Supplementary Information

Deletion of SIRT1 from Hepatocytes in Mice Disrupts Lipin-1 Signaling and Aggravates Alcoholic Fatty liver

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Abbreviations used in the paper: ALD, alcoholic liver disease; AH, alcoholic hepatitis, ACC, acetyl CoA carboxylase; ADH, alcohol dehydrogenase; ALDH2, aldehyde dehydrogenase 2, ALT, alanine aminotransferase; AST, aspartate aminotransferase; AOX, acyl-CoA oxidase; β-OHB, beta-hydroxybutyate; CYP7A1, cholesterol 7α-hydroxylase; DAG, diacylglycerol; CPT1a, carnitine palmitoyltransferase 1a; FAS, fatty acid synthase; Fld, fatty liver dystrophic; FOXO1, forkhead transcription factor O 1; Lcn 2, HDAC, histone deacetylase, Lipocalin 2; MDA, malondialdehyde; MCP-1, monocyte chemoattractant protein 1; NALD, non-alcoholic liver disease, NFATc4, nuclear factor of activated T cells c4; NF-κB, nuclear transcription factor-κB; PPAR, peroxisome proliferator-activated receptor; PGC-1α, peroxisome proliferator-activated receptor γ co-activator-1alpha; PA, phosphatidate; SIRT1, sirtuin 1; SREBP-1, sterol regulatory element-binding protein 1; SCD1, stearoyl-coenzyme A desaturase 1; TIMP1, tissue inhibitor of metalloproteinase 1; TNF-α, tumor necrosis factor-alpha; TAG, Triacylglyceride; TGF-β, transforming growth factor beta 1; SMA, Smooth Muscle Actin

Supplementary Material and Methods

Plasmids, antibodies, and reagents. Most chemicals and supplies were purchased from Sigma Chemical (St. Louis, MO), Schleicher and Schuell, GIBCO-BRL, and DuPont NEN Research Products. Plasmids for wild-type SIRT1 (SIRT1wt) and SIRT1 deacetylase domain mutation (SIRT1H363Y) and SIRT1siRNA were purchased from Addgene Inc. Plasmids for SFRS10wt and SFRS10siRNA were purchased from Origene.

Measurement of Hepatic Lipid. Total liver or cellular triglyceride and cholesterol were measured using a colorimetric assay kit from Cayman Chemical.

Histopathological analysis. Formalin-fixed liver samples were processed, and paraffin sections of 4-μm thickness were used for staining assays. Sections of the liver were stained with hematoxylin and eosin (H&E). Hepatic collagen content was analyzed by Sirius red staining of paraffin sections. α-SMA and $F4/80^+$ staining assays were performed by pathology services at H. Lee Moffitt Cancer Center (Tampa, FL 33612) using anti-α-SMA antibody (Abcam Inc. Cambridge, MA) or anti-$F4/80^+$ antibody (Novus Biologicals, Littleton, CO). The paraffin sections were imaged by an Olympus BX53 digital upright microscope. Stained sections were quantified by histomorphometry using NIH imaging software (Image, NIH, Bethesda, Maryland). A minimum of four independent fields were quantified and results were expressed as percentage of area occupied by immunostaining. The H&E-stained slides were evaluated for scoring the inflammation. Scoring of tissue inflammation was performed on a 0–3 scale by a trained pathologist in the same institute (pathology services at H. Lee Moffitt Cancer Center; Tampa, FL33612) through blind review. The scores recorded as degree of inflammation on a 4-digit scale (0, none; 1, mild; 2, moderate; 3, severe).

Biochemical assays. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using kits from BioVision (Mountain View, CA). Plasma fatty acids, triglyceride and cholesterol were determined using the Sigma Diagnostics Triglyceride and Infinity Cholesterol Reagent.

mRNA analysis. Total RNA was prepared from mouse livers or hepatocytes using Trizol extraction. Real-time quantitative polymerase chain reaction (qRT-PCR) was performed in an iCycler Spectrofluorometric thermal cycler (Bio-Rad Laboratories, Hercules, CA). The relative amount of target mRNA was calculated using the comparative cycle threshold (C_t) method by normalizing target mRNA C_t values to those for GAPDH. Primer sets were either purchased or designed (Supplementary Table II).

XBP-1 Splicing Assay. XBP-1 splicing assay was performed as described elsewhere.[1] Briefly, total RNA was prepared from mouse livers using Trizol extraction and the first-strand cDNA was synthesized using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). The primer set for the XBP-1 splicing assay was as follows: 5-ACACGCTTGGGAATGGACAC-3′ and 5'-CCATGGGAAGATGTTCTGGG-3. The primer set encompassing the missing sequences in Xbp1s was used for the PCR amplification with Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Beverly,MA). The PCR

products were separated by electrophoresis on a 3% agarose gel (Bio-Rad Laboratories, Richmond, CA) and visualized by ethidium bromide staining.

Measurement of Hepatic Hydroxyproline Content. Hepatic hydroxyproline content was measured a portion of a liver lobe (>50 mg liver samples) by commercially available kit (BioVision, Mountain View, CA) according to the manufacturer's instruction.

Western Blot Analysis. Western blot analyses were performed using liver cytosolic extracts, nuclear extracts or whole liver extracts separated by 1D gel electrophoresis. SIRT1 antibody is obtained from Cell Signaling. SFRS10, and lipin-1, NFATc4 antibodies were obtained from Santa Cruz, and PGC-1α antibody was obtained from Calbiochem (San Diego, CA). Bip, p-NK, JNK, p-AMPKα and AMPKα were visualized with antibodies (Cell Signaling Technology, Danvers, MA). Polyclonal rabbit anti-Actin β antibody (Sigma) or Lamin A (Santa Cruz) was used to normalize the signal obtained for total liver or nuclear protein extracts.

Liver Nuclear Isolation and Immunofluorescence Imaging Assays. Immunofluorescence imaging using isolated mouse liver nuclei was performed as described.[2]

Patients with Alcoholic Hepatitis (AH) and Selection of normal Control Livers. Patients admitted to the Liver Unit, Hospital Clínic of Barcelona with clinical, analytical and histological features of AH from 2007 to 2010 were prospectively included in the study.[3-5] The inclusion criteria have been previously described.[6] All patients had histological diagnosis of AH (n=34). Patients with hepatocellular carcinoma or any other potential cause of liver disease were excluded from the study. Liver biopsy was obtained using a transjugular approach. As controls, fragments of normal liver tissue (n=6) were obtained from optimal cadaveric liver donors (n=3) or resection of liver metastases (n=3) as described in detail previously.[6] All liver specimens were analyzed by an expert pathologist and a part of the biopsy was submerged into a RNA stabilization solution (RNAlater, Ambion, Austin, Texas, USA). The protocol was approved by the Ethics Committee of the Hospital Clinic and all patients gave informed consent.

Real-time PCR Analysis of human liver samples. Quantitative real-time PCR reactions for human liver samples were carried out in a StepOnePlus™ Real-Time PCR System using commercial primer-probe pairs (Applied Biosystems, Foster City, California, USA). mRNA levels for human LPIN1α, LPIN1β, SIRT1 and SFRS10 were measured (Supplementary Table II). 18S RNA was used as the endogenous control. Gene expression values were calculated based on the ΔΔCt method. The results were expressed as $2^{-\Delta\Delta Ct}$ referred as fold increase compared with the mean expression quantified on normal livers.

In vitro experiments. Mouse AML-12 hepatocytes (American Type Culture Collection, Manassas, VA) were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomysin, 63 μg/ml penicillin G, 0.1 μM dexamethasone, and insulin-transferrin-selenium (ITS; Gibco-BRL). Transfection studies were performed using lipofectamin 2000 Reagent (Invitrogen) according to the manufacturer's protocol.

Supplementary References

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Table S1. Selected parameters in WT and Sirt1LKO mice following chronic-binge ethanol administration.

8-12 week-old male WT and Sirt1LKO (LKO) mice were divided into 4 groups as follows: (**1**) low fat pair-fed WT control (WT); (**2**) ethanol-fed WT ethanol-containing diet (5% w/v ethanol); (**3**) Sirt1LKO control; (**4**) ethanol-fed Sirt1LKO (5% w/v ethanol). Ethanol groups were fed for 10 days. At day 11, mice in ethanol groups were gavaged a single dose of ethanol (5 g/kg body weight, 20% ethanol), while mice in control groups were gavaged an isocaloric dose of dextrin maltose. The mice were euthanized and blood and tissue samples were collected 9 h post-gavage. The animals were sacrificed after 4-wks. MDA, malondialdehyde; NEFA, non-esterified fatty acids.

Parameters	**WT**	**WT+EtOH**	**LKO**	**LKO+EtOH**
Starting Body (g)	28.9±0.7^{a}	30.2±1.8^{a}	28.8±0.9^{a}	28.4±1.6^{a}
Final Body (g)	30.2±1.3^{a}	31.4±2.5^{a}	29.9±1.6^{a}	29.4±1.5^{a}
Liver (g)	1.4±0.3^{b}	1.8±0. 3^{a}	1.4±0.2^{b}	1.9±0.4^{a}
Liver/body (%)	4.5±0.8^{b}	5.6±1.0 ^{ab}	4.6±0.4^{b}	6.3±1.4^{a}
Liver Pyruvate (pmol/mg protein)	181.0±9.3 ^{a}	90.5±14.1 ^{c}	147.9±20.6 ^{b}	69.6±8.1 ^{d}
Liver Lactate (pmol/mg protein)	43.1±1.9 ^{c}	55.0±3.0 ^{b}	47.5± 5.6 ^{c}	70.8±3.9 ^{a}
Liver MDA (nmol/mg protien)	152.3±10.9 ^{c}	167.4±19.7 ^{c}	242.1±28.8 ^{b}	814.6±57.3 ^{a}
Plasma				
NEFA (mg/dL)	330.5±12.3^{a}	326.4±7.3^{a}	325.2±25.5^{a}	331.5±24.6^{a}
Triglycerides (mg/dL)	94.1±7.9^{b}	156.8±16.3^{a}	76.9±7.8^{c}	143.3±22.2^{a}
Cholesterol (mg/dL)	88.3±4.5^{c}	98.6±4.6^{b}	116.4±11.5^{a}	123.4±7.7^{a}

Results are expressed as means±SEM of 4-8 mice. Means without a common letter differ, $p<0.05$.

Table S2. Primers used in this study

Target Gene	Direction	Sequence (5'-3')
ACC	Forward	TGAGGAGGACCGCATTTATC
	Reverse	GAAGCTTCCTTCGTGACCAG
AOX	Forward	TGGTATGGTGTCGTACTTGAATGAC
	Reverse	AATTTCTACCAATCTGGCTGAAC
CD36	Forward	ATGGGCTGTGATCGGAACTG
	Reverse	GTCTTCCCAATAAGCATGTCTCC
CD68	Forward	TGTCTGATCTTGCTAGGACCG
	Reverse	GAGAGTAACGGCCTTTTTGTGA
CHOP	Forward	CTGGAAGCCTGGTATGAGGAT
	Reverse	CAGGGTCAAGAGTAGTGAAGGT
Collagen-1α	Forward	ACCTCAAGATGTGCCACTC
	Reverse	TGCTCTCTCCAAACCAGAC
CPT-1α	Forward	CTCCGCCTGAGCCATGAAG
	Reverse	CACCAGTGATGATGCCATTCT
FAS	Forward	GGAGGTGGTGATAGCCGGTAT
	Reverse	TGGGTAATCCATAGAGCCCAG
F4/80	Forward	TGACTCACCTTGTGGTCCTAA
	Reverse	CTTCCCAGAATCCAGTCTTTCC
Fibronectin	Forward	GCTCAGCAAATCGTGCAGC
	Reverse	CTAGGTAGGTCCGTTCCCACT
GAPDH	Forward	CTTCACCACCATGGAGAAGGC
	Reverse	GGCATGGACTGTGGTCATGAG
Lipin-1	Forward	CCCTCGATTTCAACGTACCC
	Reverse	GCAGCCTGTGGCAATTCA
Lipin-1α	Forward	GGTCCCCCAGCCCCAGTCCTT
	Reverse	GCAGCCTGTGGCAATTCA
Lipin-1β	Forward	CAGCCTGGTAGATTGCCAGA
	Reverse	GCAGCCTGTGGCAATTCA
Lipocalin-2	Forward	TGGCCCTGAGTGTCATGTG
	Reverse	CTCTTGTAGCTCATAGATGGTGC
MCP-1	Forward	TTAAAAACCTGGATCGGAACCAA
	Reverse	GCATTAGCTTCAGATTTACGGGT
Mip-1á	Forward	TTCTCTGTACCATGACACTCTGC
	Reverse	CGTGGAATCTTCCGGCTGTAG
PGC-1α	Forward	TTGCTAGCGGTTCTCACAGA
	Reverse	GGCTCTTCTGCCTCCTGA
PPARα	Forward	AGAGCCCCATCTGTCCTCTC
	Reverse	ACTGGTAGTCTGCAAAACCAAA
PPARγ	Forward	CATTTGTATGACTCATACATAAAGT
	Reverse	CGGATGGCCACCTCTTTGCTCTG
SAA-1	Forward	GCGAGCCTACACTGACATGA

	Reverse	TTTTCTCAGCAGCCCAGACT
SCD1	Forward	CCTCATCATTGCCAACACCAT
	Reverse	AGCCAACCCACGTGAGAGAA
Sfrs10	Forward	GTGGACAACCTGACCTACCG
	Reverse	TCCTTGGTGTAGCGATCCC
SIRT1	Forward	ATCGGCTACCGAGACAAC
	Reverse	GTCACTAGAGCTGGCGTGT
SREBP-1	Forward	GATGTGCGAACTGGACACAG
	Reverse	CATAGGGGGCGTCAAACAG
Timp-1	Forward	TGCCTGCTGCGATTACAACC
	Reverse	GGAATGGTGTGGTGATGCATGG
TNF-α	Forward	CTGAACTTCGGGGTGATCGG
	Reverse	GGCTTGTCACTCGAATTTTGAGA
XBP-1	Forward	AGCAGCAAGTGGTGGATTTG
	Reverse	GAGTTTTCTCCCGTAAAAGCTGA

Supplementary Figure Legend

Figure S1. **Effects of ethanol feeding in WT and Sirt1LKO mice.** Sirt1LKO (LKO) mice were fed chronically ethanol containing diets for 10 days followed by single gavage of ethanol. WT Control mice were pair-fed control diets without ethanol for 10 days followed by single gavage of maltose. (**A**) Representative Western blot analysis of SIRT1. (**B**) Serum ALT levels. (**C**) Serum AST levels.

Figure S2. Liver-specific deletion of SIRT1 leads to liver fibrosis in ethanol-fed mice. Mice were fed as described in Figure S1. Relative hepatic mRNA levels of CD11b, CD68 and Fibronectin. Results are expressed as means±SEM (n=4-8 mice). Means without a common letter differ, $p< 0.05$.

Figure S3. Liver-specific deletion of SIRT1 leads to exacerbated liver injury and inflammation responses in ethanol-fed mice. Mice were fed as described in Figure S1. (**A**) Relative hepatic mRNA levels of TNF-α, MCP-1, Mip-1α and F4/80^{+}. (**B**) Relative liver mRNA levels of lipocalin-2 and SAA1. (**C**) Semiquantification of histological inflammation. The scores recorded as degree of inflammation on a 4-digit scale (0, none; 1, mild; 2, moderate; 3, severe). Results are expressed as means±SEM (n=4-8 mice). Means without a common letter differ, $p< 0.05$

Figure S4. **Liver-specific deletion of SIRT1 promotes ethanol-mediated impairment of lipin-1 signaling in mice.** Mice were fed as described in Figure S1. (**A**) Relative liver mRNA levels of total (T) lipin-1, lipin-1α, lipin-1β, and SFRS10. (**B**) Relative liver SFRS10 protein levels. (**C**) Relative hepatic lipin-1 protein levels. Results are expressed as means±SEM (n=3-8 mice). Means without a common letter differ, $p< 0.05$

Figure S5. Liver-specific deletion of SIRT1 augments ethanol-mediated activation of SREBP-1 signaling in mice. Mice were fed as described in Figure S1. (**A**) Representative Western blot analysis of hepatic nuclear SREBP-1(nSREBP-1) protein levels. (**B**) Relative nSREBP-1 protein levels. (**C**) Isolated mouse liver nuclei of mice with immunofluorescence for SREBP-1 (green) and DAPI (blue). Original magnification: ×200. (**D** Relative mRNA expression levels of SREBP-1, ACC, SCD1, FAS. Results are expressed as means±SEM (n=3-8 mice). Means without a common letter differ, $p< 0.05$

Figure S6. Liver-specific deletion of SIRT1 exacerbates ethanol-mediated suppression of PGC-1α signaling in mice. Mice were fed as described in Figure S1. (**A**) Representative Western blot analysis of hepatic PGC-1α protein levels. (**B**) Relative PGC-1α protein levels. (**C**) Relative mRNA expression levels of PGC-1α. AOX, CPT1α, CD36, PPARα, and PPARγ. Results are expressed as means±SEM (n=4-8 mice). Means without a common letter differ, $p< 0.05$

Figure S7. Liver-specific deletion of SIRT1 promotes ethanol-mediated elevated XBP-1 mRNA splicing in mice. Mice were fed as described in Figure S1. (**A**) Splicing analysis of

XBP-1. (**B**) Relative hepatic mRNA levels of sXBP1. (**C**) Representative Western blots analysis of sXBP1. (**D)** Relative hepatic sXBP1 protein levels. Results are expressed as means±SEM (n=4-8 mice). Means without a common letter differ, $p< 0.05$

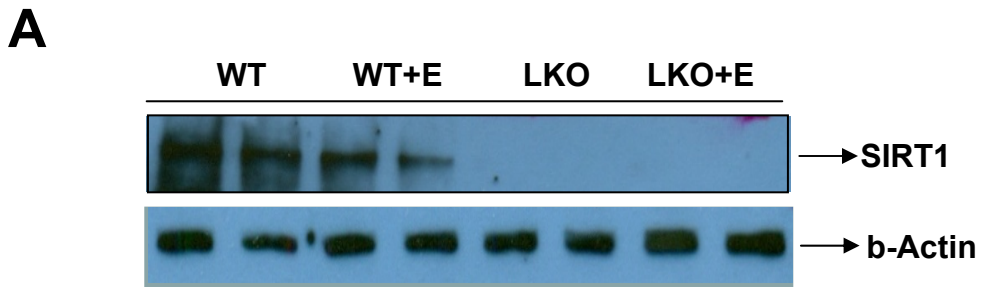
Figure S8. Liver-specific deletion of SIRT1 promotes ethanol-mediated elevated ER stress in mice. Mice were fed as described in Figure S1. (**A**) Hepatic relative mRNA levels of CHOP and XBP-1. (**B**) Representative Western analysis of Bip. P-JNK, and JNK. (**C)** Hepatic relative Bip protein levels. (**D**) Hepatic relative p-JNK/JNK levels. Results are expressed as means±SEM (n=4-8 mice). Means without a common letter differ, $p< 0.05$

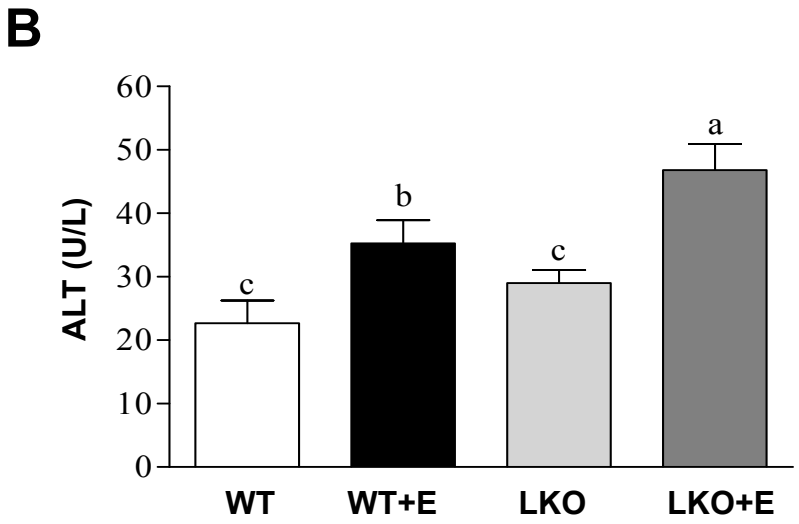
Figure S9. Ethanol induces TG accumulation via SIRT1 inhibition in AML-12 hepatocytes. AML-12 cells were transfected with control vector, SIRT1wt, SIRT1H363Y, SIRT1siRNA, SFRS10wt, or SFRS10siRNA. 48 h after transfection, ethanol (50 mM) was added. After incubation for 24 h, cellular TG content was measured by Nile Red Staining. All data are means±SEM from at least 3 experiments. Means without a common letter differ, $p< 0.05$.

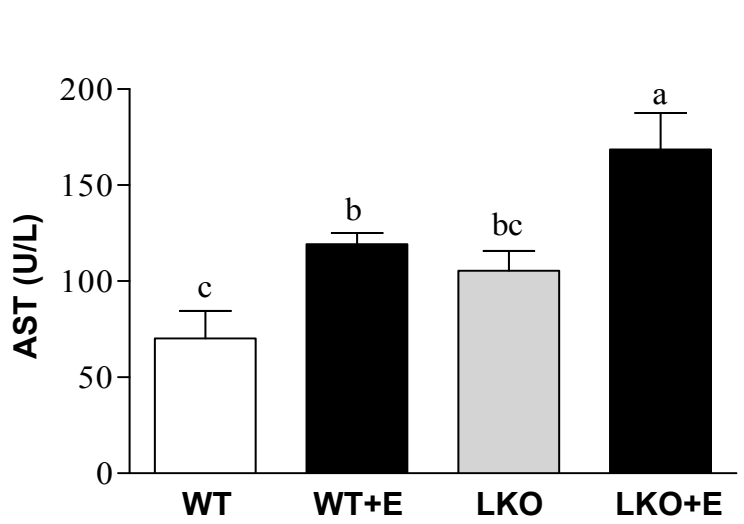
Figure S10. Ethanol induces TG accumulation via SIRT1-SFRS10 axis inhibition in AML-12 hepatocytes. (**A**) AML-12 cells were transfected with control vector, SIRT1wt, SIRT1H363Y, SIRT1siRNA, SFRS10wt, or SFRS10siRNA. 48 h after transfection, ethanol (50 mM) was added. After incubation for 24 h, cellular TG content was measured by a cellular enzymatic kit. (**B**) Hepatic *Lpin 1*β/α ratio. All data are means±SEM from at least 3 experiments. Means without a common letter differ, $p< 0.05$.

Figure S11. Ethanol perturbs *Lpin 1* alternative splicing via SIRT1-SFRS10 axis in AML-12 hepatocytes. AML-12 hepatocytes were treated as described in Figure S10. Relative mRNA levels of lipin-1, lipin-1α, lipin-1β and SFRS10. All data are means±SEM from at least 3 experiments. Means without a common letter differ, $p< 0.05$.

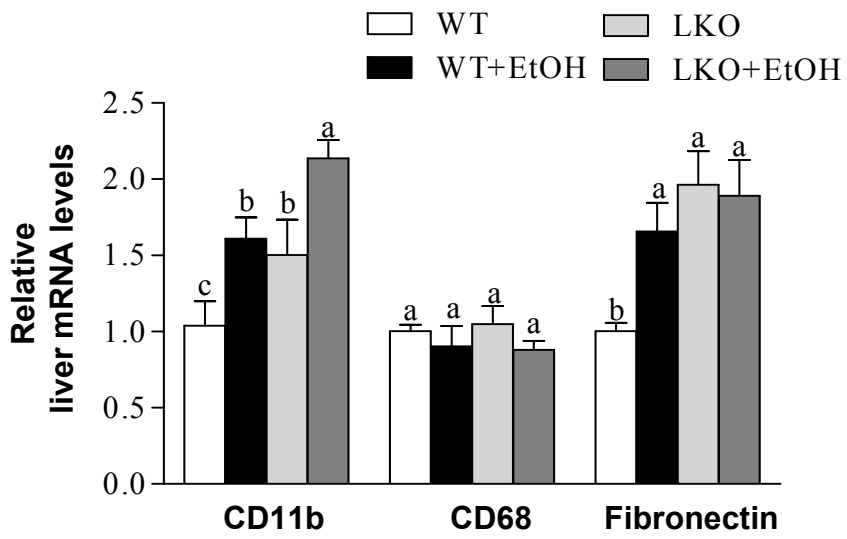
Figure S12. Effects of ethanol feeding in WT and Sirt1LKO mice. (**A**) Western blots analysis of p-AMPK, AMPK and p-ACC. (**B**) Relative hepatic miR-217 levels. (**C**) Western blots analysis of NFATc4 from freshly prepared RAW 264.7 macrophages and AML-12 hepatocytes. Results are expressed as means±SEM (n=3-8 mice). Means without a common letter differ, $p< 0.05$







Supplementary Figure 1



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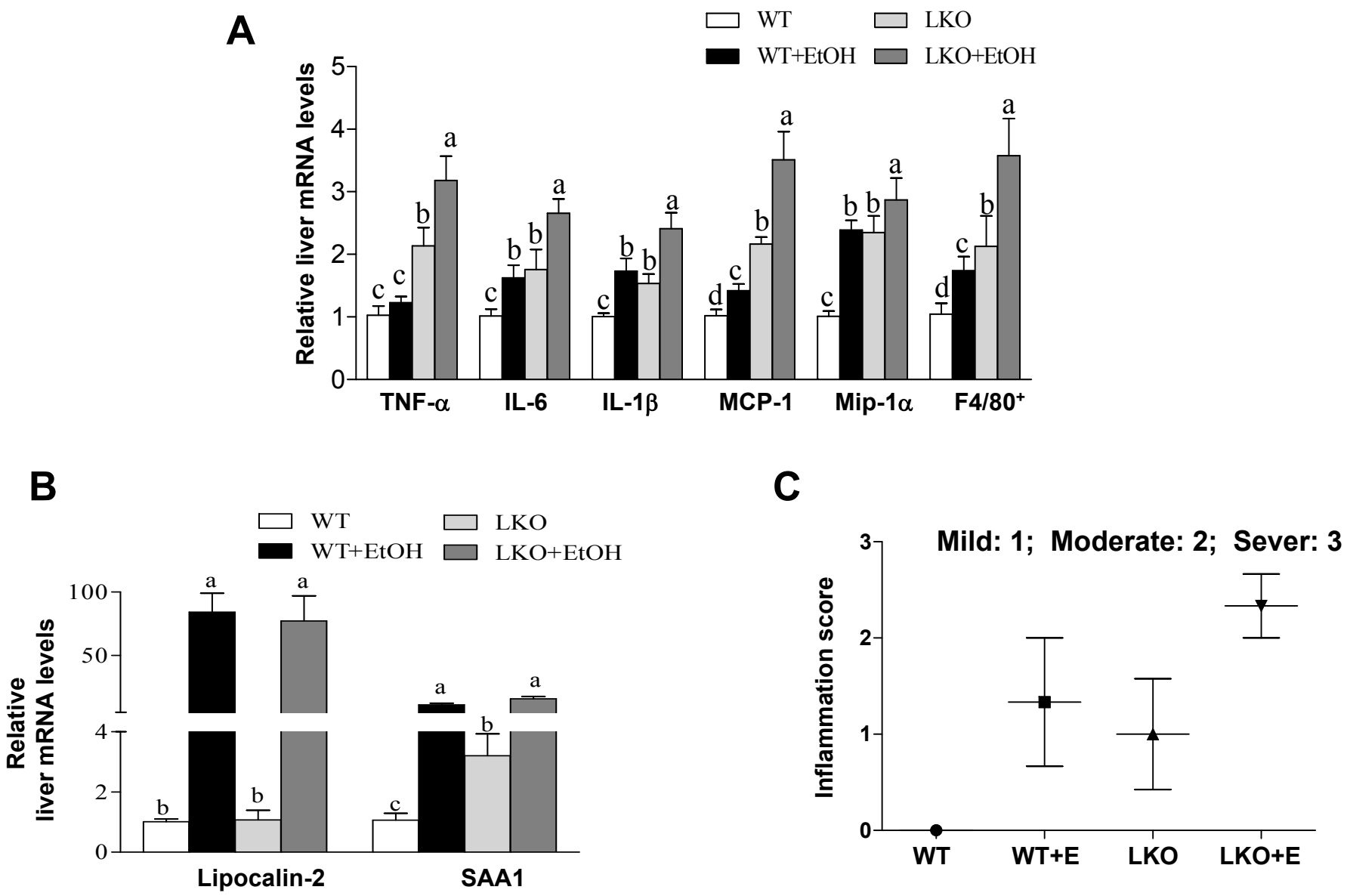

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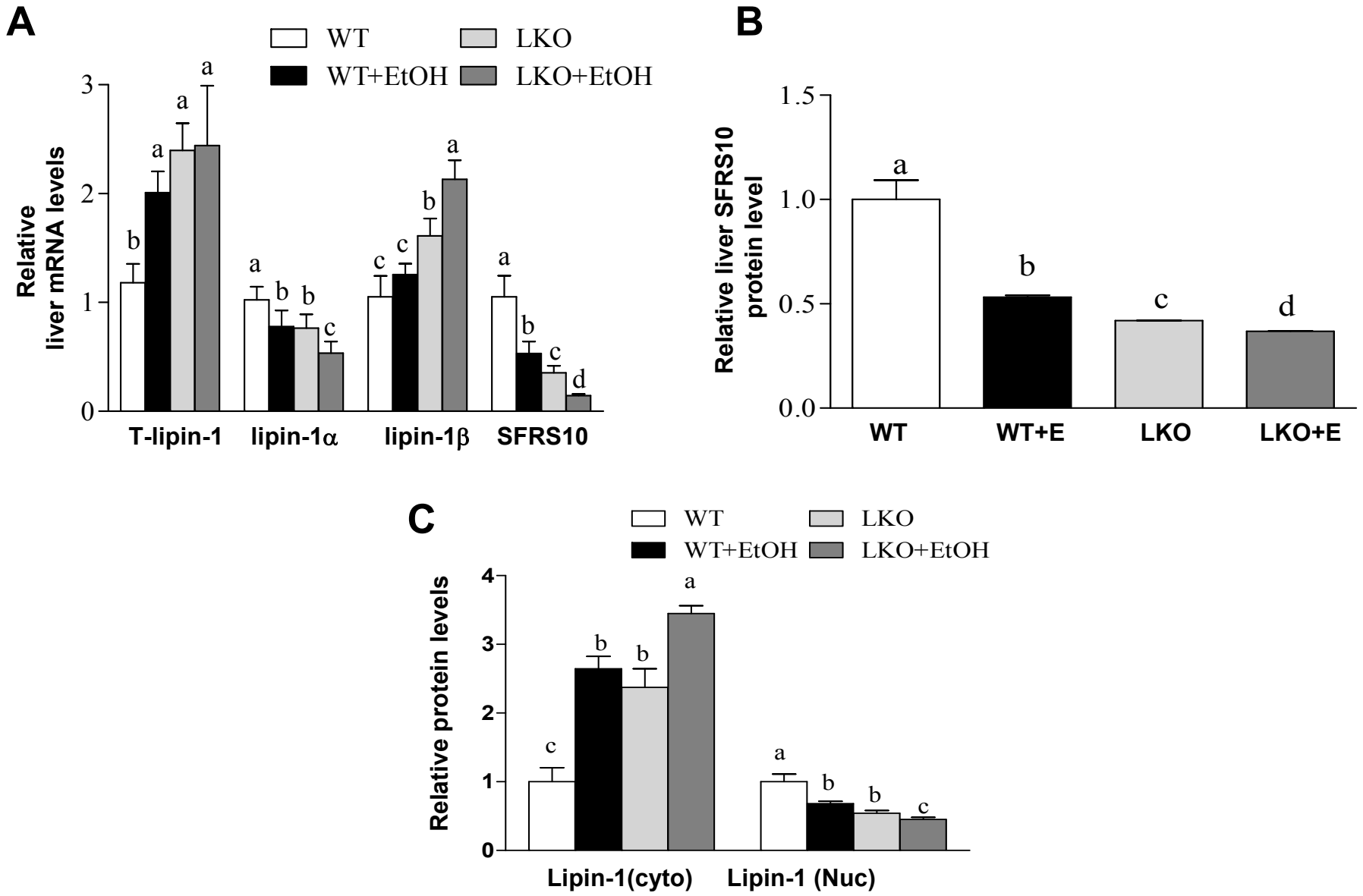

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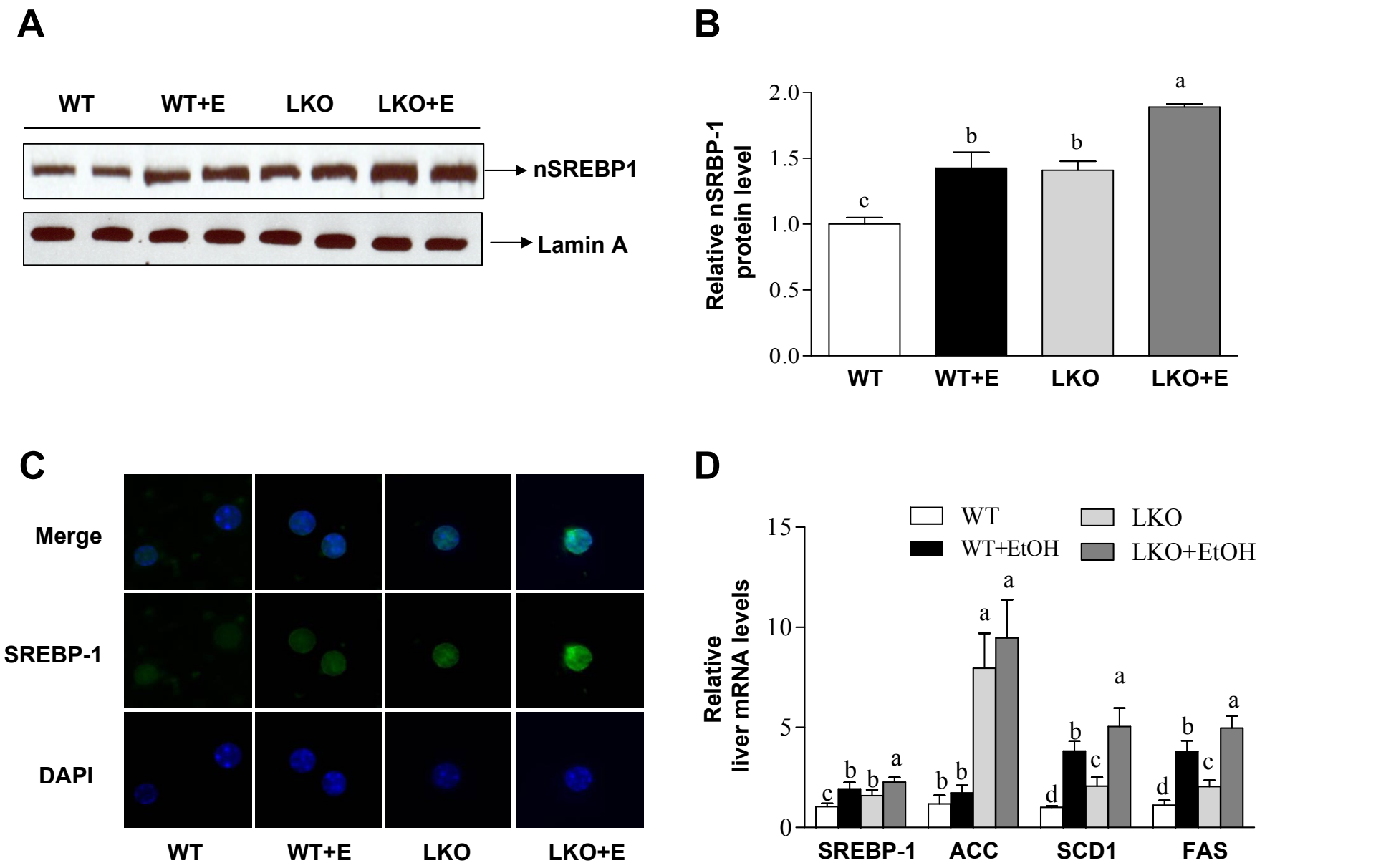

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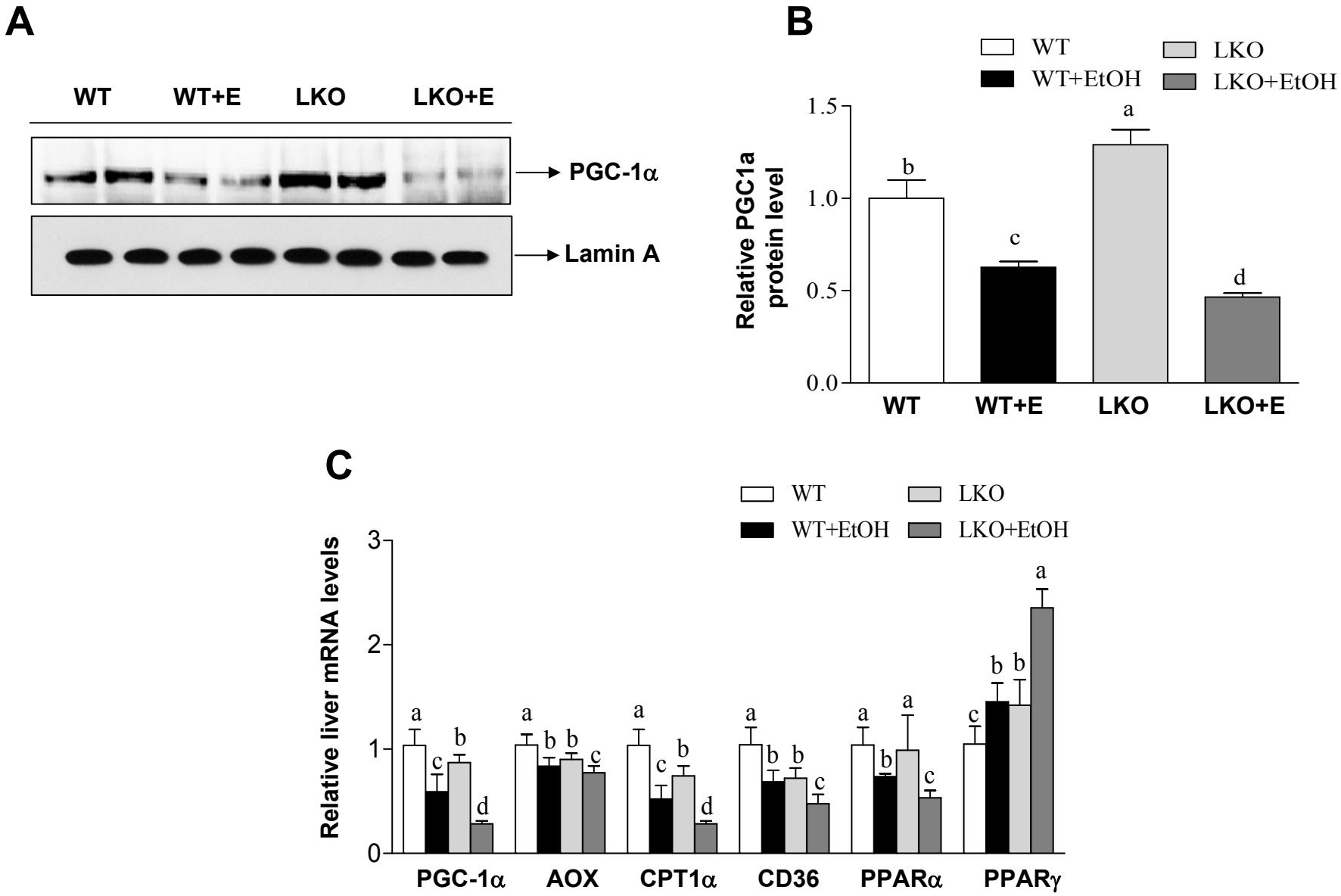

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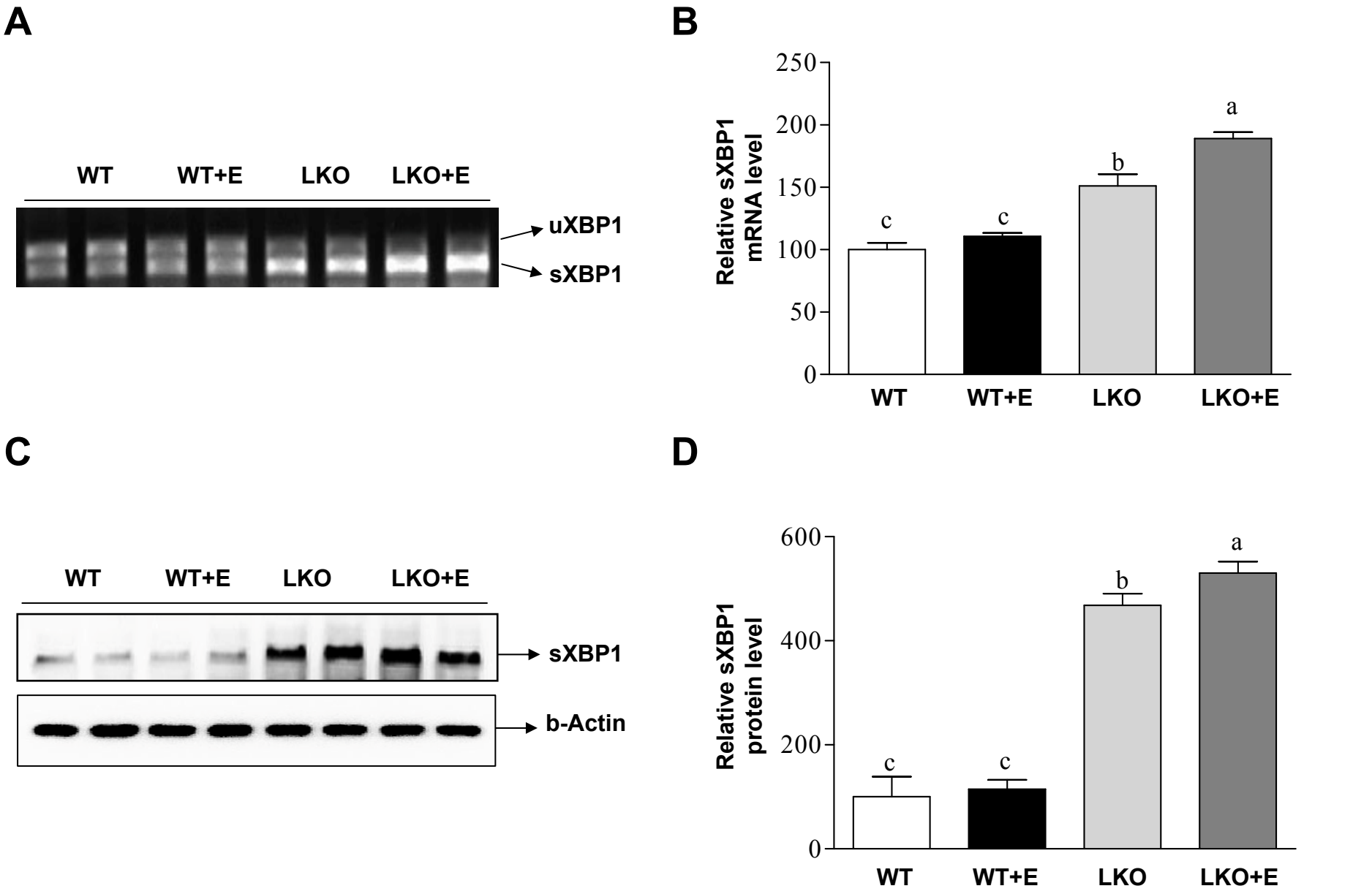
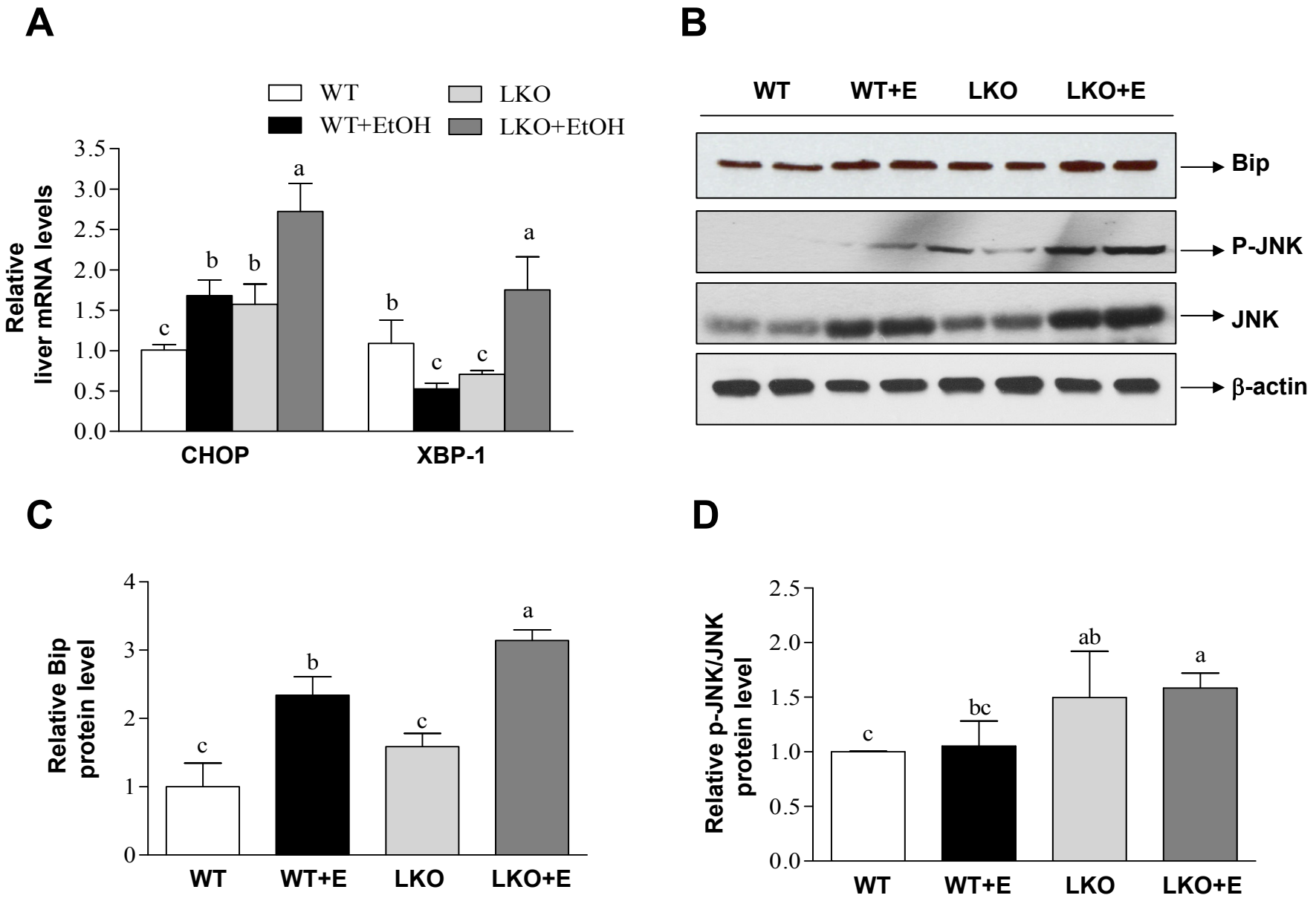


Supplementary Figure 7



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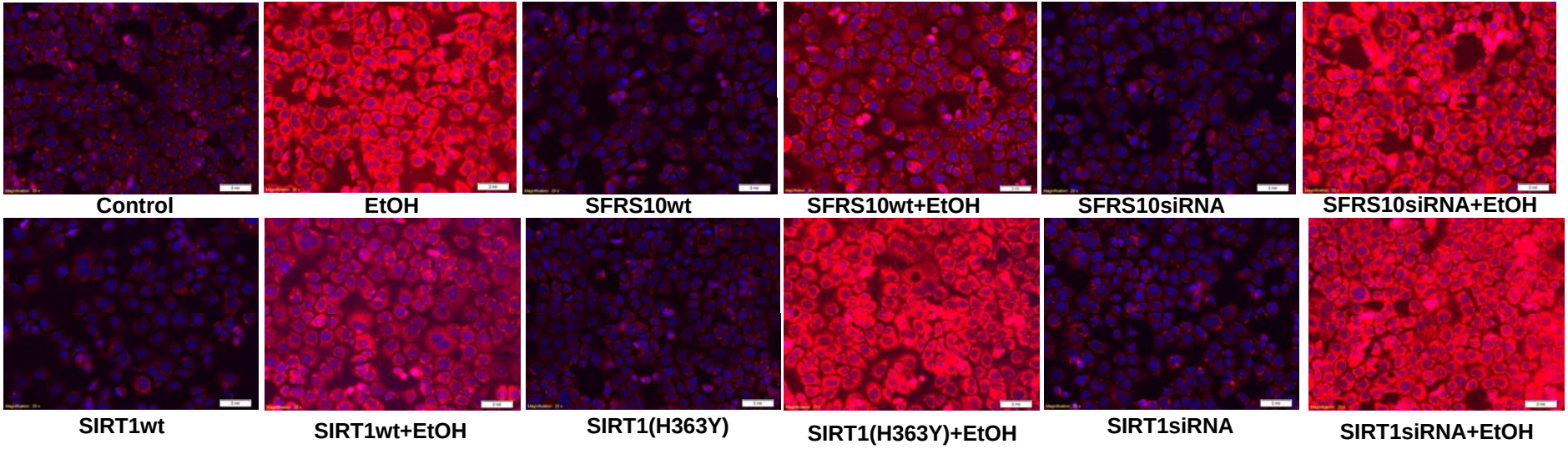
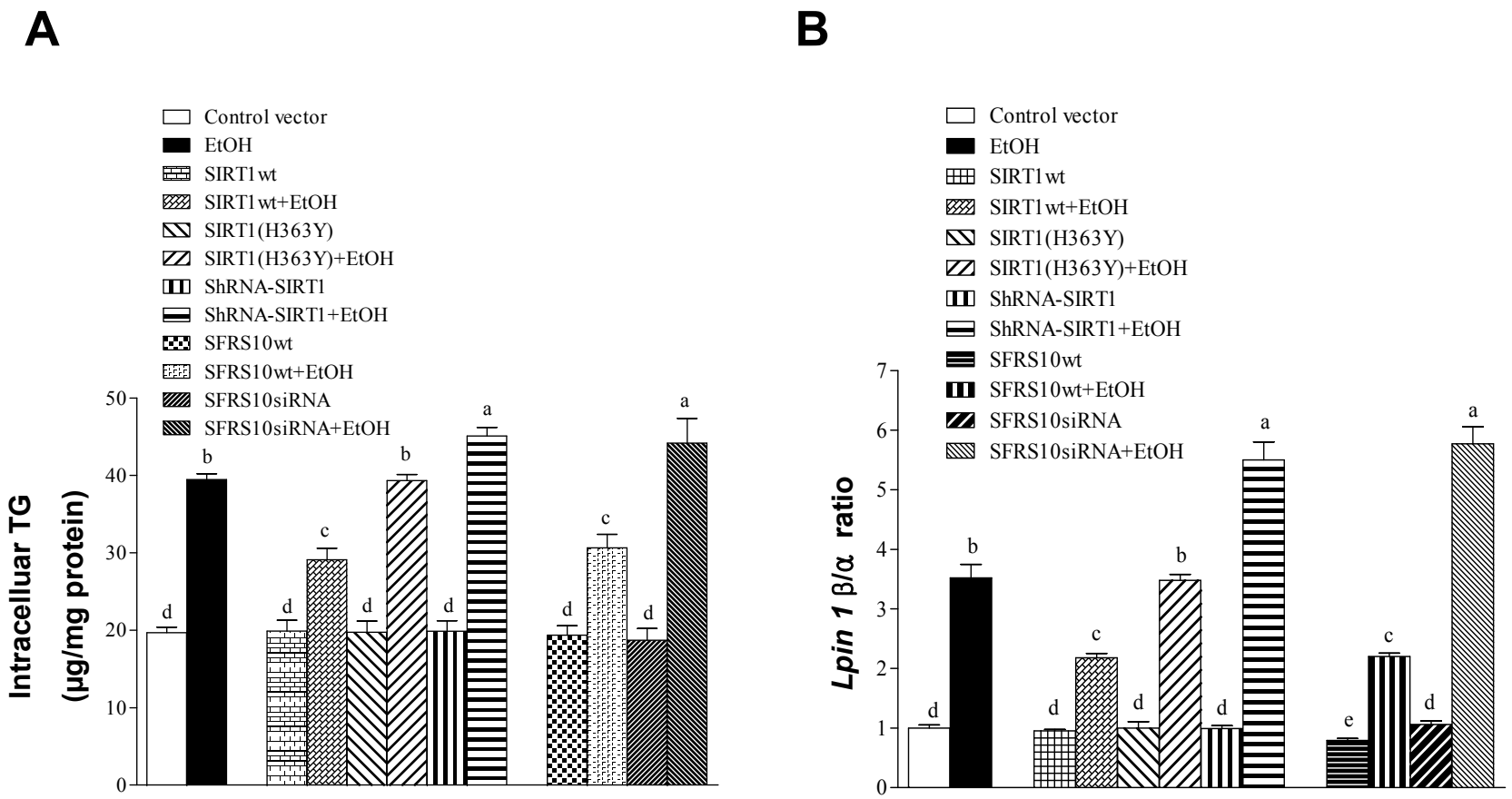
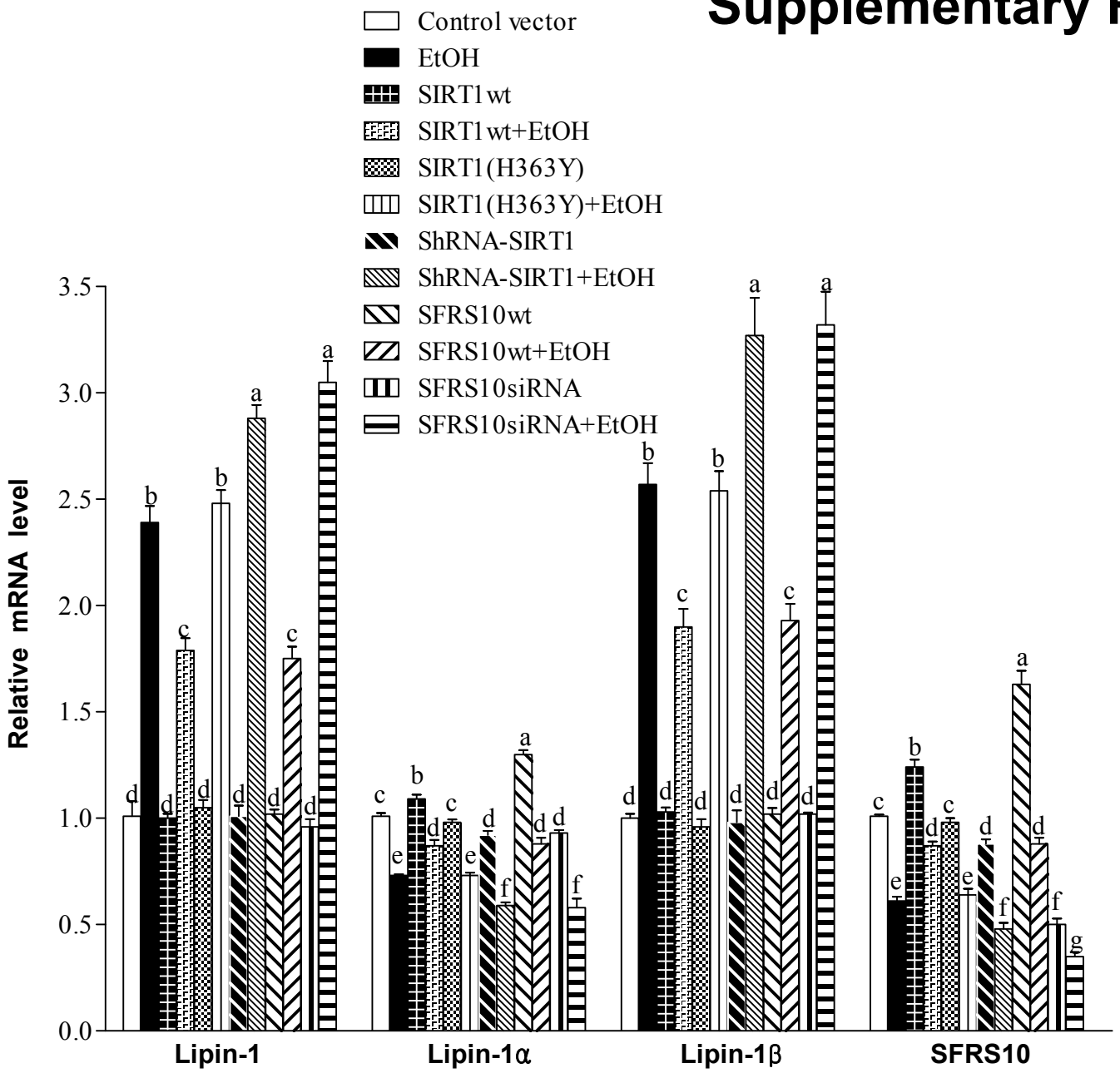


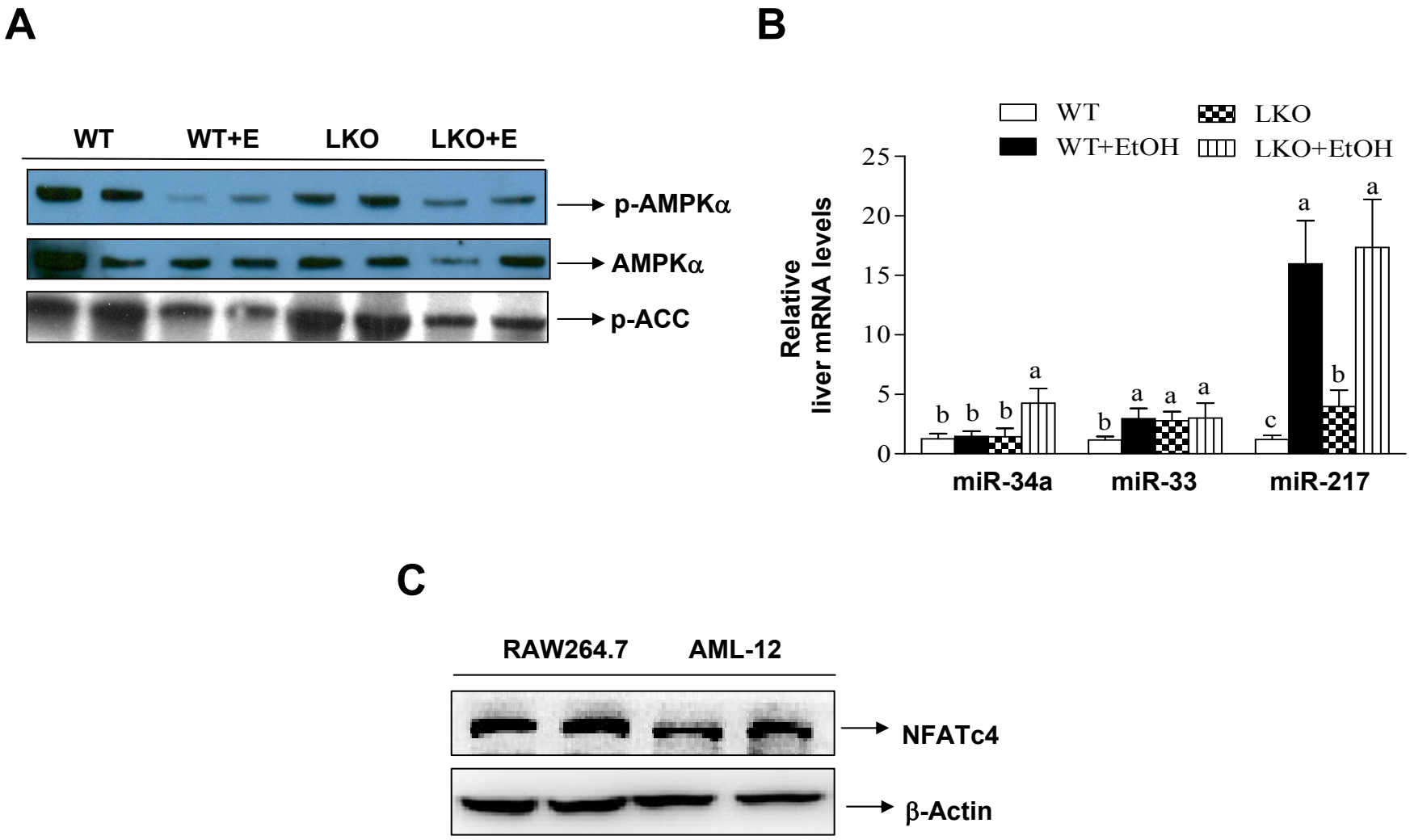
Supplementary Figure 9



Supplementary Figure 10

Supplementary Figure 11





Supplementary Figure 12