
Supplementary information for

Integrated screening identifies GPR31 as a key driver and druggable target for metabolic dysfunction-associated steatohepatitis

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Supplementary Tables

Table S1. The physical indexes for monkeys at the baseline (0W) and 12 weeks (12W) following G4451 administration.

	0W		12W	
	Vehicle (n=4)	G4451 (n=4)	Vehicle (n=4)	G4451 (n=4)
ALT(U/L)	28.35±13.49	49.33±10.46	41.85±11.58	56.30±23.14
AST(U/L)	27.53±3.54	31.23±1.98	32.28±3.83	37.35±5.58
CREA(uM)	88.14±8.53	108.46±9.14	89.22±6.81	97.2±6.49
UREA(mM)	3.41±0.59	5.74±0.29	3.40±0.82	2.97±0.49
TC(mM)	13.39±3.00	7.12±1.07	17.10±2.65	21.29±0.91
TG(mM)	0.83±0.49	0.26±0.04	1.03±0.57	1.09±0.45
LDH(U/L)	289.80±42.16	305.33±64.29	276.43±33.64	317.68±7.50
CK(U/L)	106.90±45.13	187.65±95.12	75.18±20.42	64.63±4.86
HDL-c(mM)	2.29±0.55	1.07±0.20	1.69±0.44	1.25±0.15
LDL-c(mM)	6.00±2.02	2.26±0.44	8.04±2.10	9.91±0.75

Table S2. Sequence for shRNA.

Gene	Forward primer (5'-3')
<i>Gpr31</i>	GCCTCATCGTGTCTGCAATG
<i>Gnas</i>	GCCAAGTACTTCATTTCGAGAT
<i>Gnai3</i>	GCAGGAGTGATTAAACGGTTA
<i>Gnb1</i>	GCCATTTGCTTCTTTCCAAAT
<i>Gnb2</i>	GCCCTGCCCATGCCACACTA
<i>Stt3a</i>	GCATGCTACTCACCTACTACA
<i>Stt3b</i>	GCAGGTGCTGTGTTCTTAGT

Table S3. Primers used for qPCR.

Human.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>ACTB</i>	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC
<i>GPR31</i>	ACCAGGTGCCACAGTTTCTAC	CGGAGTCTTTTCTGGAGAGCC
<i>IL6</i>	GAGTAGTGAGGAACAAGCCAGA	AAGCTGCGCAGAATGAGATGA
<i>IL8</i>	CTAGGACAAGAGCCAGGAAGAA	GGGGTGGAAAGGTTTGGAGTA
<i>TNF</i>	TGGCGTGAGCTGAGAGATA	TGATGGCAGAGAGGAGGTTG

46 **Table S4. The physical indexes for clinical individuals**

	no MASLD	MASL	MASH
Man	7	7	7
Woman	7	7	7
Age	37.5±3.08	35.14±2.92	36.93±2.67
BMI	31.72±0.67	34.11±1.06 ^{###}	38.25±1.14 ^{***}
Systolic pressure (mmHg)	110±3.75	110.85±0.85	110±0
Diastolic pressure (mmHg)	72.38±1.85	74.85±0.15	75±0
Total bilirubin (μmol/L)	11.12±1.08	15.04±2.04	13.71±1.7
Direct bilirubin (μmol/L)	3.44±0.74	2.51±0.25	2.36±0.28
Indirect bilirubin (μmol/L)	8.09±1.03	12.52±1.82	11.36±1.44
Alanine aminotransferase ALT (U/L)	30.07±5.7	28.5±3.9 [#]	54.79±10.94 [*]
Aspartate aminotransferase AST (U/L)	37.11±12.05	24.71±3.36	32.64±4.83
Alkaline phosphatase ALP (U/L)	88.4±9.95	85.36±5.46	87.29±6.63
Gamma glutamyltransferase (U/L)	31.77±4.85	30.93±4.35	49.86±8.9
Total cholesterol (mmol/L)	4.06±0.41	4.83±0.23 [#]	5.32±0.17 [*]
Triglycerides (mmol/L)	1.29±0.25	1.58±0.22	2.12±0.3 ^{**}
HDL-c (mmol/L)	1.01±0.13	1.23±0.08	1.08±0.07
LDL-c (mmol/L)	2.52±0.36	3.1±0.2	3.52±0.17
Fasting blood glucose (mmol/L)	5.2±0.24	5.93±0.6	7.94±1.11

47 * represent comparison to no MASLD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.48 # represent comparison between MASL and MASH. #, $P < 0.05$; ###, $P < 0.001$.

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Table S5. The sgRNA information of GPR31b and multiple pseudogenes

Official Symbol	Gene ID	sgRNA1+PAM	sgRNA2+PAM
Gpr31b	436440	GGCCTCAGAGGAAGTAAATTGGG	AGGAAGGTGATGCATGCCACCGG

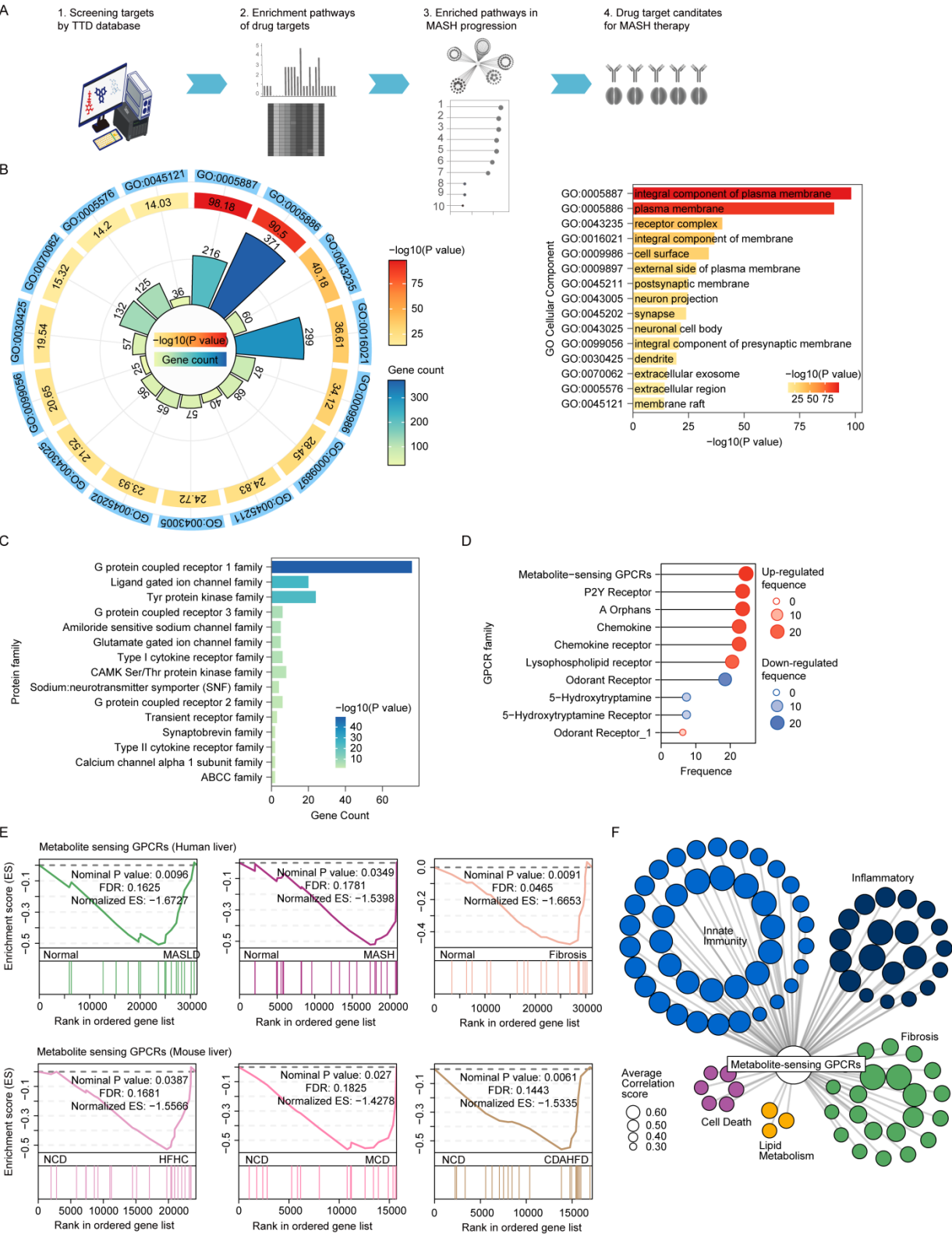
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Table S6. The identification information and genotyping report for GPR31b-HepKO

PCR No.	Primer	Sequence	Band Size	Sample	Genotype
① LOXP1	F1	CAATCGACTTGGTCTCCTTCCTTG	WT: none Targeted: 434bp	Toe	<i>Gpr3 1b-flox</i>
	R1	CCAAGTACCTTGGGCAAGAACAT			
② LOXP2	F2	GCATTCTACGAAGCATGGTATCC	WT: 158bp Targeted: 261bp		
	R2	TCACAACAGCCAAAAGGCATGAG			
③ LOXP1	F3	AGAGGAAGTAAATTGGGCAG	WT: 81bp Targeted: none		
	R3	ACATGCTCACTCATGCTACCATG			
④ LOXP2	F4	TCAGAGCTTACCCACCGGTG	WT:216bp Targeted:none		
	R4	ACCATGCGACACAGAAACTC			
⑤-1 Alb-Cre	C	TTGGCCCCTTACCATAACTG	WT:351bp Targeted:none		Alb- Cre
	W	TGCAAACATCACATGCACAC			
⑤-2 Alb-Cre	C	TTGGCCCCTTACCATAACTG	WT: none Targeted: 390bp		
	K	GAAGCAGAAGCTTAGGAAGATGG			
⑥ HepKO	F1	CAATCGACTTGGTCTCCTTCCTTG	WT: none Targeted: 575bp	Liver	<i>Gpr31b -HepK O</i>
	R2	TCACAACAGCCAAAAGGCATGAG			
⑦ Cyc	F5	ATGTGCCATTTACCGTGAC	WT: none Targeted: 649bp		
	R5	CCTCAATCCCAGAGTCTGCTTT			

52 **Supplementary Figure**

Figure S1



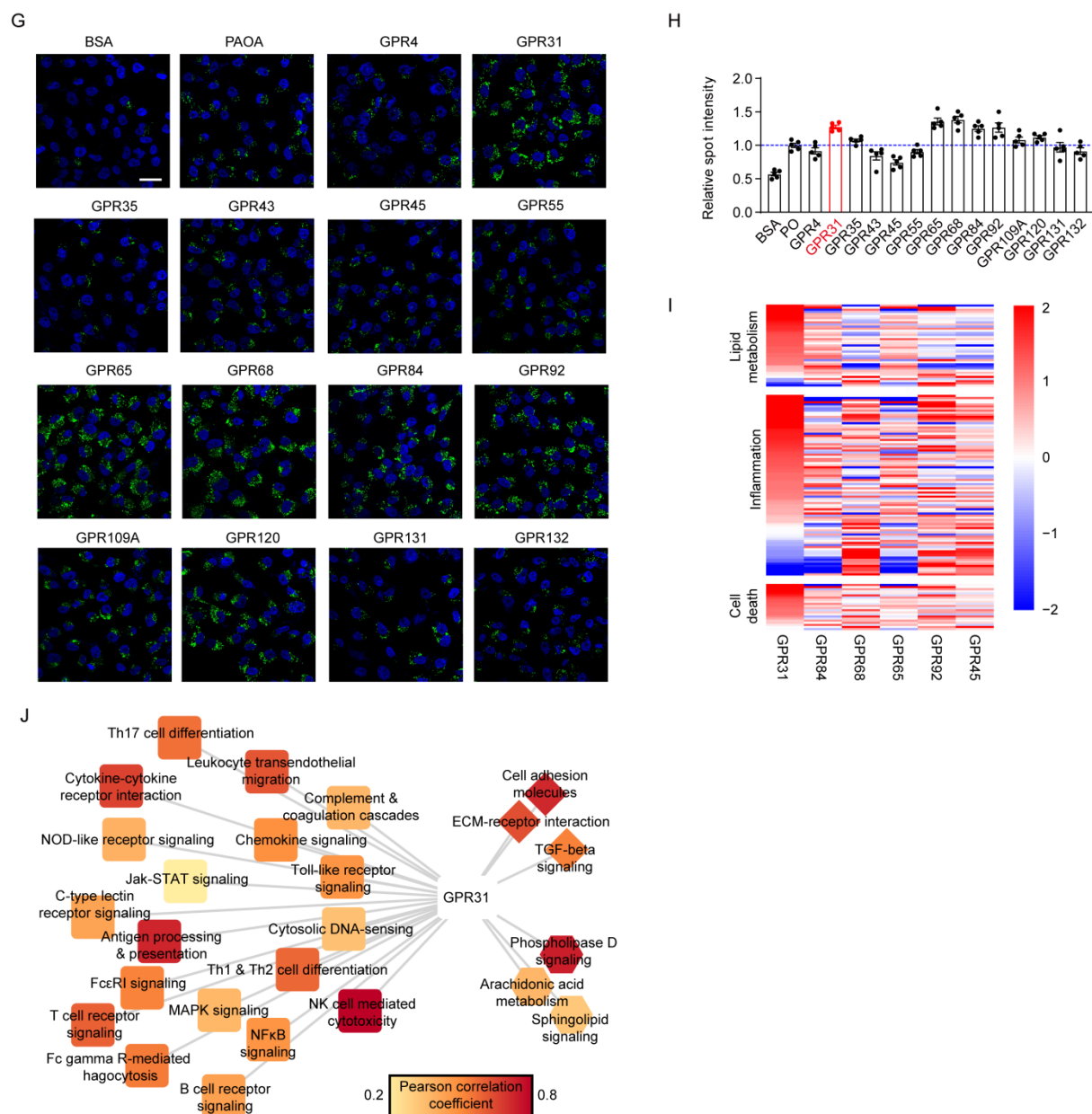


Figure S1. The metabolite-sensing GPCR subfamily was identified as the subfamily with the most significant correlation with MASH

(A) Screening strategy to identify the druggable target protein family in MASH progression.

(B) Druggable targets profile based on Therapeutic Target Database.

(C) Protein families about plasma membrane proteins based on Therapeutic Target Database.

(D) Enrichment frequency of GPCRs subfamilies in MASH transcriptomics.

(E) The enrichment of metabolite sensing GPCRs in samples from clinical individuals and mouse models by GSEA.

(F) The relationships between metabolite sensing GPCRs and the key events in MASH.

65 (G) High-content screening of tested GPCRs on lipid droplet formation in PAOA challenged
66 hepatocytes. Scale bars, 30 μ m. n = 5.
67 (H) Quantitative results for BODIPY staining of lipid droplets with tested GPCRs
68 overexpression. n = 5.
69 (I) Heatmap analysis of genes related to inflammation, lipid metabolism and cell death in
70 hepatocytes with overexpression of tested GPCRs.
71 (J) The relationships between GPR31 and the key events in MASH.
72 Data are shown as mean $\pm$ SEM.

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Figure S2

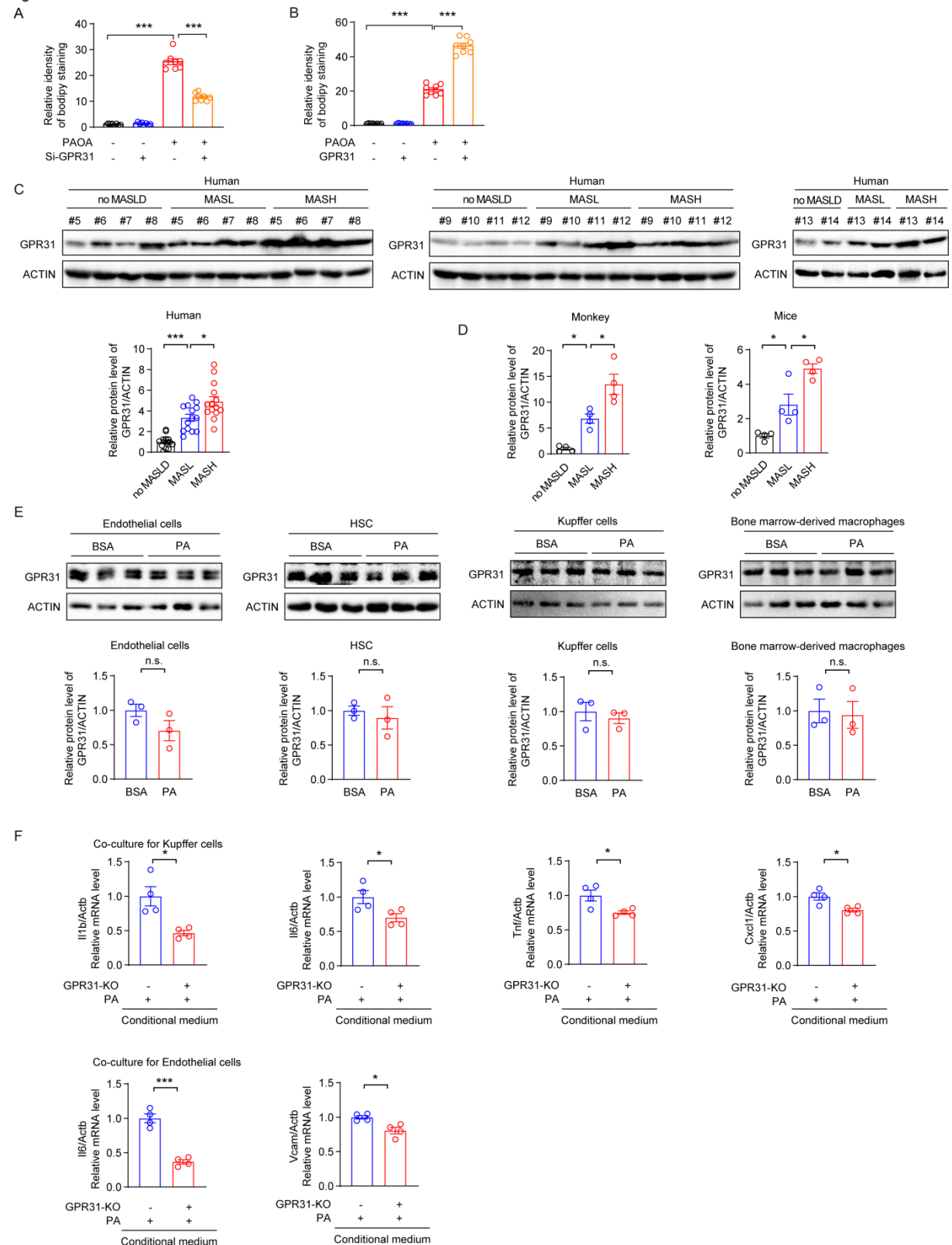


Figure S2 GPR31 function in liver cells

(A) Quantification of BODIPY staining for figure 2B. n = 3 with 8 images quantified.

(B) Quantification of BODIPY staining for figure 2F. n = 3 with 8 images quantified.

(C) Protein levels and quantification of hepatic GPR31 in a cohort of MASH patients and MASLD patients against a control group of age- and gender- matched healthy subjects. n = 14 persons.

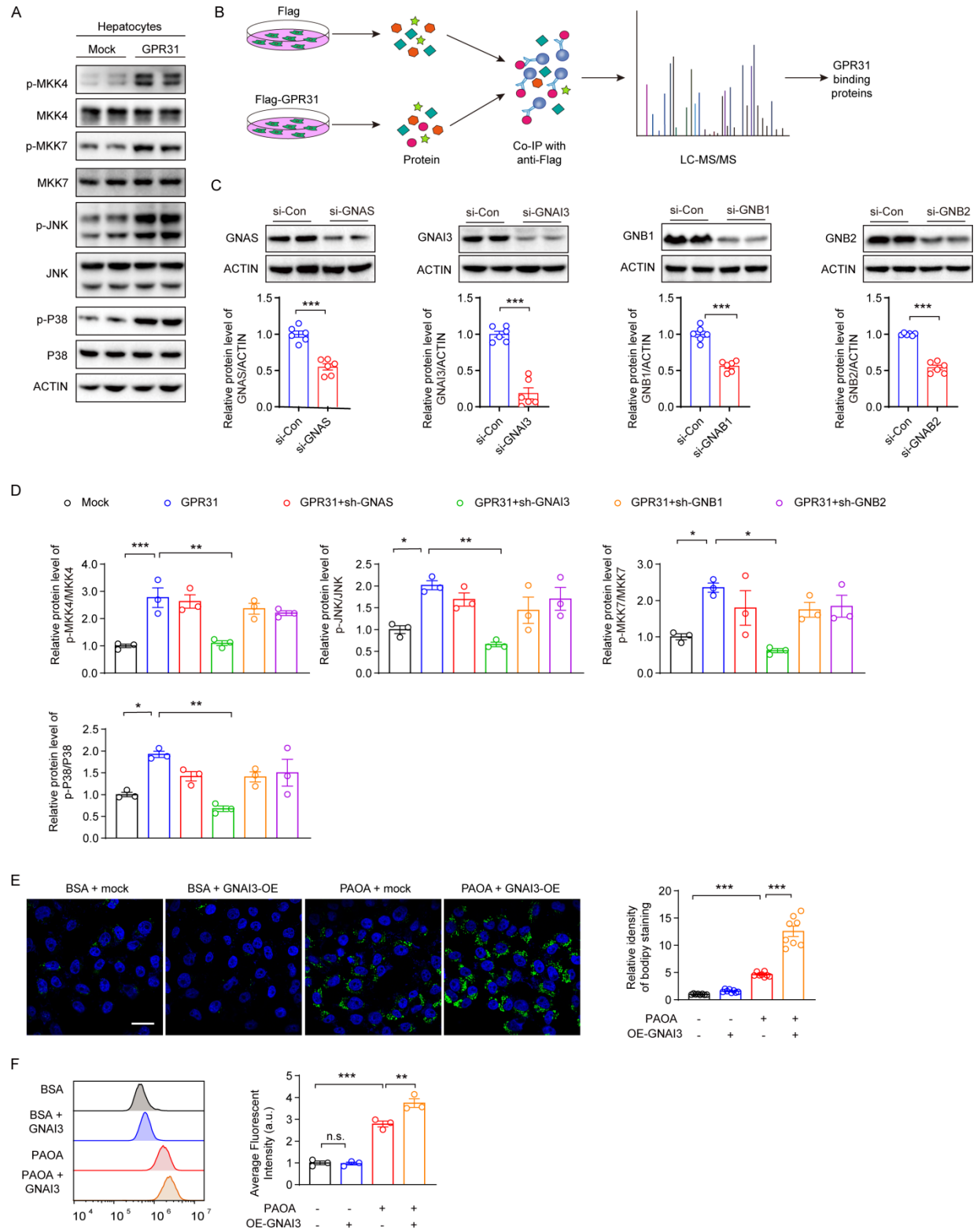
(D) Quantification of GPR31 protein levels in Figure 2L and M. n = 4.

(E) Protein levels and quantification of GPR31 in mouse primary hepatic endothelial cells, hepatic stellate cells (HSC), kupffer cells and bone marrow-derived macrophages treated with or without palmitic acid (PA) for 12 h. n = 3 mice.

(F) The WT or GPR31-KO mouse primary hepatocytes were first challenged with BSA or PA for 12 hours followed by incubation with fresh DMEM without PA for an additional 12 hours. The conditional medium was then collected for kupffer cells and endothelial cells incubation for another 12 hours. n = 4 mice.

Data are shown as mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., no significance, $P > 0.05$.

Figure S3



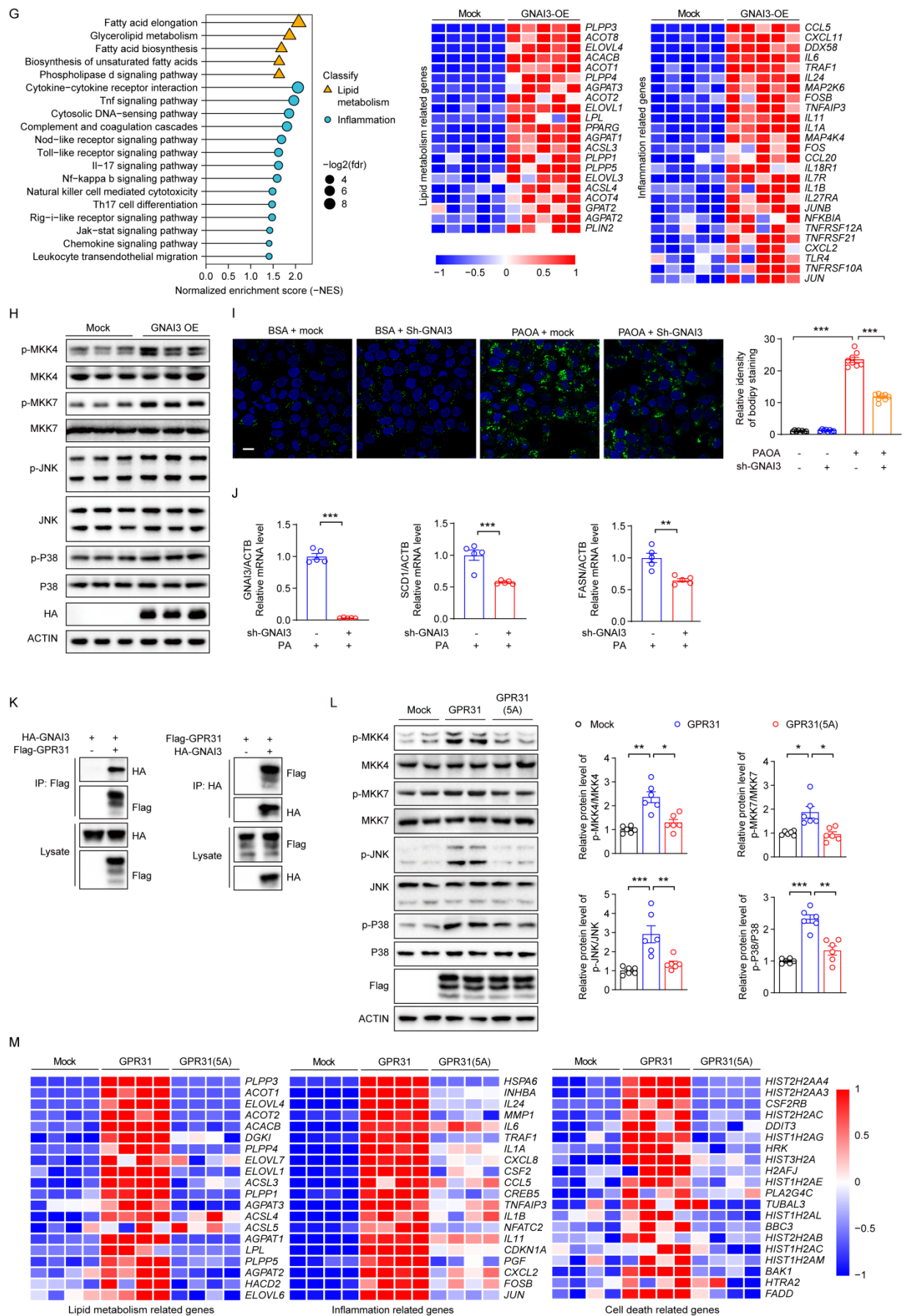


Figure S3 GNAI3 exacerbate lipid droplet formation

(A) Effect of GPR31 overexpression on the phosphorylation of key proteins in the MAPK signaling pathway in hepatocytes. n = 3.

(B) Scheme describing the procedure to identify GPR31 interacting proteins by IP-MS in hepatocytes overexpressed with Flag-GPR31.

(C) The knockdown effects of GNAS, GNAI3, GNB1 and GNB2 validated by western blotting. n = 6.

(D) Quantification of protein levels in Figure 3G. n = 3.

(E) BODIPY staining of lipid droplets in hepatocytes with or without GNAI3 overexpression. Scale bars, 30µm. n = 3 with 8 images quantified.

(F) BODIPY staining for quantification by flow cytometry in control and GNAI3 overexpressed hepatocytes. n = 3.

(G) Pathway and heatmap analysis of GNAI3 overexpressed hepatocytes. n = 5.

(H) Effect of GNAI3 overexpression on the phosphorylation of key proteins in the MAPK signaling pathway in hepatocytes. n = 3.

(I) BODIPY staining of lipid droplets in hepatocytes with or without GNAI3 knockdown. Scale bars, 30µm. n = 3 with 8 images quantified.

(J) Effect of GNAI3 knockdown on SCD1 and FASN mRNA level. n = 5.

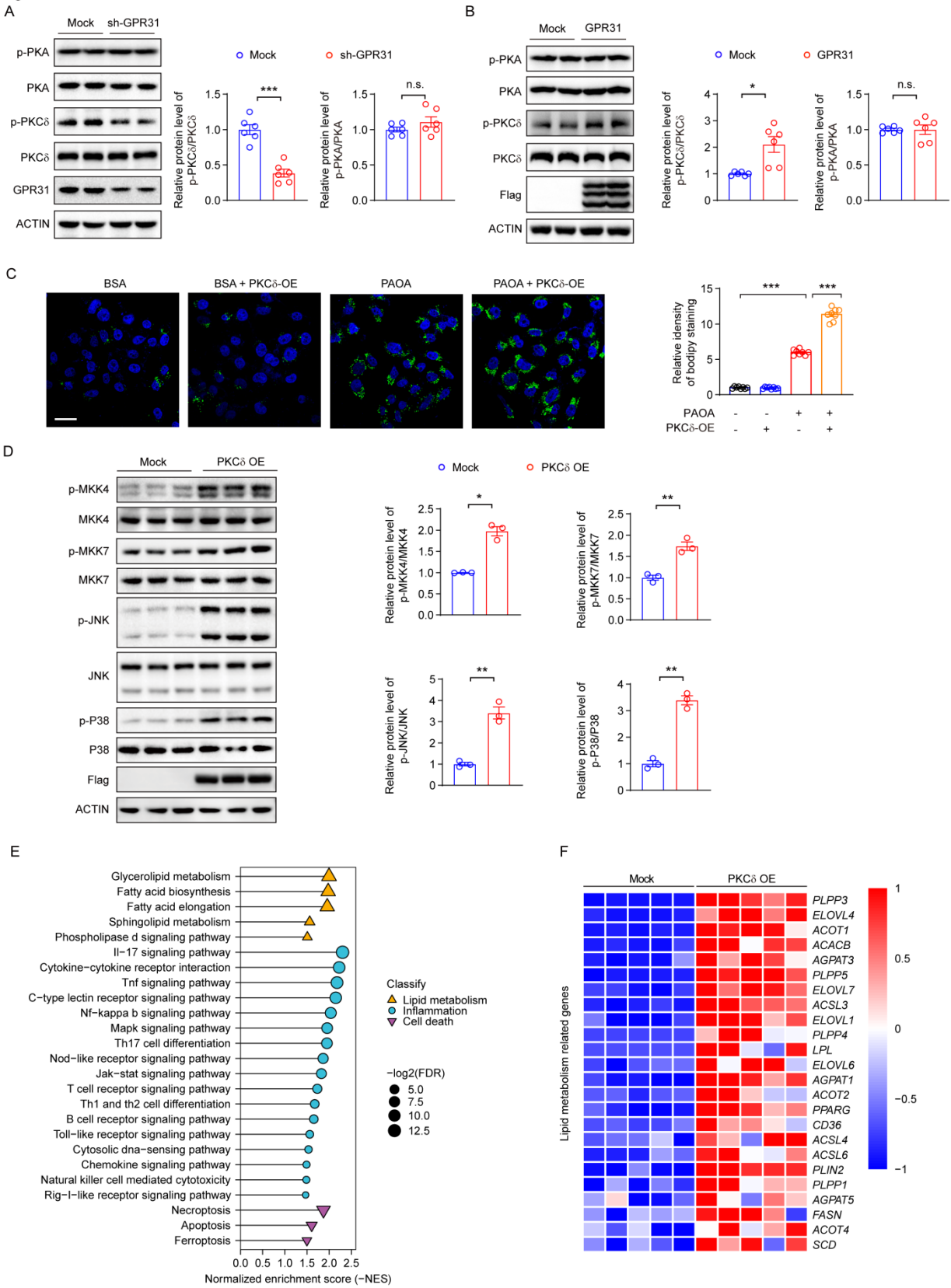
(K) Coimmunoprecipitation (Co-IP) assays were performed to examine the interaction of GPR31 and GNAI3. n = 3.

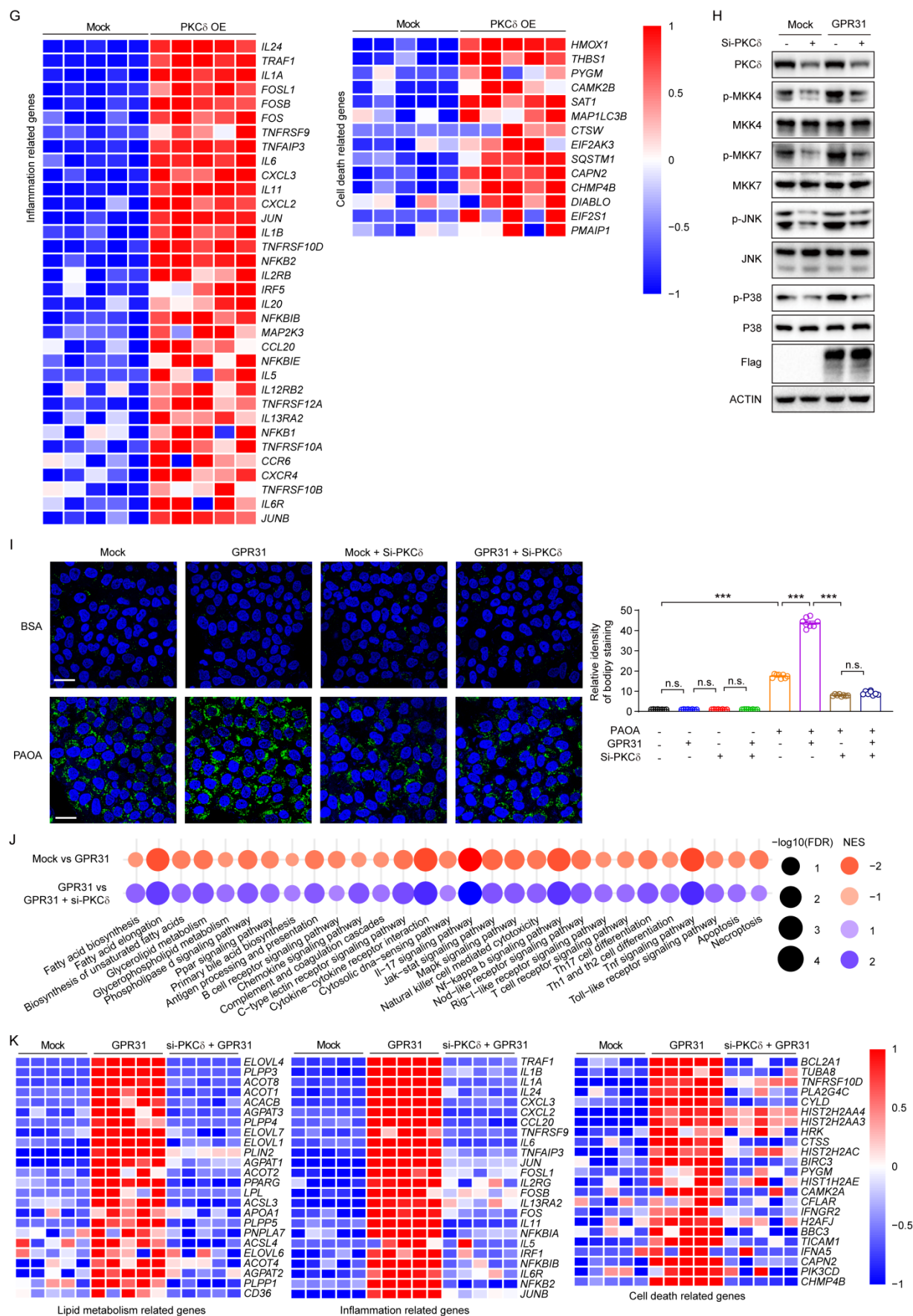
(L) Effect of GPR31 or its docking mutant GPR31(5A) overexpression on the phosphorylation of key proteins in the MAPK signaling pathway in hepatocytes. n = 6.

(M) Transcriptomic profiling of hepatocytes transfected with GPR31 or GPR31(5A). Differentially expressed genes in pathways of lipid metabolism, inflammatory response and cell death are presented. n = 4.

Data are shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., no significance, $P > 0.05$.

Figure S4





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Figure S4. GPR31 aggravates MAPK-mediated MASH in a PKC δ dependent manner

(A) Effect of GPR31 knockdown on the phosphorylation of PKA and PKC δ in hepatocytes. n = 6.

(B) Effect of GPR31 overexpression on the phosphorylation of PKA and PKC δ in hepatocytes. n = 6.

(C) BODIPY staining of lipid droplets in hepatocytes after BSA or PAOA stimulation, with or without PKC δ overexpression. Scale bars, 30 μ m. n = 3 with 8 images quantified.

(D) Effect of PKC δ overexpression on the phosphorylation of key proteins in the MAPK signaling pathway in hepatocytes. n = 3.

(E) Enriched pathway analysis of hepatocytes with or without PKC δ overexpression. n = 5.

(F) Heat maps showing different expressed genes in pathways of lipid metabolism in hepatocytes with or without PKC δ overexpression. n = 5.

(G) Heat maps showing different expressed genes in pathways of inflammatory response and cell death in hepatocytes with or without PKC δ overexpression. n = 5.

(H) Western blotting of the phosphorylation of key proteins in the MAPK signaling pathway in hepatocytes with or without GPR31 overexpression and PKC δ depletion. n = 3.

(I) BODIPY staining of lipid droplets in hepatocytes with or without GPR31 overexpression and PKC δ depletion. Scale bars, 30 μ m. n = 3 with 8 images quantified.

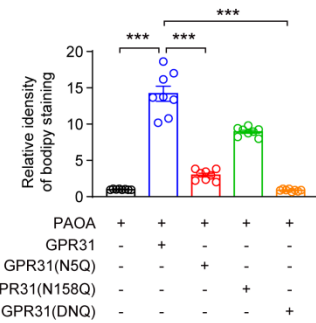
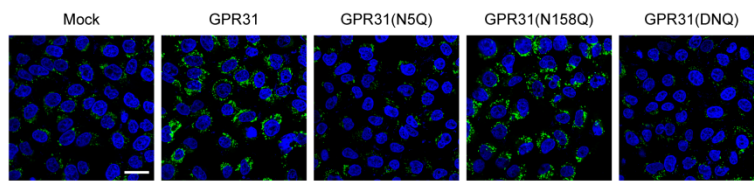
(J) Enriched pathway analysis of hepatocytes with or without GPR31 overexpression and PKC δ depletion. n = 5.

(K) Heat maps showing different expressed genes in hepatocytes with or without GPR31 overexpression and PKC δ depletion. n = 5.

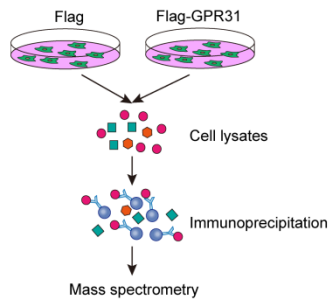
Data are shown as mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., no significance, $P > 0.05$.

Figure S5

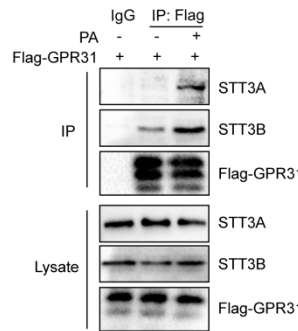
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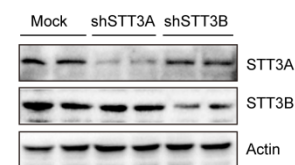
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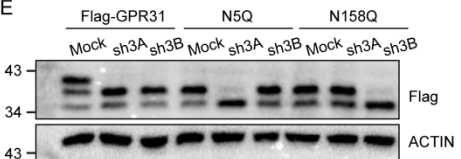
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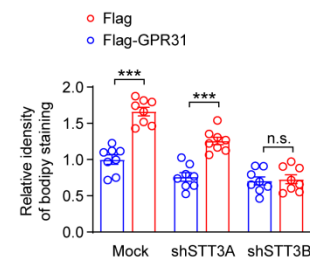
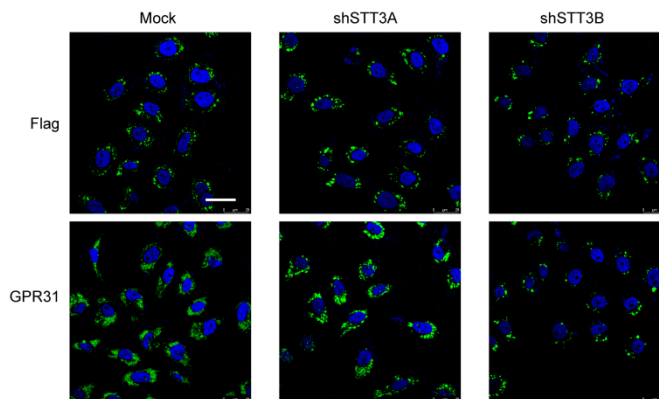
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E



F



G

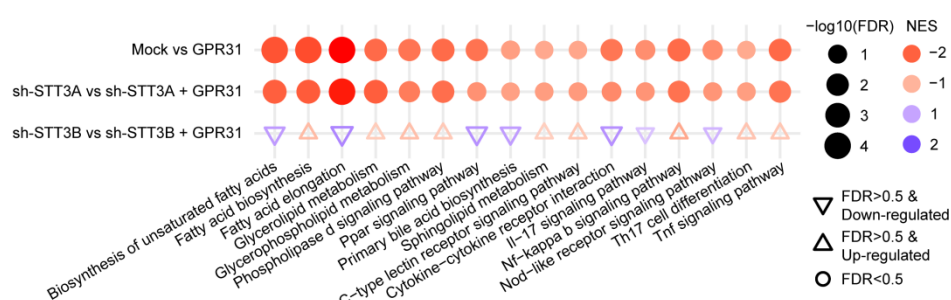


Figure S5. Glycosylation of GPR31 at Asn5 is mediated by STT3B and determines its cytomembrane localization

(A) BODIPY staining of lipid droplets in GPR31-wild type and its nonglycosylatable mutants overexpressed hepatocytes. Scale bars, 30μm. n = 3 with 8 images quantified.

(B) Schematic of the interaction proteomics assayed by cells transfected with Flag or Flag-GPR31.

(C) The interaction of GPR31 and two conventional oligosaccharyltransferases STT3A and STT3B. n = 3.

(D) The protein levels of STT3A and STT3B after STT3A or STT3B knockdown. n = 3.

(E) The immunoblot of GPR31 in cells transfected with GPR31-wild type or its nonglycosylatable mutants after STT3A or STT3B knockdown. n = 3.

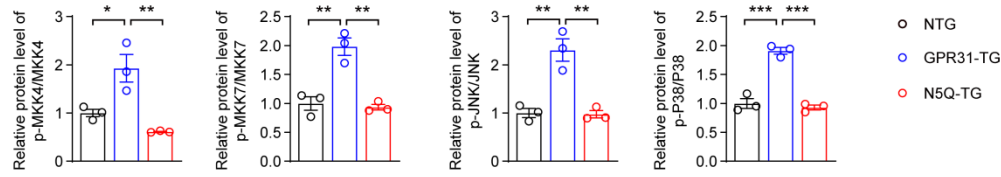
(F) BODIPY staining and quantitative results of lipid droplets in Flag or Flag-GPR31 overexpressed hepatocytes after STT3A or STT3B knockdown. Scale bars, 30 μ m. n = 3.

(G) Enriched pathway analysis of Flag or Flag-GPR31 overexpressed hepatocytes after STT3A or STT3B knockdown.

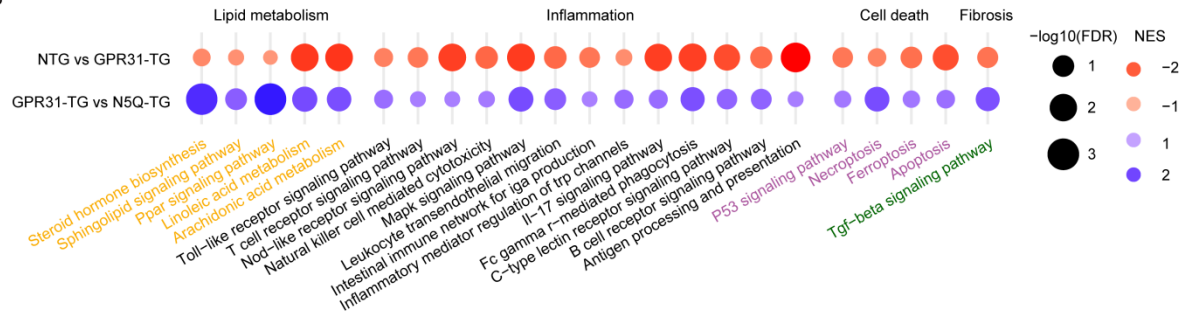
Data are shown as mean $\pm$ SEM. ***, $P < 0.001$; n.s., no significance, $P > 0.05$.

Figure S6

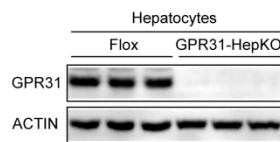
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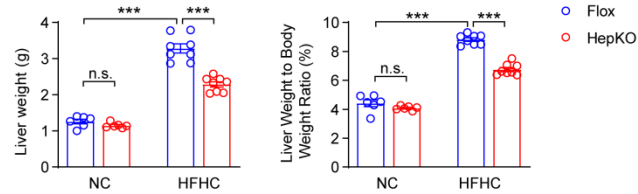
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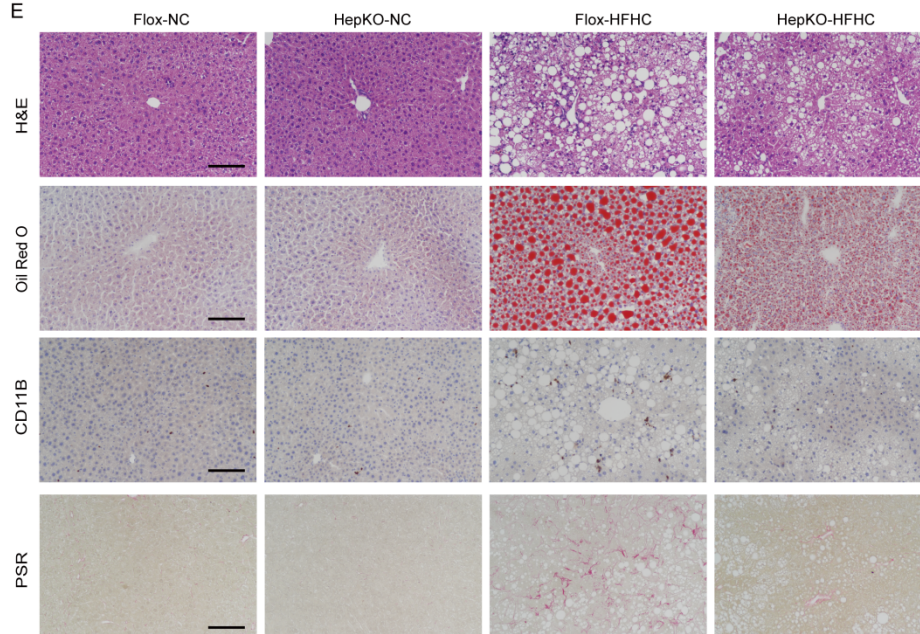
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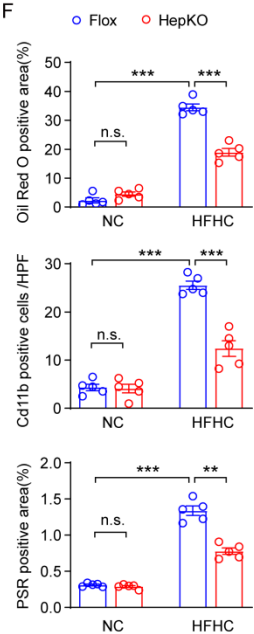
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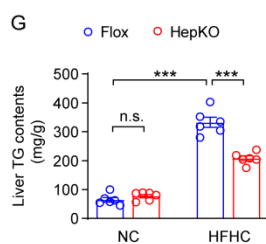
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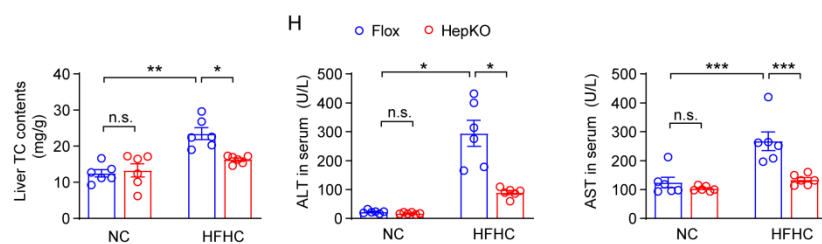
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G



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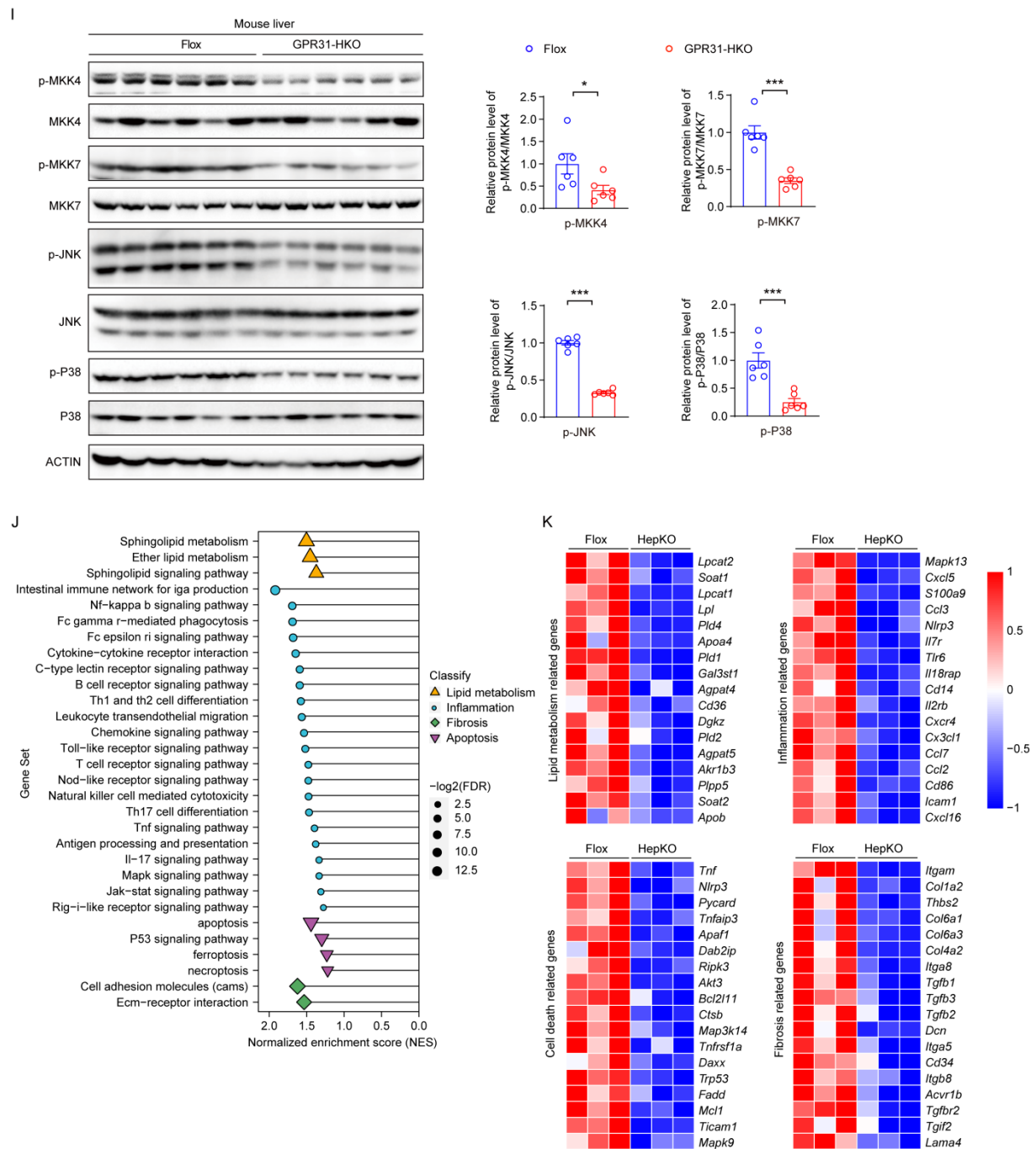


Figure S6 Targeted deletion of GPR31 in hepatocytes protects against MASH in mice

(A) Quantification of the protein levels in Figure 5F. n = 3 mice.

(B) Dot blot representing pairwise GSEA comparison of RNA-seq data obtained from GPR31-WT and GPR31-N5Q transgenic mouse livers. n = 3 mice.

(C) Validation of GPR31-flox and GPR31-HepKO mutant mice by western blot analysis. n = 3 mice.

(D) Liver weight and liver weight/body weight ratio in Flox (GPR31^{flox/flox}) and GPR31-HKO mice. n = 8 mice.

(E) H&E, oil red O, CD11B and picosirus red (PSR) staining of the liver sections in Flox and

GPR31-HKO mice. n = 5 mice. Scale bars, 100 μ m.

(F) Quantitative results of oil red O, CD11B and picosirus red (PSR) staining in (D). n = 5 mice.

(G) Hepatic TG and TC contents of the mice in the indicated group. n = 6 mice.

(H) Serum ALT and AST levels of the mice in the indicated group. n = 6 mice.

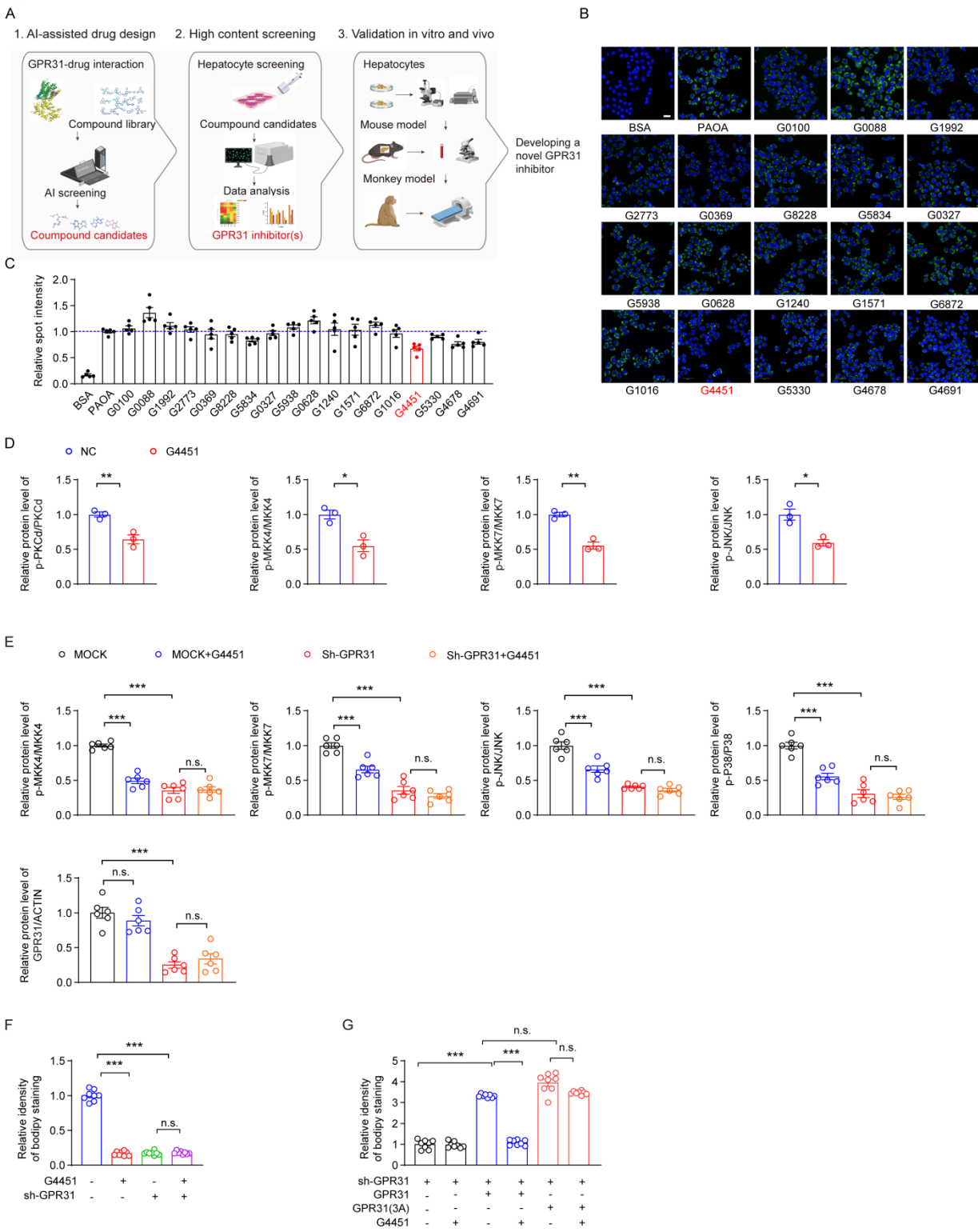
(I) Effect of GPR31-HKO on the phosphorylation of key proteins in the MAPK signaling pathway in mouse liver. n = 6 mice.

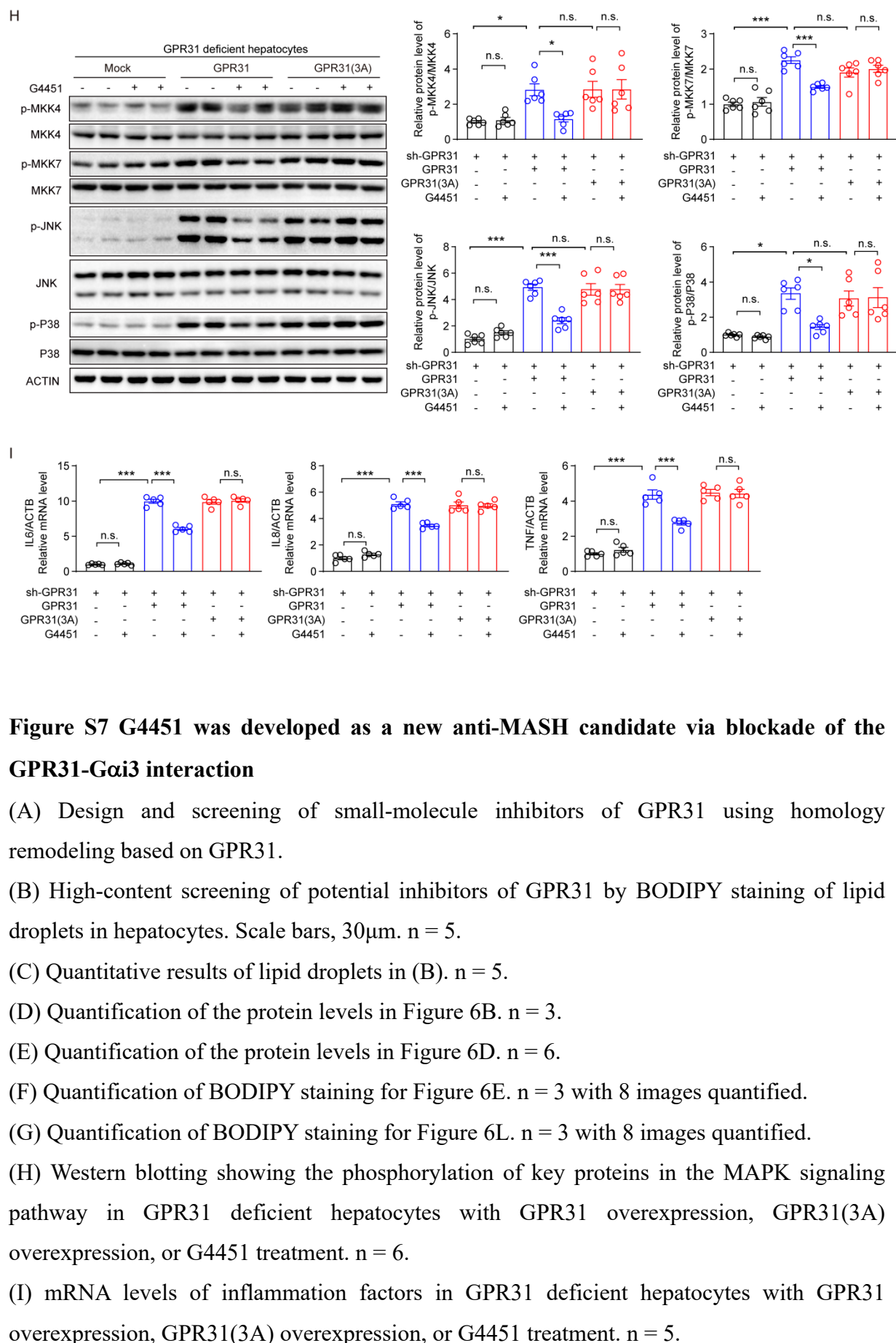
(J) Enriched pathway analysis of liver tissues from HFHC-16W Flox and GPR31-HKO mice. n = 3 mice.

(K) Heat maps showing different expressed genes in pathways of lipid metabolism, inflammatory response, cell death and fibrosis in liver tissues from HFHC-16W Flox and GPR31-HKO mice. n = 3 mice.

Data are shown as mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., no significance, $P > 0.05$.

Figure S7





216 Data are shown as mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., no
217 significance, $P > 0.05$.
218

Figure S8

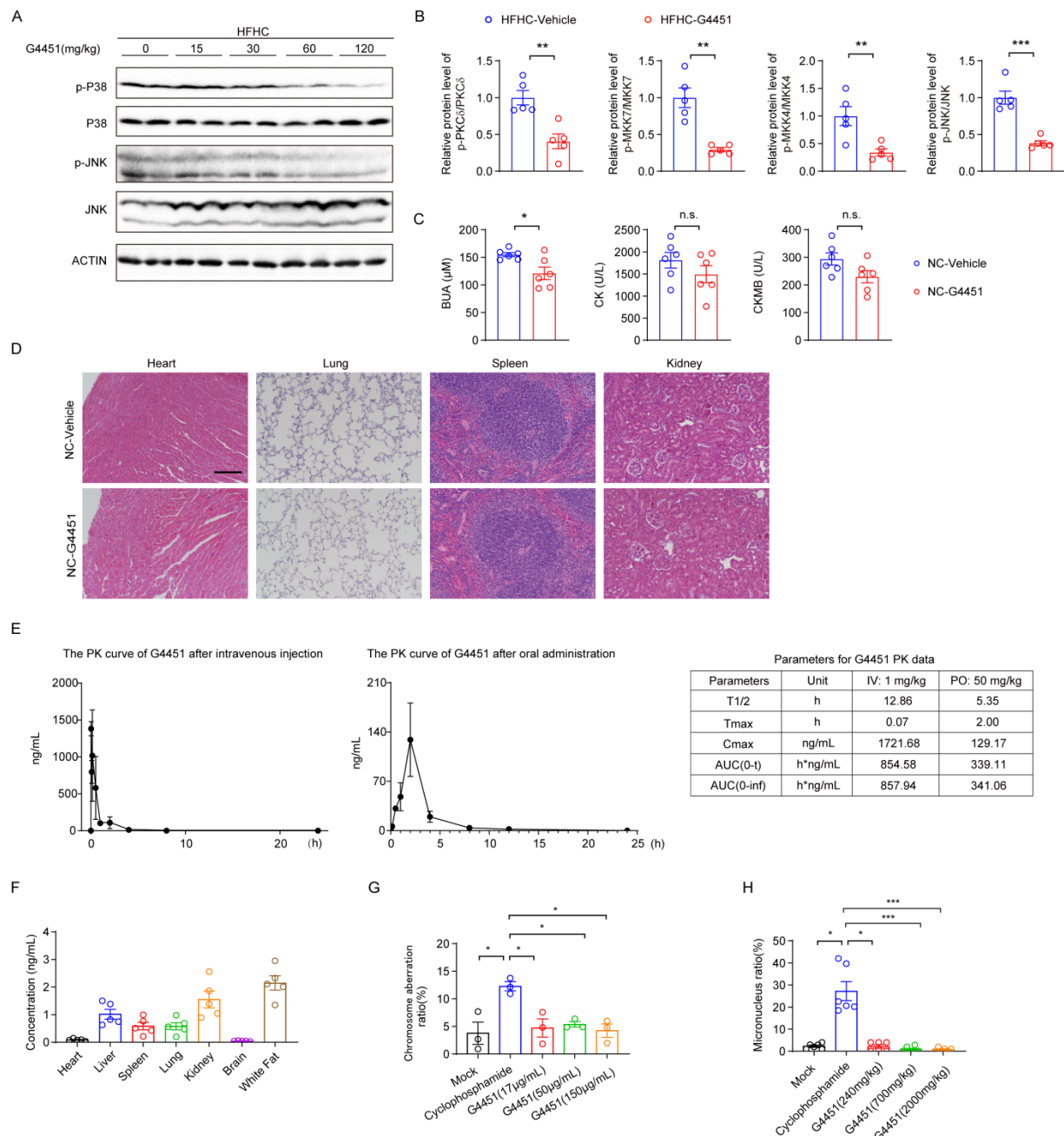


Figure S8 G4451 has no side effect in mice

(A) Western blotting showing the phosphorylation of key proteins in the MAPK signaling pathway by G4451 treatment.

(B) Quantification of the protein levels in Figure 7D. $n = 5$ mice.

(C) Serum BUA, CK and CKMB contents in mice treated with vehicle or G4451. $n = 6$ mice.

(D) Representative images of H&E staining on mouse heart, lung, spleen and kidney sections in G4451- and vehicle-treated mice fed a NC diet. $n = 6$ mice. Scale bars, 100 μm .

(E) The PK data of G4451 in Wistar rats. $n = 3$ rats.

228 (F) The tissue accumulation of G4451 by oral administration. n = 5 mice.
229 (G) The chromosomal aberration assay of G4451 in CHO cells. n = 3.
230 (H) The bone marrow micronucleus assay of G4451 in mouse. n = 6.
231 Data are shown as mean $\pm$ SEM. *, $P < 0.05$; ***, $P < 0.001$; n.s., no significance, $P > 0.05$.
232

Figure S9

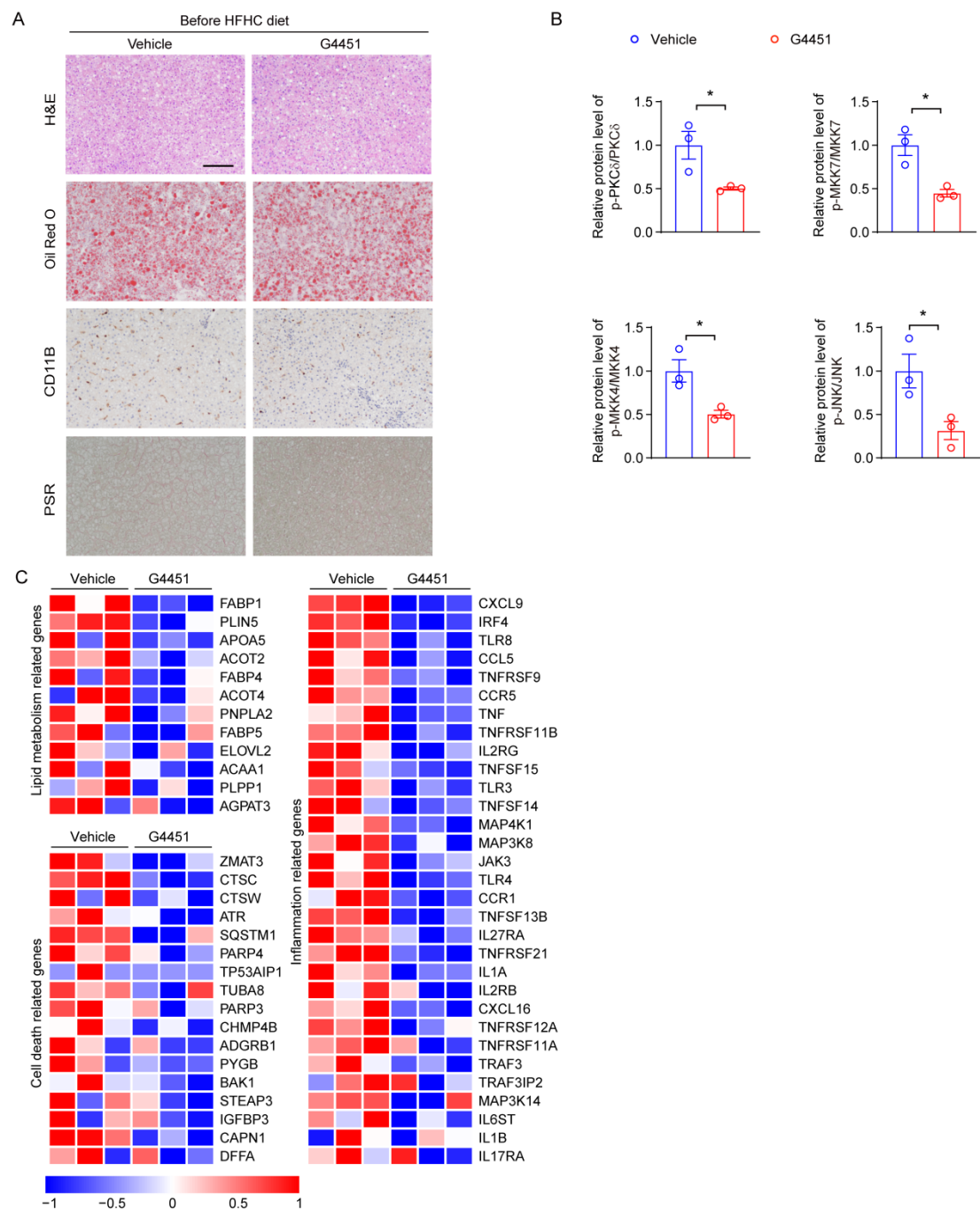


Figure S9 G4451 effectively blocks MASH in monkeys

(A) Representative images of H&E, Oil Red O, CD11B and picosirus red (PSR) staining on monkey liver sections before HFHC diet. Scale bars, 100 μ m. n = 4 monkeys.

(B) Quantification of the protein levels in Figure 8E. n = 3 monkeys.

(C) Heat maps showing different expressed genes in pathways of lipid metabolism, inflammatory response and fibrosis in liver tissues from HFHC monkeys treated with vehicle or G4451. n = 3 monkeys.

Data are shown as mean $\pm$ SEM. *, $P < 0.05$.

Figure S10

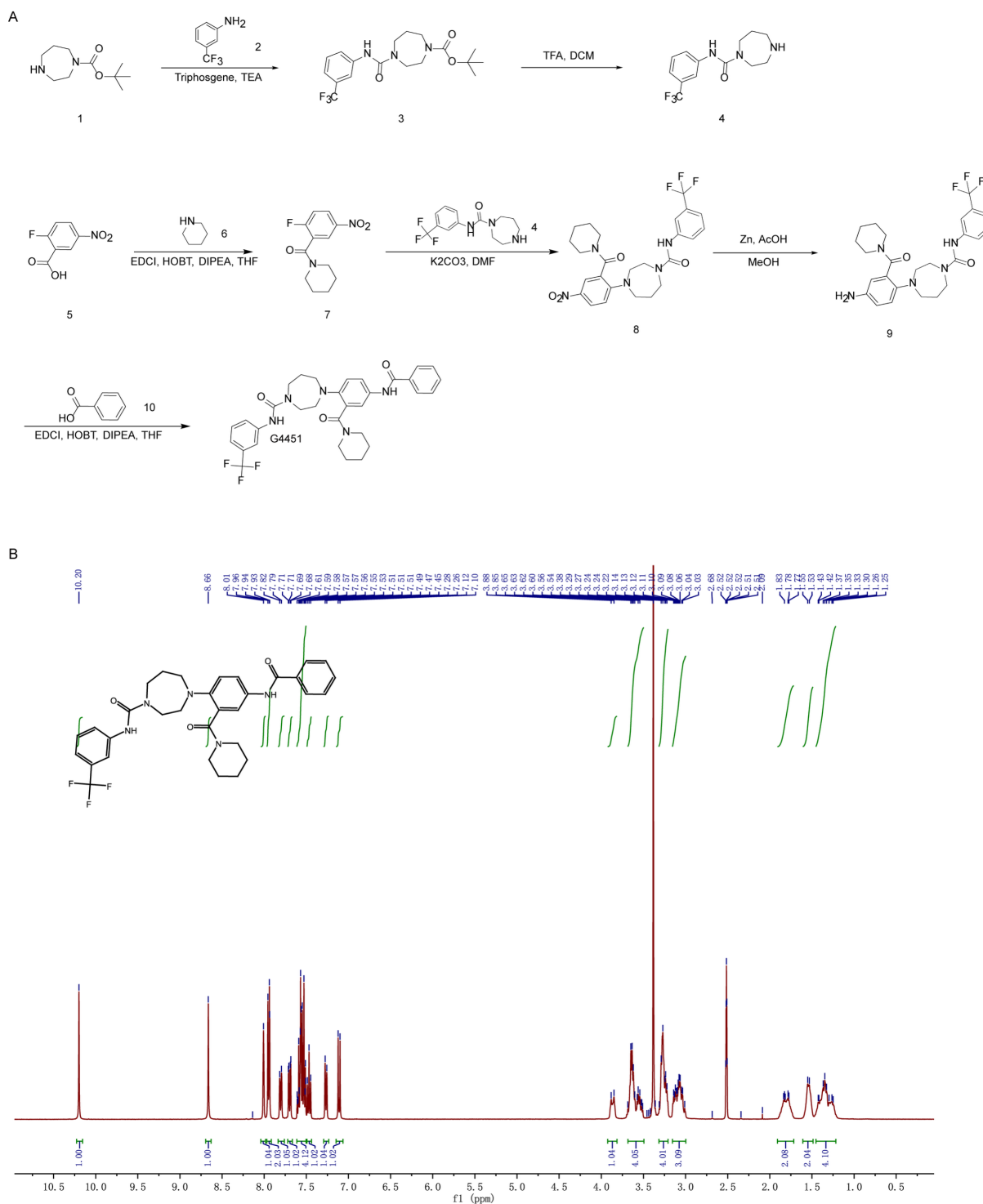


Figure S10 The structure information and synthesis procedure of G4451

(A) The synthetic procedure of G4451. 1-Boc-hexahydro-1,4-diazepine and 3-Aminobenzotrifluoride reacted by condensation reaction to form compound 3, then deprotected by TFA to give intermediate 4. Amide 7 was obtained from 2-Fluoro-5-nitrobenzoic acid and piperidine, then reacted with intermediate 4 by substitution

248 reaction to give compound 8, then reduced to give aniline 9. Finally, G4451 was obtained
249 from aniline 9 and benzoic acid through acid amine condensation.

250 (B) The ^1H NMR of G4451.

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