Supplementary information

Hepatocyte TonEBP promotes metabolic stress-induced hepatic fibroinflammation involving transcriptional activation of ELR$^+$ CXC chemokines

Supplementary Table 1. Primers used in qPCR for gene expression analysis and ChIP assay

Primers for gene expression analysis

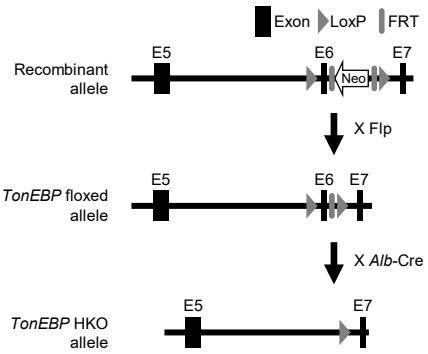
Species	Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession No.*
Mouse	*α-Sma*	AAACAGGAATACGACGAAG	CAGGAATGATTTGGAAAGGA	NM_007392.3
	Ccl2	AACTGCATCTGCCCTAAGGT	AGTGCTTGAGGTGGTTGTGGAA	NM_011333
	Col15a1	TGCGTGTTTACCCAGTCATAG	AGGATTCTGTGCTGTTTCTCTC	NM_009928.3
	Col1a1	GCTTGAAGACCTATGTGGGTATAA	GGTGGAGAAAGGAGCAGAAA	NM_007742.4
	Col1a2	CCAGAGTGGAACAGCGATTAC	GATGCAGGTTTCACCAGTAGAG	NM_007743.3
	Col3a1	GGCTGCAAGATGGATGCTATAA	GAATCTGTCCACCAGTGCTTAC	NM_009930.2
	Col4a2	CATCAAAGGAGTCAAGGGAGAC	GAATCCTGGAAACCCGGTAAT	NM_009932.5
	Col5a2	AGCCCAGGAACAAGAGAATG	CACAGATCTGACATGGAGAAGG	NM_007737.2
	Col6a3	CAGTACATCCGTACCCTGATTG	CATCTTGGAGTCCTCACTGAAC	NM_001243009.1
	Ctgf	ACCTGTGCCTGCCATTAC	GTCCCTTACTTCCTGGCTTTAC	NM_010217.2
	Cxcl1	GTGTCTAGTTGGTAGGGCATAAT	CAGTCCTTTGAACGTCTCTGT	NM_008176.3
	Cxcl2	TAAGCACCGAGGAGAGTAGAA	GTCCAAGGGTTACTCACAACA	NM_009140.2
	CypA	CAGCCATGGTCAACCCCACCG	CTGCTGTCTTTGGAACTTTGTCTG	NM_008907
	E-selectin	CAGGGAACAGGAAGTGTGATAG	GCAGTTTCTCACAGTCTCTCTC	NM_011345.3
	F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG	NM_010130
	Icam1	GTGATGGCAGCCTCTTATGT	GGGCTTGTCCCTTGAGTTT	NM_010493.3
	IL-1β	CAAAGGCGGCCAGGATATAA	CTAGGGATTGAGTCCACATTCAG	NM_000576
	Mmp2	GTTCAACGGTCGGGAATACA	GCCATACTTGCCATCCTTCT	NM_008610.3
	Mmp7	GAGTATGCAGATCCCACTGAAC	CTCTCCTTGCGAAGCCAATTA	NM_010810.6
	Mmp9	CTGGAACTCACACGACATCTT	TCCACCTTGTTCACCTCATTT	NM_013599.5
	Mpo	GATCATCACATACCGGGACTAC	GGGTCTACTGAGTCGTTGTAAG	NM_010824.2
	Pdgfa	TCCAGCGACTCTTGGAGATA	TCTCGGGCACATGGTTAATG	NM_001363271.1
	Pdgfd	AGTCAATTACCTGCCAGTTCTAC	GGGCCTGGCTTACTTCTTATT	NM_027924.3
	Pdgfra	CTCGTGCTTGGTCGGATTT	TCTTCACAGCCACCTTCATTAC	NM_001347719.1
	Pdgfrb	GTTGTTGCTGTCCGTGTTATG	AAGGTGCTGGAGATGTTGAG	NM_008809.2
	Tgfb1	GTGGAAATCAACGGGATCAG	ACTTCCAACCCAGGTCCTTC	NM_011577.2
	Timp1	TCCTAGAGACACACCAGAGATAC	TACAGGCCTTACTGGAAGCTA	NM_011593.3
	TNFα	TGGGACAGTGACCTGGACTGT	TTCGGAAAGCCCATTTGAGT	NM_013693
	TonEBP	AAGCAGCCACCACCAAACATGA	AAATTGCATGGGCTGCTGCT	NM_133957
	Vcam1	GCACTCTACTGCGCATCTT	CACCAGACTGTACGATCCTTTC	NM_011693.3
	Vm	CCCTGAACCTGAGAGAAACTAAC	CTCTGGTCTCAACCGTCTTAATC	NM_011701.4
Human	*CXCL1*	CCTGCCCTTATAGGAACAGAAG	AAGCGATGCTCAAACACATTAG	NM_001511.4
	CXCL2	GCATCGCCCATGGTTAAGA	TCAGGAACAGCCACCAATAAG	NM_002089.4
	CypA	TTCATCTGCACTGCCAAGAC	TCGAGTTGTCCACAGTCAGC	NM_021130.5
	IL-8	AAATCTGGCAACCCTAGTCTG	GTGAGGTAAGATGGTGGCTAAT	NM_000584.4
	TonEBP	AGCTGTTGTTGCTGCTGATGCT	TCCACTTGCATAGCCTTGCTGT	NM_006599

* Accession No. refer to Genebank (NCBI)

Primers for ChIP-qPCR

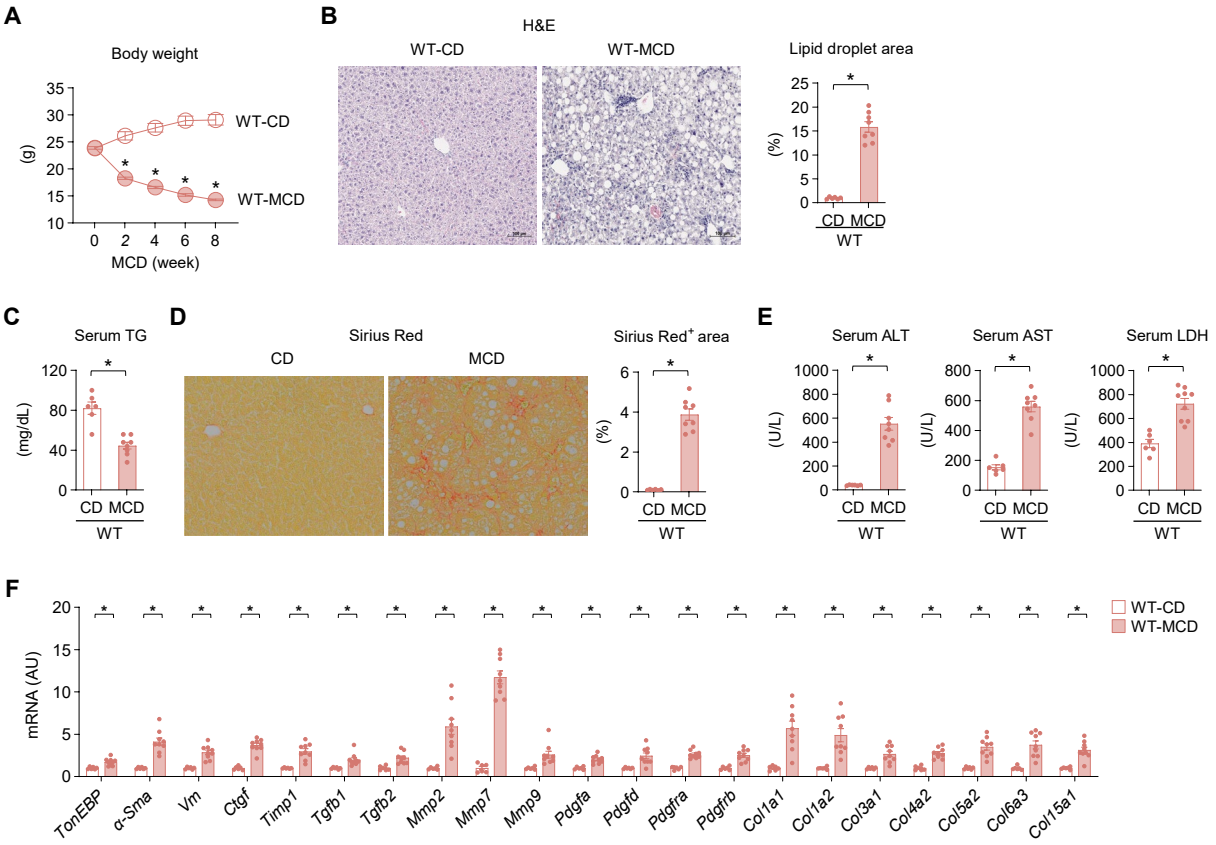
Species	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Human	*IL-8*	GGAAGTGTGATGACTCAGGTT	GAGAACTTATGCACCCTCATCT
	CXCL1	TTCCGGACTCGGGATCG	GCTCTGTGGCTCTCCGA
	CXCL2	CGCCTTCCTTCCGAACTC	CTCCGAGAACGGCGAAC

Supplementary Fig. 1



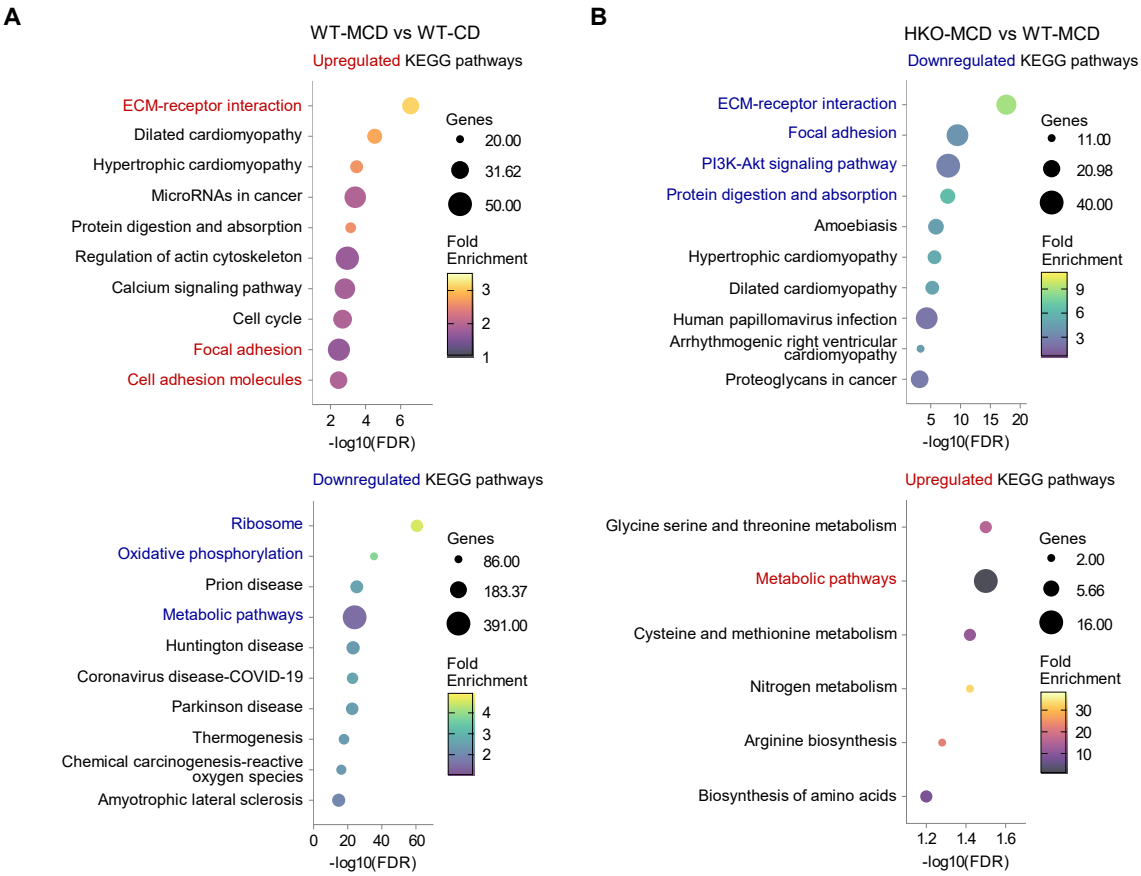
Supplementary Fig. 1.
Breeding strategy to generate *Alb-Cre;TonEBP*$^{flox/flox}$ (hepatocyte -specific TonEBP knockout, HKO) mice.

Supplementary Fig. 2 (related to Fig. 1)



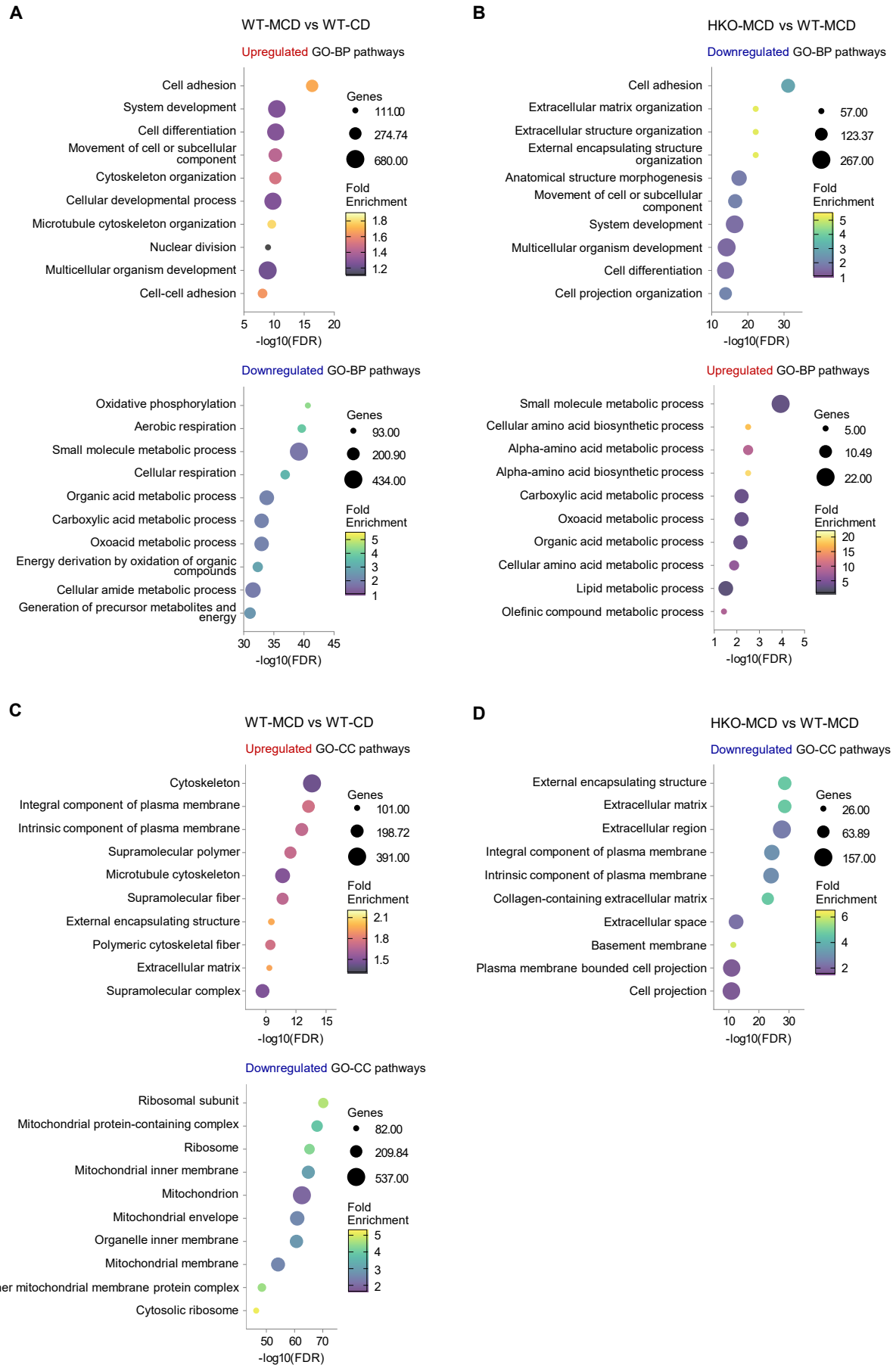
Supplementary Fig. 2 (related to Fig. 1).
MCD diet induces liver injury and fibrosis in mice.
Male C57BL/6 mice (8 weeks old) were fed a chow diet (CD, *n* = 6) or methionine-choline deficient (MCD, *n* = 9) diet for 8 weeks. (A) Changes in body weight. (B) Representative H&E-stained liver sections with quantification. (C) Serum triglyceride levels. (D) Sirius Red-stained images with quantification. (E) Serum ALT, AST, and LDH activities. (F) RT-qPCR analysis of fibrosis-related gene expression in the liver (*n* = 9). Data are presented as mean ± SD. One-way ANOVA with Tukey's test; *p < 0.05 vs. CD.

Supplementary Fig. 3 (related to Fig. 2)

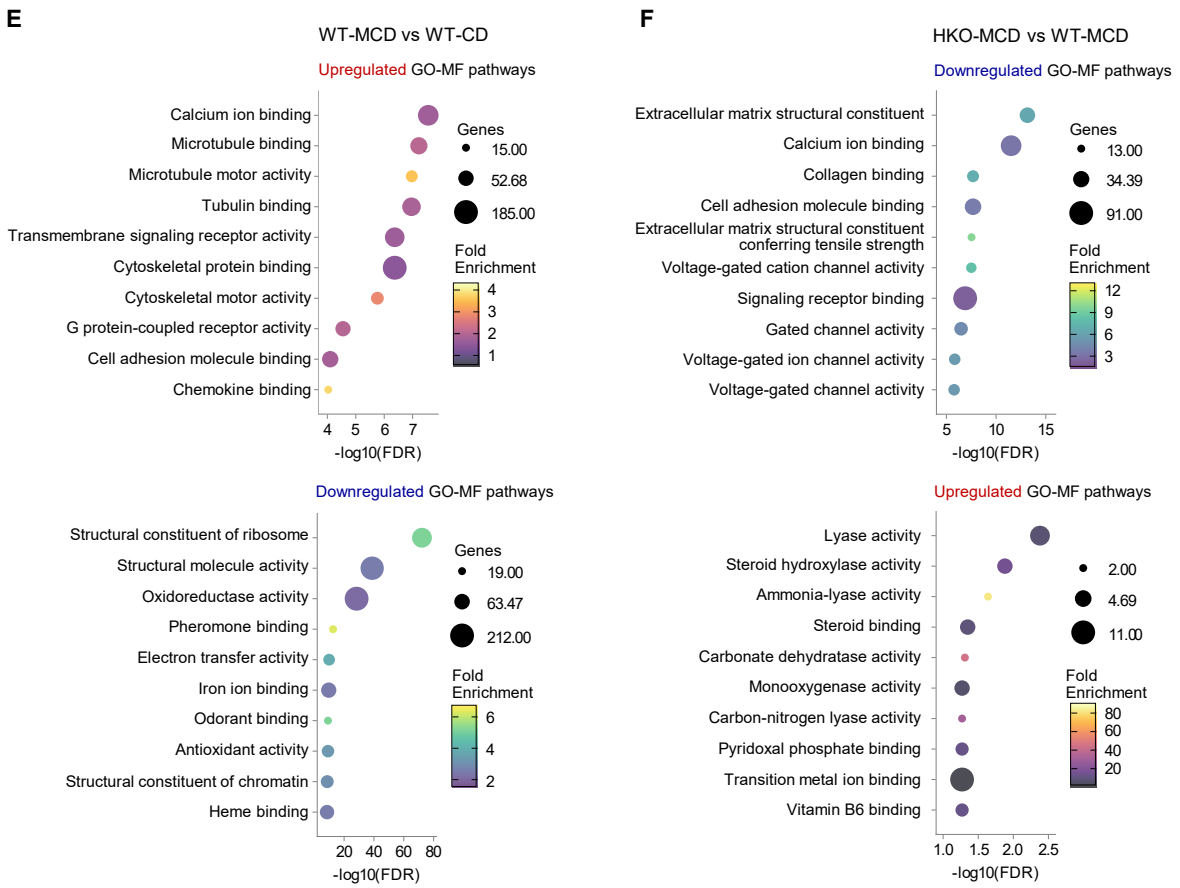


Supplementary Fig. 3 (related to Fig. 2).
Transcriptomic enrichment analyses.
Significantly (FDR <0.05) enriched KEGG pathways of DEGs identified by DESeq2 and edgeR (|log2FC| > 2) from RNA-sequencing data of liver samples from male HKO and WT mice fed a CD (*n* = 3) or MCD (*n* = 5) for 8 weeks. (A) WT-MCD vs WT-CD. (B) HKO-MCD vs. WT-MCD.

Supplementary Fig. 4 (related to Fig. 2)

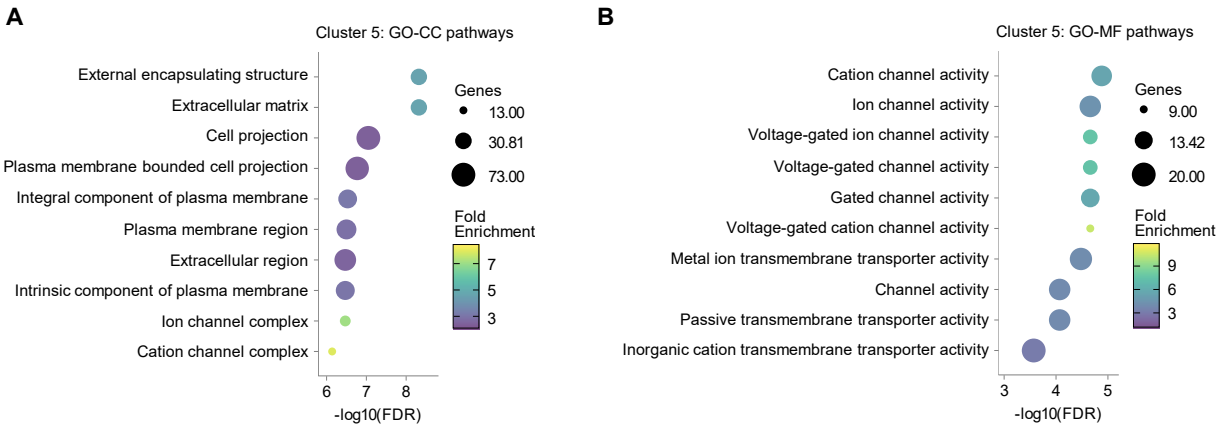


Supplementary Fig. 4 (related to Fig. 2)



Supplementary Fig. 4 (related to Fig. 2).
Transcriptomic enrichment analyses.
Gene Ontology (GO) term enrichment analysis of DEGs from RNA-sequencing data of liver samples from male HKO and WT mice fed a CD (*n* = 3) or MCD (*n* = 5) for 8 weeks. (A, B) Significantly (FDR <0.05) enriched GO Biological Process (BP) terms: (A) WT-MCD vs. WT-CD. (B) HKO-MCD vs. WT-MCD. (C, D) Significantly enriched GO cellular component (CC) terms: (C) WT-MCD vs. WT-CD. (D) HKO-MCD vs. WT-MCD. (E, F) Significantly enriched GO Molecular Functions (MF): (E) WT-MCD vs. WT-CD. (F) HKO-MCD vs. WT-MCD.

Supplementary Fig. 5 (related to Fig. 2)



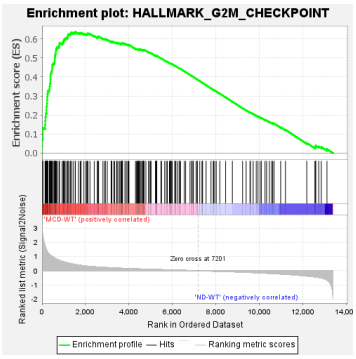
Supplementary Fig. 5 (related to Fig. 2).
Transcriptomic enrichment analyses.
K-means clustering of the top 2,000 variable genes and GO terms enrichment analysis of cluster 5 from RNA-sequencing data of liver samples from male HKO and WT mice fed a CD (*n* = 3) or MCD (*n* = 5) for 8 weeks. (A) Significantly enriched GO-CC terms. (B) Significantly enriched GO-MF terms. False discovery rate (FDR) <0.05.

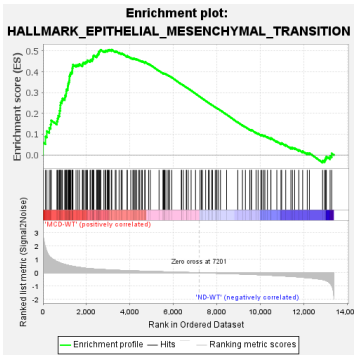
Supplementary Fig. 6 (related to Fig. 2)

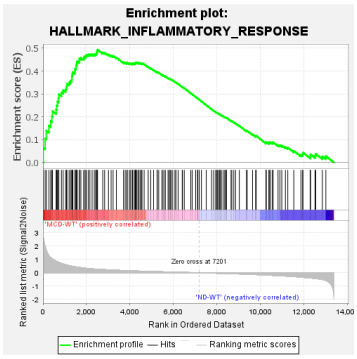
A

WT-MCD vs. WT-CD

GSEA: Hallmark Gene Sets	SIZE	NES	NOM p-val	FDR q-val
G2M_CHECKPOINT	189	2.00	0.000	0.000
E2F_TARGETS	190	1.90	0.000	0.000
MITOTIC_SPINDLE	194	1.86	0.000	0.001
ANGIOGENESIS	28	1.62	0.013	0.020
EPITHELIAL_MESENCHYMAL_TRANSITION	159	1.61	0.001	0.019
KRAS_SIGNALING_UP	161	1.61	0.000	0.016
INFLAMMATORY_RESPONSE	149	1.58	0.000	0.020
APICAL_JUNCTION	153	1.55	0.000	0.023
UV_RESPONSE_DN	132	1.35	0.037	0.199
IL2_STAT5_SIGNALING	171	1.34	0.031	0.193

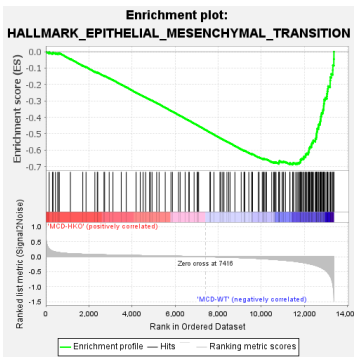


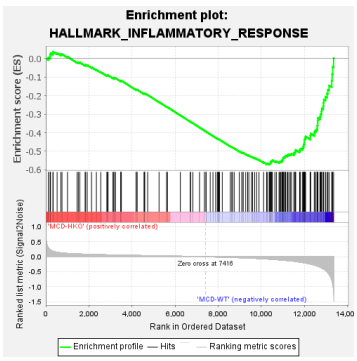


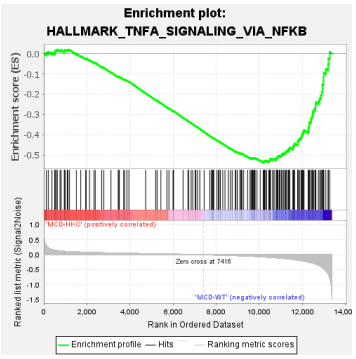


HKO-MCD vs. WT-MCD

GSEA: Hallmark Gene Sets	SIZE	NES	NOM p-val	FDR q-val
EPITHELIAL_MESENCHYMAL_TRANSITION	158	-1.90	0.000	0.000
MYOGENESIS	131	-1.78	0.000	0.000
APICAL_JUNCTION	153	-1.77	0.000	0.000
ANGIOGENESIS	28	-1.72	0.001	0.001
KRAS_SIGNALING_UP	160	-1.65	0.000	0.003
INFLAMMATORY_RESPONSE	147	-1.57	0.000	0.011
APICAL_SURFACE	30	-1.52	0.029	0.020
TNFA_SIGNALING_VIA_NFKB	167	-1.48	0.002	0.029
UV_RESPONSE_DN	133	-1.45	0.004	0.038
COAGULATION	118	-1.37	0.017	0.076
ESTROGEN_RESPONSE_EARLY	159	-1.34	0.017	0.100
P53_PATHWAY	182	-1.29	0.043	0.135





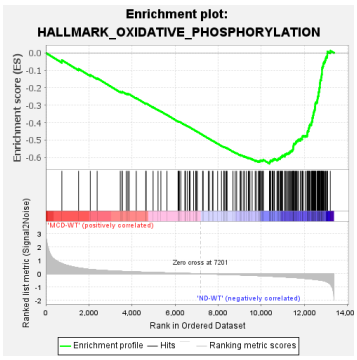


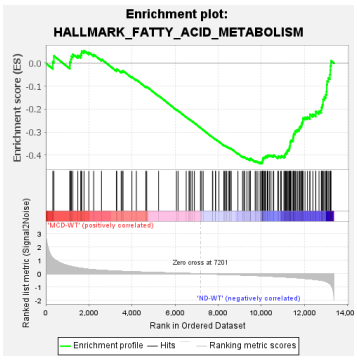
Supplementary Fig. 6 (related to Fig. 2)

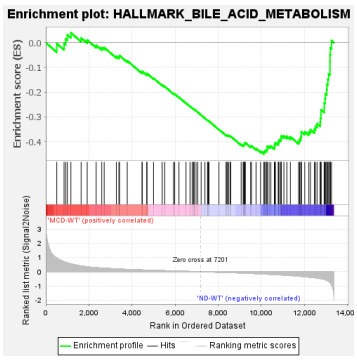
B

HKO-MCD vs. WT-MCD

GSEA: Hallmark Gene Sets	SIZE	NES	NOM p-val	FDR q-val
OXIDATIVE_PHOSPHORYLATION	193	-2.857	0.000	0.000
FATTY_ACID_METABOLISM	140	-1.918	0.000	0.000
BILE_ACID_METABOLISM	106	-1.902	0.000	0.000
ADIPOGENESIS	192	-1.663	0.000	0.009
XENOBIOTIC_METABOLISM	186	-1.611	0.000	0.013
DNA_REPAIR	144	-1.370	0.000	0.075

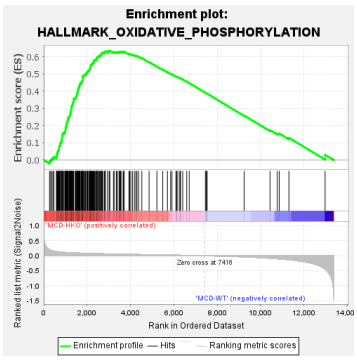


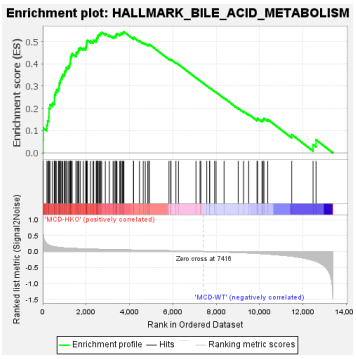


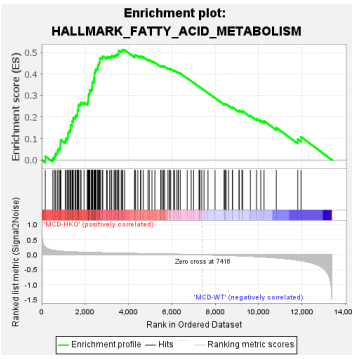


HKO-MCD vs. WT-MCD

GSEA: Hallmark Gene Sets	SIZE	NES	NOM p-val	FDR q-val
OXIDATIVE_PHOSPHORYLATION	193	2.69	0.000	0.000
BILE_ACID_METABOLISM	106	2.42	0.000	0.000
FATTY_ACID_METABOLISM	140	2.27	0.000	0.000
MYC_TARGETS_V1	191	1.98	0.000	0.000
ADIPOGENESIS	192	1.52	0.000	0.072
XENOBIOTIC_METABOLISM	186	1.42	0.000	0.111
PEROXISOME	90	1.30	0.033	0.183
INTERFERON_ALPHA_RESPONSE	91	1.29	0.000	0.166

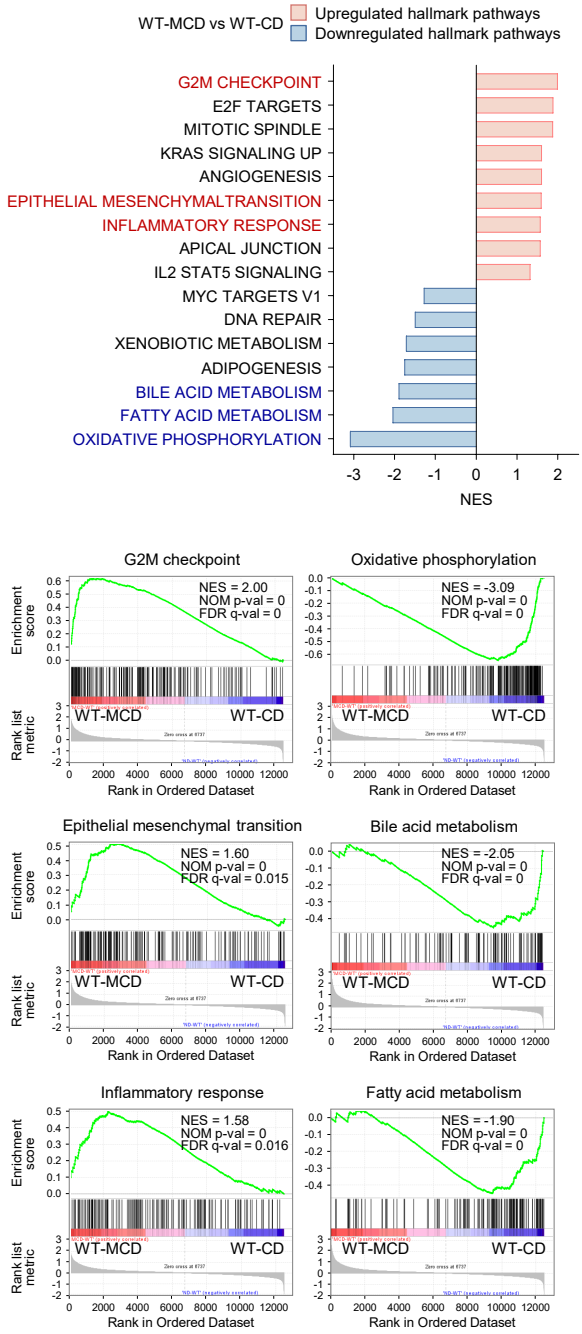






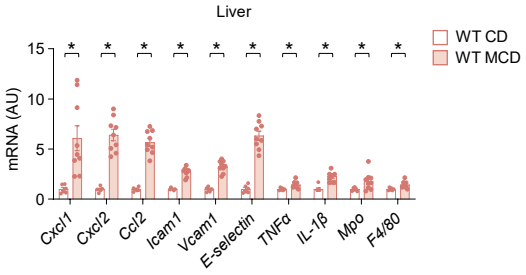
Supplementary Fig. 6 (related to Fig. 2).
Transcriptomic enrichment analyses.
GSEA analysis using MSigDB hallmark gene sets from RNA-sequencing data of liver samples from male HKO and WT mice fed a CD (*n* = 3) or MCD (*n* = 5) for 8 weeks. (A) The pathways significantly downregulated in HKO-MCD compared to WT-MCD. (B) The pathways significantly upregulated in HKO-MCD compared to WT-MCD.

Supplementary Fig. 7 (related to Fig. 2)



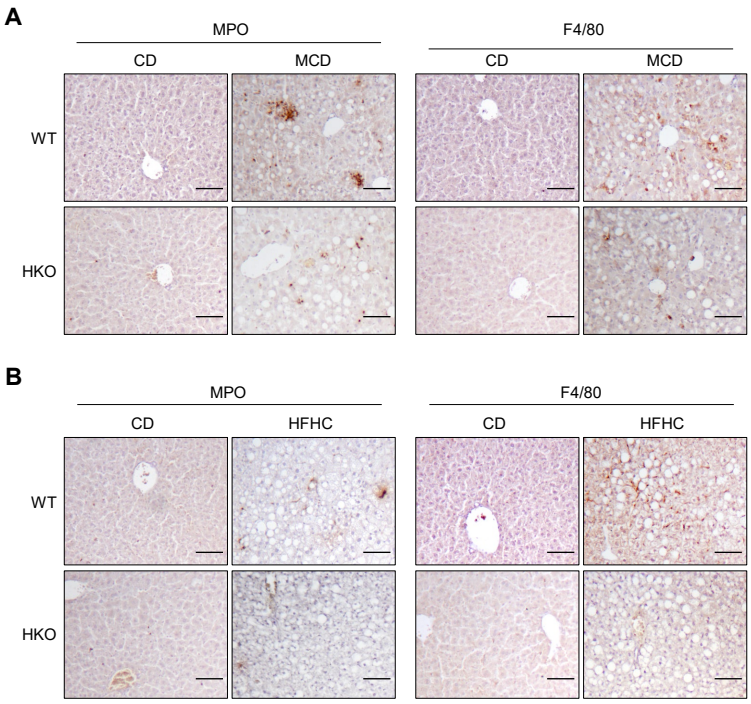
Supplementary Fig. 7 (related to Fig. 2).
Transcriptomic enrichment analyses.
GSEA of KEGG pathways after mapping mouse genes to human orthologs from RNA-sequencing data of liver samples from male HKO and WT mice fed a CD (*n* = 3) or MCD (*n* = 5) for 8 weeks.

Supplementary Fig. 8 (related to Fig. 4)



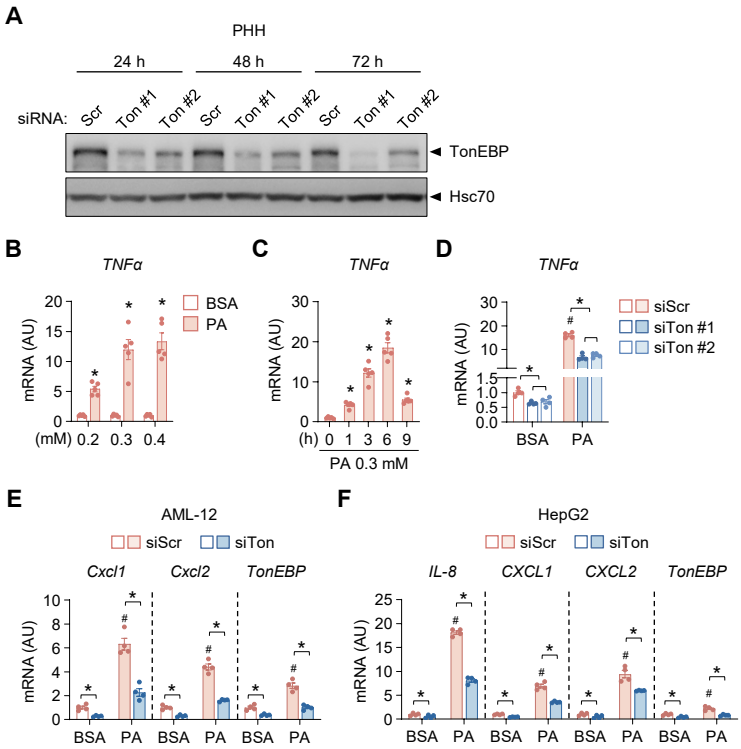
Supplementary Fig. 8 (related to Fig. 4).
MCD diet induces hepatic expression of inflammation-related genes in mice.
RT-qPCR analysis of inflammation-related genes in liver samples from HKO (*n* = 9) and WT (*n* = 6) mice fed an MCD for 8 weeks.
Data are presented as mean ± SEM. Statistical analysis was performed by an ANOVA with Tukey's test. *p < 0.05 vs. WT CD.

Supplementary Fig. 9 (related to Fig. 5)



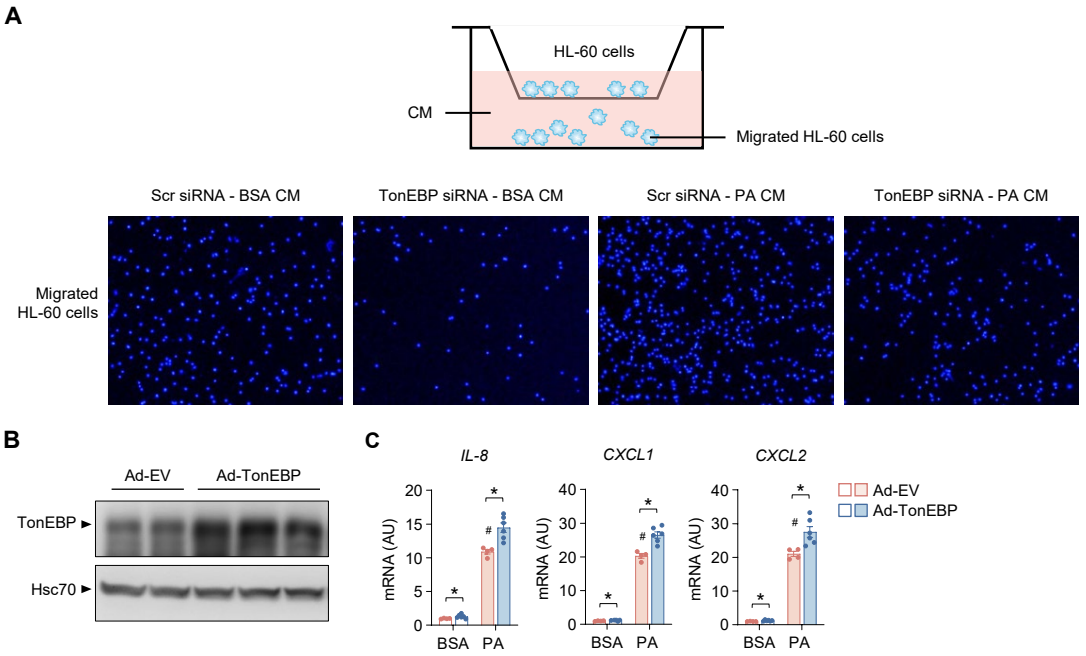
Supplementary Fig. 9 (related to Fig. 5).
MCD and HFHC diets promote neutrophil and macrophage infiltration into the liver, which is attenuated by hepatocyte TonEBP depletion.
(A, B) Representative immunohistochemistry images of MPO (neutrophils) and F4/80 (macrophages) in liver sections from WT and HKO mice fed a control diet (CD), MCD (8 weeks) (A), or HFHC (16 weeks) (B). Scale bar: 50 μm.

Supplementary Fig. 10 (related to Fig. 5)



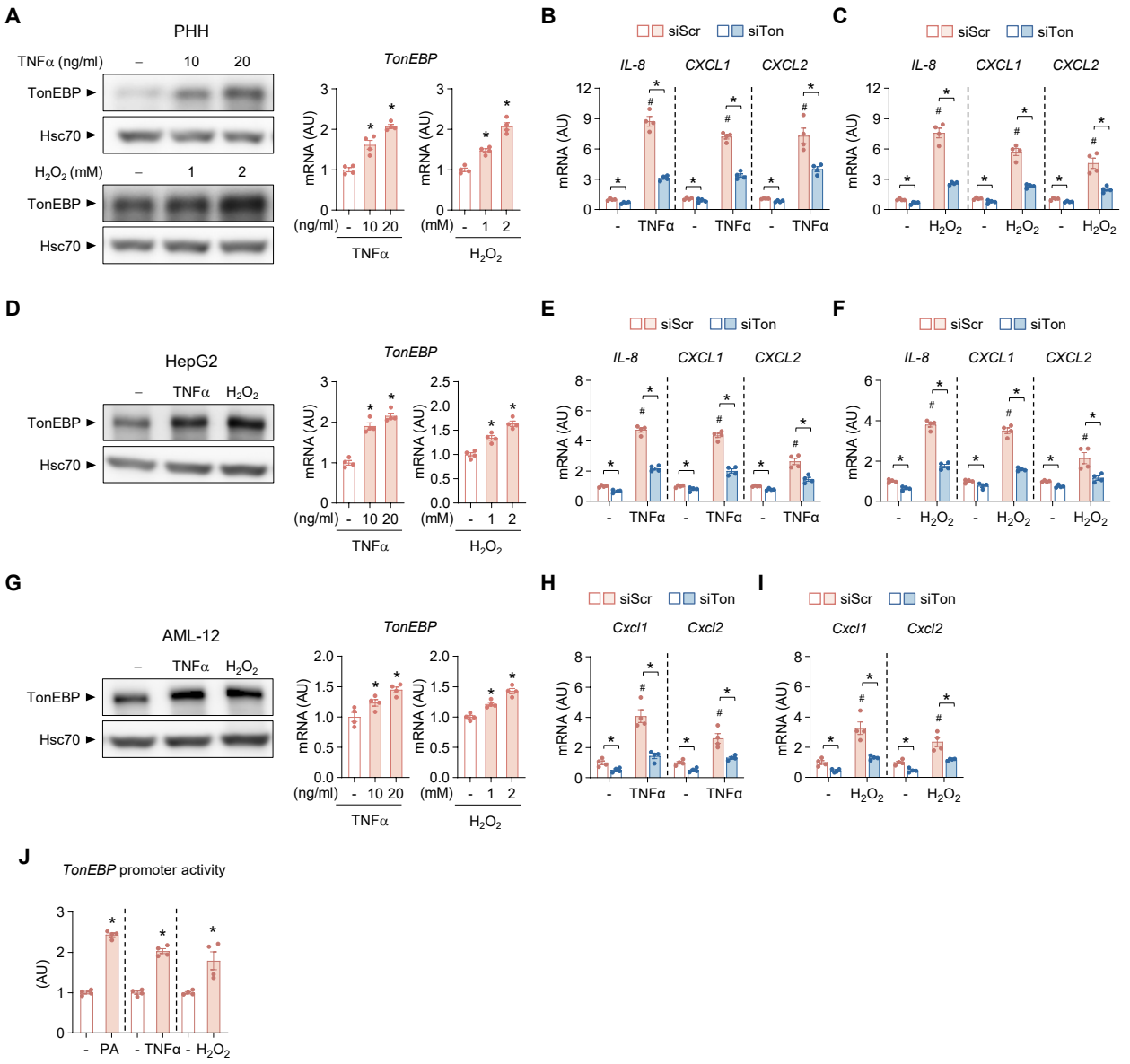
Supplementary Fig. 10 (related to Fig. 5).
TonEBP regulates CXC chemokine expression in response to palmitate.
(A) Immunoblot analysis of TonEBP expression in PHHs transfected with scrambled siRNA (siScr) or TonEBP siRNAs (#1 or #2) for 24, 48, or 72 h. (B–D) RT-qPCR analysis of *TNFα* mRNA expression in primary human hepatocytes (PHHs) treated with BSA or palmitate (PA) at the indicated concentrations for 3 h (B), at the indicated time after PA (0.3 mM) treatment (C), and in PHHs transfected with siTon or siScr and treated with BSA or PA for 6 h (D). (E–F) RT-qPCR of *IL-8* (PHHs only), *CXCL1, CXCL2*, and *TonEBP* in AML-12 cells (E), and HepG2 (F) transfected with siTon or siScr and treated with BSA or PA for 3 h. Data are presented as mean ± SD. One-way ANOVA with Tukey's test. *p < 0.05 vs. BSA (B) or time zero (0 h) (C); #p < 0.05 vs. BSA.

Supplementary Fig. 11 (related to Fig. 5)



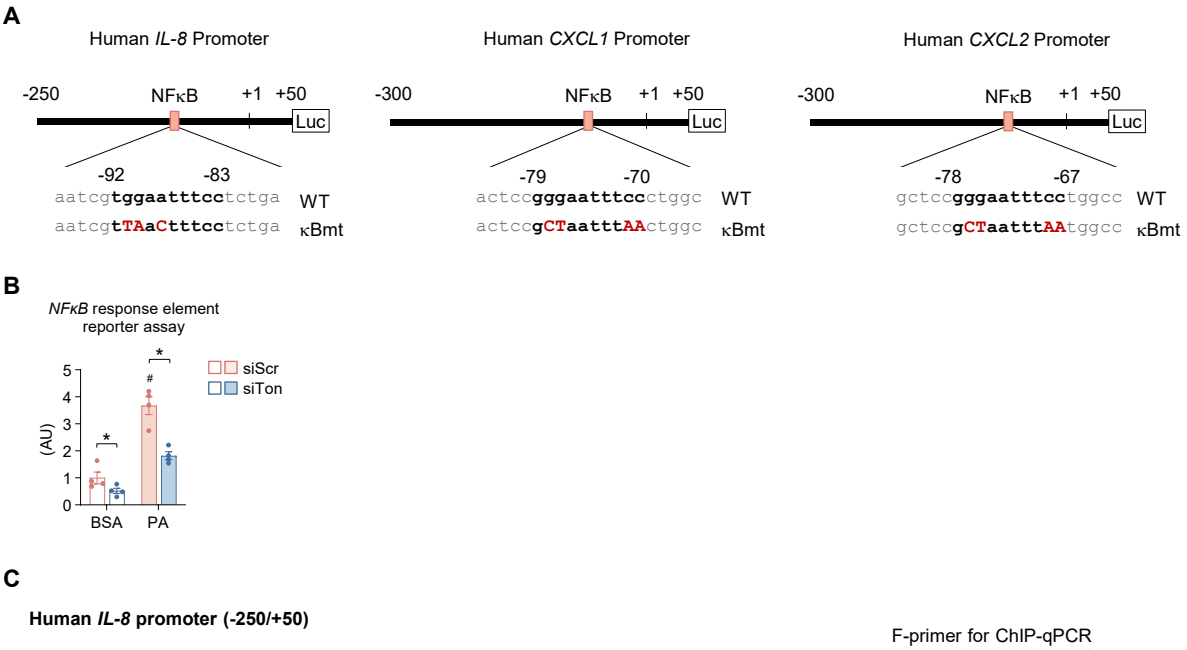
Supplementary Fig. 11 (related to Fig. 5).
TonEBP regulates neutrophil chemotaxis via CXC chemokines.
(A) Neutrophil chemotaxis assay using HL-60 cells exposed to conditioned media (CM) from HepG2 cells transfected with siScr or TonEBP siRNA (siTon) and treated with BSA or palmitate (PA, 0.3 mM) for 16 h. Migrated HL-60 cells were visualized by DAPI staining after 16 h in Transwell chambers. (B) Immunoblot analysis of TonEBP in HepG2 cells infected with adenovirus expressing TonEBP (Ad-TonEBP) or empty vector (Ad-EV). (C) RT-qPCR analysis of *IL-8*, *CXCL1*, and *CXCL2* expression in HepG2 cells infected with Ad-TonEBP or Ad-EV and treated with PA (0.3 mM) for 3 h (n = 5). Data are presented as mean ± SD. One-way ANOVA with Tukey's test. *p < 0.05 vs. Ad-EV; #p < 0.05 vs. Ad-EV–BSA.

Supplementary Fig. 12 (related to Fig. 5)



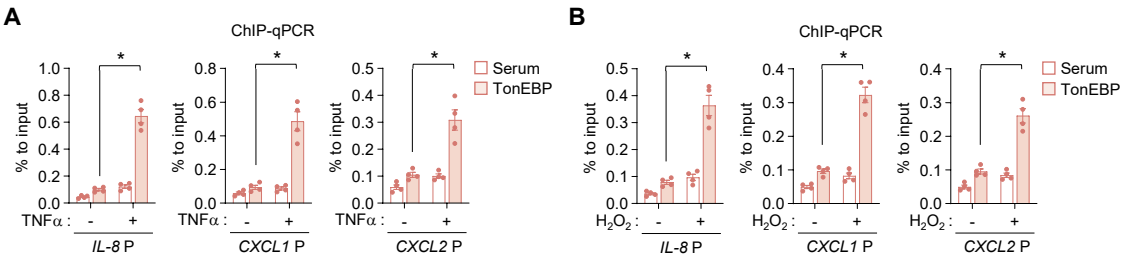
Supplementary Fig. 12 (related to Fig. 5).
TonEBP is required for chemokine induction in response to inflammatory stimuli.
(A) Immunoblot (left) and RT-qPCR (right) analysis of TonEBP protein and mRNA levels in primary human hepatocytes (PHH) treated with TNFα (10, 20 ng/mL) or H_2O_2 (1, 2 mM) for 16 h for protein analysis, and for 6 h for mRNA analysis. (B, C) RT-qPCR analysis of *IL-8*, *CXCL1*, and *CXCL2* in PHHs transfected with control siRNA (siScr) or TonEBP siRNA (siTon) and treated with TNFα (10 ng/mL) (B) or H_2O_2 (1 mM) (C) for 6 h. (D) TonEBP protein (left) and mRNA (right) levels in HepG2 cells treated with TNFα (10 ng/mL) or H_2O_2 (1 mM) for 16 h for protein analysis, and TNFα (10, 20 ng/mL) or H_2O_2 (1, 2 mM) for 6 h for mRNA analysis. (E, F) RT-qPCR analysis of *IL-8*, *CXCL1*, and *CXCL2* in HepG2 cells transfected with siScr or siTon and treated with TNFα (E) or H_2O_2 (F) as in (B, C). (G) TonEBP protein (left) and mRNA (right) levels in AML-12 cells treated with TNFα or H_2O_2 as in (D). (H, I) RT-qPCR analysis of *Cxcl1* and *Cxcl2* in AML-12 cells transfected with siScr or siTon and treated with TNFα (H) or H_2O_2 (I) as in (B, C). (J) Luciferase assay for TonEBP promoter activity in HepG2 cells treated with palmitate (PA, 0.3 mM), TNFα (10 ng/mL) or H_2O_2 for 12 h. Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. #p < 0.05 vs. vehicle (–); *p < 0.05 vs. indicated control.

Supplementary Fig. 13 (related to Fig. 6)



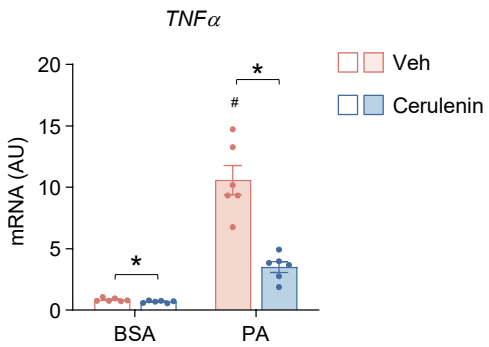
Supplementary Fig. 13 (related to Fig. 6).
TonEBP is required for NF-κB recruitment to *IL-8*, *CXCL1*, and *CXCL2* promoters.
(A) Schematic representation of luciferase reporter constructs containing the wild-type (WT) or NF-κB binding site-mutant (κBmt) promoter regions of *IL-8*, *CXCL1*, and *CXCL2*. (B) NF-κB response element luciferase assay in HepG2 cells transfected with siScr or TonEBP siRNA (siTon) and treated with BSA or palmitate (PA, 0.3 mM) for 9 h. One-way ANOVA with Tukey's test. #$p < 0.05$ vs. siScr–BSA; *$p < 0.05$.(C) NF-κB binding sites and ChIP-qPCR amplicon regions in the *IL-8*, *CXCL1*, and *CXCL2* promoters.

Supplementary Fig. 14 (related to Fig. 6)



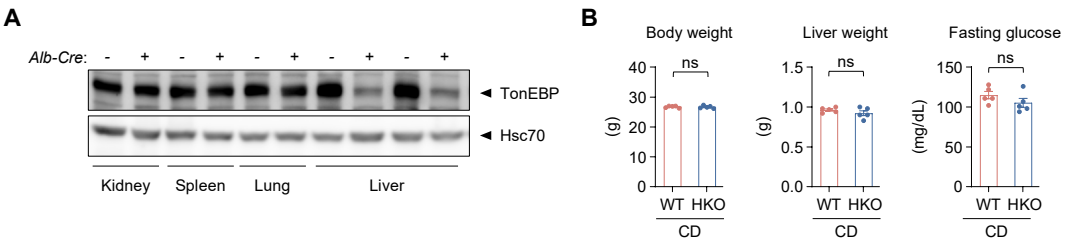
Supplementary Fig. 14 (related to Fig. 6).
TonEBP is required for NF-κB recruitment to CXC chemokine promoters.
(A, B) ChIP-qPCR analysis of TonEBP binding to the κB regions of the *IL-8*, *CXCL1*, and *CXCL2* promoters in HepG2 cells treated with TNFα (10 ng/mL) (A) or H_2O_2 (1 mM) (B) for 9 h (n = 4). Data are presented as mean ± SD. One-way ANOVA with Tukey's test. *p < 0.05.

Supplementary Fig. 15 (related to Fig. 7)



Supplementary Fig. 14 (related to Fig. 7).
Disruption of the TonEBP–NF-κB interaction suppresses *TNFα* mRNA expression.
RT-qPCR analysis of *TNFα* expression in HepG2 cells pretreated with vehicle (Veh) or cerulenin (5 μM) for 1 h, followed by stimulation with BSA or palmitate (PA, 0.3 mM) for 3 h.
Data are presented as mean ± SEM. One-way ANOVA with Tukey's test. #$p < 0.05$ vs. Veh–BSA; *$p < 0.05$.

Supplementary Fig. 16

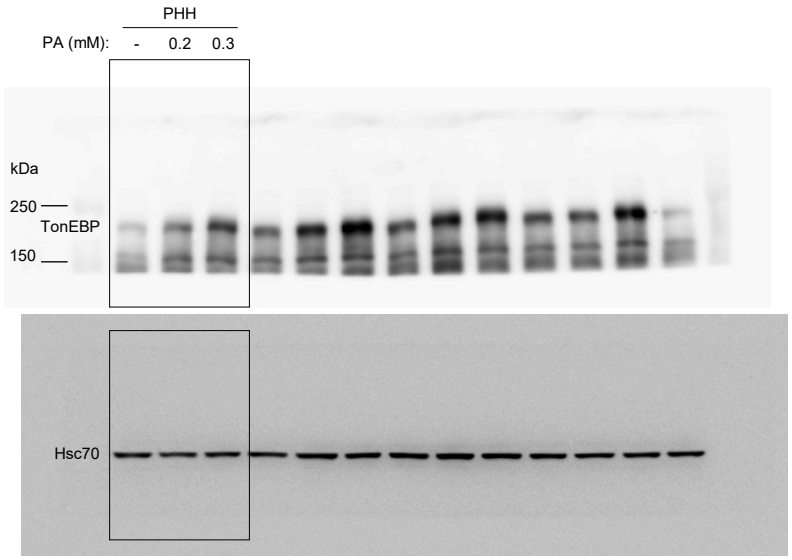


Supplementary Fig. 16.
Validation of hepatocyte-specific TonEBP deletion and phenotypic neutrality under chow diet.
(A) Western blot of TonEBP expression in various tissues. (B) Physiological assessment of 14-week-old mice on chow diet (CD). No significant differences were observed (*n* = 5 per group).

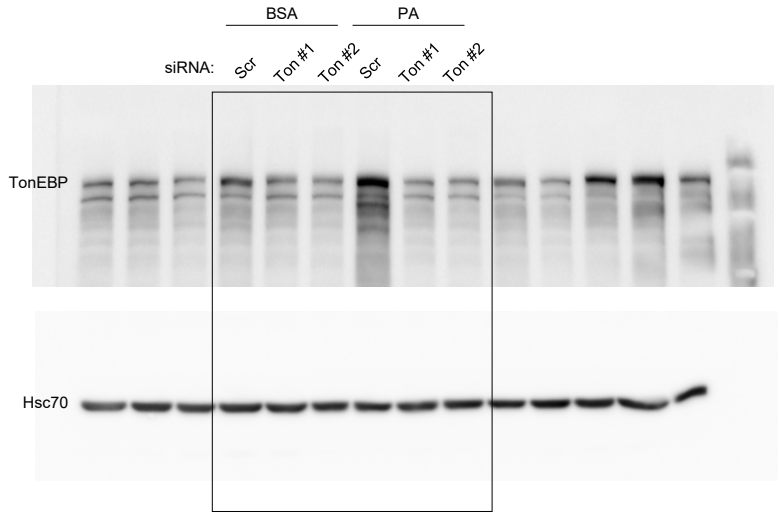
Uncropped blots of Western blots

Fig. 5

F



H



Uncropped blots of Western blots

Fig. 6

E

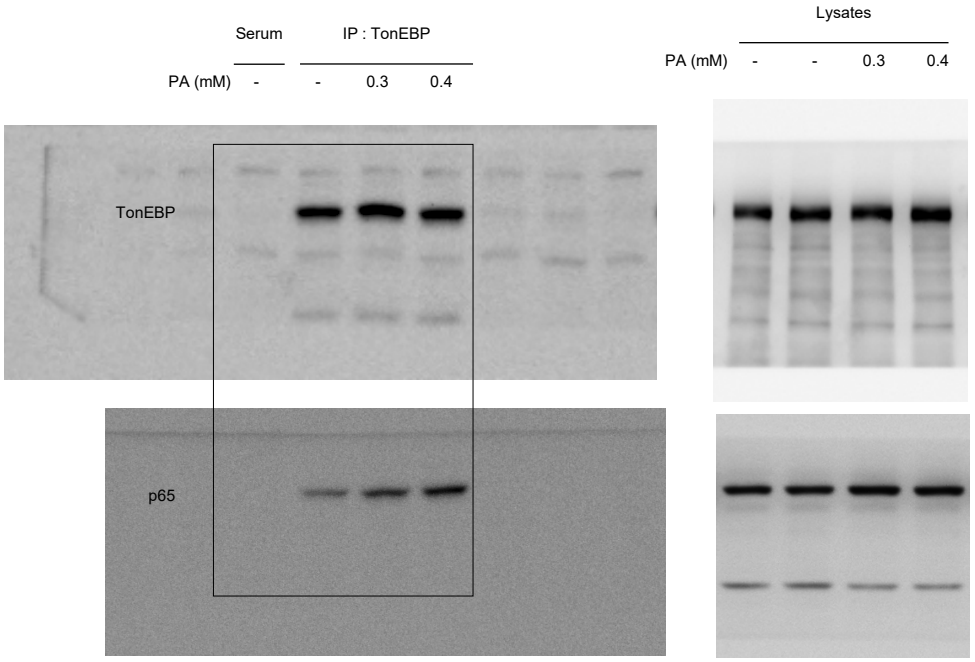


Fig. 7

A