Supplementary Appendix

Loss of long-chain acyl-CoA synthetase 1 promotes hepatocyte death in alcohol-induced steatohepatitis

Running title: ACSL1 deficiency mediates ALD

Authors: Haibo Dong[a], Wei Zhong [a, b], Wenliang Zhang [a], Liuyi Hao [a], Wei Guo [a], Ruichao Yue [a], Xinguo Sun [a], Zhaoli Sun [c], Ramon Bataller [d], Zhanxiang Zhou [a, b*]

Affiliations: [a] Center for Translational Biomedical Research, the University of North Carolina at Greensboro, North Carolina Research Campus, Kannapolis, NC, USA.

[b] Department of Nutrition, the University of North Carolina at Greensboro, Greensboro, NC, USA.

[c] Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

[d] Department of Medicine, University of Pittsburgh, Pittsburgh, PA, USA

[*] **Corresponding author:** Zhanxiang Zhou, Center for Translational Biomedical Research, University of North Carolina at Greensboro, North Carolina Research Campus, 600 Laureate Way, Suite 2203, Kannapolis, North Carolina 28081. Phone: 704-250-5800. Fax: 704-250-5809. E-mail: z_zhou@uncg.edu.

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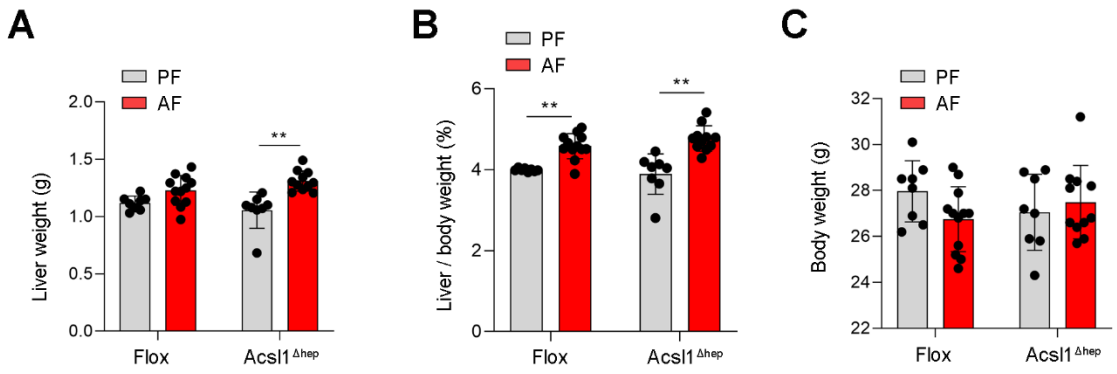
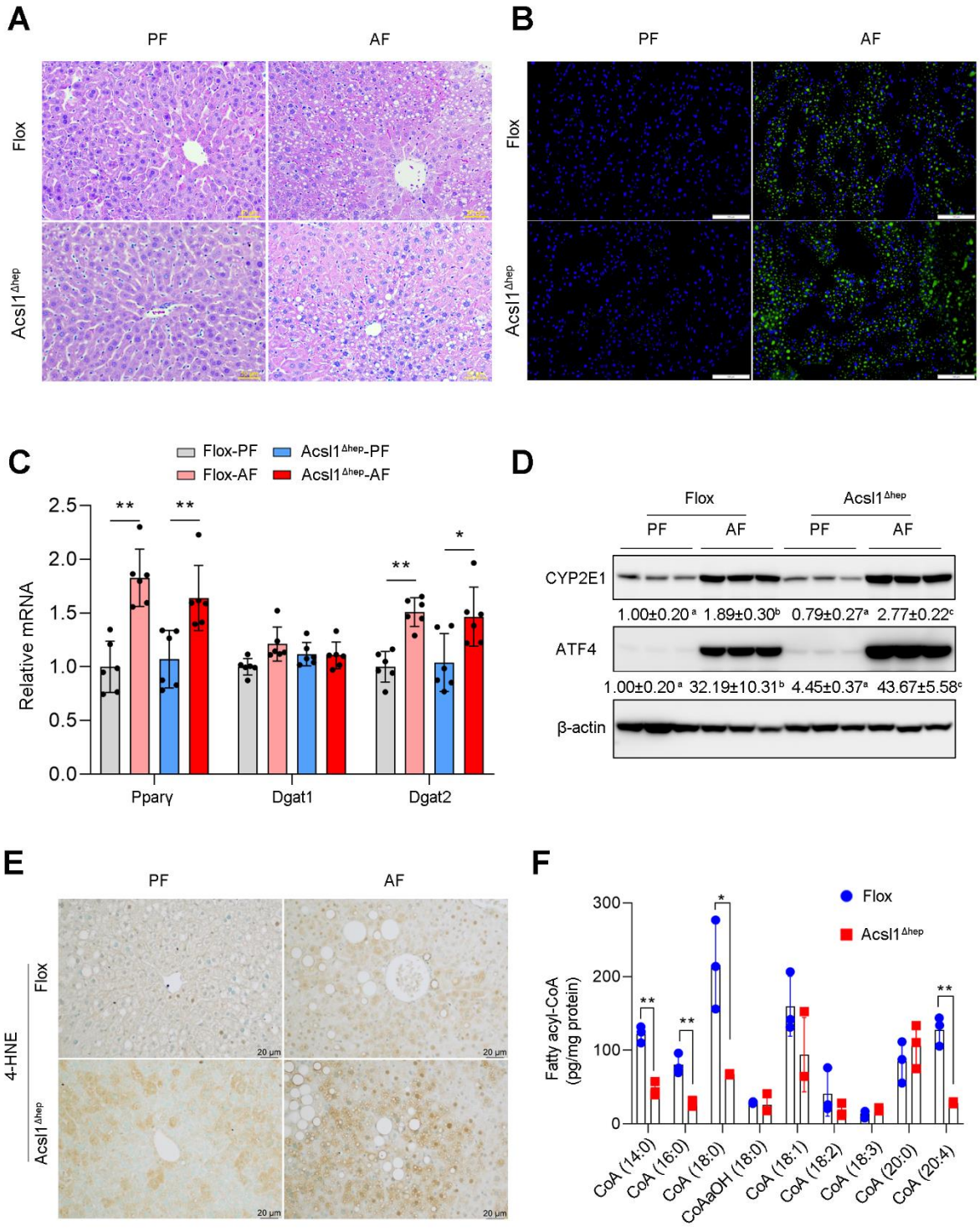
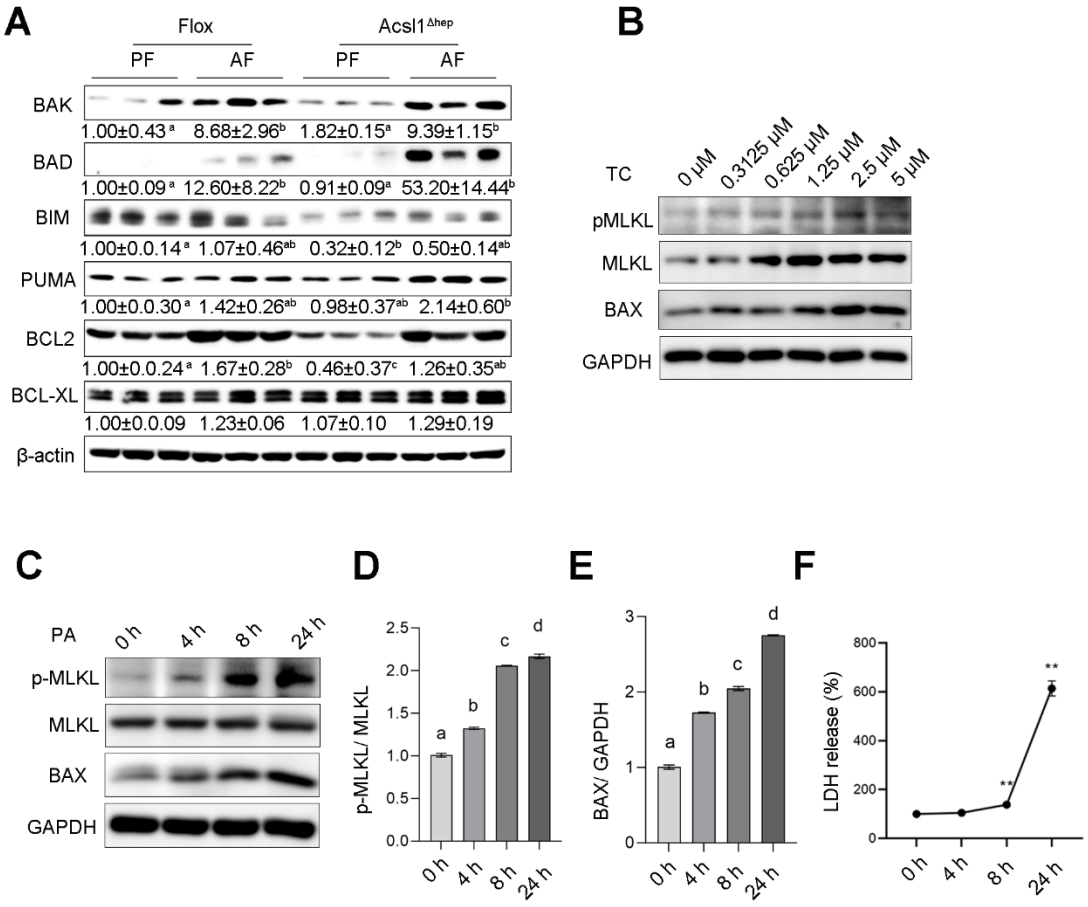


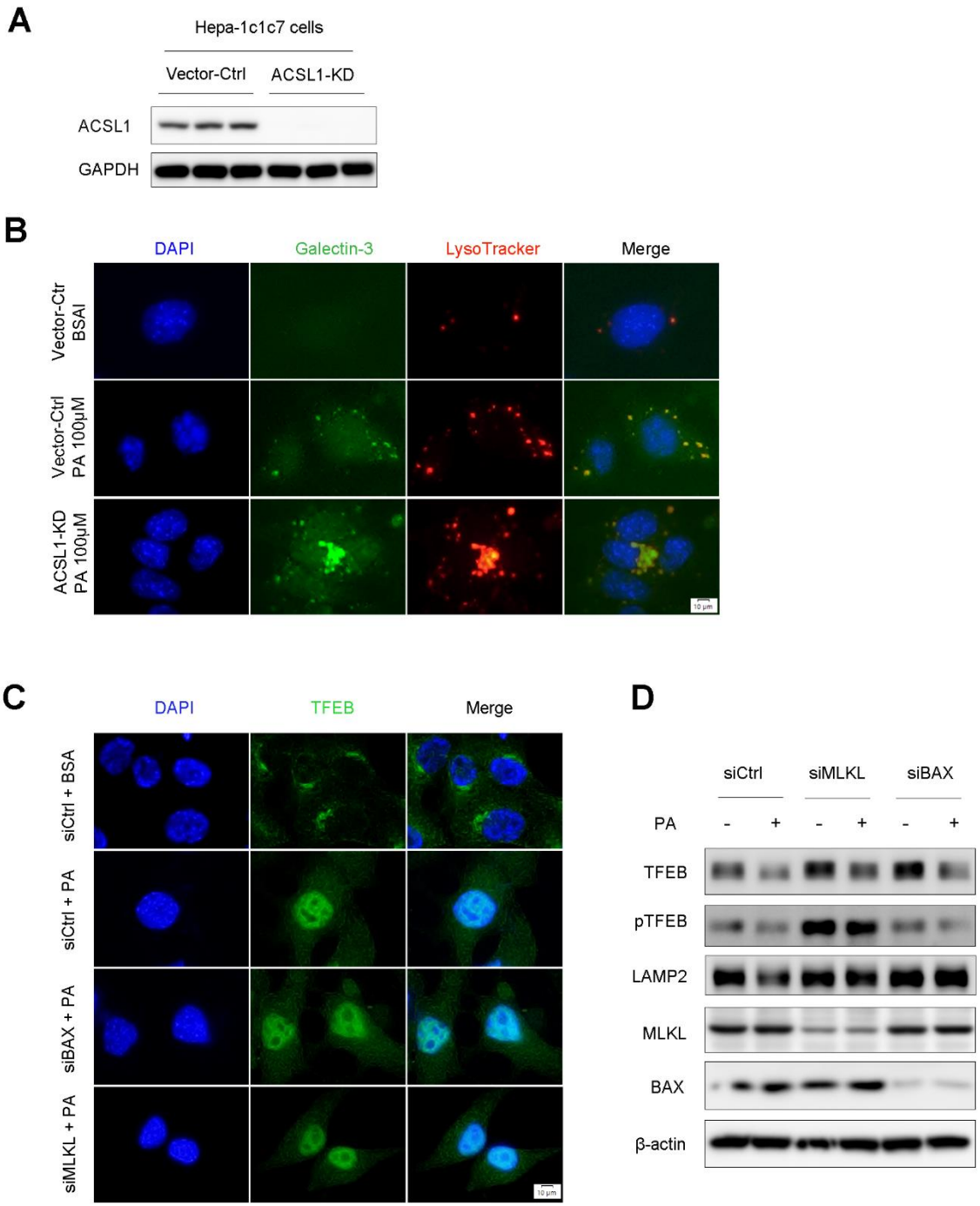
Supplementary Fig. S1 Effect of hepatocyte-specific ACSL1 deletion on liver weight, body weight, and the ratio of liver weight to body weight. **(A)** Changes in liver weight, **(B)** liver weight to body weight ratio, and **(C)** body weight (n = 8-12/group). Data are presented as mean ± SD. $^{**}P < 0.01$.


A
PF
AF
Flox
Acsl1Δhep
B
PF
AF
Flox
Acsl1Δhep
C
Flox-PF
Acsl1Δhep-PF
Flox-AF
Acsl1Δhep-AF
Relative mRNA
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
*
Pparγ
Dgat1
Dgat2
D
Flox
Acsl1Δhep
PF
AF
PF
AF
CYP2E1
1.00±0.20a 1.89±0.30b 0.79±0.27a 2.77±0.22c
ATF4
1.00±0.20a 32.19±10.31b 4.45±0.37a 43.67±5.58c
β-actin
E
PF
AF
4-HNE
Flox
Acsl1Δhep
20 μm
20 μm
20 μm
20 μm
F
Flox
Acsl1Δhep
Fatty acyl-CoA (pg/mg protein)
300
200
100
0
**
**
*
**
CoA (14:0)
CoA (16:0)
CoA (18:0)
CoAaOH (18:0)
CoA (18:1)
CoA (18:2)
CoA (18:3)
CoA (20:0)
CoA (20:4)

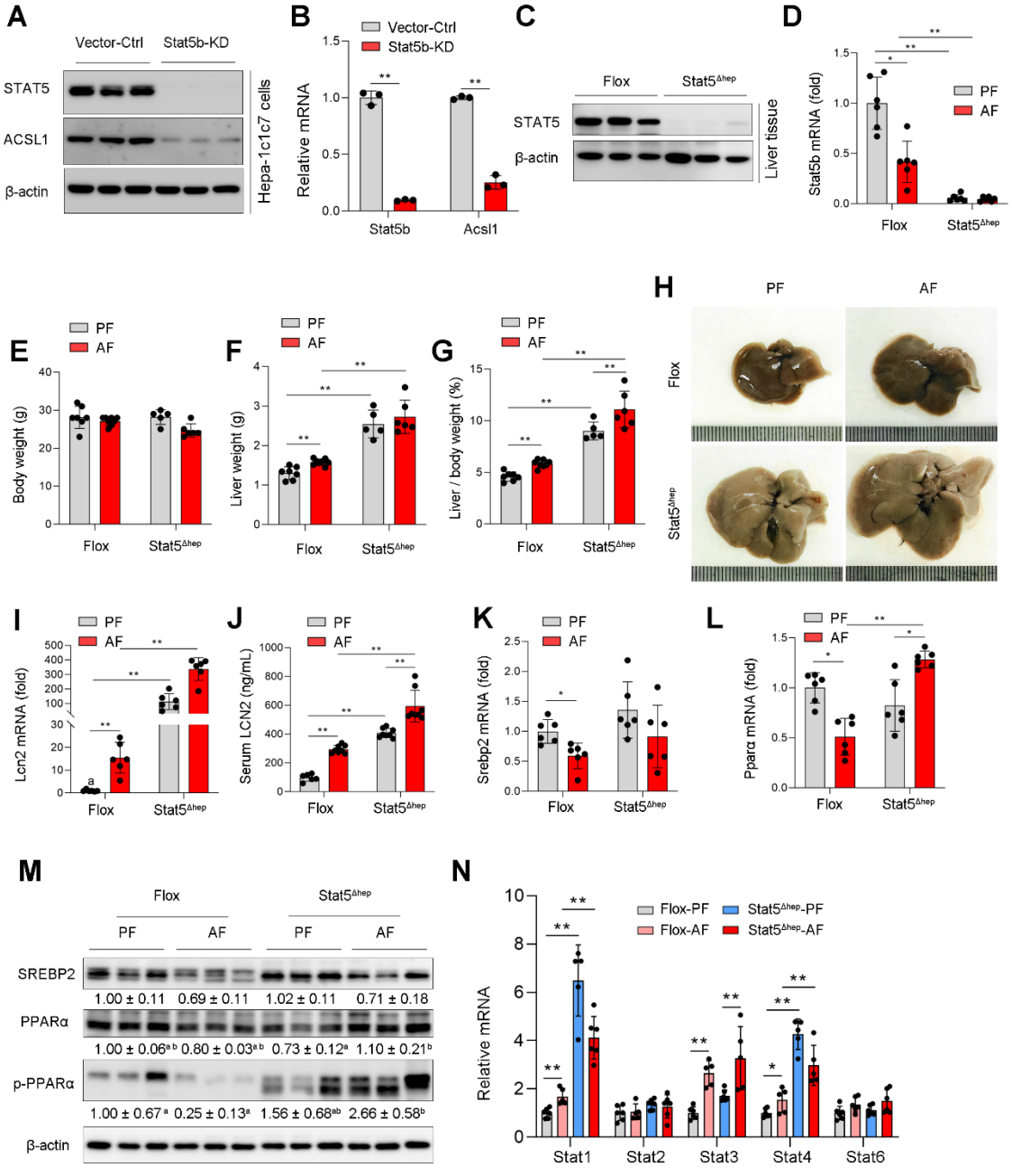
Supplementary Fig. S2 ACSL1 deficiency induces fatty acids accumulation and promotes oxidative damage in mouse liver. (A) Representative H&E staining of the liver sections of alcohol-fed mice from Flox mice and Acsl1$^{\Delta hep}$ mice. Scale bars = 50 μm. **(B)** Representative BODIPY staining of neutral lipids of the liver sections of alcohol-fed mice from Flox mice and Acsl1$^{\Delta hep}$ mice. Scale bars = 100 μm. **(C)** The mRNA levels of *Ppar γ*, *Dgat1*, and *Dgat2* in liver tissues of alcohol-fed Flox mice and Acsl1$^{\Delta hep}$ mice (n = 6/group). (**D)** The protein levels of CYP2E1 and ATF4 (n = 3/group). **(E)** IHC staining of hepatic 4-HNE from Flox mice and Acsl1$^{\Delta hep}$ mice. Scale bars = 20 μm. **(F)** Lipidomics analysis of fatty acyl-CoA in liver tissues from Flox mice and Acsl1$^{\Delta hep}$ mice (n =3/ group). Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, values with different superscripts are significantly different.


A
Flox
Acsl1Δhep
PF
AF
PF
AF
BAK
1.00±0.43a 8.68±2.96b 1.82±0.15a 9.39±1.15b
BAD
1.00±0.09a 12.60±8.22b 0.91±0.09a 53.20±14.44b
BIM
1.00±0.0.14a 1.07±0.46ab 0.32±0.12b 0.50±0.14ab
PUMA
1.00±0.0.30a 1.42±0.26ab 0.98±0.37ab 2.14±0.60b
BCL2
1.00±0.0.24a 1.67±0.28b 0.46±0.37c 1.26±0.35ab
BCL-XL
1.00±0.0.09 1.23±0.06 1.07±0.10 1.29±0.19
β-actin
B
TC
0 μM
0.3125 μM
0.625 μM
1.25 μM
2.5 μM
5 μM
pMLKL
MLKL
BAX
GAPDH
C
PA
0 h
4 h
8 h
24 h
p-MLKL
MLKL
BAX
GAPDH
D
p-MLKL/ MLKL
E
BAX/ GAPDH
F
LDH release (%)

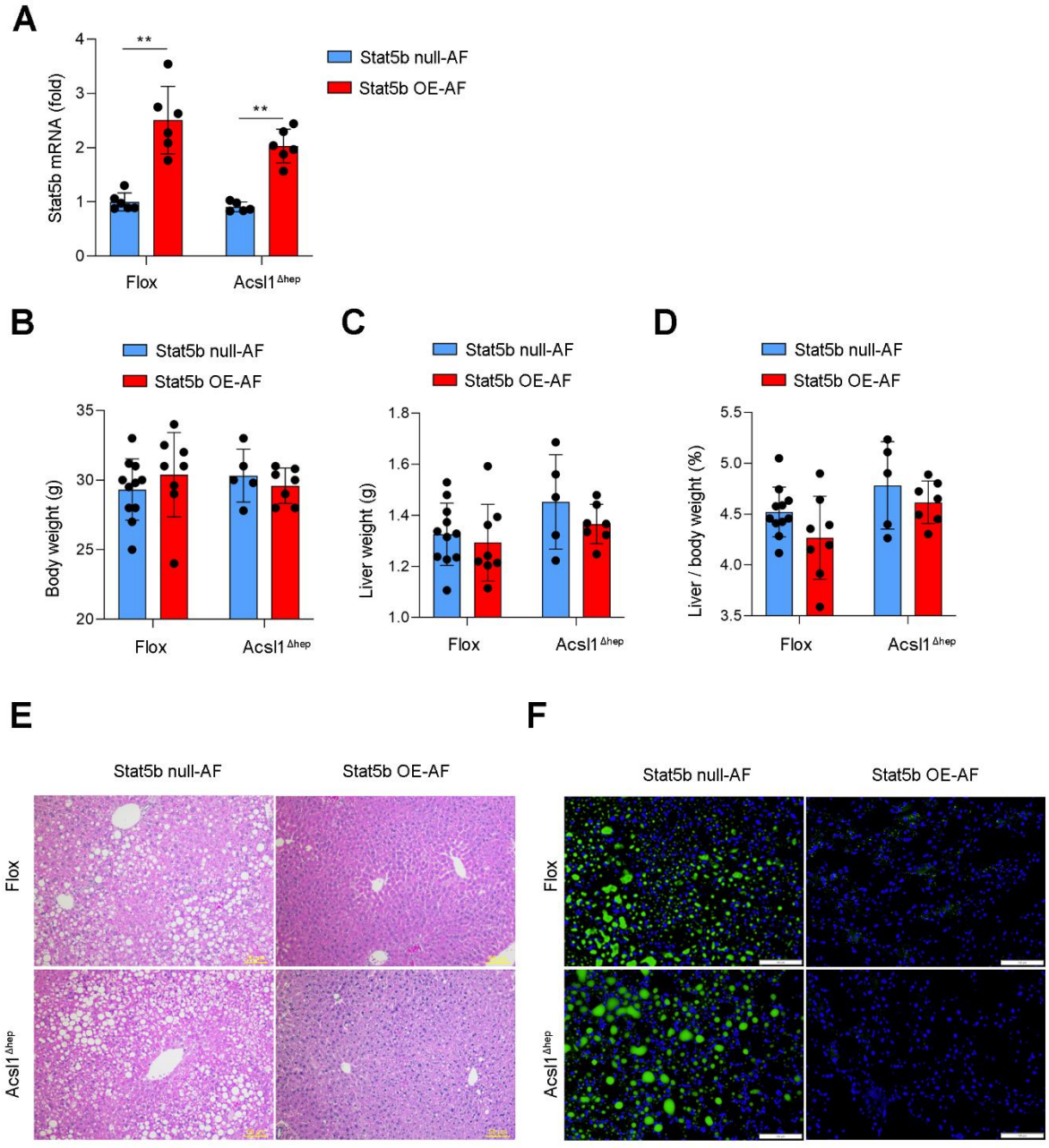
Supplementary Fig. S3 ACSL1 deficiency induces cell apoptosis and necroptosis (A) Protein levels of apoptotic proteins in the liver from Flox mice and hepatocyte-specific ACSL1 knockout mice ($Acsl1^{\Delta hep}$) with or without alcohol feeding for 8 weeks. **(B)** Protein levels of p-MLKL, MLKL, and BAX were analyzed by immunoblot after triacsin-c (TC, an ACSL1 inhibitor) treatment for 24h. **(C-E)** Protein levels of p-MLKL, MLKL, and BAX were analyzed by immunoblot under PA stimulation for 0 h, 4 h, 8 h, and 24 h (C). Quantification of p-MLKL (D) and BAX (E). **(F)** LDH release from Hepa-1c1c7 cells was determined in culture medium after PA stimulation for 0 h, 4 h, 8 h, or 24 h. Data are presented as mean ± SD. $^{**}P < 0.01$, values with different superscripts are significantly different.


A
Hepa-1c1c7 cells
Vector-Ctrl
ACSL1-KD
ACSL1
GAPDH
B
DAPI
Galectin-3
LysoTracker
Merge
Vector-Ctr BSAl
Vector-Ctrl PA 100μM
ACSL1-KD PA 100μM
C
DAPI
TFEB
Merge
siCtrl + BSA
siCtrl + PA
siBAX + PA
siMLKL + PA
D
siCtrl
siMLKL
siBAX
PA
- + - + - +
TFEB
pTFEB
LAMP2
MLKL
BAX
β-actin

Supplementary Fig. S4 ACSL1 knockdown aggravates PA-induced lysosome dysfunction, but Bax and MLKL do not mediate lysosome biogenesis. (A) Protein levels of ACSL1 were confirmed in ACSL1 knockdown Hepa-1c1c7 cells. **(B)** Colocalization of galectin-3 and lysosome tracker was examined by immunofluorescence microscopy in Hepa-1c1c7 cells with/without PA (100 μM) stimulation for 8 h. Scale bars = 10 μm. **(C)** IF analysis of TFEB nuclear translocation in Hepa-1c1c7 cells after BAX or MLKL knockdown by siRNAs, respectively, followed by PA (100 μM) stimulation for 24 h. Scale bars = 10 μm. **(D)** Western blot analysis of lysosome biogenesis in Hepa-1c1c7 cells after BAX or MLKL knockdown by siRNAs, respectively, followed by PA (100 μM) stimulation for 24 h.


A
Vector-Ctrl
Stat5b-KD
STAT5
ACSL1
β-actin
Hepa-1c1c7 cells
B
Vector-Ctrl
Stat5b-KD
Relative mRNA
Stat5b
Acsl1
C
Flox
Stat5Δhep
STAT5
β-actin
Liver tissue
D
Stat5b mRNA (fold)
PF
AF
Flox
Stat5Δhep
E
PF
AF
Body weight (g)
Flox
Stat5Δhep
F
Liver weight (g)
G
Liver / body weight (%)
H
PF
AF
Flox
Stat5Δhep
I
Lcn2 mRNA (fold)
J
Serum LCN2 (ng/mL)
K
Srebp2 mRNA (fold)
L
Pparα mRNA (fold)
M
Flox
Stat5Δhep
PF
AF
SREBP2
1.00 ± 0.11
0.69 ± 0.11
1.02 ± 0.11
0.71 ± 0.18
PPARα
1.00 ± 0.06a b
0.80 ± 0.03a b
0.73 ± 0.12a
1.10 ± 0.21b
p-PPARα
1.00 ± 0.67a
0.25 ± 0.13a
1.56 ± 0.68ab
2.66 ± 0.58b
β-actin
N
Flox-PF
Stat5Δhep-PF
Flox-AF
Stat5Δhep-AF
Relative mRNA
Stat1
Stat2
Stat3
Stat4
Stat6

Supplementary Fig. S5 Hepatocyte-specific STAT5 Deletion Exacerbates Alcohol-induced Liver Injury. (A) Western blot analysis of ACSL1 after STAT5b knockout in Hepa-1c1c7 cells (n = 3/ group). **(B)** qPCR analysis of ACSL1 after STAT5b knockout in Hepa-1c1c7 cells (n = 3/ group). **(C)** Western blot analysis of STAT5 in liver tissues of Flox mice and $Stat5^{\Delta hep}$ mice (n =6/group, 3 replicates). **(D)** qPCR analysis of STAT5 in liver tissues of Flox mice and $Stat5^{\Delta hep}$ mice (n =6/group). **(E-G)** Body weight (E), liver weight (F), and liver weight to body weight ratio (G) (n = 5-7/group). **(H)** Representative liver images of Flox and $Stat5^{\Delta hep}$ mice with/without alcohol feeding for 8 weeks. **(I)** Hepatic mRNA expression of *Lcn2* in Flox mice and $Stat5^{\Delta hep}$ mice with/without alcohol feeding for 8 weeks (n = 6/group). **(J)** Serum LCN2 levels in Flox mice and $Stat5^{\Delta hep}$ mice with/without alcohol feeding for 8 weeks (n = 6-8/group). **(K)** Hepatic mRNA expression of *Srebp2* in Flox mice and $Stat5^{\Delta hep}$ mice with/without alcohol feeding for 8 weeks (n = 6/group). **(L)** Hepatic mRNA expression of *PPARα* in Flox mice and $Stat5^{\Delta hep}$ mice with/without alcohol feeding for 8 weeks (n = 6/group). **(M)** Western blot analysis of SREBP2, PPARα and p-PPARα in liver tissues of Flox mice and $Stat5^{\Delta hep}$ mice (n =6/group, 3 replicates). **(N)** mRNA levels of *Stat1*, *Stat2*, *Stat3*, *Stat4*, and *Stat6* in liver tissues of alcohol-fed Flox mice and $Stat5^{\Delta hep}$ mice, (n =5-6/group). Data are presented as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, values with different superscripts are significantly different.


A
Stat5b mRNA (fold)
Stat5b null-AF
Stat5b OE-AF
**
**
Flox
Acsl1 Δhep
B
Body weight (g)
C
Liver weight (g)
D
Liver / body weight (%)
E
F

Supplemental Fig. S6 STAT5b overexpression ameliorates alcohol-induced TG accumulation. (A) qPCR analysis of Stat5b in liver tissues of alcohol-fed mice from Flox mice and Acsl1$^{\Delta hep}$ mice with/without Stat5b overexpression (n =5-6/group). **(B-D)** Body weight (B), liver weight (C), and liver weight to body weight ratio (D) (n = 5-7/group). **(E)** Representative H&E staining of the liver sections of alcohol-fed mice from Flox mice and Acsl1$^{\Delta hep}$ mice with/without Stat5b overexpression. Scale bars = 50 μm. **(F)** Representative BODIPY staining of neutral lipids of the liver sections of alcohol-fed mice from Flox mice and Acsl1$^{\Delta hep}$ mice with/without Stat5b overexpression. Scale bars = 100 μm. Data are presented as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$.

Supplementary Key Resources Table 1. Primer sequences for real-time PCR in mouse samples.

Genes	Forward	Reverse
Acsl1	AGGACTCGGCATGTGACAAA	ACACCGCAGCAGAATCAGAA
Acsl3	CCTGTCAGTTCCAAACCGGA	CCAAAGTCAAGGGCTCGGAT
Acsl4	AGAGTCCAAAGCGAGGGAGA	TCTCTCCAGTTCCCAAACGC
Acsl5	TTAAACTTGGCGGGGTGAGA	CTGTGTAGCTCCTTTCGCCA
Cpt1α	CTCCGCCTGAGCCATGAAG	CACCAGTGATGATGCCATTCT
Cyp4a10	TTCCAGCAGTTCCCATCACC	ACGCACCATTAGCCTTTGGA
Cyp4a12	CAAGACAGCCCAGCTCTACC	TGAATGCGCACTTTGCTTCC
Cyp4a14	CTGGGTGATGGAACCTCTGTC	TCCCAATGCAGTTCCTTGATCC
Acox1	CGCACATCTTGGATGGTAGT	GGCTTCGAGTGAGGAAGTTATAG
Srebp2	GCAGCAACGGGACCATTCT	CCCCATGACTAAGTCCTTCAACT
Pparα	AGAGCCCCATCTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
Cd36	ATGGGCTGTGATCGGAACTG	GTCTTCCCAATAAGCATGTCTCC
Stat5b	GTACTACACACCGGTCCCCT	CCCACTGGTATCAAGGACGG
Pparγ	GAACCTGCATCTCCACCTTATT	TGGAAGCCTGATGCTTTATCC
Dgat1	TCCGCCTCTGGGCATTC	GAATCGGCCCACAATCCA
Dgat2	CTGGCTGATAGCTGCTCTCTACTT	TGTGATCTCCTGCCACCTTTC
Stat1	TGGCCCTGATGGTCTTATTC	GGGAGCTCTCACTGAATCTAAG
Stat2	CTGGTAAGATCCCTTTCTGGAC	GCTCCACCCAAGAACAAGTA
Stat3	GTCCTCTATCAGCACAACCTTC	TCCAACATCTGCTGCTTCTC
Stat4	CCTGCGAGACTACAAGGTTATC	GGAGAAGGTCTGAAGGAGATTG
Stat6	GGCTTTCCGGAGTCACTATAAG	TGGATGGACTGTGGAGGA
RPS17	GGAGATCGCCATTATCCCCA	ATCTCCTTGGTGTCGGGATC

Supplementary Key Resources Table 2. Key resources in this study

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti ACSL1	Cell Signaling Technology	Cat# 4047, RRID: AB_2222411
Rabbit monoclonal anti STAT5	Cell Signaling Technology	Cat# 9358, RRID: AB_659905
Rabbit polyclonal anti p-STAT5	Cell Signaling Technology	Cat# 9351S, RRID: AB_ 2315225
Mouse monoclonal anti MLKL	Proteintech	Cat# 66675-1-Ig, RRID:AB_2882029
Rabbit monoclonal anti p-MLKL	Abcam	Cat# ab196436, RRID:AB_2687465
Rabbit polyclonal anti BAX	Cell Signaling Technology	Cat# 2774, RRID:AB_490806
Mouse monoclonal anti BAX (6A7)	BD Biosciences	Cat# 556467, RRID:AB_396430
Rabbit monoclonal anti BIM	Cell Signaling Technology	Cat# 2933, RRID:AB_1030947
Mouse monoclonal anti BID	Cell Signaling Technology	Cat# 2006, RRID:AB_2065507
Rabbit polyclonal anti PUMA	Cell Signaling Technology	Cat# 4976, RRID:AB_2064551
Rabbit monoclonal anti cleaved-caspase 3	Cell Signaling Technology	Cat# 9579, RRID:AB_10897512
Rabbit monoclonal anti Caspase 8	Cell Signaling Technology	Cat# 4790, RRID:AB_10545768
Rabbit polyclonal anti BCL-2	Cell Signaling Technology	Cat# 2876, RRID:AB_2064177
Rabbit monoclonal anti BCL-XL	Cell Signaling Technology	Cat# 2764, RRID:AB_2228008
Rabbit polyclonal anti CPT1 α	Proteintech	Cat# 15184-1-AP, RRID:AB_2084676
Rabbit polyclonal anti ACOX1	Proteintech	Cat# 10957-1-AP, RRID:AB_2221670
Rabbit polyclonal anti ACOX2	Thermo Fisher Scientific	Cat# PA5-50297, RRID:AB_2635750
Mouse monoclonal anti CYP4A	Santa Cruz Biotechnology	Cat# sc-271983, RRID:AB_10715105
Rabbit polyclonal anti CYP2E1	Abcam	Cat# ab28146, RRID:AB_2089985
Rabbit monoclonal anti ATF4	Cell Signaling Technology	Cat# 11815, RRID:AB_2616025
Rabbit polyclonal anti p-PPAR α	Thermo Fisher Scientific	Cat# PA1-820, RRID:AB_325817
Rabbit polyclonal anti PPAR α	Thermo Fisher Scientific	Cat# PA1-32484, RRID:AB_1959487
Rabbit polyclonal anti SREBP2	Abcam	Cat# ab30682, RRID:AB_779079
Rabbit monoclonal anti LAMP1	Cell Signaling Technology	Cat# 3243, RRID:AB_2134478

Rat monoclonal anti LAMP2	Thermo Fisher Scientific	Cat# MA1-165, RRID:AB_2609332
Mouse monoclonal anti 4-HNE	R and D Systems	Cat# MAB3249, RRID:AB_664165
Rabbit polyclonal anti MPO	LSBio (LifeSpan)	Cat# LS-B6699-250, RRID:AB_11143601
Mouse monoclonal anti Galectin-3	Santa Cruz Biotechnology	Cat# sc-32790, RRID:AB_627657
Rabbit monoclonal anti Cathepsin B	Abcam	Cat# ab214428, RRID:AB_2848144
Rat monoclonal anti CD45	BioLegend	Cat# 103108, RRID:AB_312973
Rat monoclonal anti CD11b	BioLegend	Cat# 101208, RRID:AB_312791
Rat monoclonal anti Ly6G	BioLegend	BioLegend Cat# 127618, RRID:AB_1877261
Rabbit polyclonal anti β-actin	Abcam	Cat# ab70165, RRID:AB_1209525
Rabbit monoclonal anti GAPDH	Cell Signaling Technology	Cat# 2118, RRID:AB_561053
Bacterial and virus strains		
AAV8-Null vectors	Vector Biolabs	Cat# 7077
AAV8-TBG-Stat5b vectors	Vector Biolabs	Lot.# 210816#41
Stat5b-Double Nickase Plasmid	Santa Cruz Biotechnology	Cat# SC-423179-Nic-2
ACSL1-Double Nickase Plasmid	Santa Cruz Biotechnology	Cat# SC-420278-Nic-2
Control Double Nickase Plasmid	Santa Cruz Biotechnology	Cat# SC-423179
Chemicals, peptides, and recombinant proteins		
Alcohol	Millipore Sigma	Cat# 64-17-5
Sodium Palmitate	Millipore Sigma	Cat# 408-35-5
Bax inhibitor peptide V5	Millipore Sigma	Cat# 196810-5mg
Necrosulfonamide	Selleckchem	Cat# S8251
7-AAD	BioLegend	Cat# 420404
DAPI	Thermo Fisher Scientific	Cat# P36935
BODIPY 493/503	Thermo Fisher Scientific	Cat# D3922
LysoTracker DND-99	Thermo Fisher Scientific	Cat# L7528
Pierce™ Protein A/G Agarose	Thermo Fisher Scientific	Cat# 20421
Lipofectamine 3000	Invitrogen	Cat# L3000008
Collagenase Type IV	Millipore Sigma	Cat# C4-22-1G
Critical commercial assays		
Mouse LCN2 ELISA	R&D systems	Cat# MLCN20
Lysosome enrichment kit	Thermo Fisher Scientific	Cat# 89839
Dual-luciferase reporter assay	Promega	Cat# E1910
ALT assay	Cayman Chemical	Cat# 700260
AST assay	Cayman Chemical	Cat# 701640
Triglyceride assay	Biovision	Cat# K622
Free fatty acid assay	Biovision	Cat# K612

TUNEL assay	Millipore Sigma	Cat# S7100
Seahorse XF Cell Mito Stress Test Kit	Agilent Technologies	Cat# 103015-100
Seahorse XF Real-time ATP Rate Kit	Agilent Technologies	Cat# 103592-100
Retrieved data source		
Gene microarray of human alcoholic hepatitis patient liver tissue	NCBI Gene Expression Omnibus	GEO: GSE28619
RNAseq analysis of human alcoholic hepatitis patient liver tissue	NCBI Gene Expression Omnibus	GEO: GSE143318
Experimental models: Cell lines		
Mouse: Hepa-1c1c7	ATCC	Cat# CRL-2026
Mouse: STAT5b knockdown of Hepa-1c1c7 cell line	This paper	N/A
Mouse: Acsl1 knockdown of Hepa-1c1c7 cell line	This paper	N/A
Experimental models: Organisms/strains		
ACSL1 Flox mice	Dr. Rosalind Coleman	N/A
STAT5 Flox mice	Jackson Laboratory	Strain #: 032053-JAX
Albumin-Cre mice	Jackson Laboratory	Strain #: 003574
Hepatocyte-specific ACSL1 knockout mice	This paper	N/A
Hepatocyte-specific Stat5 knockout mice	This paper	N/A
Hepatocyte-specific Stat5b overexpression mice	This paper	N/A
Oligonucleotides		
siRNA of BAX	Santa Cruz Biotechnology	Cat# SC-421913
siRNA of MLKL	Santa Cruz Biotechnology	Cat# SC-149468
siRNA control	Santa Cruz Biotechnology	Cat# SC-36869
Primers for genes see Table S1	This paper	N/A
Recombinant DNA		
WT clone of mouse ACSL1	GeneCooeia.Inc.	Cat# CS-MPRM39476-pGL3-01
MUT1 clone of mouse ACSL1 mutant TTCTAAGAA to AACTAAGAA	GeneCooeia.Inc.	Cat# CS-MPRM39476-pGL3-02
MUT2 clone of mouse ACSL1 mutant TTCTGGCAA to AACTGGCAA	GeneCooeia.Inc.	Cat# CS-MPRM39476-pGL3-03
MUT3 clone of mouse ACSL1 mutant both TTCTAAGAA to AACTAAGAA and TTCTGGCAA to AACTGGCAA	GeneCooeia.Inc.	Cat# CS-MPRM39476-pGL3-04
Software and algorithms		
Flowjo 10.1 software	BD Life Sciences	https://www.flowjo.com
GEO dataset analysis software	HOME for Researchers	https://www.aclbi.com/static/index.html#/
Figdraw software	HOME for Researchers	https://www.figdraw.com
R software	R Core Team	https://www.r-project.org/
TFBIND	T.Tsunoda and T.Takagi,1999	https://tfbind.hgc.jp/

GraphPad Prism 8	GraphPad	https://www.graphpad.com/
IBM SPSS Statistics	IBM	http://www.ibm.com/analytics/us/en/technology/spss/
Other		
BD FACSMelody™ Cell Sorter	BD Life Sciences	N/A
Promega GloMax 96 Microplate Luminometer	Promega	Cat# E6521