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Supplemental Data

Prevention of Steatosis by Hepatic JNK1

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Supplemental Experimental Procedures

RNA analysis.
The expression of mRNA was examined by quantitative PCR analysis using a 7500 Fast Real Time PCR machine. Taqman© assays were used to quantitate *Acacα* (Mm01304289_m1), *Acacβ* (Mm01204683_m1), *Acot3* (Mm00652967_m1), *Acsl1* (Mm00484217_m1), *Acsl4* (Mm00490331_m1), *Cd68* (Mm00839636_g1), *Ceacam1* (Mm00442360_m1), *Cyp2e1* (Mm00491127_m1), *Dgat1* (Mm00515643_m1), *Fas* (Mm00662322_g1), *Foxo1* (Mm0049072_m1), *G6p* (Mm00839363_m1), *Gyk* (Mm00433896_m1), *Hnf4α* (Mm00433964_m1), *Icam1* (Mm00516023_m1), *Il6* (Mm_00446190_m1), *InsR* (Mm00439693_m1), *Lysozyme* (Mm00727183_m1), *Mttp* (Mm00435015_m1), *Pepck* (Mm00440636_m1), *Jnk1* (Mm00489514_m1), *Jnk2* (Mm004442341_m1), *Pgc1α* (Mm 00447183_m1), *Scd1* (Mm00772290_m1) and *Tnfa* (Mm_00443258_m1), (Applied Biosystems). The amount of *Pgc1β* mRNA (TACATGCATACCTACTGCCTGCCT & TTGGGCCAGAAGTTCCCTTAGGAT) and *Srebp1* mRNA (GATGTGCGAACTGGACACCAG & CATAGGGGGCGTCAAACAG) was examined by quantitative RT-PCR using Syber Green detection. The relative mRNA expression was normalized by measurement of the amount of *18S* RNA in each sample using Taqman© assays (430449011032, Applied Biosystems).

Protein kinase assays.
JNK activity was measured using an *in vitro* protein kinase assay with the substrates cJun and [γ-^{32}P]ATP as substrates (Whitmarsh and Davis, 2001).

Glucose tolerance and insulin tolerance tests.
The mice were fed a standard chow diet or a high fat diet (Iso Pro 3000, Purina and F3282, Bioserve Inc.) for 16 wks. Glucose tolerance tests and insulin tolerance tests were performed using methods described previously (Mora et al., 2005).

Insulin clearance assays.
Mice were administered human insulin (1.5 mU/g; Novolin; Novo Nordisk. Princeton NJ) by intraperitoneal injection. The amount of human insulin in the blood at different times post-injection was measured by ELISA (Luminex 200 machine, Millipore).

Hyperinsulinemic-euglycemic clamp studies.
The clamp studies were performed at the Penn State Diabetes & Obesity Mouse Phenotyping Center and the UMass Mouse Phenotyping Center. Briefly, mice were fed a HFD diet (55% fat by calories; Harlan Teklad TD93075) or chow diet for 3 weeks, and whole body fat and lean mass were non-invasively measured using ^{1}H-MRS (Echo Medical Systems). Following an overnight fast (~15 hr), a 2-hr hyperinsulinemic-euglycemic clamp was conducted in awake mice with a primed and continuous infusion of human insulin (150 mU/kg body weight priming followed by 2.5 mU/kg/min; Humulin; Eli Lilly), and 20% glucose was infused at variable rates to maintain euglycemia (Kim et al., 2004). Whole body glucose turnover was assessed with a continuous infusion of [3-^{3}H]glucose and 2-deoxy-D-[1-^{14}C]glucose (PerkinElmer) was

administered as a bolus (10 μCi) at 75 min after the start of clamps to measure insulin-stimulated glucose uptake in individual organs. At the end of the clamps, mice were anesthetized, and tissues were taken for biochemical analysis (Kim et al., 2004).

Metabolic cages.
Male mice were housed under controlled temperature and lighting with free access to food and water. The food /water intake, energy expenditure, respiratory exchange ratio, and physical activity were performed (3 days) using metabolic cages (TSE Systems, Bad Homburg, Germany).

Fat absorption.
Fat absorption in mice was determined by the University of Cincinnati Mouse Metabolic Phenotyping Center (Lipid, Lipoprotein and Glucose Metabolism Core; Dr P. Tso, Director) using the sucrose polybehenate method (Jandacek et al., 2004). The mice were fed the test diet for 5 days. Individually housed mice were transferred to new cages on day 3 and fecal pellets were collected each day. The fecal pellets collected on day 4 and day 5 were examined for the fatty acid content by gas chromatography. Since behenic acid is essentially absent from the fat sources used in the test diet and is entirely excreted when given as sucrose polybehenate, absorption is calculated from the difference between diet and feces in the ratio behenate/total fatty acid.

Measurement of hepatic triglyceride.
Hepatic triglyceride content was measured using livers from mice starved overnight. Total lipids were extracted from liver samples (50 mg) using an 8:1 mixture of chloroform and methanol (4 hrs). The extracts were mixed with 1N sulfuric acid and centrifuged (10 mins). The amount of triglyceride was measured using a kit purchased from Sigma.

Hepatic lipogenesis.
The mice were fed a standard chow diet and starved for 6 hours. The mice were administered 20 μCi of [3-^{3}H]glucose (PerkinElmer) by intraperitoneal injection and euthanized after one hour. Lipids were extracted from samples of the liver (50 mg) using an 8:1 mixture of chloroform and methanol. The amount of radioactivity incorporated into lipid was measured by liquid scintillation counting.

Blood lipid analysis.
Total Cholesterol, HDL, LDL, and triglyceride were measured using a Cardiocheck PA (PTS, Inc.). The concentration of free fatty acids was measured using a kit purchased from Roche.

Analysis of tissue sections.
Sections (7 μm) prepared from tissue frozen in O.C.T. compound (Tissue-Tek) were stained with Oil-red-O (Sigma).

Supplemental References

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Mora, A., Sakamoto, K., McManus, E.J., and Alessi, D.R. (2005). Role of the PDK1-PKB-GSK3 pathway in regulating glycogen synthase and glucose uptake in the heart. FEBS Lett *579*, 3632-3638.

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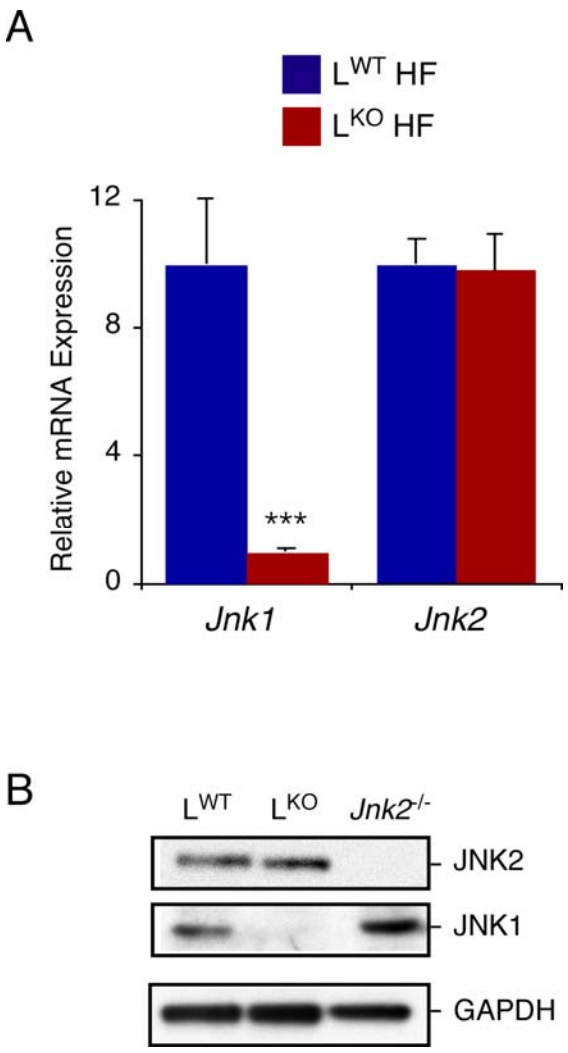

Figure S1. Expression of the JNK group of protein kinases in the liver of L^{WT} and L^{KO} mice.

(**A**) The expression of *Jnk1* and *Jnk2* mRNA in the liver of L^{KO} and L^{WT} mice is presented (mean ± SD; n = 7). Statistically significant differences between L^{KO} and L^{WT} mice are indicated (***, $P < 0.001$). (**B**) Extracts prepared from the liver of L^{WT}, L^{KO} and *Jnk2*$^{-/-}$ mice were probed with antibodies to JNK1 and GAPDH. JNK1-immunodepleted extracts were examined by immunoblot analysis by probing with antibodies to JNK1/2 to detect JNK2.

Ablation of the *Jnk1* gene in hepatocytes caused decreased expression of JNK1, but no changes in the expression of JNK2. No expression of the neuronal isoform JNK3 was detected in the liver.

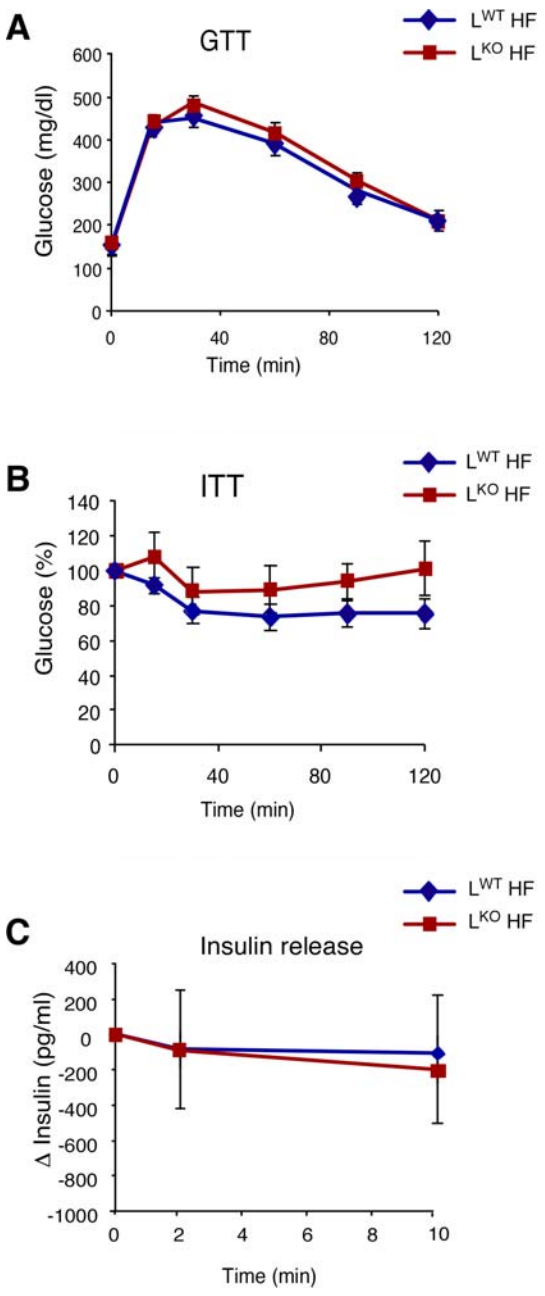

Figure S2. Metabolic phenotyping of HFD-fed L^{WT} and L^{KO} mice.

L^{KO} and L^{WT} mice were maintained in high fat diet (HF) for 16 wk. (**A**) Glucose tolerance test (GTT). Mice fasted overnight were injected intraperitoneally with glucose (1g / kg). Blood glucose concentration was measured at the indicated times (mean ± SD; n = 10). (**B**) Insulin tolerance test (ITT). Mice fed *ad libitum* were injected intraperitoneally with insulin (0.75 mU/g). Blood glucose concentration was measured at the indicated times (mean ± SD; n = 10). (**C**) Insulin release . The effect of administration of glucose (2g / kg body mass) by intraperitoneal injection on blood insulin concentration was examined (mean ± SD; n = 10).

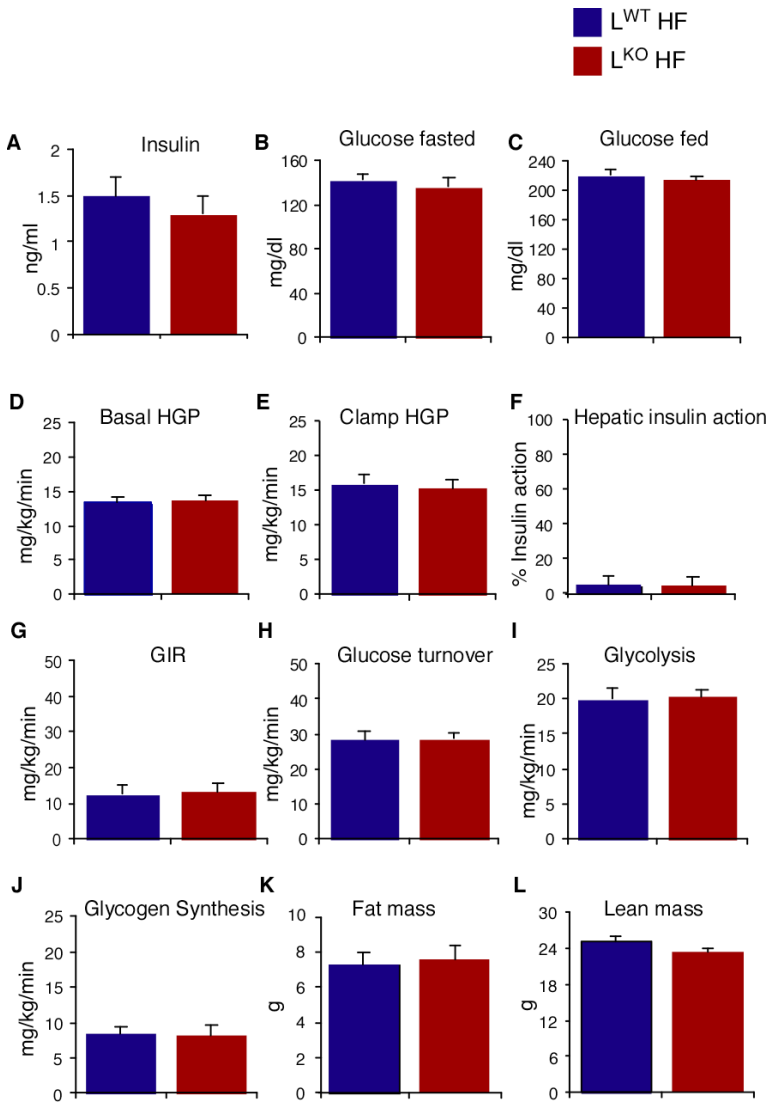

Figure S3. Hyperinsulinemic-euglycemic clamp studies of HFD-fed L^{WT} and L^{KO} mice.

(**A-C**) L^{KO} and L^{WT} mice were maintained on a high-fat diet (HFD) for 16 wk. Resting blood insulin (A) and glucose (B) were measured in mice that were fasted overnight (mean ± SD, n =10). Blood glucose concentration was also examined in mice fed *ad libitum* (C). No statistically significant differences between L^{KO} mice and L^{WT} mice were detected ($P > 0.1$).

(**D-L**) Hyperinsulinemic-euglycemic clamp studies. L^{KO} and L^{WT} mice were maintained on a HFD for 3 wks. (D) Basal hepatic glucose production (HGP). (E) Insulin-stimulated rate of HGP. (F) Hepatic insulin action, expressed as insulin-mediated percent suppression of basal HGP. (G) Steady-state glucose infusion rate (GIR) to maintain euglycemia during clamps. (H) Insulin-stimulated whole body glucose turnover. (I) Whole body glycolysis. (J) Whole body glycogen synthesis. (K) Whole body fat mass. (L) Whole body lean mass. The data presented are the mean ± SE for 8 experiments. No statistically significant differences between L^{KO} mice and L^{WT} mice were detected.

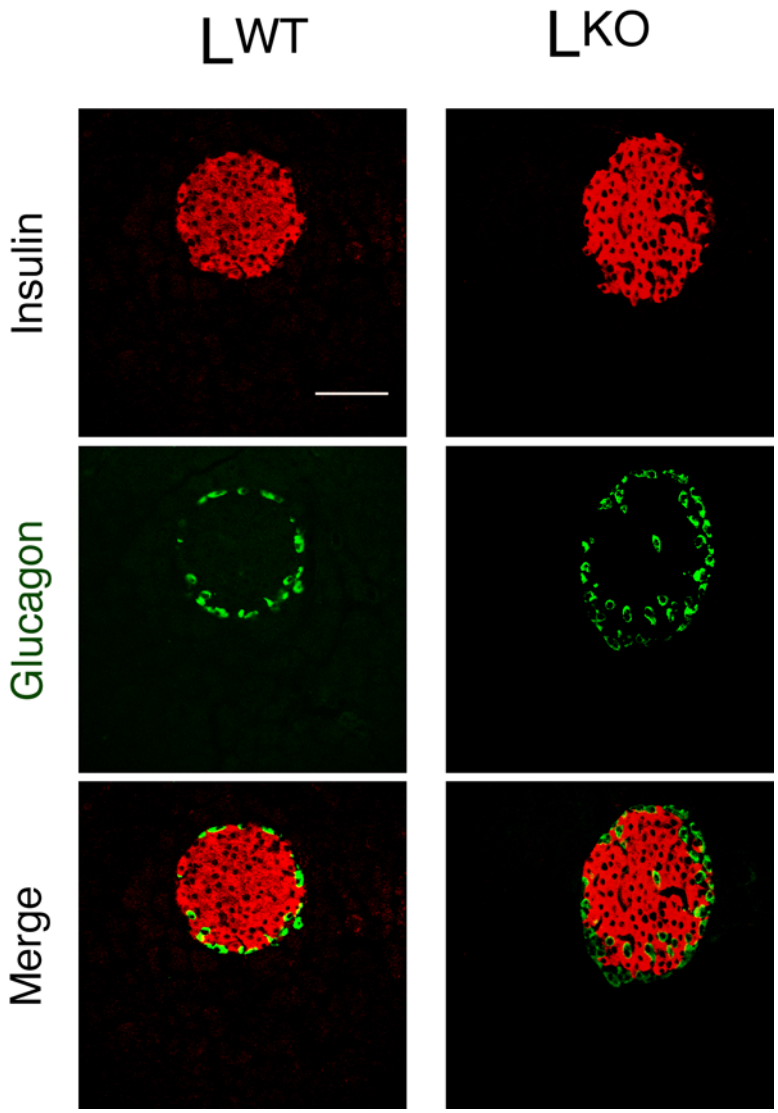

Figure S4. Morphology of pancreatic islets.

Mice were starved overnight. Sections of pancreas prepared from L^{KO} and L^{WT} mice were stained with antibodies to insulin (red) and glucagon (green). The compensatory increase in secretion by L^{KO} islets (Figure 2) was not associated with substantial islet hypertrophy. Representative images of L^{KO} and L^{WT} islets are presented. Scale bar, 75 µm.

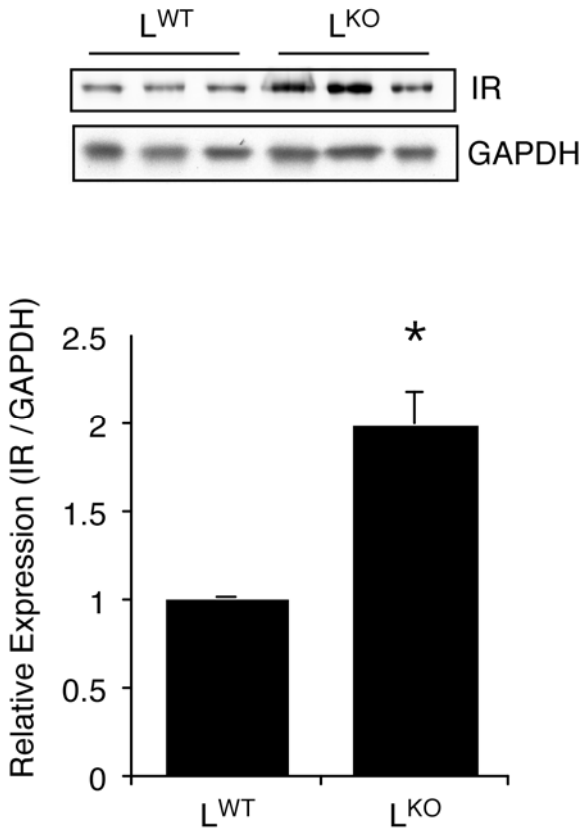

Figure S5. Analysis of hepatic insulin receptor expression in L^{WT} and L^{KO} mice.

L^{KO} and L^{WT} mice (24 weeks old) were maintained on a chow diet and fasted overnight. The amount of insulin receptor β subunit and GAPDH was examined by immunoblot analysis (mean ± SD; n = 6). Statistically significant differences between L^{WT} mice and L^{KO} mice are indicated (*, $P < 0.05$).

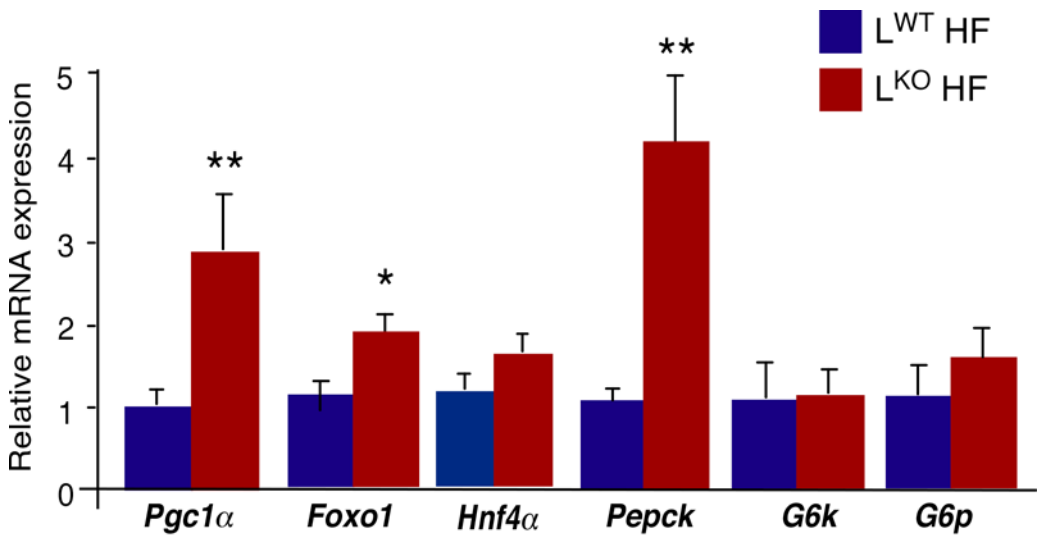

Figure S6. Comparison of the expression of genes associated with gluconeogenesis in the liver of chow-fed L^{WT} and L^{KO} mice.

The expression of the different mRNA in liver fasted over night was examined by quantitative RT-PCR analysis (Taqman©) and is presented as relative mRNA expression (mean ± SD; n = 6). Statistically significant differences between L^{KO} mice and L^{WT} mice are indicated (*, $P < 0.05$; **, $P < 0.01$).

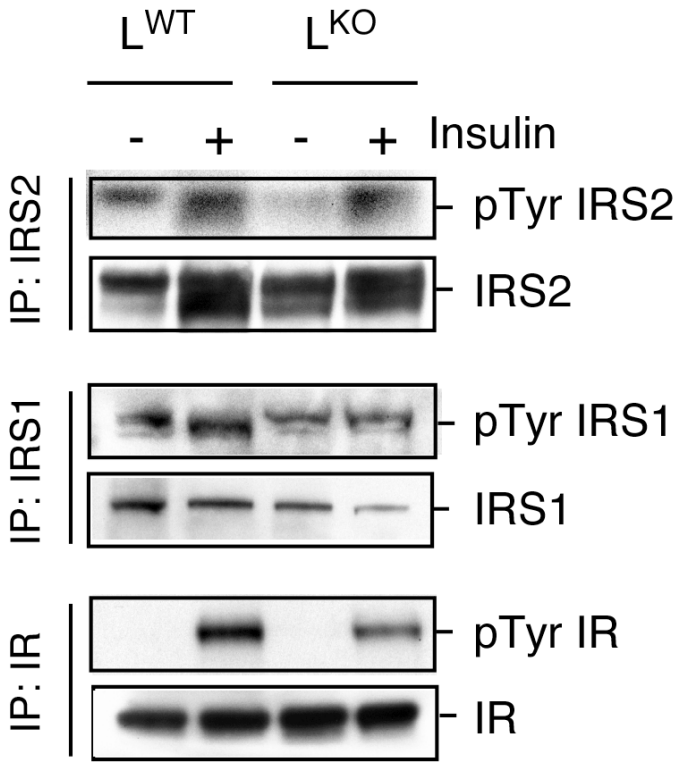

Figure S7. Insulin-stimulated tyrosine phosphorylation in the liver of L^{WT} and L^{KO} mice.

Chow-fed L^{KO} and L^{WT} mice were administered insulin (5 U/ kg body mass) by intraperitoneal injection (10 mins). Liver extracts were examined by immunoblot analysis of immunoprecipitates (IP) using antibodies to phosphotyrosine, IRS1, IRS2, and insulin receptor (IR).

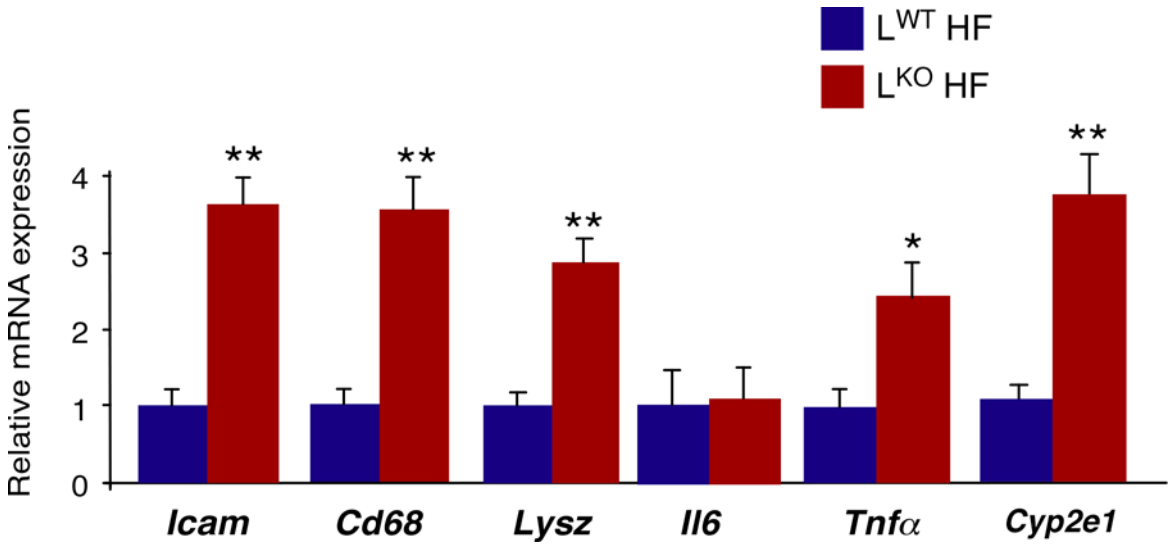

Figure S8. Comparison of the expression of genes associated with inflammation in the liver of chow-fed L^{WT} and L^{KO} mice.

The expression of the different mRNA in liver fasted overnight was examined by quantitative RT-PCR analysis and presented as relative mRNA expression (mean ± SD; n = 6). Statistically significant differences between L^{KO} mice and L^{WT} mice are indicated (*, $P < 0.05$; **, $P < 0.01$).

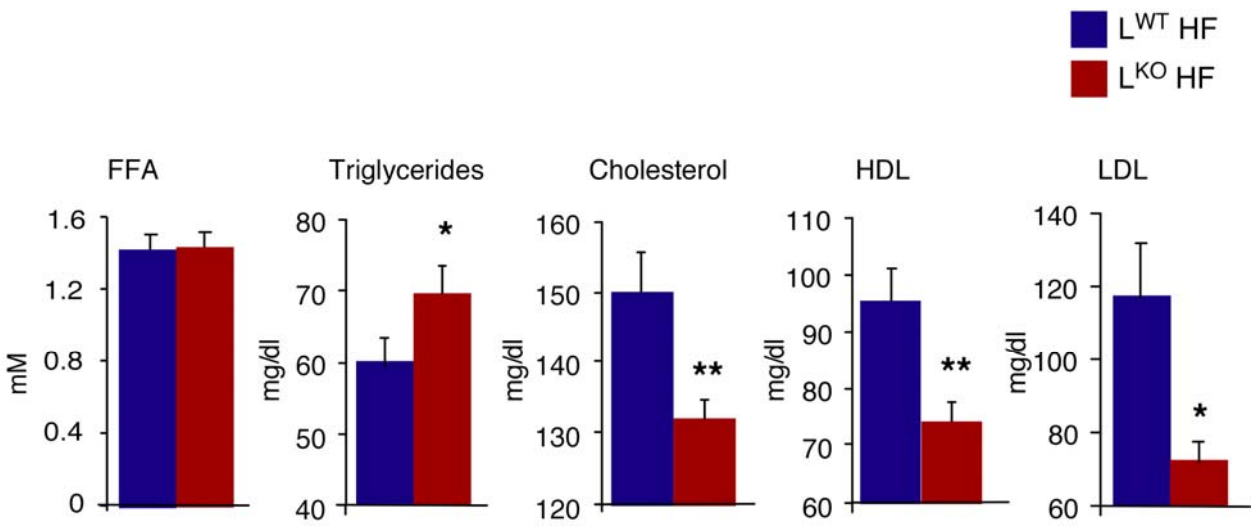

Figure S9. Comparison blood lipids in chow-fed L^{WT} and L^{KO} mice.

Blood lipids were examined in mice fasted overnight (mean ± SD; n = 8). Statistically significant differences between L^{KO} mice and L^{WT} mice are indicated (*, $P < 0.05$; **, $P < 0.01$).

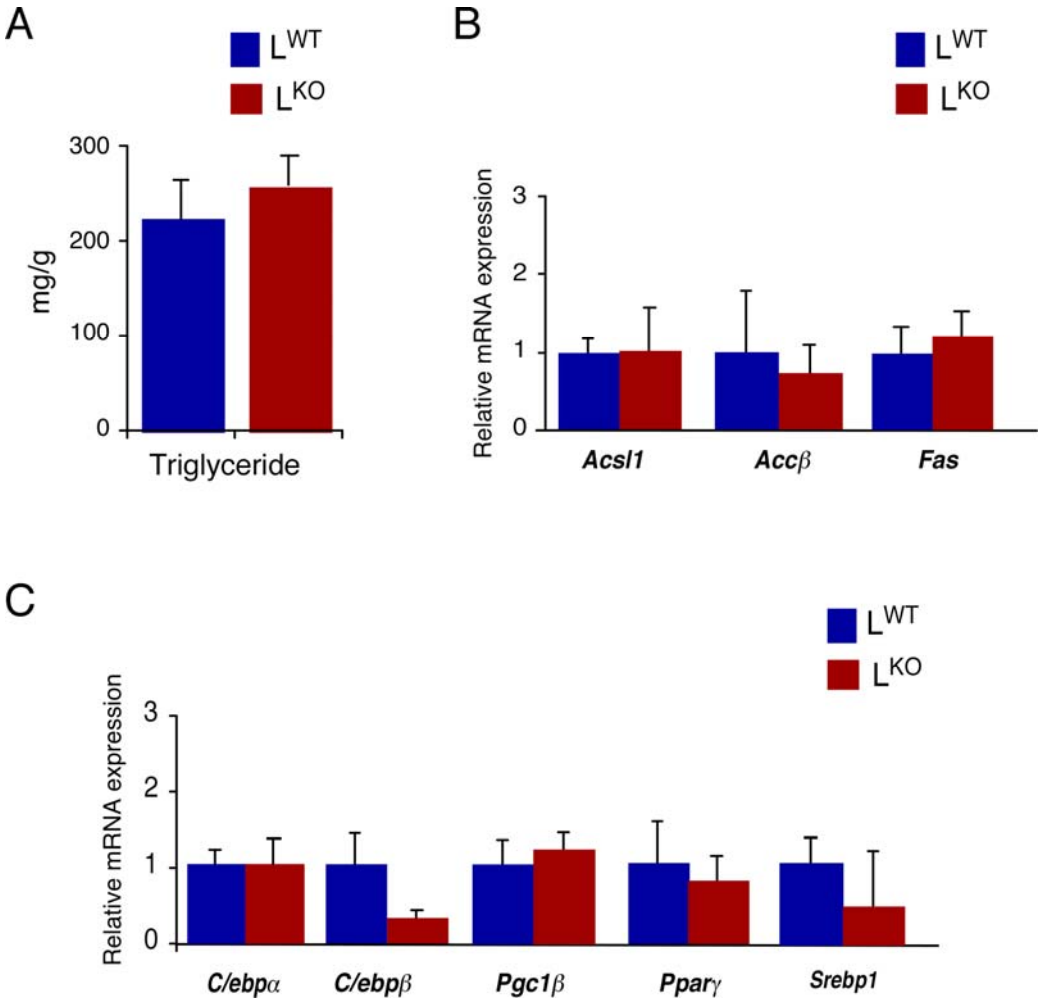

Figure S10. Hepatic triglyceride accumulation in HFD-fed L^{KO} and L^{WT} mice.

(**A**) The amount of hepatic triglyceride was measured in HFD-fed L^{KO} and L^{WT} mice that were fasted overnight (mean ± SD; n = 8). No statistically significant differences between L^{KO} and L^{WT} were detected ($P > 0.05$). (**B**) The expression of genes that encode enzymes that promote lipogenesis in the liver of HFD-fed L^{KO} and L^{WT} mice that were fasted overnight (*Acsl1, acetyl-CoA synthetase long chain family member 1; Accβ, acetyl-CoA carboxylase β*; *Fas*, *Fatty acid synthase*) was measured by quantitative RT-PCR assays of the amount of mRNA and was normalized to the amount of *18S* RNA in each sample (mean ± SD; n = 7-9). No statistically significant differences between L^{KO} and L^{WT} were detected ($P > 0.05$). (**C**) The expression of genes that encode lipogenic transcription factors and co-activators in the liver of HFD-fed L^{KO} and L^{WT} mice that were fasted overnight (*C/ebpα*, *C/ebpβ*, *Pgc1β*, *Pparγ*, and *Srebp1*) was measured by quantitative RT-PCR (Taqman©) assays of the amount of mRNA and was normalized to the amount of *18S* RNA in each sample (mean ± SD; n = 7-9). No statistically significant differences between L^{KO} and L^{WT} were detected ($P > 0.05$).

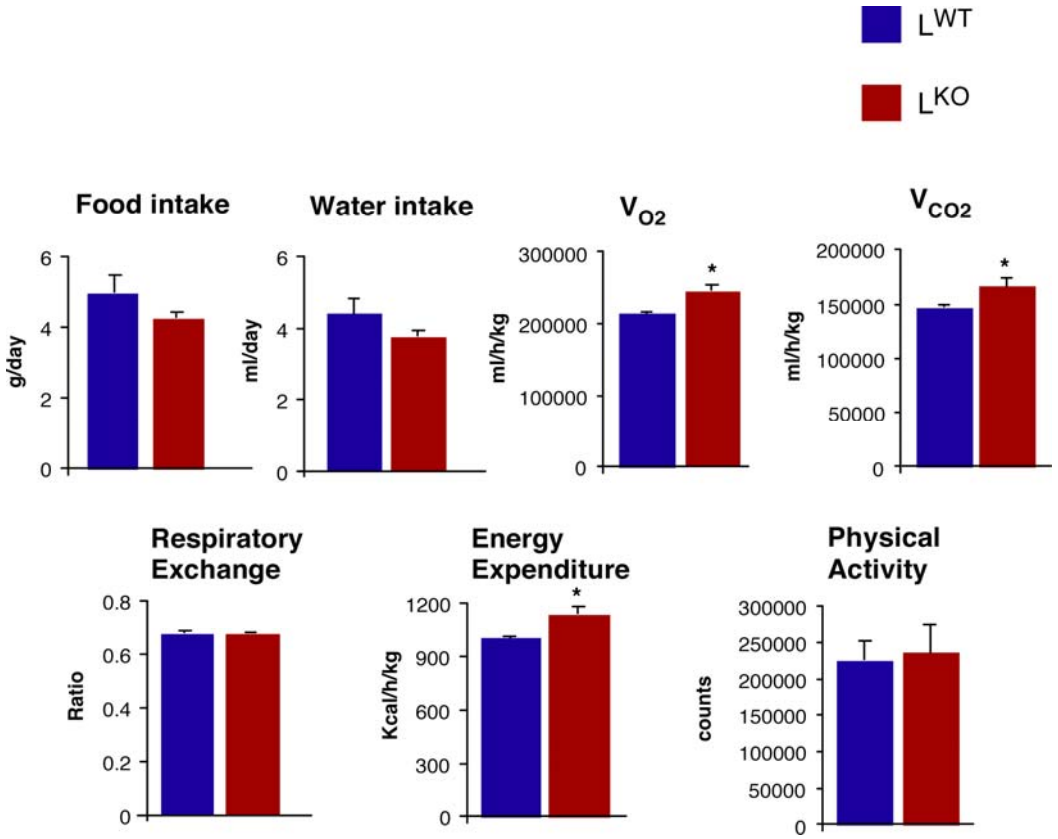

Figure S11. Comparsion of energy balance in chow-fed L^{WT} and L^{KO} mice using metabolic cages.

Groups of 4 mice were examined during a 3 day period to measure the mean food and water consumption, gas exchange (V_{O2} and V_{CO2}), respiratory exchange quotient [V_{CO2}]/[V_{O2}], energy expenditure, and physical activity (mean ± SE; n = 6). Statistically significant differences between L^{KO} mice and L^{WT} mice are indicated (*, $P < 0.05$).

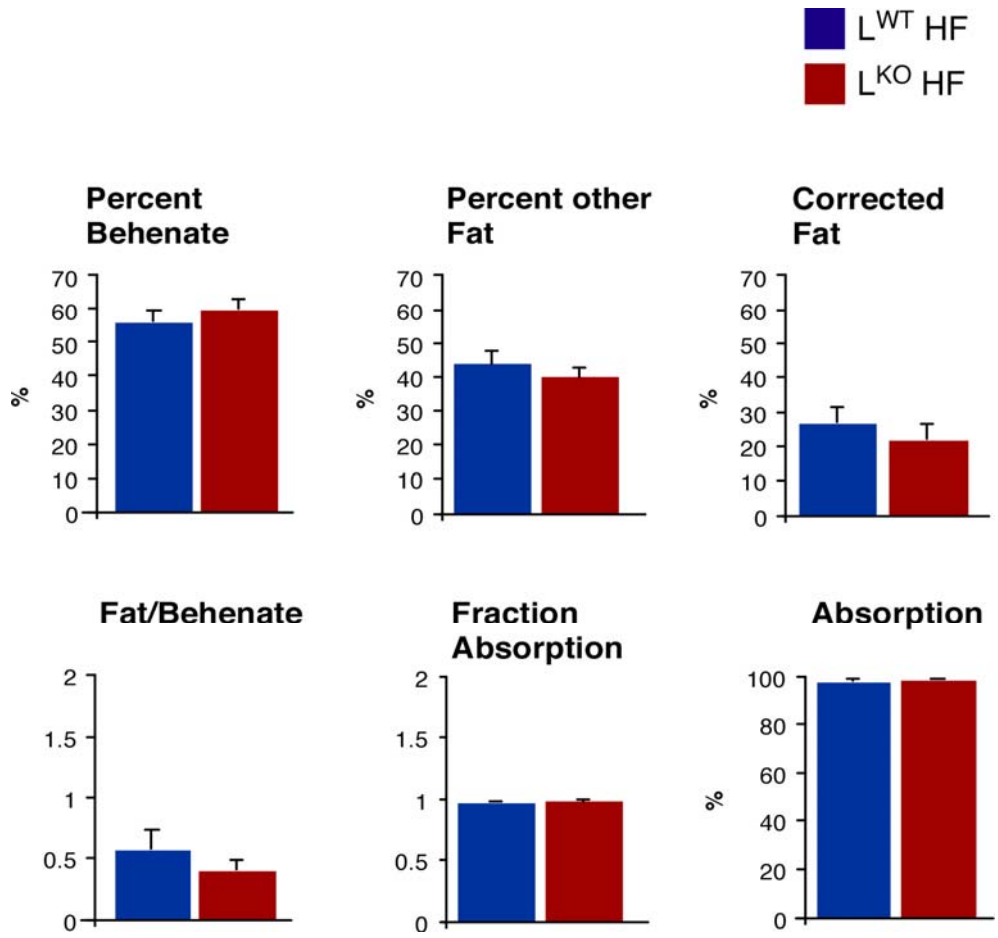

Figure S12. Absorption of dietary fat.

Mice were fed the test diet and fecal pellets were collected on days 4 - 5 were collected. The fatty acid content and composition of excreted lipids were determined by gas chromatography (mean ± SD; n = 10). No statistically significant differences between L^{KO} mice and L^{WT} mice were detected.

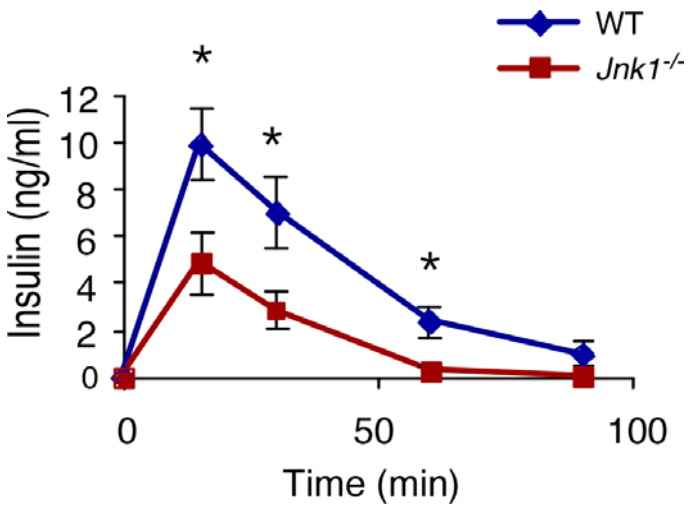

Figure S13. Comparison of insulin clearance between wild-type and *Jnk1*$^{-/-}$ mice.

WT and *Jnk1*$^{-/-}$ mice were maintained in chow diet. Mice were injected intraperitoneally with human insulin (1.5 mU/g). The blood concentration of human insulin was measured at the indicated times (mean ± SD; n = 8).