Supplementary Materials and Methods

Ethics statement

Human specimens used in this study were taken from patients with cholecystitis who underwent liver biopsy at the First Affiliated Hospital of Guangzhou University of Chinese Medicine. Written informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the ethics committees of the First Affiliated Hospital of Guangzhou University of Chinese Medicine (NO. K [2019]082). In the field of animal experiments, all animal care and experiments were conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health (China), and approved by the Ethics Committee of Guangzhou University of Chinese Medicine (20190508002).

Animals and experimental design

Male C57BL/6J mice, C57BL/KsJ mice, *ob/ob* mice and *db/db* mice aged 6-8 weeks were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). Klf16$^{fl/fl}$ mice were generated by the CRISPR/Cas9 system. The gRNA to mouse Klf16 gene, the donor vector containing 2 loxP sites flanked exon1, and Cas9 mRNA were co-injected into fertilized mouse eggs to generate targeted conditional knockout offspring. F0 founder animals were identified by PCR followed by sequence analysis, and bred with wildtype mice to generate germline transmission F1 founders. F1 founders were genotyped by tail genomic PCR/DNA sequencing and Southern blot examination was performed to further confirm correct genotype.

gRNA target sequence (http://crispor.tefor.net/)

gRNA-A1 (matching forward strand of gene): CGTGTCTCCGCGTCCTTGCCCGG

gRNA-A2 (matching forward strand of gene): GGGTCGGTAGATCCGGGTTACGG

Albumin-Cre mice were provided by Hongbing Zhang (Institute of Basic Medical Sciences, Peking Union Medical College). Hepatic-specific Klf16 knockout mice were generated by crossing Klf16$^{fl/fl}$ mice with Albumin-Cre mice (called Klf16$^{alb-/-}$). Age-matched male littermates Klf16$^{fl/fl}$ mice were used as controls.

Pparα$^{-/-}$ mice were generated by the CRISPR/Cas9 system at Cyagen. The exon 4~6 have been selected as the target site. Briefly, gRNA sequences were designed by the CRISPR design tool (http://crispr.mit.edu) to target upstream and downstream from the coding region exon 4~6, respectively, and then were screened for off-target activity using a Universal CRISPR Activity Assay (UCA) (Biocytogen). Based on a sgRNA activity assay, these gRNAs targeting vectors were selected.

gRNA target sequence:

gRNA1 (matching reverse strand of gene): ATATTACAGGACCTCCACGGGGG

gRNA2 (matching forward strand of gene): TAATACCTTACCTAGTATCGGGG

These gRNA vectors were constructed and confirmed by sequencing, then were transcribed into short RNA by in vitro transcription. Then the Cas9 mRNA, gRNA were co-microinjected into the cytoplasm of 2-cell stage fertilized C57BL/6N eggs. The injected zygotes were transferred into oviducts of ICR surrogate females to generate F0 mice. F0 mice with the expected genotype confirmed by tail genomic DNA PCR and sequencing were mated with C57BL/6N mice to establish germline-transmitted F1 founders.

All mice were housed and maintained on a 12-h-light-dark cycle with a regular unrestricted diet. To establish NAFLD model, mice were fed a high-fat diet (Rodent Diet with 60 Kcal% Fat) ad libitum for 12 weeks without water limitation. To generate alcohol fatty liver models, mice were subjected to NIAAA alcohol model protocol. Briefly, mice were acclimated to a control liquid diet (Bio-Serv, F1259SP) for one day, followed by feeding with a liquid diet containing a gradual increased ethanol concentration from 1-4% (vol/vol) for day 2 to day 5. From day 6, the mice fed a liquid diet containing 5% ethanol for 10 days, and control groups were pair-fed a control diet for 10 days. At day 11, mice were gavaged with a single dose of ethanol (5g/kg, 30% ethanol), while the control groups were gavaged with isocaloric dextrin maltose. All mice were sacrificed 9h after gavage.

Male C57BL/6J mice, *db/db* mice aged 8 weeks or male C57BL/6J mice fed with HFD for 12 weeks were injected i.v. through the tail vein with adeno-associated virus expressing green fluorescent protein (AAV-*egfp*) or adeno-associated virus expressing KLF16(AAV-*Klf16*)(8.45E+12GC/ml). After four weeks of infection,

experiments were conducted. For Pparα overexpression, male Klf16$^{alb-/-}$ on HFD for 12 weeks were injected i.v. through the tail vein with adenovirus expressing green fluorescent protein (Ad-*egfp*) or adenovirus expressing Pparα(Ad-*Pparα*) (1.0×10^9 active viral particles in 200μl saline [54 mmol/l NaCl]). After one week of infection, experiments were conducted. All mice were fasted for 6h before sacrificed. Livers and serum were collected for further analysis. All mice were randomly assigned, and the experimenter was blind to the assignment of the groups and the evaluation of the results. No samples, animals or data, were excluded.

Preparation of recombinant adeno-associated virus (AAV)

To generate KLF16 overexpression adeno-associated virus, the primers sequence containing restriction sites used as follows:

KLF16-SacI-fw, 5'- CGAGCTCGCCACCATGTCGGCGGCCGTGGCGTG-3'

KLF16-EcoRI-rv, 5'- CGGAATTCTCAGGGCAAGGCGGAGCCGGA-3'

AAV2/8-KLF16-P2A-ZsGreen and AAV2/8-ZsGreen were further packaged (DongBio.Co.Ltd, Shenzhen, China) and the final titers for use were 1×10^{12} vector genome (v.g.)/ml. A total amount of $0.5\text{-}1\times10^{11}$ v.g. diluted in 200 μl of PBS was delivered to mice via tail vein injection.

Preparation of expression plasmids and recombinant adenoviruses

The full-length mouse Klf16 gene was amplified by PCR from C57BL/6J mouse liver cDNA, and myc-tagged Klf16 was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) using the following PCR primer pairs:

F: 5′-ACGCAGATCGAATTCATGTCGGCGGCCGTGGCGTGTGTGGATTACTT-3′

R: 5′-AAACAAGTTGCTCGAAGTCGACGAGCTCAAGCTTTCAGGGCAAGGCG-3′

Recombinant Adenovirus expressing vectors were purchased from Promega (Madison, WI, USA) and generated as previously described. For Klf16 overexpression, the full-length mouse KLF16 gene was amplified by PCR from the C57BL/6J mouse liver cDNA library and then was subcloned into pAd-Track-CMV vector. The primers sequence as follows:

F:5'-GAATTCATGTCGGCGGCCGTGGCGTGTGTGGATTACTT-3'

R:5'-CAAGCCAGCAGGAGCTGGGCTGGGGACAGGGCTAG -3'

For KLF16 knockdown, short-hairpin RNA (shRNA)-encoding DNA sequences were synthesized by Invitrogen (Carlsbad, CA) and constructed into adenovirus plasmids (pAdTrack-U6 vectors), The primers sequence as follows:

F:5'-AAGCTCCGGCCAAAGCCTATTACAAGTCTTCTCGAGAAGACTTGTAATAGGCTTTGGTTTTTG-3'

R:5'-GGTACAAAAACCAAAGCCTATTACAAGTCTTCTCGAGAAGACTTGTAATAGGCTTTGGCCGGA-3'

Adenovirus expressing Pparα(Ad-*Pparα*) was kindly provided by prof.Chang lab.

RNA extraction and quantitative (q) real-time PCR

Total RNA was extracted from mouse liver or cells with TRIzol (Takara), and then reverse-transcribed to cDNA with a high-capacity cDNA reverse transcription kit (Applied Biological Materials Inc, Vancouver, Canada). qPCR (PowerUp^{TM} SYBR^{TM} Green Master Mix) was performed by using gene-specific primers and SYBR Green. All gene expression data were normalized to β-actin expression levels. The data was analyzed using the □□CT cycle threshold method, and the mRNA transcription levels of target genes in each group of experiments were expressed as a multiple relative to the control groups. The specific primer sequences are listed in electronic supplementary material (ESM) Table 1.

Western blot analysis

Proteins were extracted from frozen liver samples or cultured hepatocytes in cell lysis buffer, and the total protein concentrations were determined by BSA method. In total, 80-100μg of protein was loaded onto a 10% SDS–polyacrylamide gel, and then separated proteins were transferred to polyvinylidene difluoride membranes. Western blot assays were performed using antibodies specific for rabbit anti-PPARα, rabbit anti-CPT1a (Servicebio, Wuhan, China), rabbit anti-KLF16 (Bioss, Beijing, China), and mouse anti-β-actin (Cwbio, Beijing, China). Antibodies against p-Akt(Ser473), Akt, p-GSK3β, GSK3β were purchased from Cell signaling

Technology.

Luciferase reporter gene assay

HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in 24-well plates using DMEM Medium containing 10% FBS (Invitrogen). Luciferase reporter genes were then co-transfected into cells, together with the indicated expression plasmids (pcDNA3.1 and pcDNA3.1-Klf16 plasmids, Series of luciferase reporter constructs containing the PPARα gene promoter fragment were kindly provide by prof. Chang), Plasmid containing PPARα gene promoter mutant fragment were constructed by PCR using -1286Luc as a template. The primers used for mutant fragment are F:CTGAGCCGGGGCCCGGGCCT; R:AGGCCCGGGCCCCGGCTCAG). The Ramlila luciferase expression vector pCMV-RL-TK (Promega) was used as an internal control. After 48h, cells were harvested and evaluated for luciferase activity using the Dual Luciferase Reporter Assay System (Promega). Relative luciferase activity was corrected for Renilla luciferase activity of pCMV-RL-TK, and normalized to the activity of the control.

Mitochondrial DNA copy number

The mitochondrial DNA (mtDNA) copy numbers are used as qPCR markers for mitochondrial density. In Brief, total DNA was isolated from mice liver using a Universal Genomic DNA Extraction kit (Tiangen), as described by the manufacturer. Mitochondrial DNA copy number is expressed by the COXII (mitochondrial-encoded gene)/Rps18 ratio. The primer sequences of COX II and Rps18 are presented in (ESM) Table 2.

Histology and immunohistochemistry

For H&E straining, liver tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, and cut into 7μm sections. For Oil red O staining, liver tissue was frozen in liquid nitrogen and cut into 10μm sections. Sections were stained and analyzed at 100X magnification with Masson's trichrome staining being used to determine collagen deposition.

Assessment of mitochondrial membrane potential

Mitochondrial membrane potential of MPHs was detected using JC-1assay kit (C2006, Beyotime, Shanghai,

China). In Brief, Primary hepatocytes were seeded in 6-well plates and transfected with pcDNA3.1-Klf16 or pcDNA3.1 using lipo3000 according to its manufacturer. After 24h, medium was replaced with OA&PA containing fresh medium for another 24h. MPHs were fixed with 4% paraformaldehyde for 20 minutes and mitochondrial membrane potential was measured according to the manufacturer's instructions and illustrated by fluorescence microscopy imaging (Leica Microsystems Ltd., Wetzlar, Germany).

Supplementary Tables

Table 1. Primer information for gene amplification

Primer	Sequences
KLF16(m)	F1: GTGTACCAAGCGGTTCACC
	R1: CAGGTCGTCGCAGGAGTTC
PPARα(m)	F1:AACATCGAGTGTCGAATATGTGG
	R1:CCGAATAGTTCGCCGAAAGAA
MgII(m)	F1: AGGCGAACTCCACAGAATGTT
	R1: ACAAAAGAGGTACTGTCCGTCT
HSL(m)	F1: GATTTACGCACGATGACACAGT
	R1: ACCTGCAAAGACATTAGACAGC
Atgl(m)	F1: ATGTTCCCGAGGGAGACCAA
	R1: GAGGCTCCGTAGATGTGAGTG
Acadvl(m)	F1: ACTACTGTGCTTCAGGGACAA
	R1: GCAAAGGACTTCGATTCTGCC
Acadl(m)	F1: TTTCCTCGGAGCATGACATTTT
	R1: GCCAGCTTTTTCCCAGACCT
Acads(m)	F1: GACTGGCGACGGTTACACA
	R1: GGCAAAGTCACGGCATGTC
Cpt2(m)	F1: CAGCACAGCATCGTACCCA
	R1: TCCCAATGCCGTTCTCAAAA
Cpt1a(m)	F1: TGGCATCATCACTGGTGTGTT

	R1: TCTAGGGTCCGATTGATCTTTG
Cpt1b(m)	F1: GACTTCCGGCTTAGTCGGG
	R1: GAATAAGGCGTTTCTTCCAGGA
Fabp3(m)	F1: ACCTGGAAGCTAGTGGACAG
	R1: TGATGGTAGTAGGCTTGGTCAT
β-actin(m)	F1: ATGACCCAAGCCGAGAAGG
	R1: CGGCCAAGTCTTAGAGTTGTTG
TNFα(m)	F1: CAGGCGGTGCCTATGTCTC
	R1: CGATCACCCCGAAGTTCAGTAG
IL-6(m)	F1:CTGCAAGAGACTTCCATCCAG
	R1: GTGGTATAGACAGGTCTGTTGG
IL-10(m)	F1: TTACTGACTGGCATGAGGATCA
	R1: GCAGCTCTAGGAGCATGTGG
IL-1beta(m)	F1: GAAATGCCACCTTTTGACAGTG
	R1: TGGATGCTCTCATCAGGACAG
KLF16(h)	F1: CAAGTCCTCGCACCTAAAGTC
	R1: AGCGGGCGAACTTCTTGTC
PPARα(h)	F1: ATGGTGGACACGGAAAGCC
	R1: GATGGATTGCGAAATCTCTTGG
GAPDH(h)	F1: GGAGCGAGATCCCTCCAAAAT
	R1: GGCTGTTGTCATACTTCTCATGG

Supplementary Figures

Figure Legend

Figure1. Hepatic KLF16 expression is decreased in hepatic steatosis

(A) KLF16 mRNA levels in C57BL/6J mice subjected to fasting and refeeding cycle.

(B) QPCR analyses indicated that KLF16 expression was decreased in NALFD patients (n=38) than in controls (n=28).

(C) OA&PA treatment decreased KLF16 mRNA levels in mice primary hepatocytes.

Comparable results obtained in three independent experiments.

Data are means ± SEM; n=8 mice/group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Figure2. Hepatic KLF16 deficiency worsens hepatic steatosis and increases insulin resistance

(A) QPCR data indicated a deletion of KLF16 in the liver, rather than other tissues.

(B) KLF16 deficiency increased the ratio of liver/body weight in chow diet mice.

(C) KLF16 knockout increased serum and hepatic TGs levels of mice fed on chow diet, while has no effects on the TC levels.

(D) KLF16 deletion increased the fasting blood glucose levels and insulin levels of chow diet mice.

(E) KLF16 deficiency impaired pyruvate tolerance of mice on chow diet, repeated measure two way ANOVA was used to analysis the difference of two curves.

(F) The hepatic Pepck, G6pase mRNA levels were upregulated due to KLF16 deficiency in chow diet mice.

(G&H) KLF16 deletion impaired mitochondrial biogenesis (G) and altered mitochondrial ultrastructure (H).

(I) KLF16 deletion increased hepatic ROS overproduction in chow diet mice.

(J) KLF16 deletion suppressed genes involved in anti-oxidant response.

(K) KLF16 deficiency stimulates hepatic inflammatory genes in chow diet mice.

Comparable results obtained in three independent experiments.

Data are means ± SEM; n=8 mice/group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Figure3. KLF16 overexpression alleviates hepatic steatosis in *db/db* mice

(A) QPCR data indicated KLF16 mRNA levels were not altered in other tissues in *db/db* mice.

(B) KLF16 overexpression does not alter body weight of *db/db* mice.

(C) KLF16 overexpression reduced serum and hepatic TGs levels, while has no effects on the TC levels in *db/db* mice.

(D) KLF16 overexpression decreased fasting blood glucose and insulin levels in *db/db* mice.

(E) KLF16 overexpression improved pyruvate tolerance of *db/db* mice, repeated measure two way ANOVA was used to analysis the difference of two curves.

(F) The Pepck, G6pase mRNA levels were decreased in *db/db* mice after KLF16 overexpression.

(G) KLF16 overexpression reduced serum ALT and AST levels in *db/db* mice.

(H&I) KLF16 overexpression in *db/db* mice improved mitochondrial biogenesis (H) and mitochondrial ultrastructure (I).

(J) KLF16 overexpression decreased hepatic ROS overproduction in *db/db* mice.

(K) KLF16 overexpression up-regulates genes involved in anti-oxidant response in *db/db* mice.

(L) KLF16 overexpression decreased hepatic inflammatory genes in *db/db* mice.

Comparable results obtained in three independent experiments.

Data are means ± SEM; n=8 mice/group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Figure4. KLF16 overexpression attenuated hepatic steatosis in HFD mice

(A) QPCR data indicated KLF16 mRNA levels were unchanged in other tissues in HFD mice.

(B) KLF16 overexpression does not alter body weight of HFD mice.

(C) KLF16 overexpression reduced the ratio of liver/body weight in HFD mice.

(D) KLF16 overexpression reduced serum and hepatic TGs levels of HFD mice, while has no effect on TC content.

(E) KLF16 overexpression reduced serum ALT and AST levels in HFD mice.

(F) KLF16 overexpression decreased fasting blood glucose and insulin levels in HFD mice.

(G) KLF16 overexpression reduced the expression of hepatic Pepck and G6pase in HFD mice.

(H) KLF16 overexpression decreased hepatic inflammatory genes in HFD mice.

Comparable results obtained in three independent experiments.

Data are means ± SEM; n=8 mice/group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Figure5. KLF16 affects lipid deposition in mice primary hepatocytes

(A) TG levels of mice primary hepatocytes infected with ad-*gfp* or ad-*Klf16* treated with DMSO or OA&PA for 24h. (B) TG levels of mice primary hepatocytes infected with ad-*shLuci* or ad-*shKlf16* treated with DMSO or OA&PA for 24h.

Comparable results obtained in three independent experiments.

Data are means ± SEM; n=6/group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Figure6. PPARα overexpression attenuated KLF16 deficiency-induced hepatic steatosis

(A) QPCR and WB data indicated PPARα overexpression in mice primary hepatocytes and livers.

(B) QPCR data indicated unchanged PPARα expression in other tissues of $KLF16^{alb-/-}$ mice after ad-PPARα infection.

(C&D) PPARα overexpression reduced body weight (C) and the ratio of liver/body weight (D) in $KLF16^{alb-/-}$ mice fed on HFD.

(E) PPARα overexpression increased mitochondrial biogenesis (*left panel*) and mitochondrial ultrastructure (*right panel*).

(F&G) PPARα overexpression decreased hepatic ROS overproduction (F) and inflammatory genes (G) in HFD $KLF16^{alb-/-}$ mice.

(H) PPARα overexpression reduced serum ALT and AST levels in HFD $KLF16^{alb-/-}$ mice.

(I) PPARα overexpression decreased fasting blood glucose and insulin levels of HFD $KLF16^{alb-/-}$ mice.

Comparable results obtained in three independent experiments.

Data are means ± SEM; n=8 mice/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Figure7. KLF16 affects ROS production in mice primary hepatocytes

(A) KLF16 overexpression effectively decreased OA&PA-induced ROS over-production in mice primary hepatocytes.

(B) KLF16 deletion aggravated OA&PA-induced ROS over-production in mice primary hepatocytes.

Comparable results obtained in three independent experiments.

Figure8. Hepatic KLF16 deletion increases mice sensitivity to ethanol feeding

(A) Ethanol feeding decreased hepatic KLF16 protein levels.

(B) KLF16 deletion aggravated ethanol feeding-induced elevation of serum ALT and AST levels.

(C) KLF16 deletion aggravated hepatic inflammatory genes after ethanol feeding.

(D) The mitochondrial ultrastructure was changed in $KLF16^{alb-/-}$ mice after ethanol feeding.

(E) KLF16 deletion increased hepatic ROS generation in $KLF16^{alb-/-}$ mice after ethanol feeding.

Comparable results obtained in three independent experiments.

Data are means ± SEM; n=8 mice/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Figure9. KLF16 has no effects on SREBP-1c expression

(A) KLF16 overexpression did not changed the protein levels of SREBP-1c both in cytoplasm and nucleus of MPHs.

Comparable results obtained in three independent experiments.

Figure10. The effect of KLF16 on apoptosis.

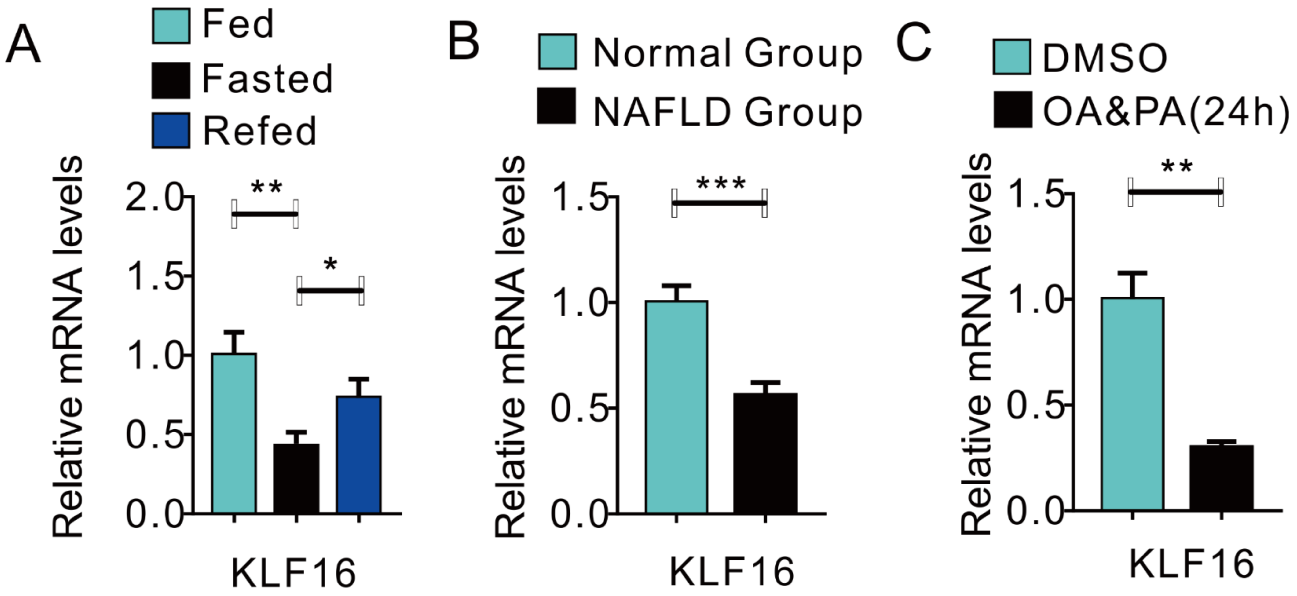
(A) KLF16 overexpression inhibits apoptosis in mice primary hepatocytes, as shown by the mitochondrial membrane potential changes of mice primary hepatocytes transfected with pcDNA3.1+KLF16 or pcDNA3.1 as control, followed by the OA&PA exposure for 24h.

(B) qPCR analysis of key genes involved in apoptosis in the liver of *db/db* and DIO mice infected with aav-*egfp* or aav-*Klf16*, as well as the liver of $Klf^{alb-/-}$ mice and $Klf16^{fl/fl}$ mice.

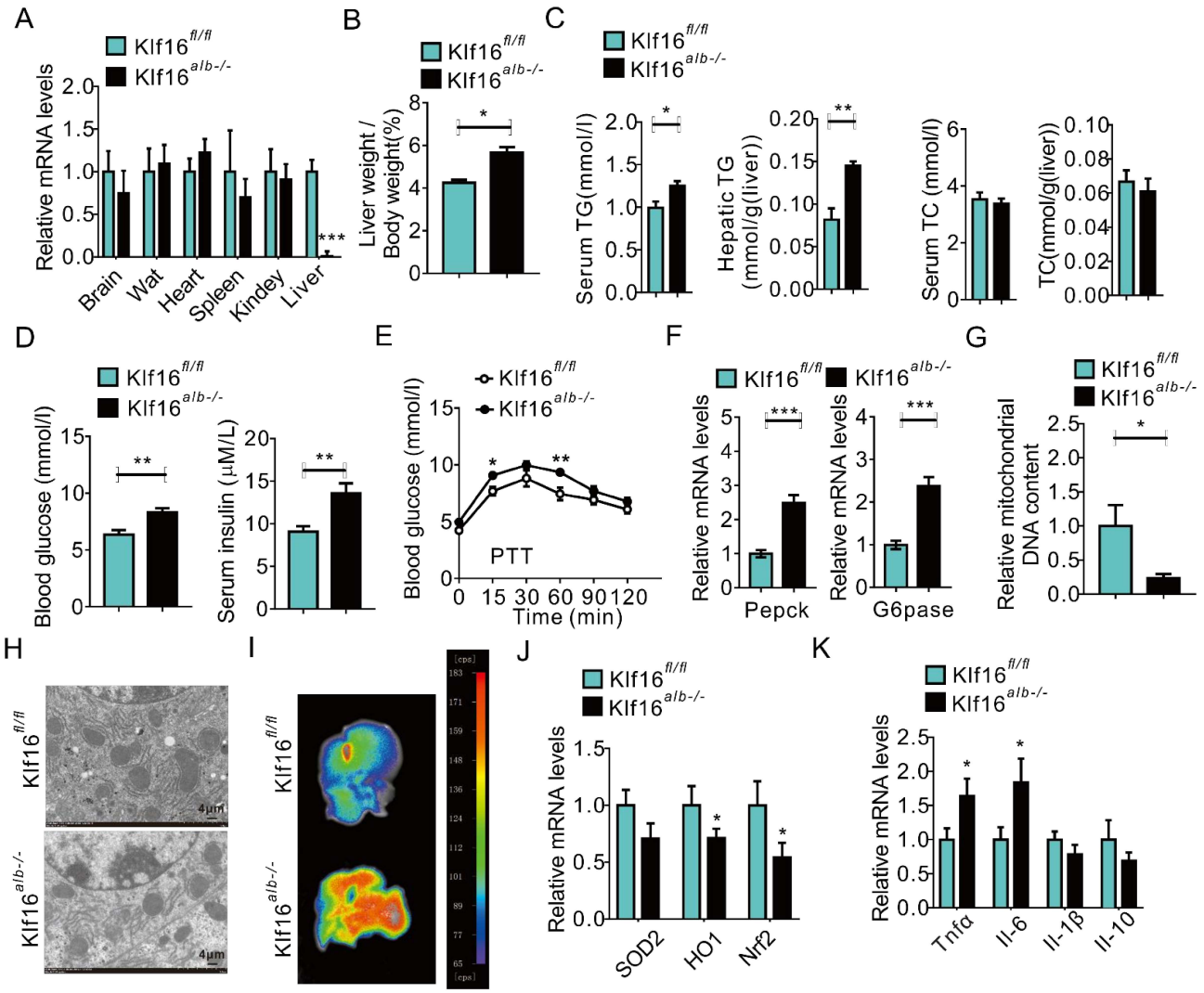
Comparable results obtained in three independent experiments.

Data are means ± SEM; $*p < 0.05$, $**p < 0.01$

Supplementary Figure 1



Supplementary Figure 2



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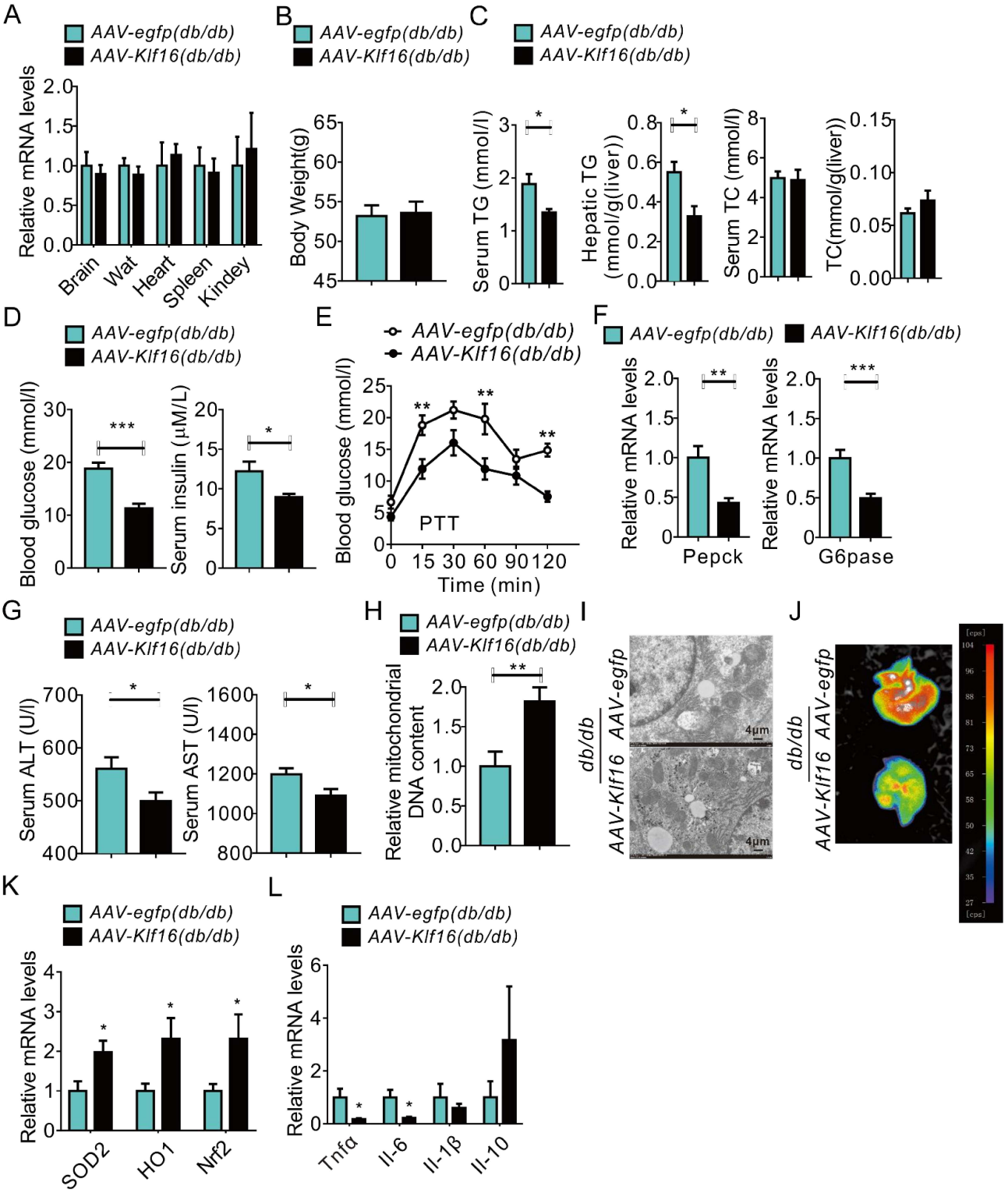

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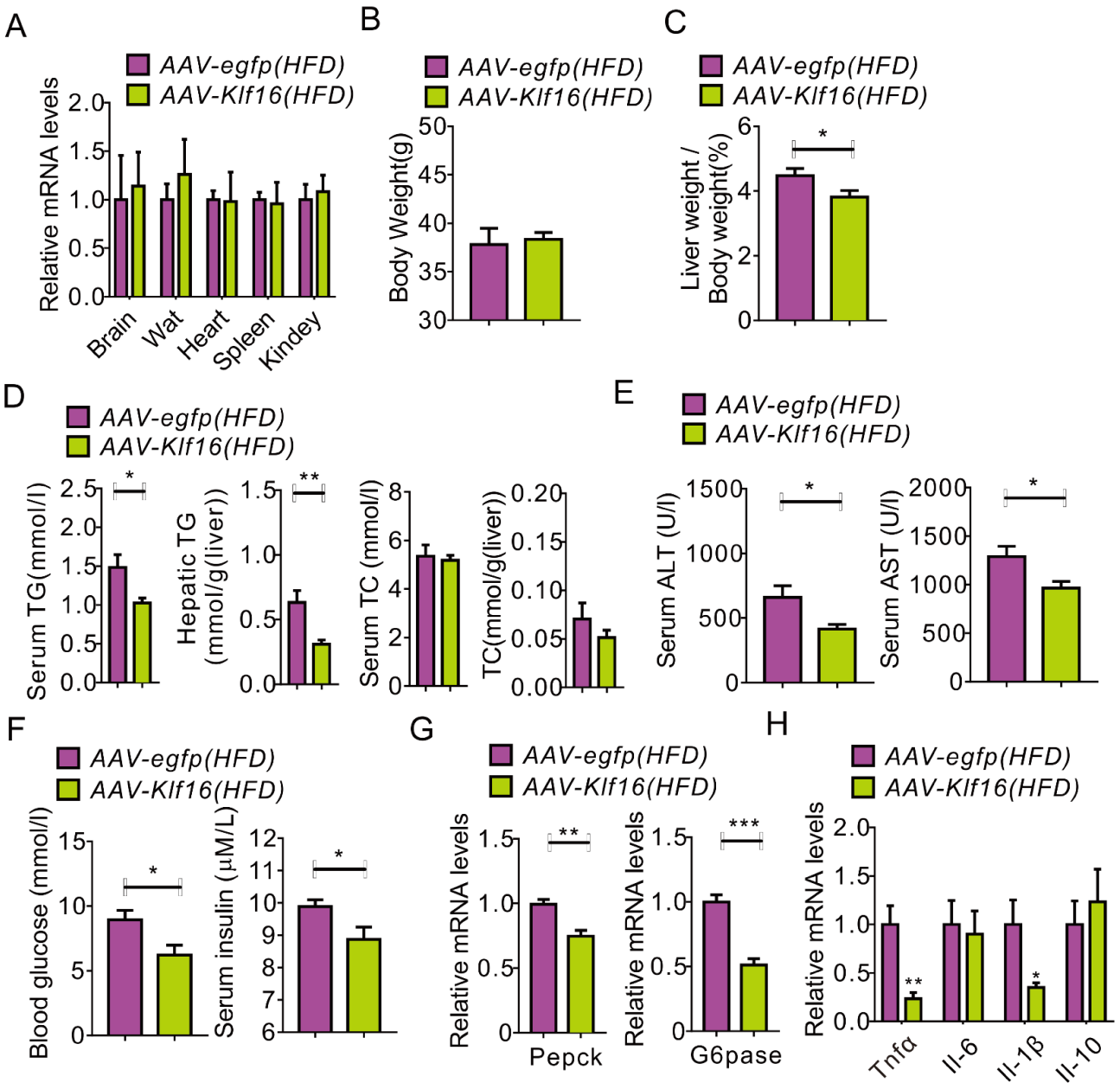
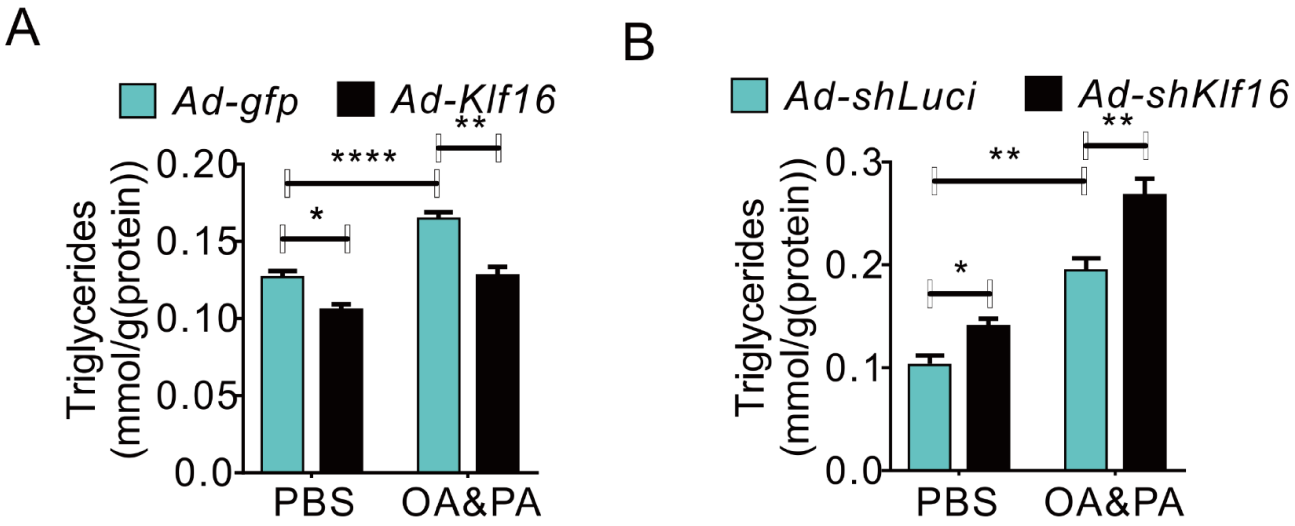
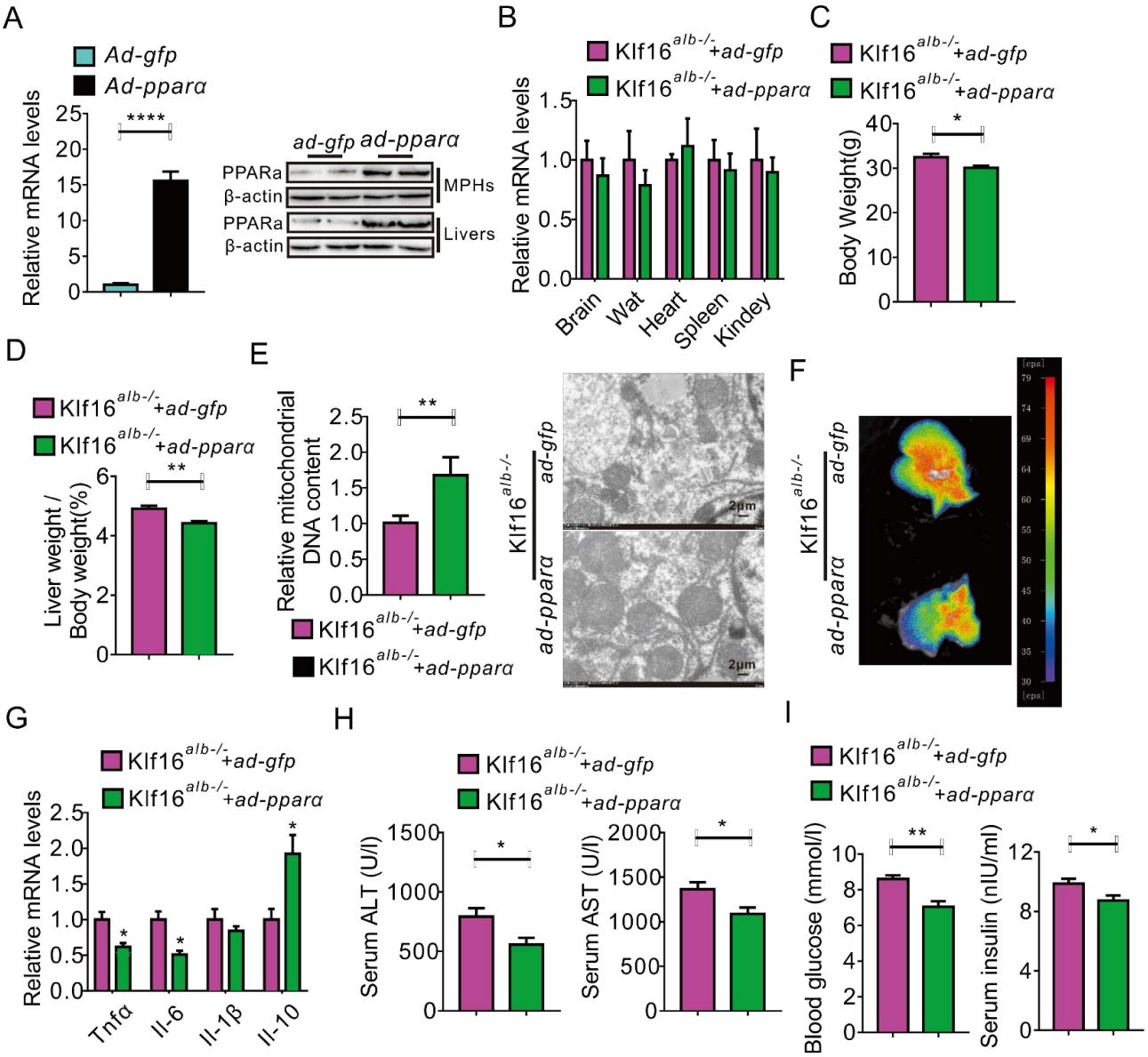


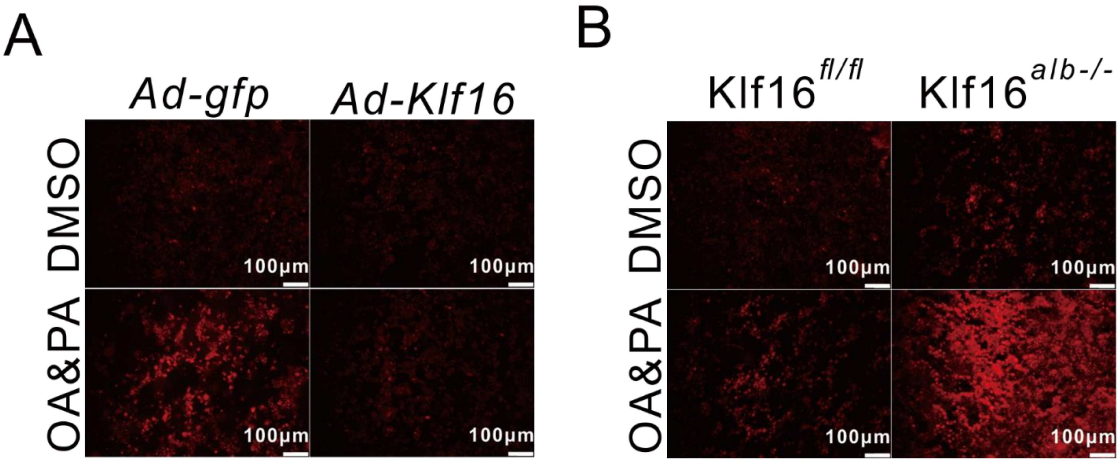
Supplementary Figure 5



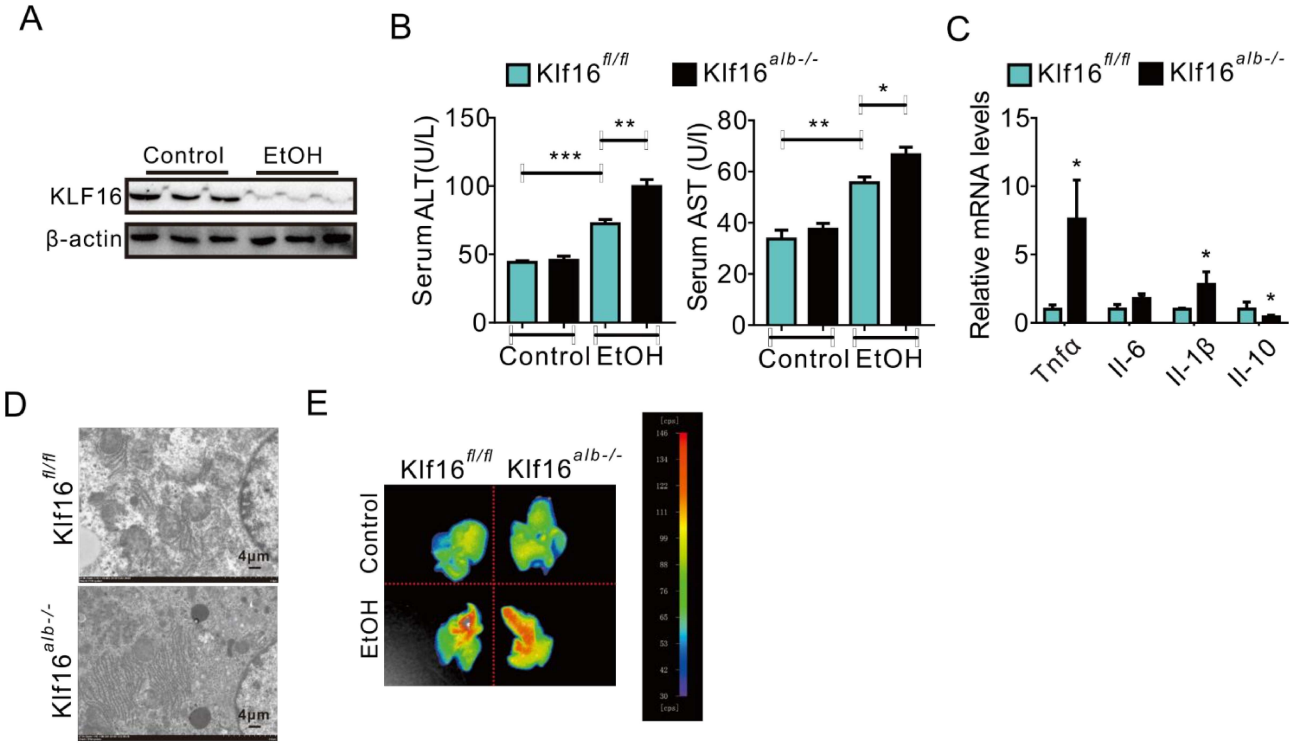
Supplementary Figure 6



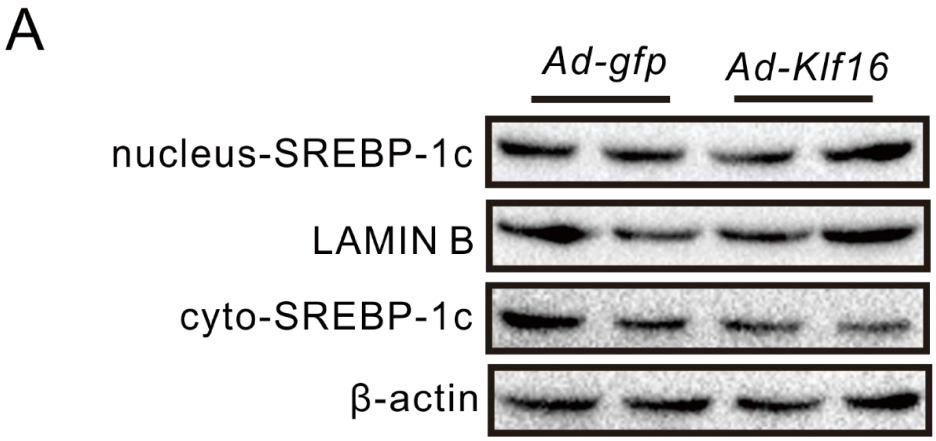
Supplementary Figure 7



Supplementary Figure 8



Supplementary Figure 9



Supplementary Figure 10