revealed that the mutant mice are glucose intolerant (Figure 2C). In addition, serum insulin levels at 15 min after glucose challenge were significantly higher (Figure 2D), suggesting peripheral insulin resistance in Prox1cKO mice.

3.3. Blood examination indicated hepatocyte injury and impaired liver function in Prox1cKO mice

Blood examination revealed Prox1cKO mice exhibited hepatic dysfunction (Figure 3). While lactate dehydrogenase (LDH) did not show significantly different levels, aspartate transaminase (AST) and alanine transaminase (ALT) were significantly higher in Prox1cKO mice, indicating hepatocyte injury. Moreover, total bilirubin (T-Bil) and direct bilirubin (D-Bil) were elevated and albumin was decreased, suggesting impaired liver function.

3.4. Tissue structural impairment and ectopically hyper-activated mitochondria in perivenous region

We next performed histological analysis. While the lining of the hepatocytes in the periportal region was normal, it became irregular in the perivenous region (Figure

4A). High magnification revealed that the perivenous hepatocytes contained abundant vacuoles in the cytoplasm, but these were not lipid droplets, as judged by negative staining with Oil-red-O (data not shown). Immunostaining for laminin and *in vivo* angiographic imaging using dextran-conjugated fluorescence revealed irregular and distorted structures of the sinusoids and blood vessels predominantly in the perivenous region (Supplementary Figure 2). We speculate that the irregular structures of the hepatocytes and vessels were secondary effects caused by the developing hepatocyte injury in the perivenous region, since very few vacuoles were detected and no apparent structural impairment was observed at younger stages (around P10; data not shown).

We noticed phenotypic changes in the mitochondria by electron microscopy analysis; in control mice, mitochondria of the periportal region were round-shaped with high electron density but elongated in the perivenous region, while in *Prox1cKO* mice the mitochondria were round-shaped and had higher electron density in both regions (Figure 4B). That is, in *Prox1cKO* mice, mitochondria in the perivenous region took a phenotype consistent of periportal mitochondria. Considering that OXPHOS is more active in the periportal region of normal liver [23-25], the phenotypic impairment of mitochondria in the perivenous region suggested ectopically hyper-activated OXPHOS that presumably lead to hepatocyte injury. Consistently, we observed 8-hydroxy-2'-deoxyguanosine (8-OHdG) positive hepatocytes in this region (Figure 4C), indicating DNA damage due to excess ROS generation.

3.5. Activated autophagy in the perivenous region of Prox1cKO liver

Our electron microscopy analysis identified autophagosomes and autolysosomes, with the former being confirmed by the positive immunostaining of LC3B (Figure 5A and 5B). The ratio of LC3B-II / LC3B-I expression was two-fold that of control mice, indicating up-regulated autophagy in Prox1cKO liver (Figure 5C).

3.6. Reduced glycolysis activity by Prox1 inactivation

Ectopically hyper-activated mitochondria and upregulated autophagy in the perivenous region led us to hypothesize that hepatocytes were unable to meet the energy demand in mutant liver. This notion prompted us to analyze the expression levels of glucose metabolism enzymes in the liver. We found that glucokinase (*Gck*) was decreased and *Pepck* and glucose 6-phosphatase (*G6Pase*) were increased, suggesting reduced glycolysis activity and increased gluconeogenic activity in mutant hepatocytes (Figure 6A). Additionally, the amount of hepatic glycogen storage was decreased in mutant mice (Figure 6B). These results are consistent with previous studies, which reported that both liver-specific GCK knockout mice and liver-specific G6Pase overexpressing mice had less hepatic glycogen storage [26,27].

We next carried out siRNA-based knockdown experiments using the Hep3B cell line to evaluate the role of Prox1 in the energy metabolism of hepatocytes. We confirmed the knockdown efficiency by western blot and qRT-PCR analysis (Figure 6C and 6D). Extracellular flux analysis revealed a significantly declined ECAR, indicating decreased glycolysis in si*PROX1*-treated cells (Figure 6E). In addition, OCR was increased upon glucose stimulation, suggesting stimulated OXPHOS activity.

4. Discussion

It is well known that obesity and hepatic injury are the main cause of human type 2 diabetes. In this report, we demonstrated that liver-specific Prox1 knockout mice exhibited glucose intolerance without obesity, and that mutant mice showed hepatic injury associated with hyperactivated OXPHOS and excess ROS production. Furthermore, we provide evidence that *Gck* was downregulated and *Pepck* and *G6Pase* were upregulated in Prox1cKO hepatocytes, indicating that Prox1 controls the balance of glycolysis and gluconeogenesis activities in hepatocytes. Previously, Harvey et al. reported that adult Prox1 heterozygous mice exhibit a glucose intolerance that is associated with obesity and hepatic lipid accumulation [7]. They proposed a model where the impaired formation of lymphatic vessels caused obesity that induced an elevation of blood insulin and leptin levels leading to glucose intolerance. Since lipid metabolism was normal in the liver, they speculated that hepatic lipid accumulation was secondary to the obesity in Prox1 heterozygous mice. While the involvement of hepatic lipid accumulation in glucose intolerance was not stressed in the Prox1 heterozygous model, recent reports have revealed a connection between obesity-induced, progressive hepatic lipid accumulation and type 2 diabetes in humans where mitochondrial function played an important role. Liver biopsies from obese diabetic patients with non-alcoholic fatty liver (NAFLD) or steatohepatitis (NASH) showed progressive lipid accumulation causes mitochondrial stress [28,29]. In NAFLD, the hepatic lipid accumulation was relatively mild such that hepatic mitochondria could adapt by increasing maximal

respiration rates. However, the hepatic lipid accumulation was higher in NASH such that the hepatic adaptation collapsed and excess ROS production and hepatocyte injury occurred. Similarly, in our liver-specific *Prox1* knockout mice, abnormally activated OXPHOS caused excess ROS and hepatocyte injury. However, our liver-specific *Prox1* knockout mice did not show obesity or hepatic fatty change, suggesting that the underlying mechanism for insulin resistance differs from that in heterozygous mutant mice. Postic et al. reported that liver-specific GCK knockout mice show hyperinsulinemic hyperglycemia [30]. In addition, liver-specific PEPCK-overexpressing transgenic mice are glucose intolerant with elevated serum insulin levels [31]. While it is unclear whether hepatic injury was observed in these mice, these findings are consistent with our *Prox1*cKO hepatocytes. Overall, these results indicate that an unbalanced glucose metabolism in the liver could contribute to the insulin resistance.

*Prox1*cKO mice showed liver dysfunction and abrogated histological appearances predominantly in the perivenous region. The hepatocyte lining is divided into three zones: periportal hepatocytes (zone 1), intermittent cells (zone 2) and perivenous hepatocytes (zone 3), and previous reports have shown zone-dependent hepatic function where cells preserve an optimal balance of the glycolytic pathway and OXPHOS to meet energy demands [23,24]. In periportal hepatocytes, OXPHOS plays a larger role in the cellular energy supply, and these cells release glucose into the blood stream by gluconeogenesis upon starvation. In contrast, perivenous hepatocytes, which have lower oxygen concentration than periportal hepatocytes, express more *Gck* to conduct more glycolysis [32]. Though immunohistochemical analyses showed no

apparent difference in the amount of expressed Prox1 among zones 1-3 (data not shown), the reduced glycolysis may explain well the zone 3-dominant hepatocyte injury in Prox1cKO mice. Consistently, qRT-PCR analysis showed less *Gck* expression in the mutant liver and Prox1 knockdown experiments *in vitro* demonstrated attenuated ECAR, demonstrating Prox1 inactivation suppressed glycolysis activity both *in vivo* and *in vitro*. Hepatocytes in zone 3, whose energy supply mainly depends on glycolysis, would be subjected to energy starvation by the reduced glycolysis activity in Prox1cKO mice. Along this scenario, the cells may compensate by stimulating autophagy and activating OXPHOS to meet their energy demand, but this response also resulted in excess ROS production, leading to hepatocyte injury.

Authors contributions

Y.K. and T.G. designed the study, analyzed the data and prepared the manuscript. T.G. performed the experiments. H.K and Y.S generated the mice. A.E., K.F., M.H., S.H., Y.A., K.T., M.S., K.H and T.M provided technical support and discussion. S.U. and Y.K. supervised the project.

Competing financial interests: The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1: Generation of liver-specific Prox1 knockout mice.

(A) Construction of the targeting vector and transgene. (B) Immunofluorescence of Prox1 and CK19. Arrows and arrowheads indicate a few Prox1 positive cholangiocytes and Prox1 positive hepatocytes that escaped *Albumin-Cre*-mediated recombination in Prox1cKO mice, respectively. (C) The relative PROX1 protein level in the liver of Prox1cKO mice was about 16% that of control mice (Prox1cKO mice, n=4; control mice, n=4). (D) The relative mRNA expression level of *Prox1* in the liver of Prox1cKO mice was about 15% that of control mice. The relative mRNA expression level of *Prox1* in the liver showed no significant difference in control mice, Prox1 floxed mice and wild type mice (Prox1cKO mice, n=4; control mice, n=4; Prox1 floxed mice, n=4; wild type mice, n=4).

Scale bars = 20 μ m. *P < 0.05. **P < 0.01. Bars represent the mean value $\pm$ SE.

Figure 2: Liver-specific Prox1 inactivation caused non-obese glucose intolerance due to hepatic insulin resistance.

(A) Body weight of Prox1cKO mice (red, n=4) and control mice (blue, n=4). (B) The appearance of visceral fat in Prox1cKO mice and control mice was almost the same. (C) Intraperitoneal glucose tolerance test (IPGTT) of Prox1cKO mice (red, n=6) and control mice (blue, n=6). (D) Plasma insulin concentrations at 15 minutes after glucose

challenge in Prox1cKO mice (red, n=6) and control mice (blue, n=6).

*P < 0.05. **P<0.01. Bars represent the mean value $\pm$ SE.

Figure 3: Hepatocyte injury and defective liver function in Prox1cKO mice.

Blood examination of Prox1cKO mice and control mice (Prox1cKO mice, n=4; control mice, n=4). Aspartate transaminase (AST), alanine transaminase (ALT), total Bilirubin (T-Bil) and direct Bilirubin (D-Bil) were higher in the blood of Prox1cKO mice, albumin was lower, and lactate dehydrogenase (LDH) showed no change.

*P < 0.05. **P<0.01. Bars represent the mean value $\pm$ SE.

Figure 4: Hepatocyte injury, ectopically hyper-activated mitochondria and excess reactive oxygen species in perivenous region of Prox1cKO mice.

(A) Hematoxylin and eosin (HE) staining showed hepatocyte injury in perivenous hepatocytes of Prox1cKO mice. The injured hepatocytes contained vacuoles (arrowheads) in the cytoplasm. (B) Electron microscopy (EM) observations of mitochondria in hepatocytes. Mitochondria in periportal hepatocytes showed no remarkable difference between Prox1cKO mice and control mice (upper panels). Mitochondria in perivenous hepatocytes of Prox1cKO mice were round-shaped and had higher electron density than control mice (lower panels). (C) 8-OHdG positive hepatocytes were observed in Prox1cKO mice. Arrows indicate stained nuclei of injured hepatocytes with vacuoles in the cytoplasm.

P, portal vein. C, central vein. Scale bars, (HE and 8-OHdG) = 50 μ m and (EM) = 1 μ m.

Figure 5: Upregulated autophagy in perivenous region of Prox1cKO mice.

(A) Electron microscopic analysis shows autophagosomes (arrows) and autolysosomes (arrowheads) in the mutant hepatocytes. (B) LC3B protein was detected in the injured hepatocytes of mutant mice (arrows). (C) The LC3B-II/ LC3B-I protein ratio in the liver of Prox1cKO mice was about twice that of control mice by Western blot analysis.

Scale bars, (EM) = 1 μm and (LC3B) = 10 μm **P < 0.01. Bars represent the mean value $\pm$ SE.

Figure 6: Downregulated glycolysis caused by Prox1 inactivation *in vivo* and *in vitro*.

(A) The relative expression levels of glucokinase (*Gck*), phosphoenolpyruvate carboxykinase (*Pepck*) and glucose 6-phosphatase (*G6Pase*) in the liver of Prox1cKO mice were approximately 50%, 741% and 150%, respectively, those of control mice. (Prox1cKO mice, n=4; control mice, n=4). (B) PAS staining shows decreased glycogen storage in the liver of Prox1cKO mice. (C) The relative PROX1 protein expression level in Hep3B cells treated with *PROX1* siRNA was about 15% that in Hep3B cells treated with *CONTROL* siRNA (*siPROX1*, n=3; *siCONTROL*, n=3). (D) The relative *PROX1* mRNA expression level in Hep3B cells treated with *PROX1* siRNA was almost 30% that in Hep3B cells treated with *CONTROL* siRNA (*siPROX1*, n=3; *siCONTROL*, n=3). (E) Extracellular Flux Analysis. Hep3B cells treated with *PROX1* siRNA (red, n=3) showed a significantly lower ratio of extracellular acidification rate (ECAR) and

significantly higher ratio of oxygen consumption rate (OCR) after glucose challenge than Hep3B cells treated with *CONTROL* siRNA (blue, n=3).

P, portal vein. C, central vein. Scale bars = 100 μm , *P < 0.05. **P<0.01. Bars represent the mean value $\pm$ SE.

Figure 1

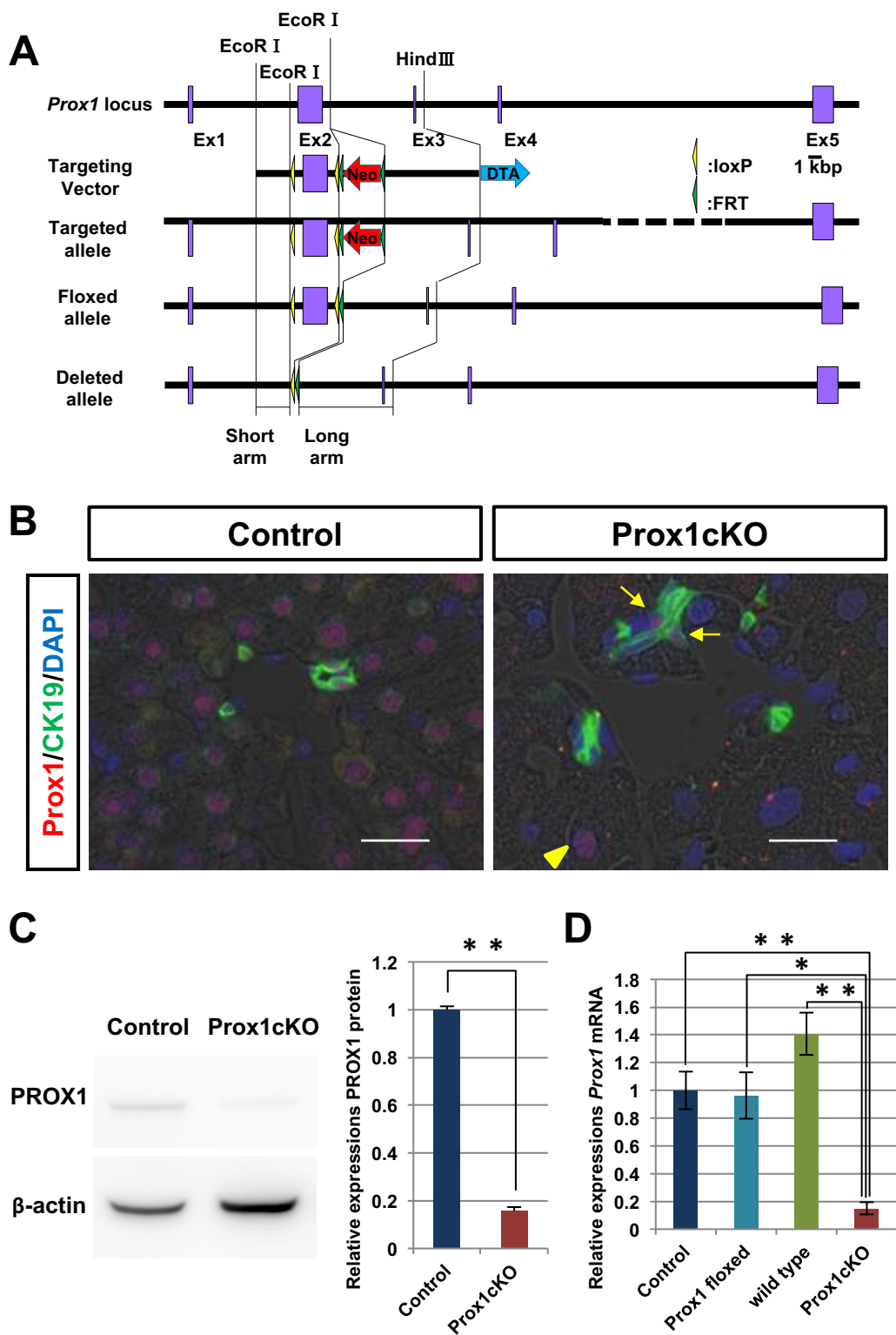
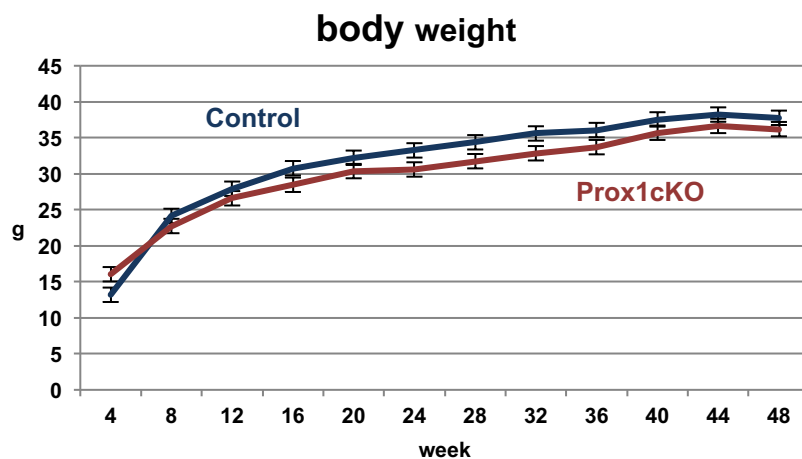
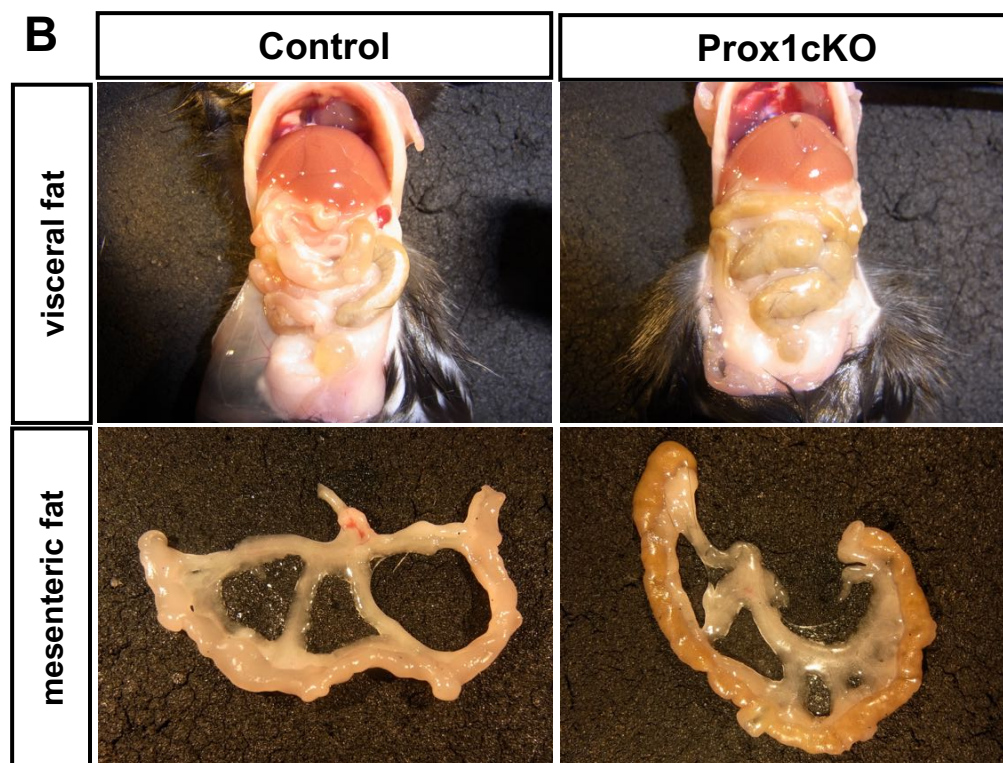


Figure 2

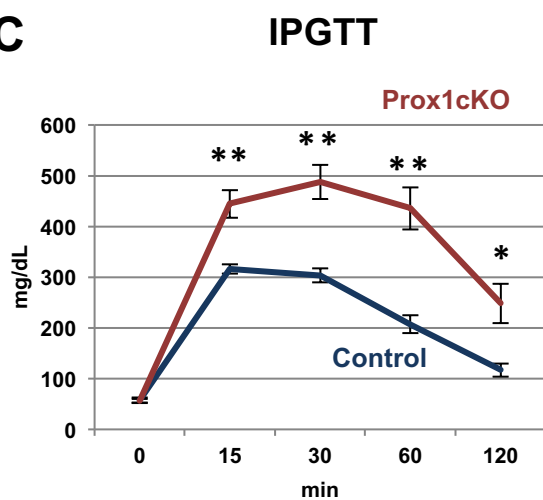
A



B



C



D

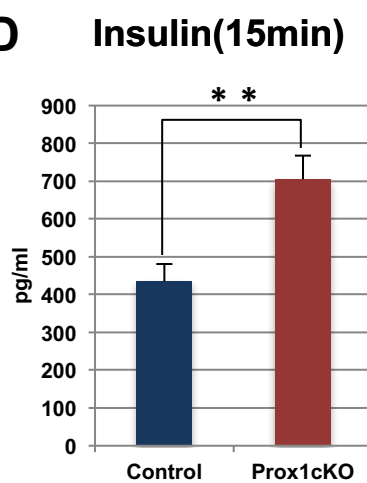


Figure 3

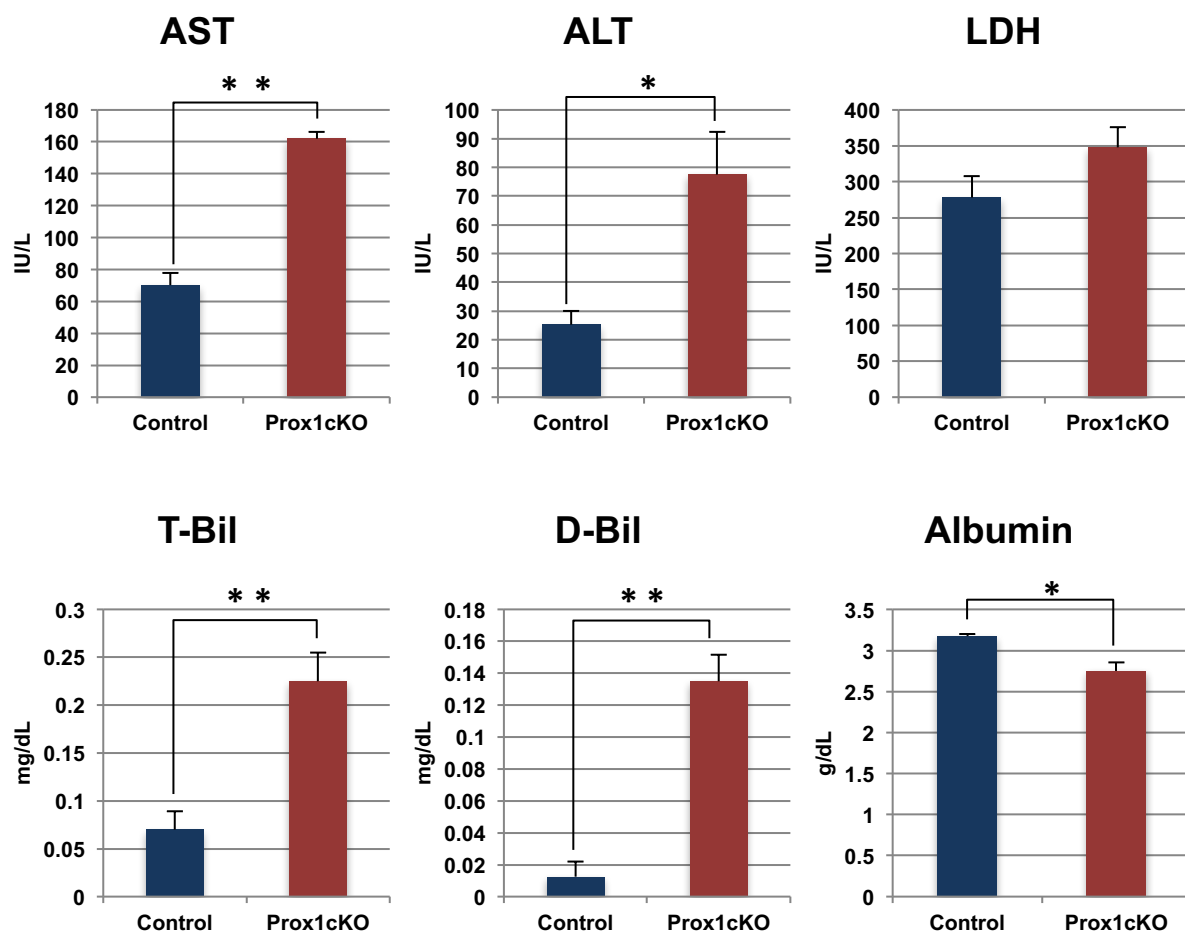


Figure 4

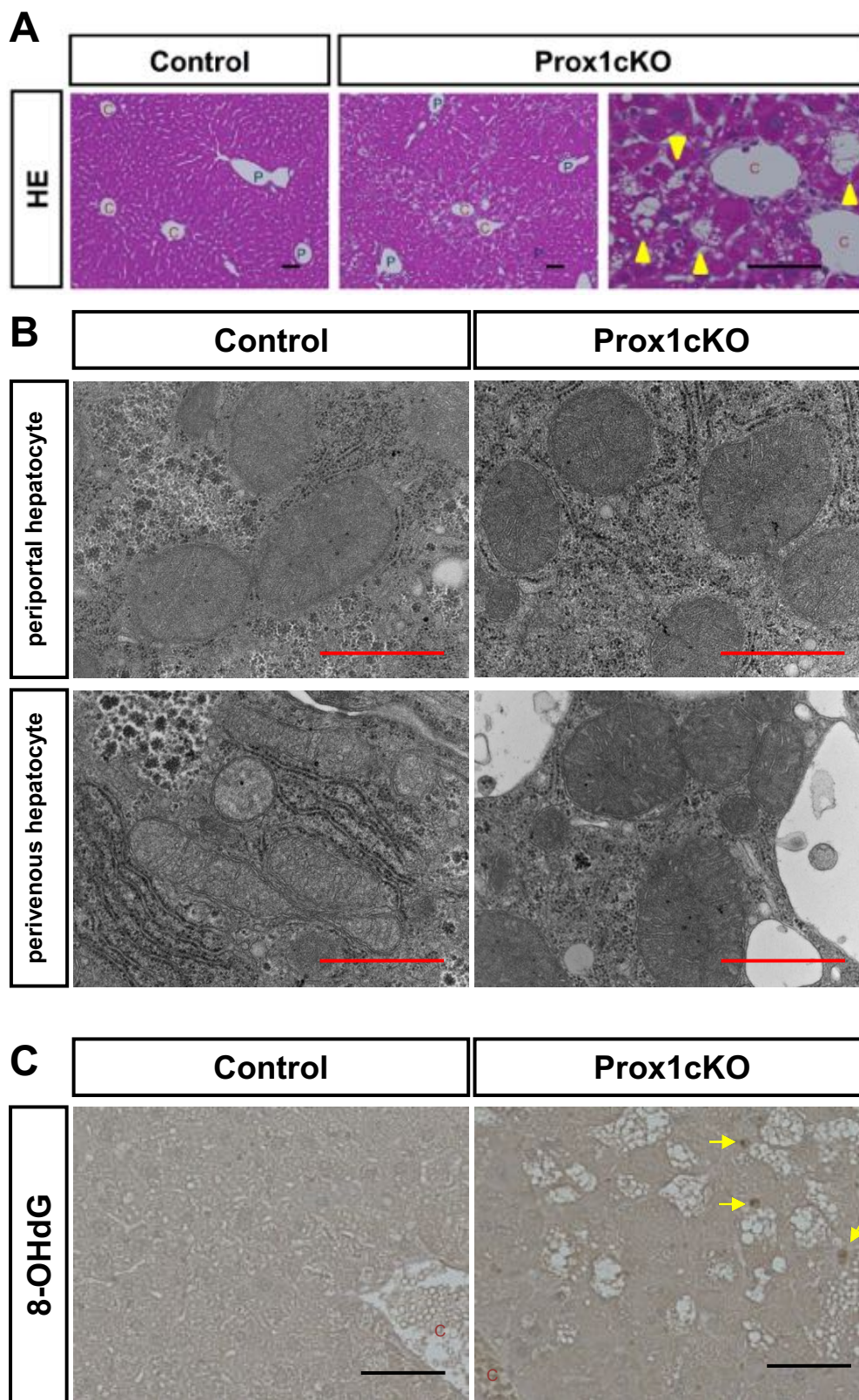


Figure 5

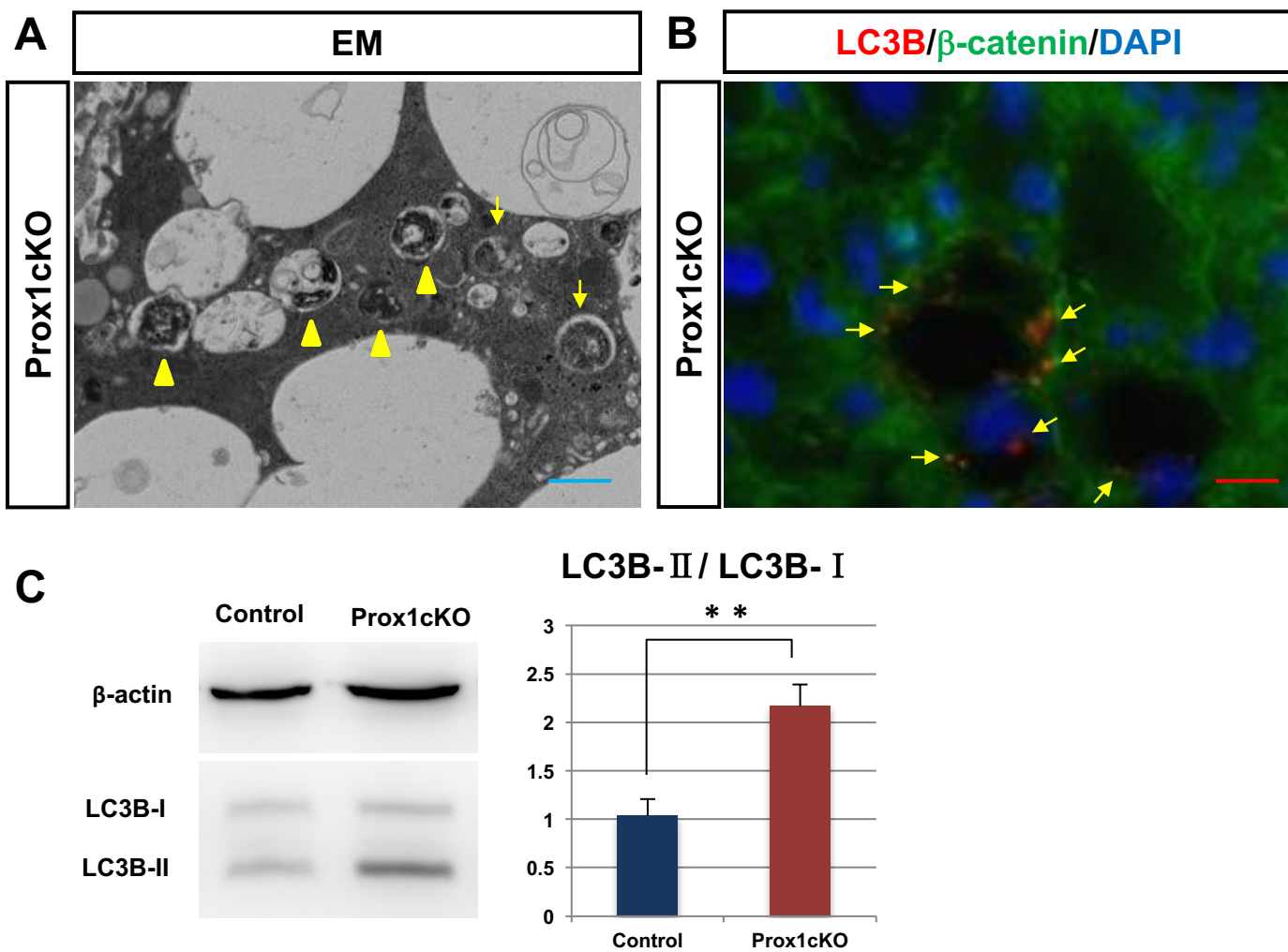
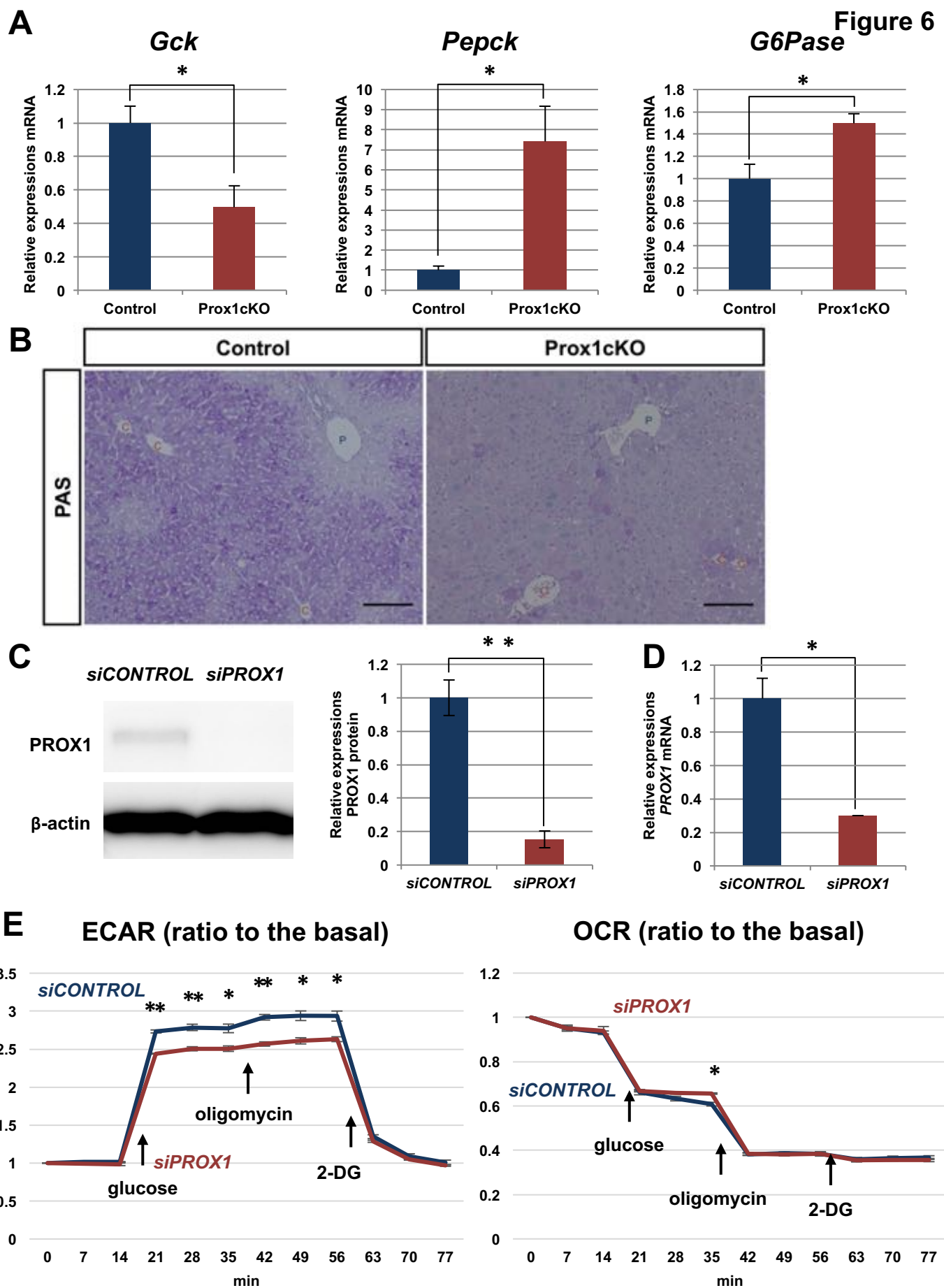


Figure 6



	Control	Prox1cKO
Glucose after starvation (mg/dl)	57 ± 5	57 ± 4
Glucose ad libitum feeding (mg/dl)	133 ± 5	119 ± 9

Table 1: Blood Glucose

Blood glucose levels of Prox1cKO mice and control mice after 16 h starvation or ad libitum feeding showed no significant difference (Prox1cKO mice, n=6; control mice, n=6).

Mean value ± SE

Supplementary Information

Liver specific Prox1 inactivation causes hepatic injury and glucose intolerance in mice

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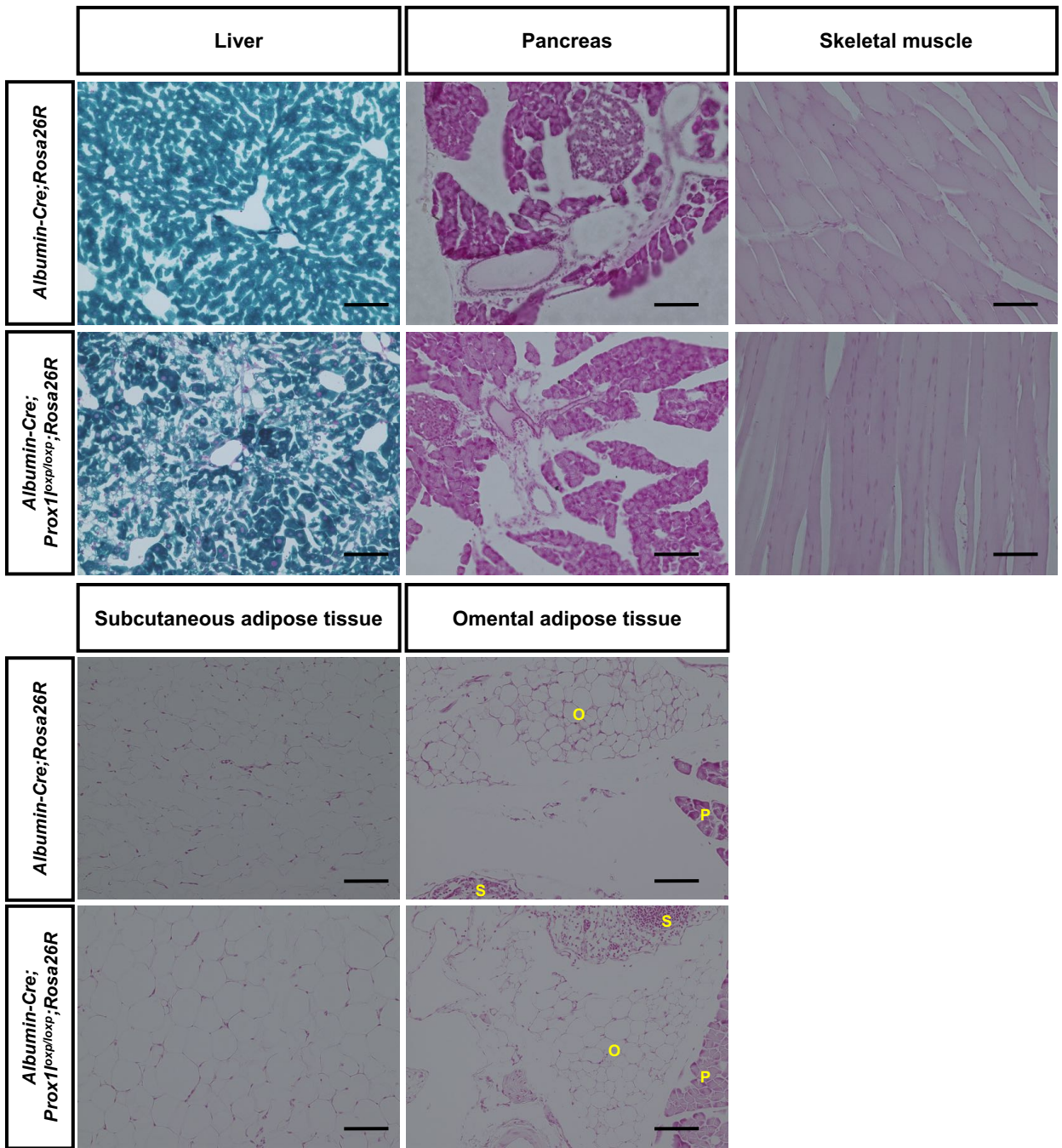


Figure S1

Negligible leaking effect of *Albumin-Cre* mice in metabolic organs.

Lineage positive cells were not detected either in the pancreas, skeletal muscle, subcutaneous adipose tissue or omental adipose tissue by X-gal staining. Liver is shown as positive control.

O, omentum. S, spleen. P, pancreas. Scale bars = 100μm.

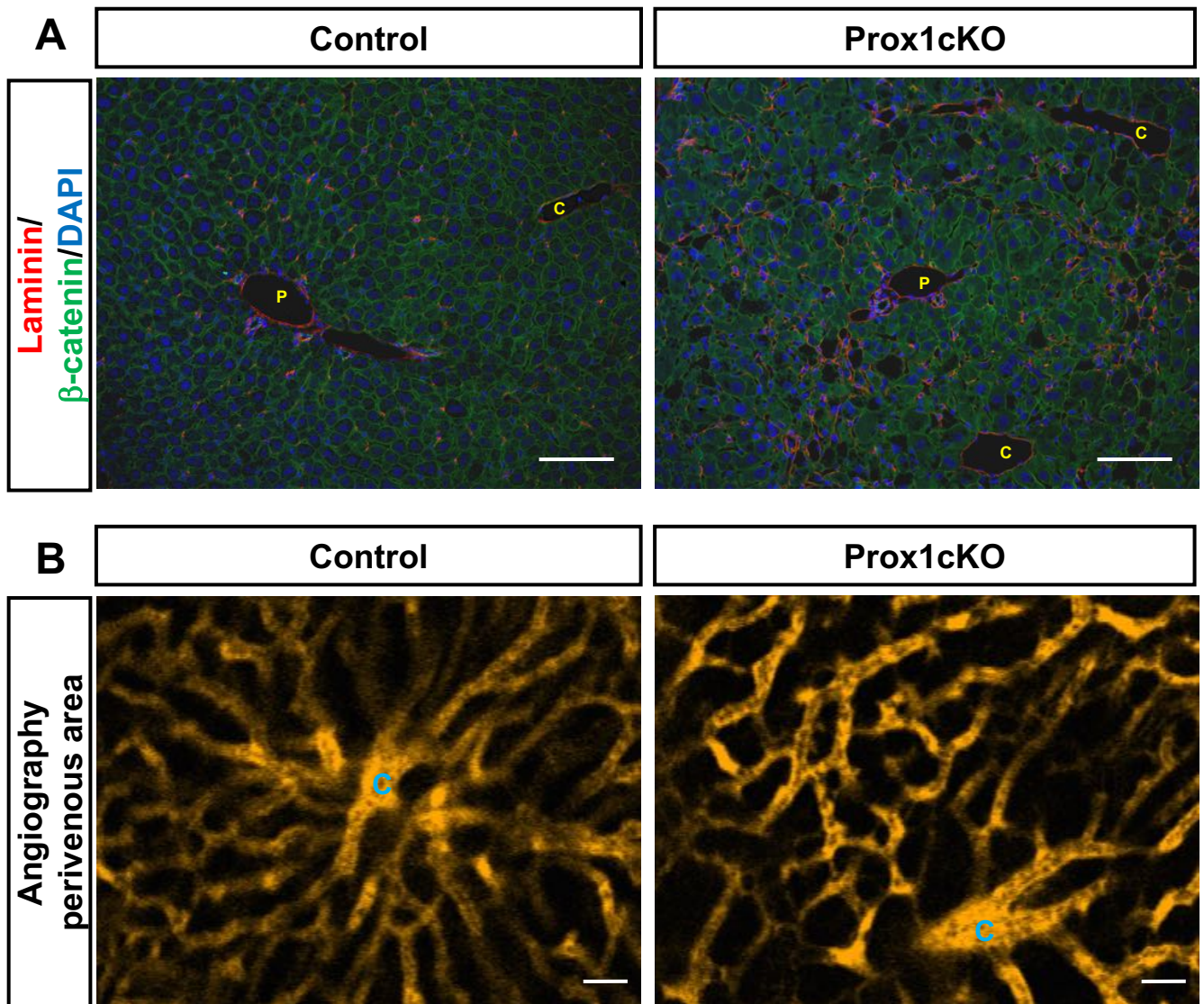


Figure S2

Prox1 absence caused the destruction of the lobular structure and impaired microcirculation.

(A) Laminin and β -catenin staining showed increased basal membrane in the sinusoids of Prox1cKO mice. (B) Angiography. The blood vessel area of Prox1cKO mice around the central vein was smaller than that of control mice.

P, portal vein. C, central vein. Scale bars = 100 μ m.

Primary antibody	Species	Dilution	Supplier
β -catenin	Mouse	1:300	BD
CK19	Goat	1:100	Santa Cruz Biotechnology
LC3B	Rabbit	1:200	
Laminin	Rabbit	1:25	Sigma-Aldrich
Prox1	Rabbit	1:125	Proteintech
8-OHdG	Mouse	1:10	JaICA

Table S1. Primary Antibodies

Secondary antibody	Conjugate	Species	Dilution	Supplier
Goat IgG	Alexa Fluor 488	Donkey	1:1000	Thermo Fisher Scientific
Mouse IgG	Alexa Fluor 488	Goat	1:1000	Molecular Probes
Rabbit IgG	Cy3	Donkey	1:500	Chemicon

Table S2. Secondary Antibodies

Gene	Primer Sequence	Probe Sequence
<i>Prox1</i>	For 5'-CAGCGGACTCTCTAGCACAG-3' Rev 5'-GCCTGCCAAAAGGGGAAAGA-3'	5'-AACTCTTCAAGAGACAGCCCC-3'
<i>Gck</i>	For 5'-CTGTTAGCAGGATGGCAGCTT-3' Rev 5'-TTTCCTGGAGAGATGCTGTGG-3'	5'-TCCGGGAAGAGAAGCAAGCCAC-3'
<i>G6Pase</i>	For 5'-TGGTAGCCCTGTCTTTCTTTG-3' Rev 5'-TTCCAGCATTACACTTTCCT-3'	5'-AGGTGAATTTTACAAAGCTTACAGGGCT-3'
<i>Pepck</i>	For 5'-ATCACCGCATAGTCTCTGAA-3' Rev 5'-ACACACACACATGCTCACAC-3'	5'-GGGTTTTGGTTCCACTTCAA-3'

Table S3. qRT-PCR primer and probe sets

Supplementary Method

In vivo imaging

Rhodamine B isothiocyanate-Dextran (average mol wt ~70000; R9379, SIGMA-ALDRICH) was used as the fluorescent probe. Nikon A1R MP was used as the multiphoton microscope. In vivo imaging of liver microcirculation was performed as described previously³⁶. Dextran solution was infused through a jugular venous catheter.

Supplementary Reference

[1] Marques, P.E., Antunes, M.M., David, B.A., Pereira, R.V., Teixeira, M.M. and Menezes, G.B. (2015). Imaging liver biology in vivo using conventional confocal microscopy. Nat Protoc 10, 258-68.