ONLINE APPENDIX

Fat-cell specific ablation of *rictor* in mice impairs insulin-regulated fat cell and whole body glucose and lipid metabolism

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Supplementary methods, figures, references

SUPPLEMENTARY METHODS

Isolation of fat cells. Mice were anesthetized by intraperitoneal injection of 0.1 ml/20g body weight of a mixture of ketamine/xylazine/acepromazine in saline and epididymal or parametrial fat pads were removed. Fat cells were isolated from these fat pads by collagenase digestion (1) as follows. Fat pads were added to Krebs Ringer HEPES (KRH) –BSA buffer containing collagenase (type I from Worthington Biochemical Corp., 1 mg/ml, 2 mg/g of tissue). The fat pads in the collagenase solution were minced with scissors and incubated in a 37°C shaking water bath (100 rpm) for 1 h. The fat cells were separated from non-fat cells and undigested debris by filtration through a 0.4 mm Nitex nylon mesh (Tetko) and then washed four times by flotation with KRH-BSA. The isolated fat cells were used in glucose transport assays and in preparation of cell lysates for immunoblotting to determine the levels of phosphorylated as well as total levels of cellular proteins.

Cell counting. Fat cell number was determined from measurements of cell sizes and lipid content in aliquots of the adipocyte suspensions used for the transport assays (2). Cell sizes of 100 or more cells from the 5% cell suspension were measured using the Nikon objective micrometer scale and Scion imaging software. Lipid was extracted from 100 µl aliquots of the 5% cell suspension in quadruplicate as described in (1). Briefly, 100 µl aliquots of the 5% cell suspension were mixed with 1.35 ml of 40:10:1 isopropanol/heptane/1 N H_2SO_4, followed by the addition of 0.9 ml of heptane and 0.5 ml of water. The mixture was vortexed and centrifuged briefly. A 1.0 ml aliquot of the organic layer was evaporated and the remaining dry lipid was weighed.

Isolation of Plasma membrane. Briefly, parametrial fat pads were isolated and rinsed with KRH-BSA and then incubated in same buffer at 37° C for 3 hours. The fat pads were then incubated without or with 10 nM insulin for 15 min. These fat pads were snap-frozen and crushed on dry ice, homogenized in ice-cold sucrose buffer (250 mM sucrose, 20 mM HEPES, 1 mM EDTA, 100 µM phenylmethylsulfonyl fluoride, pH 7.4) using a Polytron device (setting 3; 30 s), and then subjected to 10 strokes of homogenization in a glass Teflon pestle. After centrifugation (16,000 × *g* for 30 min at 4°C), the top fat layer was discarded and the pellet was used for preparation of the plasma membrane-enriched fraction (3). The pellet was resuspended in sucrose buffer, applied to a 1.12 M sucrose cushion (20 mM HEPES and 1 mM EDTA, pH 7.4), and centrifuged (100,000 ×

g for 60 min at 4°C) to obtain the interface, which was resuspended in 10 ml of the sucrose buffer and centrifuged (48,000 × *g* for 30 min at 4°C) to obtain a pellet containing the plasma membrane.

Immunoblotting. Protein extracts were prepared from isolated fat cells, muscles and liver. For incubation with insulin, fat cells isolated by the method described above were washed by flotation with KRH buffer (without BSA), a 30% cell suspension was prepared and incubated without or with insulin (10 nM). After 15 min of incubation with insulin, cells were quickly separated from buffer using dinonylphthalate and instantly frozen in liquid nitrogen. Protein extracts were prepared from fat cells, isolated EDL/soleus muscles (incubated without or with 20 mU insulin for 30 min), fat pads, Tibialis anterior (TA) muscles and livers from wild type and knockout animals and immunoblotted with antibodies against non-phosphorylated (total) and phosphorylated cellular proteins by previously described methods (4).

[U-^{14}C]-Glucose incorporation into glycogen. Incubations were conducted essentially as described previously (5). Briefly, extensor digitorum longus (EDL) and soleus muscles were removed from anesthetized mice. Muscles were incubated at 37°C for 30 min to remove endogenous hormones, then transferred to tubes containing 10 ml of Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM potassium phosphate, 1.2 mM $MgSO_4$, and 25 mM $NaHCO_3$, pH 7.4) with or without 20 mU of insulin and 5 mM glucose ([U-^{14}C] D-glucose, ~ 500 cpm/nmol) and incubation continued for 30 min. During these incubations, the buffer was gassed directly with a mixture of 95% O_2 and 5% CO_2. After the last incubation muscles were rinsed quickly, blotted on tissue paper, trimmed of connective tissue and frozen in liquid nitrogen. The muscles were weighed and digested in 0.5 ml of 30% KOH in a boiling water bath for 20-30 min. After adding 50 µl of a 5% glycogen solution to each sample, ethanol was added to a final concentration of 70%, and glycogen was allowed to precipitate overnight at –20°C. The samples were centrifuged at 8,900 x *g* for 20 min at 4°C to pellet the glycogen. Each glycogen pellet was resuspended in 50 µl distilled water and spotted on 2 cm squares of filter paper (Whatman 31ET-CHR). The filter paper squares were washed five times with 66% ethanol and once with acetone. The paper squares were then dried before determining the amount of ^{14}C-labeled glycogen on them by scintillation counting.

Tissue triglyceride content. For TAG extraction from skeletal muscle and liver, tissues were homogenized in distilled water (20 µl/mg tissue). TAG was extracted from equal volumes of tissue homogenates by the method of Bligh and Dyer (6). TAG concentration was determined using a TAG determination kit (Sigma-Aldrich, St. Louis, MO).

***In vivo* lipolysis.** This assay was conducted by the method described by Abel et al. (8).

***Ex vivo* lipolysis and measurement of glycerol in the medium.** This assay was conducted in isolated fat cells following the method described by Viswanadha and Londos (10). Glycerol released into the medium was determined by a radiometric method described by Viswanadha and Londos (10).

PKA activity assay. Isolated fat cells were incubated with in the absence or presence of either CL316243 alone or CL316243 and insulin for 10 min. Preparation of cell extract and assaying for PKA activity were carried out by previously described methods (11, 12). PKA activity was expressed as activity ratio, which is the ratio of activity in the absence of cAMP over activity in the presence of cAMP (total activation). The specific PKA activity in every extract was determined by subtracting the activity seen in the presence of a specific PKA inhibitor.

Quantitative RT-PCR. Total RNA was isolated from adipose tissue or liver with Trizol (Invitrogen), and treated with RNase-free DNase (Qiagen) to remove any contaminating genomic DNA. First-strand cDNA synthesis was performed using oligo-dT primers and the Omniscript RT kit (Qiagen). Real-time PCR reactions were performed on the iCycler iQ real-time detection system (BioRad) using iQ SYBR Green Super mix (BioRad). Reactions were performed in duplicate or triplicate with each sample containing 40 ng of cDNA. A no template control was included with each assay. Each sample was tested for expression of the gene of interest as well as for the housekeeping gene GAPDH. The efficiency of amplification (*E* in Pfaffl's formula) (13) of PCR for individual genes of interest as well as GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were determined by real-time PCR reactions of serial dilutions of control cDNA (ranging from 100 ng to 10 pg) and used in calculating fold change. The fold change in expression of genes of interest in FRic$^{-/-}$ tissue cDNA was calculated using the Pfaffl formula (13) as follows:

$$\frac{E^{\Delta Ct \text{ gene of interest (FRic+/+ - FRic-/-)}}}{E^{\Delta Ct \text{ GAPDH (FRic+/+ - FRic-/-)}}}$$

Primers used for real-time PCR were as follows: FAS (TGGGCCTGCAGCTGGGAGCA, GGCCTGGACTCGCTCATGGG); L-PK (CTTGCTCTACCGTGAGCCTC, ACCACAATCACCAGATCACC) and GAPDH (AGACGAATTCAAATTCAACG GCACAGTCAA, TCGTGAATTCGGTGCAGGATGCATTGCTGA).

Histochemical staining. Epididymal fat pads were first fixed in 10% formalin in normal saline overnight at 4°C and then in 70% ethanol for at least 2 hours. Fixed tissues were paraffin embedded and 5-7 μm sections were prepared. For liver sections, 5 μm sections were prepared from frozen liver. Tissue sections were stained for Hematoxylin and Eosin (H&E) in a Shandon Varistain Gemini automated system using Surgipath H&E staining solution.
For oil red O-staining, frozen liver sections were air dried for 30 min and then fixed in ice-cold 10% formalin for 5 min. Slides were rinsed with 3 changes of distilled water and placed in absolute propylene glycol for 5min. Slides were then placed in 0.5% oil red O stain solution in propylene glycol for 8 min in a 60°C oven, rinsed with 85% propylene glycol for 5 min, and then with distilled water, dehydrated, cleared and mounted with DPX mounting medium (Fluka, Buchs, Switzerland).
Glucose and insulin tolerance tests. These tests were carried out using previously described methods (4).

Body composition. Whole body fat and lean mass were non-invasively measured in awake mice using ^{1}H-MRS (proton magnetic resonance spectroctroscopy, Echo Medical Systems,Houston, TX).

Miscellaneous tests. Commercially available kits were used to determine the levels of insulin (Crystal chem, Downers Grove, IL), leptin and adiponectin (Linco-Millipore, Billerica, MA), NEFA (Wako Chemicals, Richmond, VA), Triacylglycerol (Sigma-Aldrich, St. Louis, MO Cat. No. T2449), and glycerol (Sigma-Aldrich, Cat. No. F6428) in serum.

Antibodies. Antibodies to rictor, pan-actin, pan-Cadherin, Foxo3A, PKCα, IRS1, HSL, Akt P (phospho) -S473, Akt P-T308 and P-S302 IRS1 were obtained from Cell Signaling Technology (Danvers, MA). The phospho-specific antibodies to Y-896 IRS1 and Y-972 IR were obtained

from Invitrogen (Carlsbad, CA). The peptides used for raising the antibodies to GLUT4, mTOR, Lipin 1 and Akt are described previously (4). Myo1c antibody was kindly provided by Dr. Peter Gillespie (Oregon Health and Science University). IRAP antibody was provided by Dr. Susanna Keller (University of Virginia). AS160 P-T642 antibody was obtained from Novus Biologicals (Littleton, CO). Total antibodies to AS160, GSK3β and Foxo3A P-T32 antibody were purchased from Upstate Biotechnology (Charlottesville, VA). Glycerol Kinase was purchased from MP Biomedicals (Solon, OH). CL316243, Kemptide and PKA inhibitor used in PKA assay were purchased from Sigma-Aldrich Corporation (St. Louis, MO).

SUPPLEMENTARY REFERENCES

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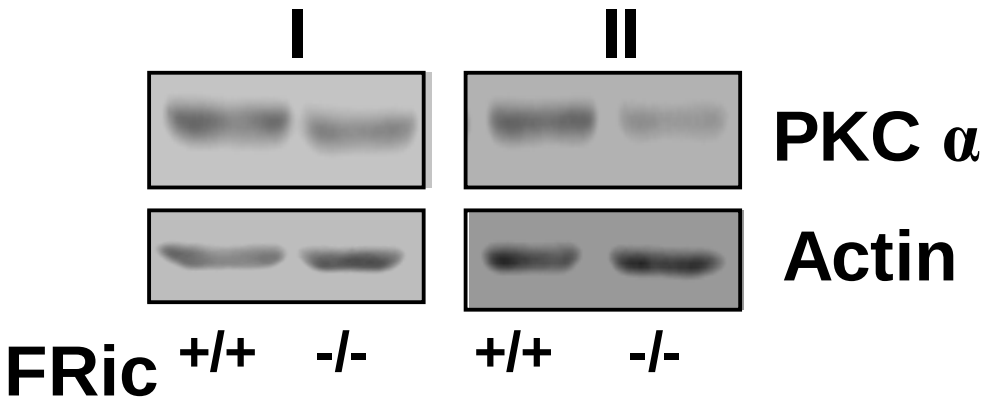
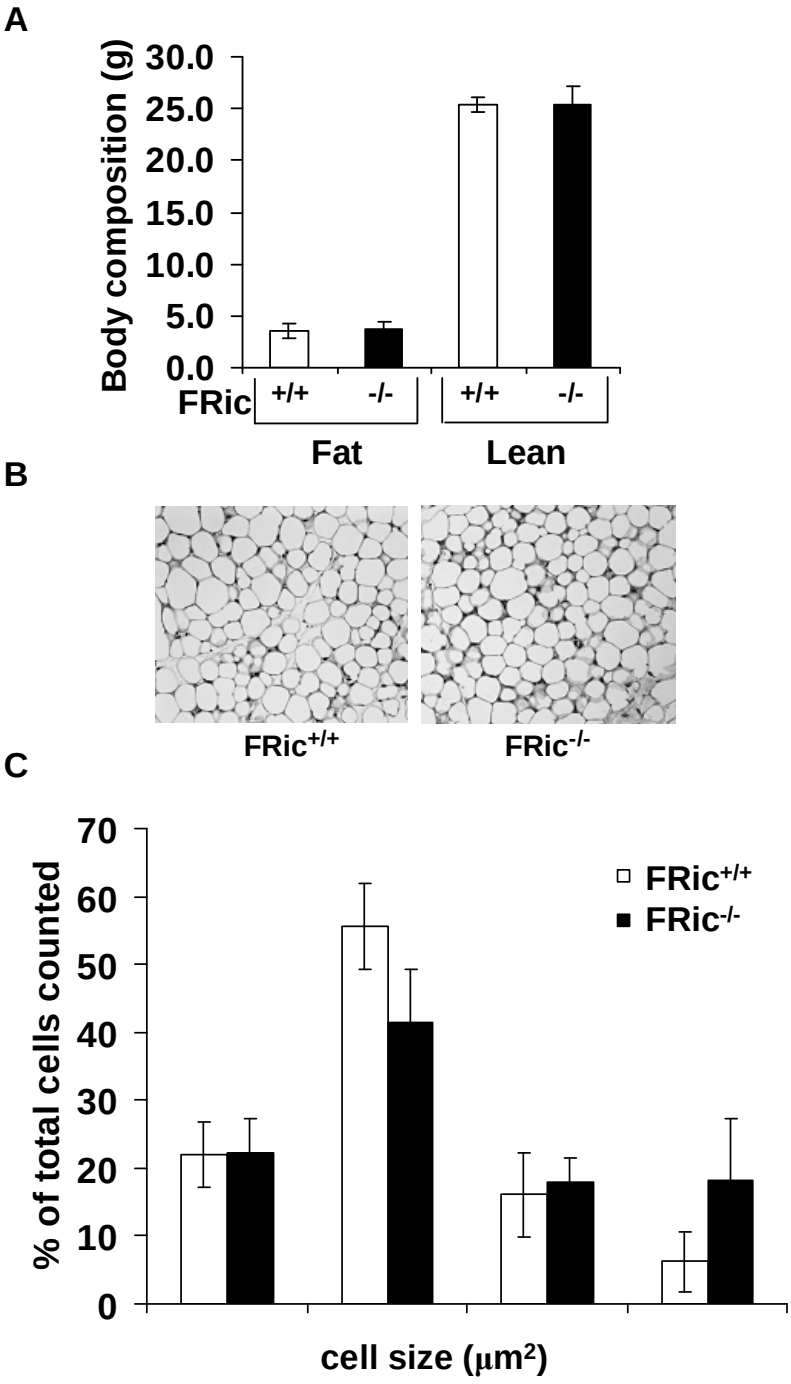
SUPPLEMENTAL FIGURES

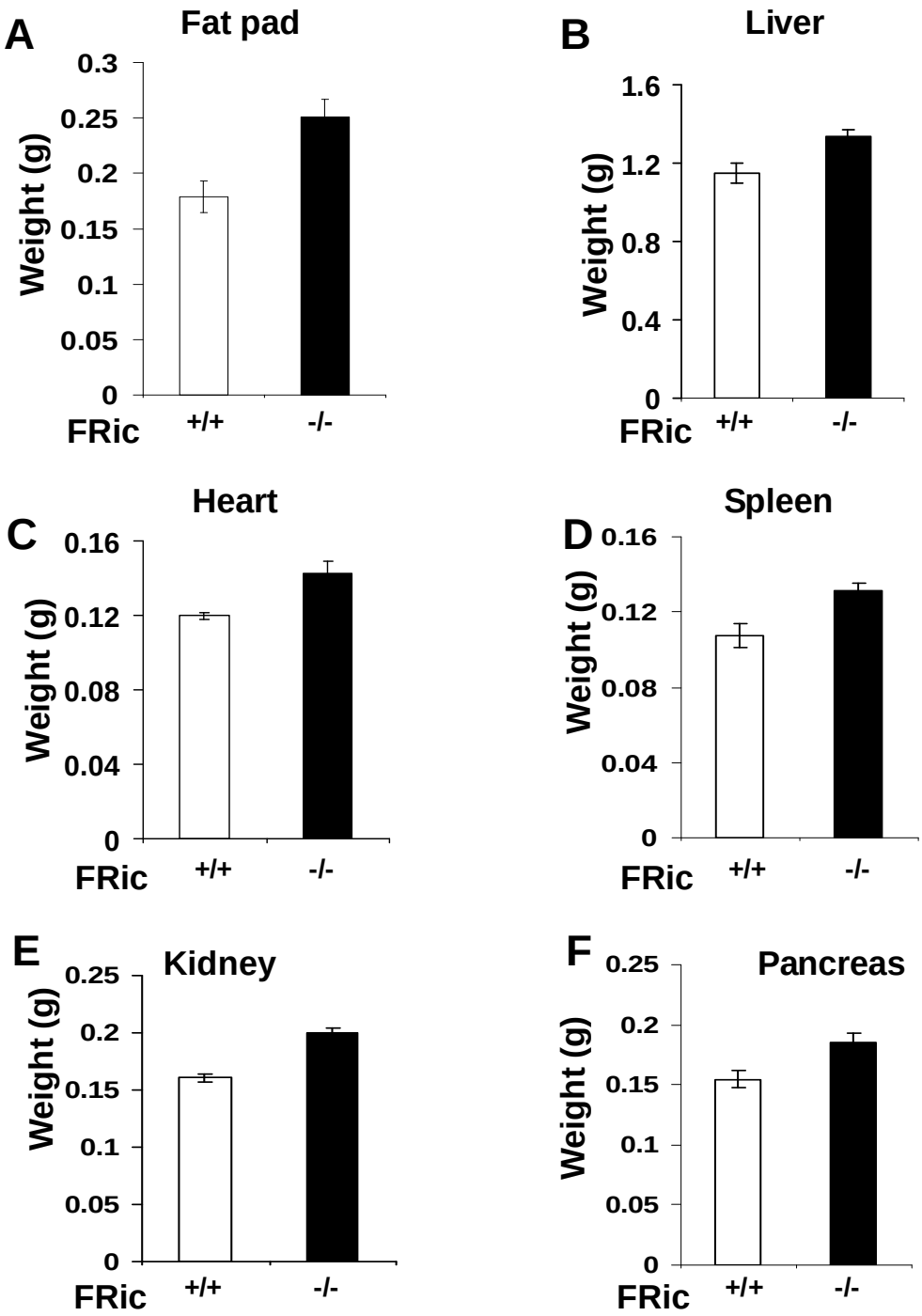
Supplemental Fig. 1. PKCα levels are reduced in FRic$^{-/-}$ fat tissue. Fat pad extracts from FRic$^{-/-}$ mice (3-5 month old) were immunoblotted for PKCα (2 sets of FRic$^{+/+}$ and FRic$^{-/-}$ mice labeled as I and II representative of a total of 6 sets).



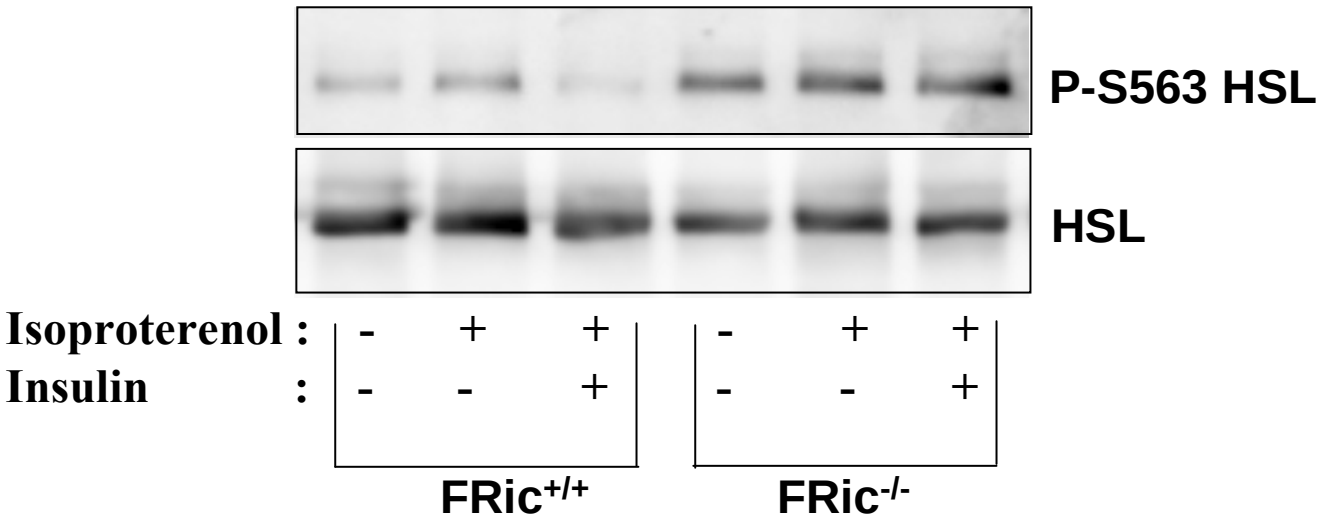
Supplemental Fig. 2. *A*) Body composition of FRic$^{+/+}$ and FRic$^{-/-}$ mice determined by ^{1}H-MRS (male, 3-5 month old, n = 8, * $p < 0.001$). *B*) Representative images of hematoxylin and eosin (H&E)-stained sections of parametrial fat pads from FRic$^{+/+}$ and FRic$^{-/-}$ mice (3-5 month old). *C*) Percentages of cells of different sizes in parametrial fat pad sections from FRic$^{+/+}$ and FRic$^{-/-}$ mice (female, 3-5 month old, n = 5).



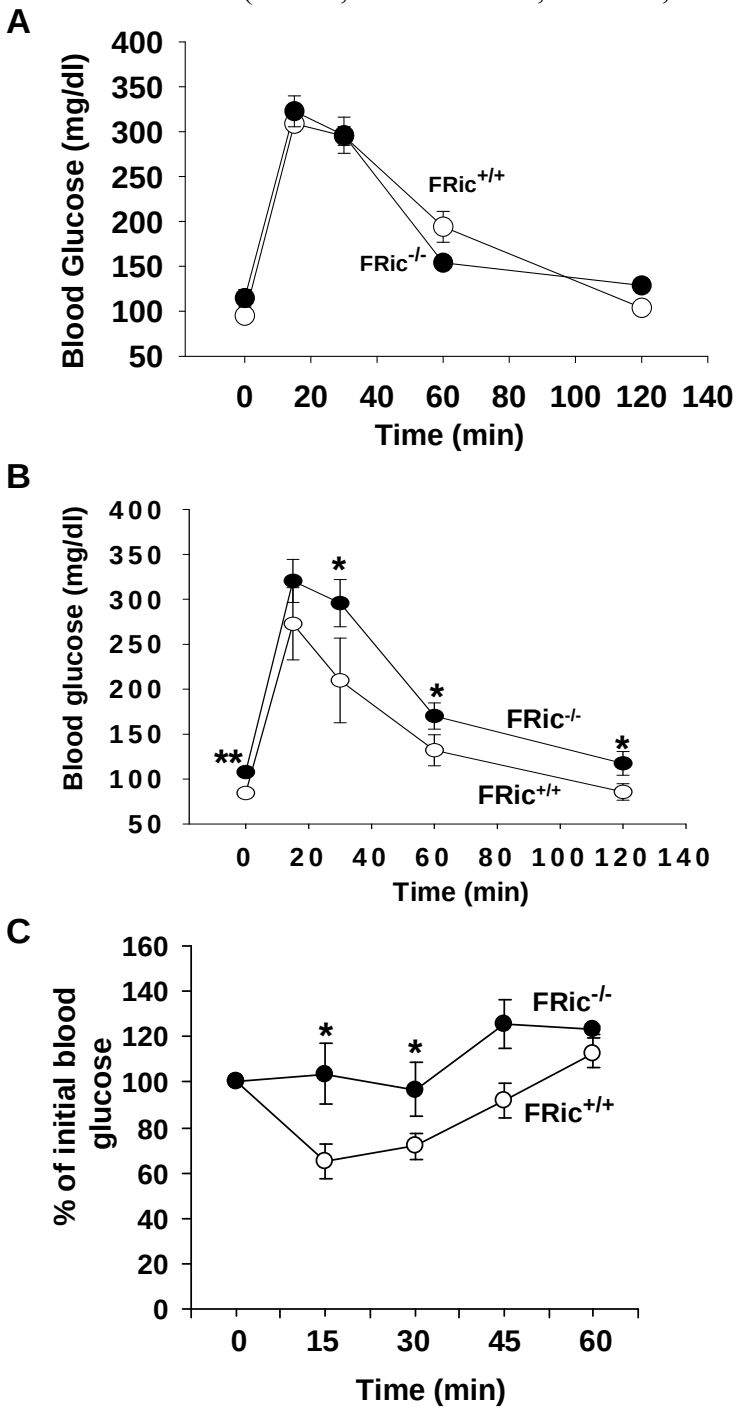
Supplemental Fig. 3. Organ weights in FRic$^{+/+}$ and FRic$^{-/-}$ mice (n = 4-6, 2-4 month old female mice).



Supplemental Fig. 4. Phosphorylation of HSL at S563 in fat pads from FRic$^{+/+}$ and FRic$^{-/-}$ mice under basal condition or stimulated with either isoproterenol alone or isoproterenol and insulin (representative immunoblots are shown, n = 4).



Supplemental Fig. 5. Glucose and insulin tolerance tests in FRic$^{-/-}$ mice. (*A* and *B*) Intraperitoneal glucose tolerance tests (IPGTT). *A*) IPGTT in young male FRic$^{-/-}$ and FRic$^{+/+}$ (2-5 month old, n = 6-8). *B*) The old FRic$^{-/-}$ mice had impaired glucose tolerance (female, more than 9 month old, n = 4, * $P < 0.05$, ** $P < 0.006$). *C*) Insulin tolerance tests (ITT) conducted in young FRic$^{-/-}$ mice showed insulin resistance (female, 2-4 month old, n = 5-11, * $P < 0.05$).



Supplemental Fig. 6. Glycogen synthesis in EDL muscles (female, more than 9 month old, n = 4-5, * $p < 0.0005$).