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Supplemental information

Activation of hepatic adenosine A1 receptor ameliorates MASH via inhibiting SREBPs maturation

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Activation of hepatic adenosine A1 receptor ameliorates MASH via inhibiting SREBPs maturation

Supplementary Figures

Fig S1

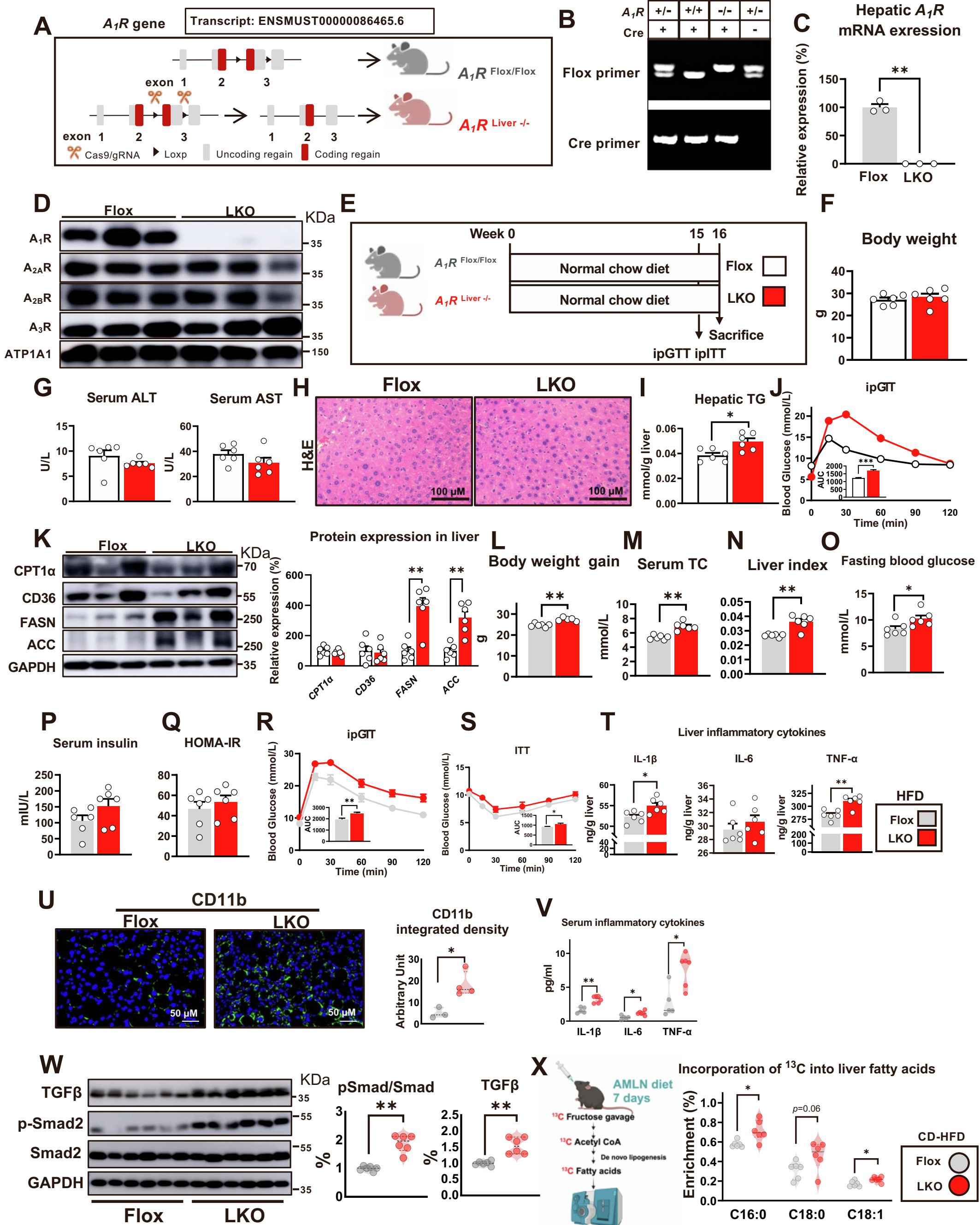
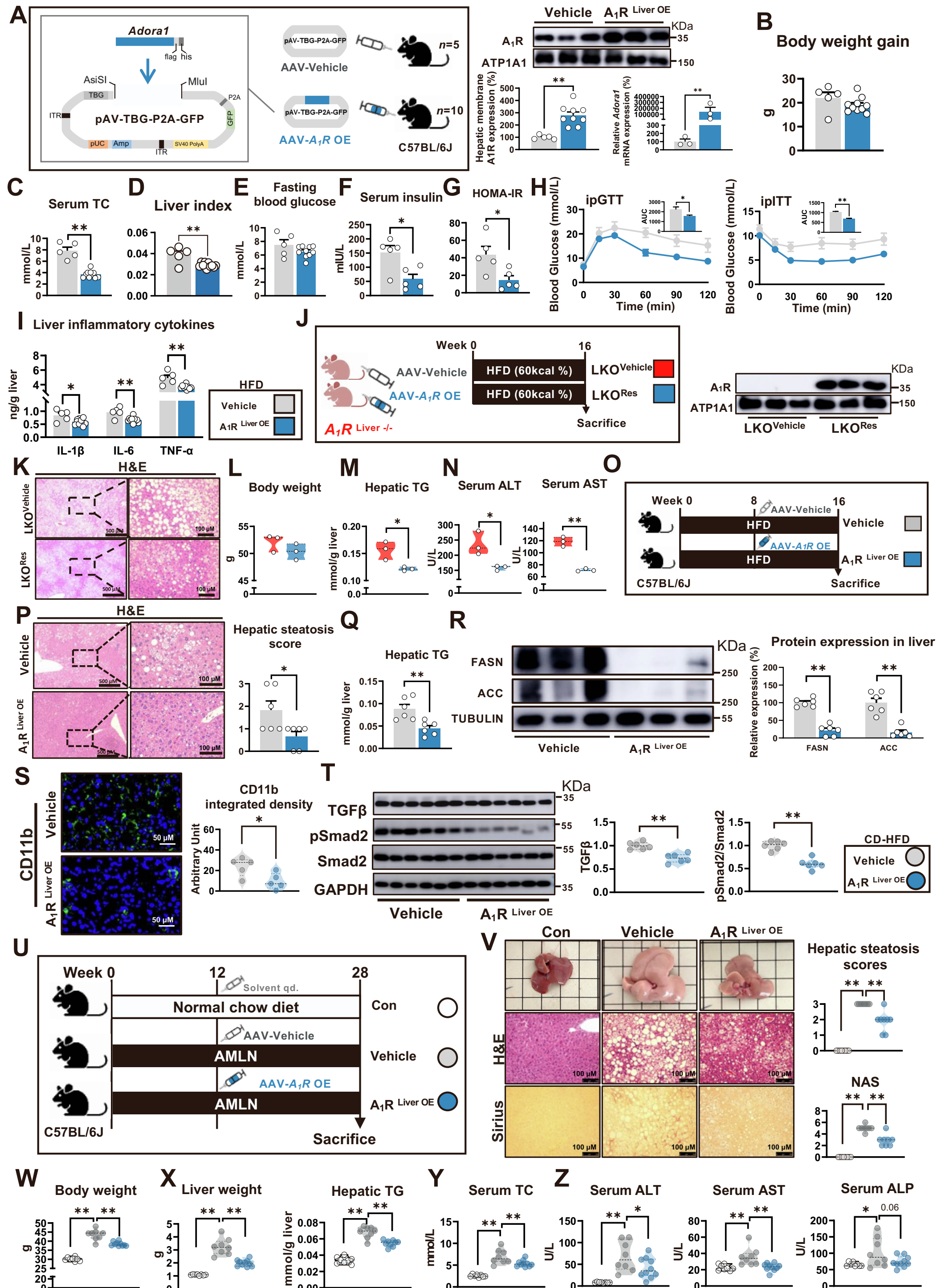


Fig. S1. Liver-specific A_1R deletion exaggerates MAFL/MASH in mice. Related to Figure 1.
(**A**) Schematic showing the CRISPR-Cas9 technology targeting *A_1R* to generate liver-specific knockout mice (LKO). (**B**) Agarose gel electrophoresis of qPCR amplification products from the mice with

various genotypes. (**C**) Hepatic A_1R mRNA expression from the Flox and LKO mice. (*n*=3) (**D**) The hepatocytes membrane proteins expression of adenosine receptor subtypes, including A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R. Flox and LKO mice were fed with normal chow diet (ND) for 16 weeks (*n*=6). ATP1A1 was used as cytomembrane protein control. (**E**) Diagram of experimental design. (**F**) Body weight. (**G**) Serum ALT and AST. (**H**) the representative image of liver H&E staining. (**I**) Quantification of hepatic TG. (**J**) Blood glucose levels during ipGTT and corresponding AUC encompassing 120 min of ipGTT. (**K**) Relative protein expression of CPT1α, CD36, FASN, and ACC. GAPDH functioned as a reference protein. (**L** to **T**) Flox and LKO mice were fed with HFD for 16 weeks (*n*=5-6). (**L**) Body weight gain. (**M**) Serum total cholesterol. (**N**) Liver index (**O**) Fasting blood glucose level. (**P**) Fasting serum insulin level. (**Q**) HOMA-IR. (**R-S**) Blood glucose levels during ipGTT or ipITT and corresponding AUC. (**T**) Hepatic inflammatory cytokines including IL-1β, IL-6, and TNF-α. (**U-W**) Flox mice and LKO mice were fed a calorie-restricted HFD (CD-HFD) for 9 weeks (**U**) Immunofluorescence of CD11b in the liver of Flox and LKO mice. (*n*=3-4) (**V**) Serum inflammation cytokines including IL-1β, IL-6, and TNF-α. (*n*=5-6) (**W**) Relative protein expression of TGF-β, pSmad2, smad2. (*n*=6) (**X**) The enrichment of ^{13}C label incorporation into hepatic fatty acids were examined by LC-MS in Flox and LKO mice fed an AMLN diet for 7 days, fasted from 9 a.m. to 7 p.m., refed for 2 hours, and force-fed ^{13}C-fructose (*n*=6). Results are representative of 1 biological replicate. Data are depicted as mean ± SEM. Student's unpaired *t*-test; *$p<0.05$, **$p<0.01$.



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2 **Fig. S2. Liver-specific A$_1$R overexpression protects mice from diet-induced NAFL/MASH.**

3 **Related to Figure 2.**

4 (**A-I**) C57BL/6J mice were inoculated with Vehicle-AAV (Vehicle) or *A$_1$R*-overexpression-AAV (A$_1$R

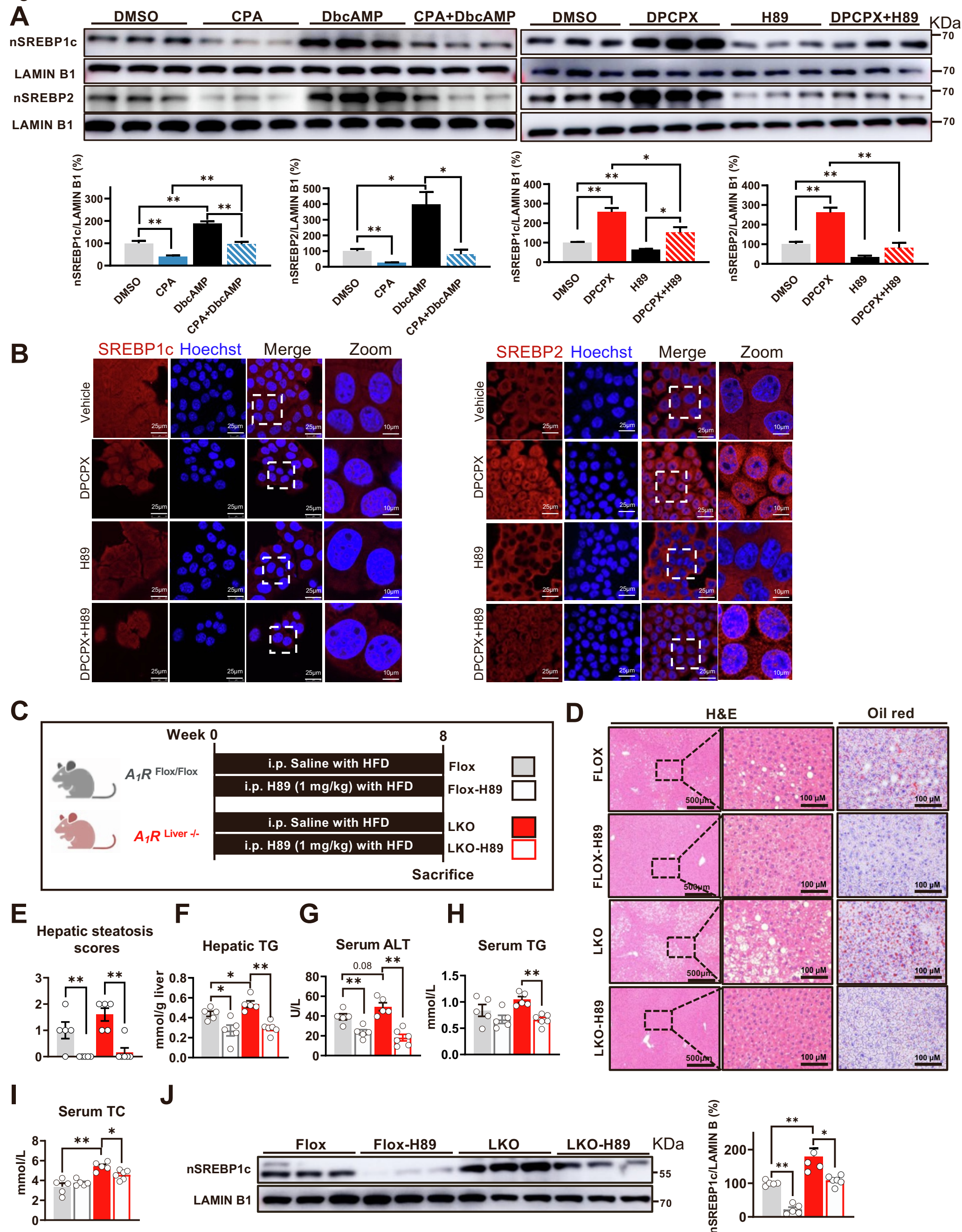
5 $^{\text{Liver OE}}$) intravenously, and then were fed with HFD for 12 weeks (*n*=5~9). AAV, adeno-associated virus.

6 (**A**) Schematic representation of construct AAV-mediated *A$_1$R* overexpression mice, with A$_1$R protein

7 expression in cell membrane of hepatocytes and *Adora1* mRNA level. (**B**) Body weight gain. (**C**)

8 Serum total cholesterol. (**D**) Liver index. (**E**) Fasting blood glucose level and (**F**) fasting serum insulin

level. (n=5) (**G**) HOMA-IR. (**H**) Blood glucose levels during ipGTT or ipITT and corresponding AUC. (**I**) Hepatic inflammatory cytokines including IL-1β, IL-6, and TNF-α. **(J-N)** LKO mice were inoculated with Vehicle-AAV ($LKO^{Vehicle}$) or A_1R-overexpression-AAV (LKO^{Res}) intravenously, and then were fed with HFD for 16 weeks (n=3). (**J**) Diagram of experimental design, and A_1R protein expression in hepatic membrane. (**K**) The representative image of liver H&E staining. (**L**) Body weight. (**M**) Quantification of hepatic triglycerides (TG). (**N**) Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). (**O-R**) Vehicle or A_1R-overexpression-AAV ($A_1R^{\text{Liver OE}}$) intravenously at 9th week during 16 weeks HFD fed (n=6). (**O**) Diagram of experimental design. (**P**) The representative image of liver H&E staining, with hepatic steatosis score. (**Q**) Quantification of hepatic TG. (**R**) Relative protein expression of FASN, and ACC. (**S-T**) Vehicle mice and $A_1R^{\text{Liver OE}}$ mice were fed a CD-HFD for 9 weeks. (**S**) Immunofluorescence of CD11b in the liver of $A1R^{\text{Liver OE}}$ mice. (n=5) (**T**) Relative protein expression of TGF-β, pSmad2, smad2. (**U-Z**) Vehicle or $A_1R^{\text{Liver OE}}$ was intravenously injected at 13th week of the 28 weeks HFD feeding (n=8-10). (**U**) Diagram of experimental design. (**V**) Representative micrographs of liver, hepatic staining of H&E and Sirius red, along with Hepatic steatosis scores and NAS scores. (**W**) Body weight. (**X**) Liver weight and Quantification of hepatic TG. (**Y**) Serum TC level. (**Z**) Serum ALT, AST, and ALP. ATP1A1 was used as cytomembrane protein control. TUBULIN or GAPDH was used as total protein control. Results are representative of 1 biological replicate. Data are depicted as mean ± SEM. Student's unpaired t-test, One-way ANOVA analysis.; *$p<0.05$, **$p<0.01$.



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2 **Fig. S3. A₁R controls the maturation of SREBPs via PKAc. Related to Figure 3.**

3 (**A**) AML-12 cells were treated with CPA (A₁R activator, 1 μM, 48 h), DbcAMP (PKA activator, 200

4 μM, 12 h), DPCPX (A₁R inhibitor, 1 μM, 48 h), H89 (PKA inhibitor, 20 μM, 4 h), co-treated with CPA

5 and DbcAMP, or co-treated with DPCPX and H89, respectively. nSREBP1c and nSREBP2 protein

6 expression are shown. LAMIN B was used as nuclear protein control. (*n*=3) **(B)** AML-12 cells were

7 treated with DPCPX (A₁R inhibitor, 1 μM, 48 h), H89 (PKA inhibitor, 20 μM, 4 h), or co-treated with

8 DPCPX and H89, respectively. Fluorescent staining of SREBP1c or SREBP2 (red) in each group was

9 performed. The nuclei were stained by hoechst (blue). **(C-J)** HFD-fed Flox and LKO mice were treated

10 with or without H89 (i.p., 1 mg/kg body weight) daily for 8 weeks (*n*=5-6). (**C**) Diagram of

11 experimental design. (**D**) The representative image of liver H&E and Oil red O staining, with hepatic

1 steatosis score. (**E**) Body weight. (**F**) Quantification of hepatic TG. (**G)** Serum ALT level. (**H**) Serum
2 TG level. (**I**) Serum TC and LDL-C level. (**J**) Protein expression of nSREBP1c. LAMIN B1 was used
3 as nuclear protein control. Results are representative of 1 biological replicate. Data are depicted as
4 mean ± SEM. One-way ANOVA analysis; $^*p<0.05$, $^{**}p<0.01$.

Fig S4

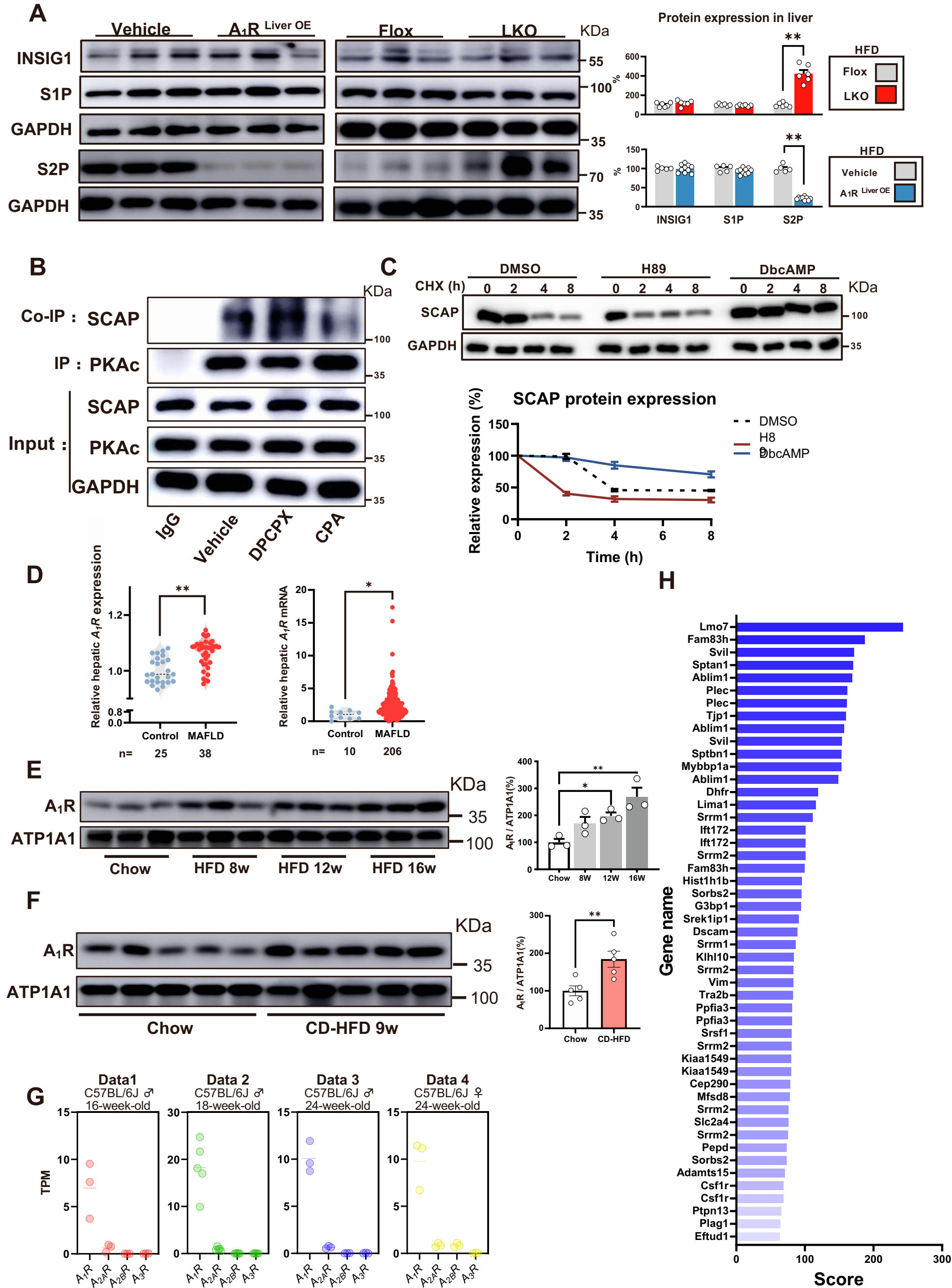

Fig. S4. A$_1$R activation accelerates SCAP protein degradation and hepatic A$_1$R expression is upgraded in MAFLD patients and mouse models. Related to Figure 3-6.
(A) Protein expression of INSIG1, S1P and S2P in liver of A$_1$R $^{\text{Liver OE}}$ (*n*=5-10) or LKO (*n*=6) mice. (**B**) Co-immunoprecipitation (co-IP) of PKAc with SCAP were performed in the AML-12 cells treated with DPCPX (1 μM) or CPA (1 μM) for 48 h. (**C**) Protein expression of SCAP in the AML-12 cells treated with H89 (20 μM, 4 h) or DbcAMP (200 μM, 12h) in the presence of cycloheximide (CHX, 50 μM) for 0, 2, 4, 8 h. GAPDH was used as total protein control. (*n*=3) **(D)** Gene and mRNA expression

of A_1R in liver of patient with MAFLD in public data (GSE89632, GSE135251). **(E-F)** A_1R protein expression in hepatocellular membrane of mice. (*n*=3-6) **(G)** mRNA expression of ARs in liver of mice. (*n*=3) ATP1A1 was used as cytomembrane protein control. (**H**) PKA-dependent phosphorylated targets in response to A_1R suppression. (Localization probability > 0.75; score diff >5 and score > 60). Results are representative of 1 biological replicate. Data are depicted as mean ± SEM. Student's unpaired *t*-test (A, D, F), One-way ANOVA analysis (G); $*p<0.05$, $**p<0.01$.

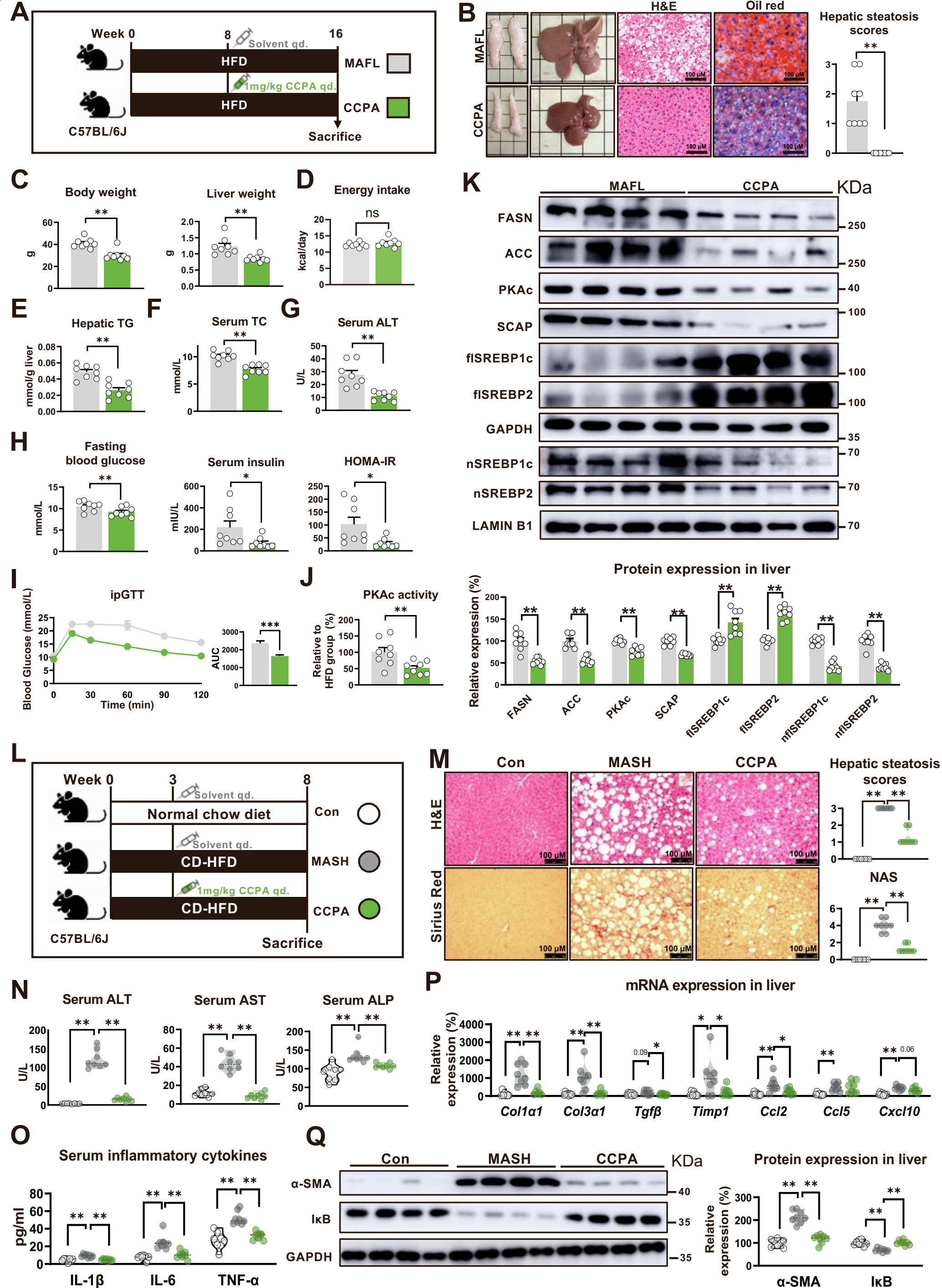

Figure S5. A_1R pharmacological activation inhibits diet-induced MAFL and MASH in mice. Related to Figure 7.

(**A** to **K**) C57BL/6J mice were fed a HFD for 16 weeks, and injected intraperitoneally 0.9% NaCl solution (NAFL) or 1 mg/kg 2-Chloro-N6-cyclopentyladenosine (CCPA) daily from 9th week. (*n*=8) (**A**) Diagram of experimental design. (**B**) Representative image of epididymis adipose, liver and liver staining of H&E and Oil red O, with hepatic steatosis scores. (**C**) Body weight and liver weight. (**D**) Energy intake (energy intake was record every week from 9th to 16th week, n=8 per group) (**E**) Quantification of hepatic TG. (**F**) Serum TC (**G**) Serum ALT. (**H**) Fasting blood glucose, serum insulin,

1 and HOMA-IR. (**I**) Blood glucose levels during ipGTT and corresponding AUC encompassing 120
2 min. (**J**) ELISA detection of active PKAc level. (**K**) Relative protein expression of PKAc, SCAP,
3 SREBPs (flSREBP1c, flSREBP2, nSREBP1c, nSREBP2), and SREBP regulated proteins (FASN,
4 ACC). GAPDH was used as total protein control, LAMIN B1 was used as nuclear protein control. (**L**
5 to **Q**) C57BL/6J mice were fed a normal chow diet (Con) or CD-HFD for 9 weeks, during the process,
6 CD-HFD mice were divided into 2 subgroups, and injected intraperitoneally 0.9% NaCl solution (CD-
7 HFD) or 1 mg/kg CCPA daily from week 4. (n=8) (**L**) Diagram of experimental design. (**M**) H&E and
8 Sirius red staining of liver sections, with histological evaluation. (**N**) Serum ALT, AST, and ALP. (**O**)
9 Serum inflammatory cytokines including IL-1β, IL-6, and TNF-α. (**P**) mRNA expression of fibrosis-
10 related genes (*Col1a1, Col3a1, Ctgf, Timp1*) and chemokine-related genes (*Ccl2, Ccl5, Cxcl10*) in liver.
11 Results are normalized for *18S*. (**Q**) Relative protein expression of α-SMA and IκB. GAPDH was used
12 as a control. LAMIN B1 was used as nuclear protein control. Results are representative of 1 biological
13 replicate. Data are depicted as mean ± SEM. Student's unpaired *t*-test (**B** to **K**); One-way ANOVA
14 analysis (**M** to **Q**); *$p<0.05$, **$p<0.01$.
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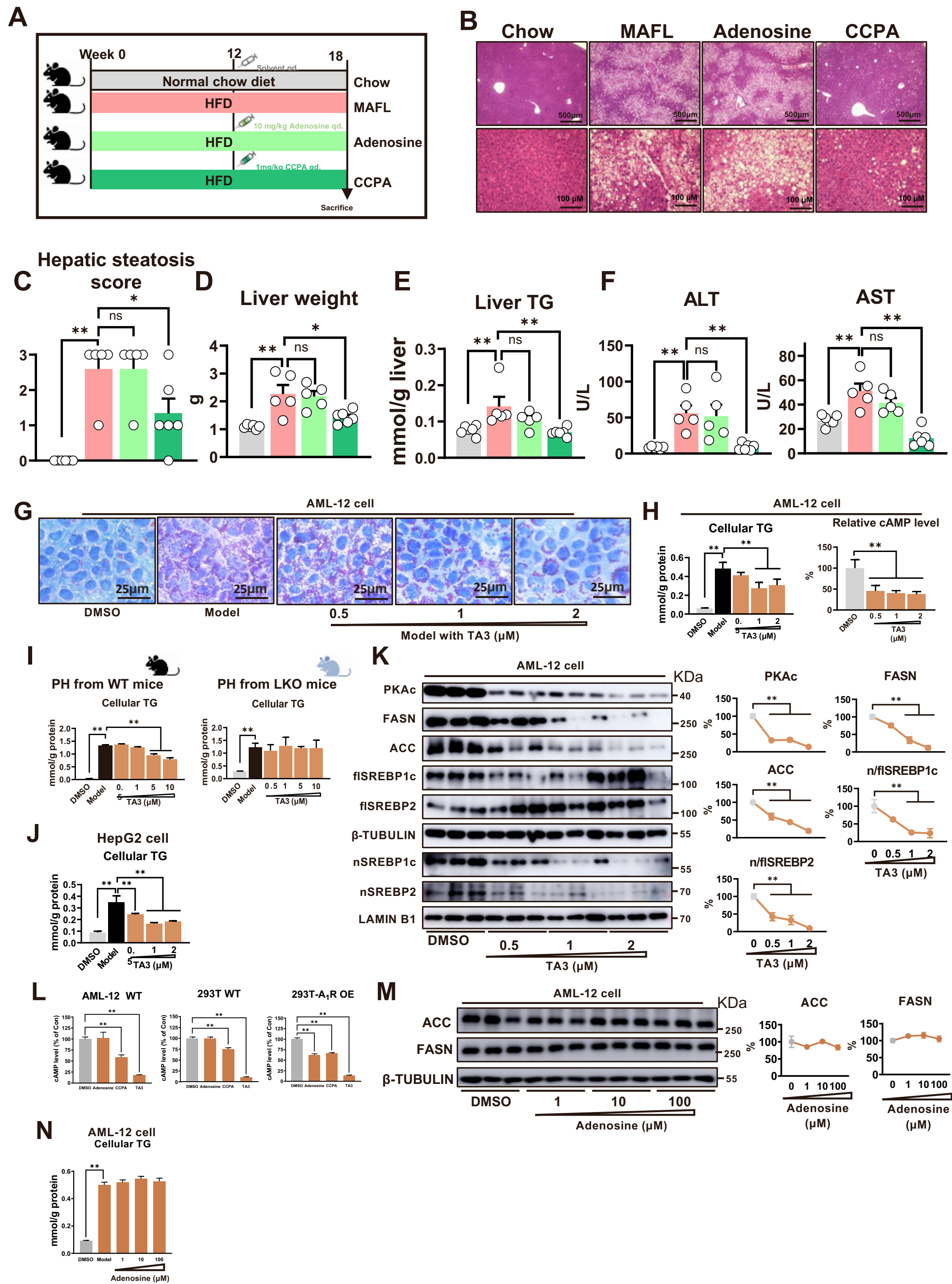

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2 **Figure S6. The anti-MAFLD test of Adenosine, and the screening and identification of A_1R**

3 **activators. Related to Figure 7.**

4 (**A** to **F**) C57BL/6J mice were fed a HFD for 18 weeks, and injected intraperitoneally 0.9% NaCl

5 solution (NAFL) , 10mg/kg adenosine or 1 mg/kg 2-Chloro-N6-cyclopentyladenosine (CCPA) daily

6 from 12th week. (*n*=5-6) (**A**) Diagram of experimental design. (**B**) Representative image of liver. (**C**)

7 Hepatic steatosis score. (**D**) Liver weight. (**E**) Quantification of hepatic triglycerides (TG). (**F**) Liver

8 injury indicators including serum alanine aminotransferase (ALT) and aspartate aminotransferase

9 (AST). Results are representative of 1 biological replicate. Data are depicted as mean ± SEM. One-way

1 ANOVA analysis; *n*=5-6, *$p<0.05$, **$p<0.01$. (**G** to **N**) **The screening and identification of A_1R**
2 **activators.** (**G-H**) AML-12 cells were treated with 0.5, 1, 2 μM timosaponin AIII (TA3) for 48 h in the
3 presence of glycerol (20 mM) and glucose (4.5 g/L) (Model). Representative micrographs and the
4 quantification of Oil Red O staining. Relative TG and cAMP level. (**I**) Cellular TG level in the primary
5 hepatocytes (PH) from WT or LKO mice treated with 0.5, 1, 5, 10 μM TA3 for 48 h in the presence of
6 glycerol (20 mM) and glucose (4.5 g/L) (Model). (**J**) Cellular TG level in HepG2 cells treated with 0.5,
7 1, 2 μM TA3 in model. (**K**) Relative protein expression of PKAc, SREBPs (flSREBP1c, flSREBP2,
8 nSREBP1c, nSREBP2), and SREBP regulated proteins (FASN, ACC). (**L**) Cellular TG level. (**M**)
9 Relative protein expression of FASN and ACC. (**N**) Cellular TG level. β-TUBULIN was used as total
10 protein control; LAMIN B1 was used as nuclear protein control. Results are representative of 1
11 biological replicate, *n*=3. Data are depicted as mean ± SEM. One-way ANOVA analysis. *$p<0.05$,
12 **$p<0.01$.
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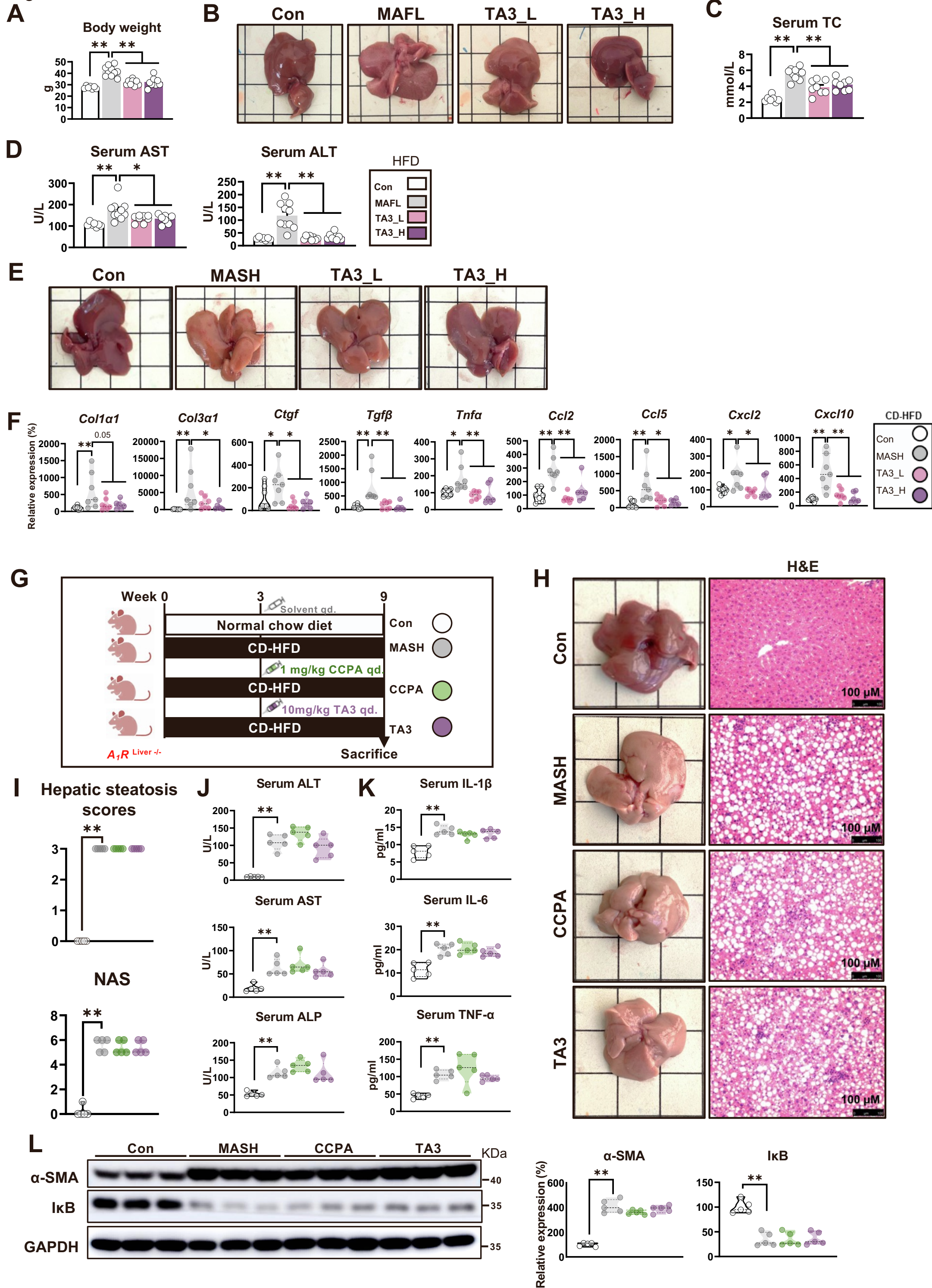

Figure S7. Timosaponin AIII inhibits diet-induced MAFL and MASH in mice in an A$_1$R-dependent manner. Related to Figure 7.

(**A** to **D**) C57BL/6J mice were fed with HFD for 16 weeks, and injected intraperitoneally 0.9% NaCl solution (HFD) or 5,10 mg/kg timosaponin AIII (TA3) daily from 9th week. (n=7-10) (**A**) Body weight. (**B**) Representative image of liver. (**C**) Serum TC. (**D**) Serum ALT and AST. (**E** to **F**) C57BL/6J mice were fed a normal chow diet (NCD, Con) or CD-HFD for 9 weeks, during the process, CD-HFD mice

1 were divided into 3 subgroups, and injected intraperitoneally with 0.9% NaCl solution (CD-HFD) or 5,
2 10 mg/kg TA3 daily from 4th week. (*n*=6-8) (**E**) Representative image of liver. (**F**) mRNA expression of
3 fibrosis-related genes (*Col1a1, Col3a1, Ctgf, Tgfb*), proinflammatory-related genes (*Tnfα*), and
4 chemokine-related genes (*Ccl2, Ccl5, Cxcl2, Cxcl10*) in liver of CD-HFD mice. (**G** to **L**) LKO mice
5 were fed a normal chow diet or CD-HFD for 9 weeks, CD-HFD mice were injected intraperitoneally
6 with 0.9% NaCl solution (MASH), 1 mg/kg 2-Chloro-N6-cyclopentyladenosine (CCPA) or 10 mg/kg
7 TA3 daily from 4th week. (n=5) (**G**) Diagram of experimental design. (**H**) Representative image of
8 Liver and hepatic H&E staining. (**I**) Histological evaluation. (**J**)Serum ALT, AST, and ALP. (**K**) Serum
9 inflammatory cytokines. (**L**) Relative protein expression of α-SMA and IκB. GAPDH was used as total
10 protein control. Results are representative of 1 biological replicate. Data are depicted as mean ± SEM.
11 One-way ANOVA analysis; *p<0.05, **p<0.01. Results are normalized for *18S*. Results are
12 representative of 1 biological replicate. Data are depicted as mean ± SEM. One-way ANOVA analysis;
13 *$p<0.05$, **$p<0.01$.
14

Table S1. Clinical and biochemical characteristics of the patients with biopsy proven MAFLD. Related to Figure 6.

Characteristics	All (*n*=30)	MAFL (*n*=22)	Control (*n*=8)	*p* value
Demographics				
Age (years)	51.3±2.2	52.7±2.4	47.6±3.9	0.3116
Gender, n (%)				
Female	20 (64.1)	14 (63.6)	6 (75.0)	
Male	10 (35.9)	8 (36.4)	2 (25.0)	
BMI (kg/m^2)	22.1±0.7	22.5±0.9	20.9±0.6	0.3242
Biological data				
AST (U/L)	55.4±14.3	48.7±13.2	72.0±39.0	0.4719
ALT (U/L)	86.4±27.6	65.5±18.7	138.6±85.7	0.2386
Triglycerides (mmol/L)	1.61±0.26	1.90±0.34	0.96±0.17	0.0894
Total cholesterol (mmol/L)	4.08±0.21	4.23±0.26	3.75±0.37	0.3030
Glucose (mmol/L)	6.75±0.60	6.58±0.72	7.25±1.12	0.6361
Histology				
NAS, n (%)				
Steatosis				
0	8 (26.7)	0 (0.0)	8 (100.0)	
1	9 (30.0)	9 (40.9)	0 (0.0)	
2	10 (33.3)	10 (45.5)	0 (0.0)	
3	3 (10.0)	3 (13.6)	0 (0.0)	
Ballooning				
0	16 (53.3)	8 (36.4)	8 (100.0)	
1	14 (46.7)	14 (63.6)	0 (0.0)	
2	0 (0.0)	0 (0.0)	0 (0.0)	
Lobular Inflammation				
0	23 (76.7)	15 (68.2)	8 (100.0)	
1	7 (23.3)	7 (31.8)	0 (0.0)	
2	0 (0.0)	0 (0.0)	0 (0.0)	
3	0 (0.0)	0 (0.0)	0 (0.0)	
Fibrosis stage				
F0	30 (100.0)	22 (100.0)	8 (100.0)	
F1	0 (0.0)	0 (0.0)	0 (0.0)	
F2	0 (0.0)	0 (0.0)	0 (0.0)	
F3	0 (0.0)	0 (0.0)	0 (0.0)	
F4	0 (0.0)	0 (0.0)	0 (0.0)	
Clinical diagnose				
Hepatic hemangioma	15 (50.0)	12 (54.5)	3 (37.5)	
Liver traumatic rupture	7 (23.3)	2 (9.1)	5 (62.5)	
Hepatolithiasis	8 (26.7)	8 (36.4)	0 (0.0)	

1 Data are presented as mean ± SEM. *P* values were obtained using Student's *t*-test for continuous
2 variables.

Table S2. Clinical and biochemical characteristics of the patients with biopsy proven MASH. Related to Figure 1.

Characteristics	All (*n*=13)	MASH (*n*=9)	Control (*n*=4)	*p* value
Demographics				
Age (years)	44.6±2.7	40.9±2.8	53.0±3.3	0.0287
Gender, n (%)				
Female	6 (46.2)	3 (33.3)	3(75.0)	
Male	7 (53.8)	6 (66.6)	1 (25.0)	
BMI (kg/m^2)	27.0±1.3	29.4±1.0	21.5±1.4	0.0007
Biological data				
AST (U/L)	62.6±10.6	70.8.0±12.5	44.3±19.3	0.2672
ALT (U/L)	99.5±19.1	126.3±20.8	39.2±19.6	0.0275
Triglycerides (mmol/L)	1.94±0.33	2.34±0.40	1.03±0.24	0.0631
Total cholesterol (mmol/L)	4.93±0.21	4.84±0.27	5.12±0.33	0.5499
Glucose (mmol/L)	5.45±0.27	5.62±0.38	5.06±0.14	0.3644
Histology				
NAS, n (%)				
Steatosis				
0	4 (30.8)	0 (0.0)	4 (100.0)	
1	2 (15.4)	2 (22.2)	0 (0.0)	
2	2 (15.4)	2 (22.2)	0 (0.0)	
3	5 (38.5)	5 (55.6)	0 (0.0)	
Ballooning				
0	4 (30.8)	0 (0.0)	4 (100)	
1	3 (23.1)	3 (33.3)	0 (0.0)	
2	6 (46.1)	6 (66.6)	0 (0.0)	
Lobular Inflammation				
0	4 (30.8)	0 (0.0)	4 (100)	
1	4 (30.8)	4 (44.4)	0 (0.0)	
2	2 (15.9)	2 (22.2)	0 (0.0)	
3	3 (23.1)	3 (33.3)	0 (0.0)	
Fibrosis stage				
F0	4 (30.8)	0 (0.0)	4 (100.0)	
F1	1 (7.7)	1 (11.1)	0 (0.0)	
F2	6 (46.1)	6 (66.7)	0 (0.0)	
F3	2 (15.4)	2 (22.2)	0 (0.0)	
F4	0 (0.0)	0 (0.0)	0 (0.0)	
Clinical diagnose				
MASH	9 (69.2)	9 (100.0)	0 (0.0)	
Hepatic hemangioma	3 (23.1)	0 (0.0)	3 (75.0)	
Hepatolithiasis	1 (7.7)	0 (0.0)	1 (25.0)	

1 Data are presented as mean ± SEM. *P* values were obtained using Student's *t*-test for continuous
2 variables.

1 **Table S3. Primers(mice) for qPCR.**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
18s forward: 5'-CCATCCAATCGGTAGTAGCG -3'	This paper	N/A
18s reverse: 5'-GTAACCCGTTGAACCCCATT-3'	This paper	N/A
A_1R forward: 5'-TGTGCCCGGAAATGTACTGG- 3'	This paper	N/A
A_1R reverse: 5'-TCTGTGGCCCAATGTTGATAAG-3'	This paper	N/A
Ccl2 forward: 5'-TACAAGAGGATCACCAGCAGC-3'	This paper	N/A
Ccl2 reverse: 5'-ACCTTAGGGCAGATGCAGTT-3'	This paper	N/A
Ccl5 forward: 5'-TGCTGCTTTGCCTACCTCTC-3'	This paper	N/A
Ccl5 reverse: 5'-TCTTCTCTGGGTTGGCACAC-3'	This paper	N/A
Cxcl10 forward: 5'-ATGACGGGCCAGTGAGAATG-3'	This paper	N/A
Cxcl10 reverse: 5'-ATGATCTCAACACGTGGGCA-3'	This paper	N/A
Cxcl2 forward: 5'-GCGCCCAGACAGAAGTCATA-3'	This paper	N/A
Cxcl2 reverse: 5'-CAGTTAGCCTTGCCTTTGTTCA-3'	This paper	N/A
Il6 forward: 5'-TAGTCCTTCCTACCCCAATTTCC-3'	This paper	N/A
Il6 reverse: 5'-TTGGTCCTTAGCCACTCCTTC-3'	This paper	N/A
Il1β forward: 5'-CCGTGGACCTTCCAGGATGA-3'	This paper	N/A
Il1β reverse: 5'-GGGAACGTCACACACCAGCA-3'	This paper	N/A
Tnfα forward: 5'-CATCTTCTCAAAATTCGAGTGACAA-3'	This paper	N/A
Tnfα reverse: 5'-TGGGAGTAGACAAGGTACAACCC-3'	This paper	N/A
Col1a1 forward: 5'-TGCTAACGTGGTTCGTGACCGT-3'	This paper	N/A
Col1a1 reverse: 5'-ACATCTTGAGGTCGCGGCATGT-3'	This paper	N/A
Col3a1 forward: 5'-ACGTAAGCACTGGTGGACAG-3'	This paper	N/A
Col3a1 reverse: 5'-CCGGCTGGAAAGAAGTCTGA-3'	This paper	N/A
Ctgf forward: 5'-TGACCCCTGCGACCCACA-3'	This paper	N/A
Ctgf reverse: 5'-TACACCGACCCACCGAAGACACAG-3'	This paper	N/A
Timp1 forward: 5'-GAGACCACCTTATACCAGCGTT-3'	This paper	N/A
Timp1 reverse: 5'-TACGCCAGGGAACCAAGAAG-3'	This paper	N/A
Actb forward: 5'-GTGACGTTGACATCCGTAAAGA-3'	This paper	N/A
Actb reverse: 5'-GCCGGACTCATCGTACTCC-3'	This paper	N/A

2