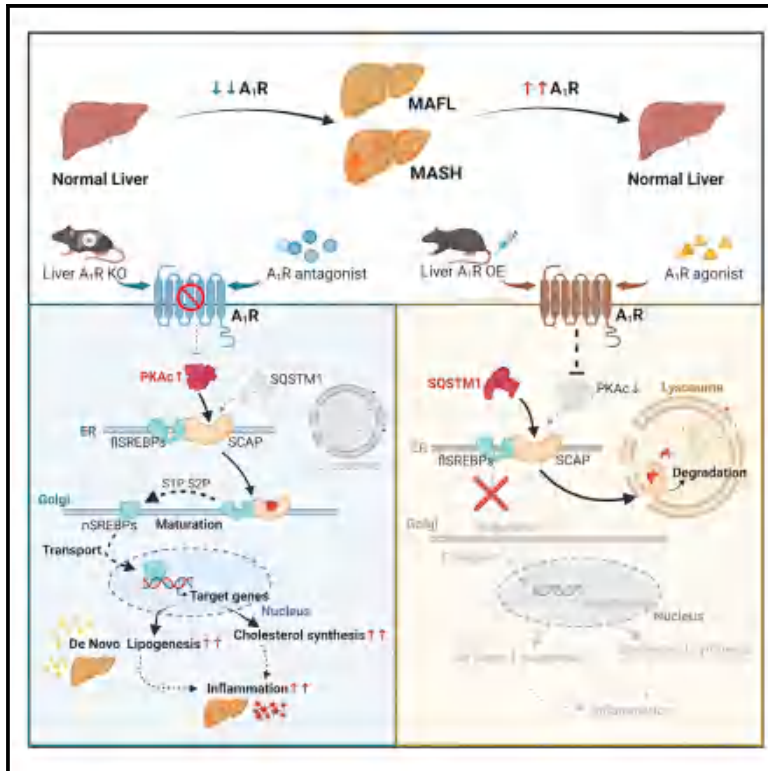


Activation of hepatic adenosine A1 receptor ameliorates MASH via inhibiting SREBPs maturation

Graphical abstract



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In brief

Zhu et al. show that hepatic A₁R activation reduces the direct binding of SCAP with PKAc but increases the formation of an SCAP-SQSTM1 complex, facilitating SCAP degradation in the lysosome, and suppressing the maturation of SREBPs. Hepatic A₁R is a promising target for MAFLD therapy.

Highlights

- Hepatic A₁R activation ameliorates MAFL and MASH in mice
- Activated hepatic A₁R reduces de novo lipogenesis via inhibiting SREBP maturation
- Activated hepatic A₁R promotes the binding of SQSTM1 to SCAP, leading to SCAP degradation
- Hepatic A₁R is a potential therapeutic target for MASH



Article

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

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SUMMARY

Metabolic (dysfunction)-associated steatohepatitis (MASH) is the advanced stage of metabolic (dysfunction)-associated fatty liver disease (MAFLD) lacking approved clinical drugs. Adenosine A₁ receptor (A₁R), belonging to the G-protein-coupled receptors (GPCRs) superfamily, is mainly distributed in the central nervous system and major peripheral organs with wide-ranging physiological functions; however, the exact role of hepatic A₁R in MAFLD remains unclear. Here, we report that liver-specific depletion of A₁R aggravates while overexpression attenuates diet-induced metabolic-associated fatty liver (MAFL)/MASH in mice. Mechanistically, activation of hepatic A₁R promotes the competitive binding of sterol-regulatory element binding protein (SREBP) cleavage-activating protein (SCAP) to sequestosome 1 (SQSTM1), rather than protein kinase A (PKA) leading to SCAP degradation in lysosomes. Reduced SCAP hinders SREBP1c/2 maturation and thus suppresses *de novo* lipogenesis and inflammation. Higher hepatic A₁R expression is observed in patients with MAFL/MASH and high-fat diet (HFD)-fed mice, which is supposed to be a physiologically adaptive response because A₁R agonists attenuate MAFL/MASH in an A₁R-dependent manner. These results highlight that hepatic A₁R is a potential target for MAFL/MASH therapy.

INTRODUCTION

Metabolic (dysfunction)-associated fatty liver disease (MAFLD) was formerly known as nonalcoholic fatty liver disease (NAFLD)^{1–4} and is the predominant chronic liver disease with a broad spectrum consisting of steatosis (fatty liver [MAFL]), steatohepatitis (metabolic-associate steatohepatitis [MASH]), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC).^{5,6} Although MAFL is the early and reversible stage of MAFLD, some MAFL patients will progress to MASH and even irreversible advanced stages.^{7,8} To date, there are no approved drugs for MASH therapy in clinic. Therefore, the identification of novel therapeutic targets of MASH is urgently needed.

Adenosine receptor (AR) belongs to the G-protein-coupled receptor (GPCR) superfamily, consisting of A₁R, A_{2A}R, A_{2B}R, and

A₃R subtypes, which are widely distributed in central nervous system and major peripheral organs with different abundance.⁹ ARs are activated upon extracellular adenosine that derives from ATP/ADP hydrolysis catalyzed by CD39 and CD73. The CD39/CD73-adenosine-ARs axis is implicated in immune and inflammation-related diseases where the increased inflammation and cellular hypoxia stimulate the generation of adenosine, resulting in diversified outcomes depending on the activated subtype of ARs and cellular distribution.^{10,11} Recent study reveals that A_{2A}R is a novel tumor suppressor of MASH-associated HCC¹² and antagonism of A_{2A}R enhances immunotherapy efficacy.^{13,14} A_{2B}R has been implicated in regulatory T cell functions and is a contributing factor in adenosine-mediated protection against inflammatory disease and organ injury.^{15,16} A₃R is involved in the inhibition of neutrophil degranulation in tissue



injury,¹⁷ and activation of A₃R reverses neuropathic pain via T cell-mediated production of interleukin (IL)-10.¹⁸ A₁R is highly expressed in central nervous system, heart atria, kidney, pancreas, adipose tissue, etc.^{9,19} Therefore, A₁R is a potential therapeutic target for diseases such as Parkinsonism, Alzheimer's disease, diabetes, and obesity,^{20–23} and several specific agonists or antagonists of A₁R are under phase II/III trials.^{24–26}

In contrast to the well-established distribution and role of A₁R in central nervous system and other peripheral organs,⁹ hepatic A₁R expression is supposed to be weak in liver under physiological condition.²⁷ Nevertheless, studies suggested that dysregulated hepatic A₁R signaling was associated with some liver diseases, such as liver fibrosis, hepatic ischemia-reperfusion injury, and intrahepatic cholestasis.^{28–30} Notably, it was reported that global A₁R knockout mice were resistant to alcohol-induced liver disease, and the underlying mechanism was supposed to be associated with the suppression of sterol-regulatory element binding protein 1c (SREBP1c)-mediated fatty acid de novo lipogenesis (DNL).³¹ However, this effect was not consistently observed in another study, which was supposed to be due to the low expression of A₁R in hepatocytes.²⁷ Paradoxically, the presence of the four subtypes of ARs in hepatocytes in either human beings or rodents has also been demonstrated, with the A₁R sequence being the most conserved.^{9,32} Thus, the exact role of hepatic A₁R in MAFLD development and progression remains uncertain.

In this study, we first investigated the role of hepatic A₁R by generating liver-specific A₁R knockout (A₁R^{Liver^{-/-}}) and overexpressed (A₁R^{Liver^{OE}}) mice in high-fat diet (HFD) and choline-deficient (CD)-HFD-induced MAFL and MASH models. Liver-specific deletion of A₁R aggravated diet-induced MAFL or MASH, which was attenuated in A₁R^{Liver^{OE}} mice. Mechanistically, our results revealed that activated A₁R suppressed the maturation of SREBPs (SREBP1c/2), leading to the inhibition of DNL via protein kinase A catalytic subunit (PKAc)/SREBP cleavage-activating protein (SCAP) pathway. Activated A₁R accelerated the degradation of SCAP protein in lysosome and reduced its anchoring at Golgi apparatus by forming SCAP-sequestosome 1 (SQSTM1) complex. Ultimately, we demonstrated that activation of hepatic A₁R by pharmacological agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA) or screened natural compound timosaponin AIII (TA3) with potent activating ability on A₁R ameliorated diet-induced MAFL and MASH in mice in an A₁R-dependent manner. Our current findings revealed a previously undetermined role of hepatic A₁R as a promising therapeutic target for MAFL and MASH.

RESULTS

Liver-specific A₁R knockout exaggerates MAFL and MASH in mice

To investigate the exact role of hepatic A₁R in MAFLD formation, we first generated liver-specific A₁R knockout (LKO) mice with no impact on the expression of other AR subtypes (Figures S1A–S1D). In the context of the normal chow diet (NCD), liver-specific A₁R deletion did not affect body weight gain, liver function, or histology (Figures S1E–S1H). Neverthe-

less, NCD-fed LKO mice showed increased hepatocyte triglyceride (TG), impaired glucose tolerance, and elevated protein expression of fatty acid synthase (FASN) and acetyl-coenzyme A (CoA) carboxylase (ACC) (Figures S1I–S1K), implying that liver-specific A₁R ablation might activate hepatic DNL. Then, we observed that liver-specific ablation of A₁R aggravated hepatic steatosis, increased liver index and serum alanine aminotransferase (ALT) level, induced dyslipidemia, and impaired glucose homeostasis in HFD-induced MAFL mice (Figures 1A–1E and S1L–S1S). Meanwhile, LKO mice showed increased liver pro-inflammatory cytokine (IL-1b, tumor necrosis factor [TNF]-a) levels and enhanced protein expression of hepatic FASN and ACC but not CPT1a or CD36 (Figures S1T and 1F).

To test whether liver-specific ablation of A₁R would aggravate MASH formation, we fed mice with CD-HFD, a commonly used diet for inducing a MASH model in relatively short time³³ (Figure 1G). Histopathological examination revealed elevated hepatic steatosis, fibrosis, macrophage marker F4/80 and CD11b, and NAFLD activity score (NAS) in LKO mice (Figures 1H and S1U). These changes were accompanied with increased serum ALT and aspartate transaminase (AST) activities and serum inflammatory cytokines (IL-1b, IL-6, and TNF-a), as well as increased gene expression of fibrosis markers, chemotactic factors, and inflammatory cytokines, suggesting enhanced liver damage and inflammation (Figures 1I, 1J, and S1V). The liver TG of LKO mice on CD-HFD was higher than Flox mice (Figure 1K). In addition, elevated protein expression of myofibroblast markers alpha smooth muscle actin (a-SMA) and inflammation pathway phosphorylated P65³⁴ but reduced Ikb³⁵ level were also observed in LKO mice (Figure 1L). We also measured the expression of TGF-b and phosphorylated Smad2 (p-Smad2)/Smad2 that are critically involved in fibrosis development.³⁶ Our results showed that hepatic A₁R knockout enhanced the expression of TGFb and the phosphorylation of Smad2 (Figure S1W). Quantification of ¹³C-incorporated fatty acids was conducted with liver tissue of mice in the context of short-term amylin liver NASH (AMLN) diet feeding. The results showed DNL was obviously higher in LKO than Flox mice (Figure S1X), which consolidated the observation of increased DNL in hepatic A₁R deletion mice. These results indicated that absence of hepatic A₁R exaggerated the diet-induced MAFL and MASH in mice.

Liver-specific A₁R overexpression protects mice from MAFL and MASH

The physiological role of hepatic A₁R was further validated by adeno-associated virus (AAV)-mediated liver-specific A₁R overexpression in mice (A₁R^{Liver^{OE}}, Figure S2A). In contrast to the observations in LKO mice, A₁R^{Liver^{OE}} mice showed dramatic improvements in HFD-induced metabolic disorders including reduced hepatic steatosis and dyslipidemia, improved liver function, and glucose homeostasis (Figures 2A–2E and S2A–S2H). In addition, A₁R^{Liver^{OE}} mice also showed decreased hepatic inflammatory cytokines, including IL-1b, IL-6, and TNF-a (Figure S2I). Moreover, overexpression of hepatic A₁R significantly suppressed CD36, FASN, and ACC protein expression (Figure 2F). In line with the protection on MAFL in wild-type (WT) C57BL/6J mice, the protective effect of hepatic A₁R on MAFL

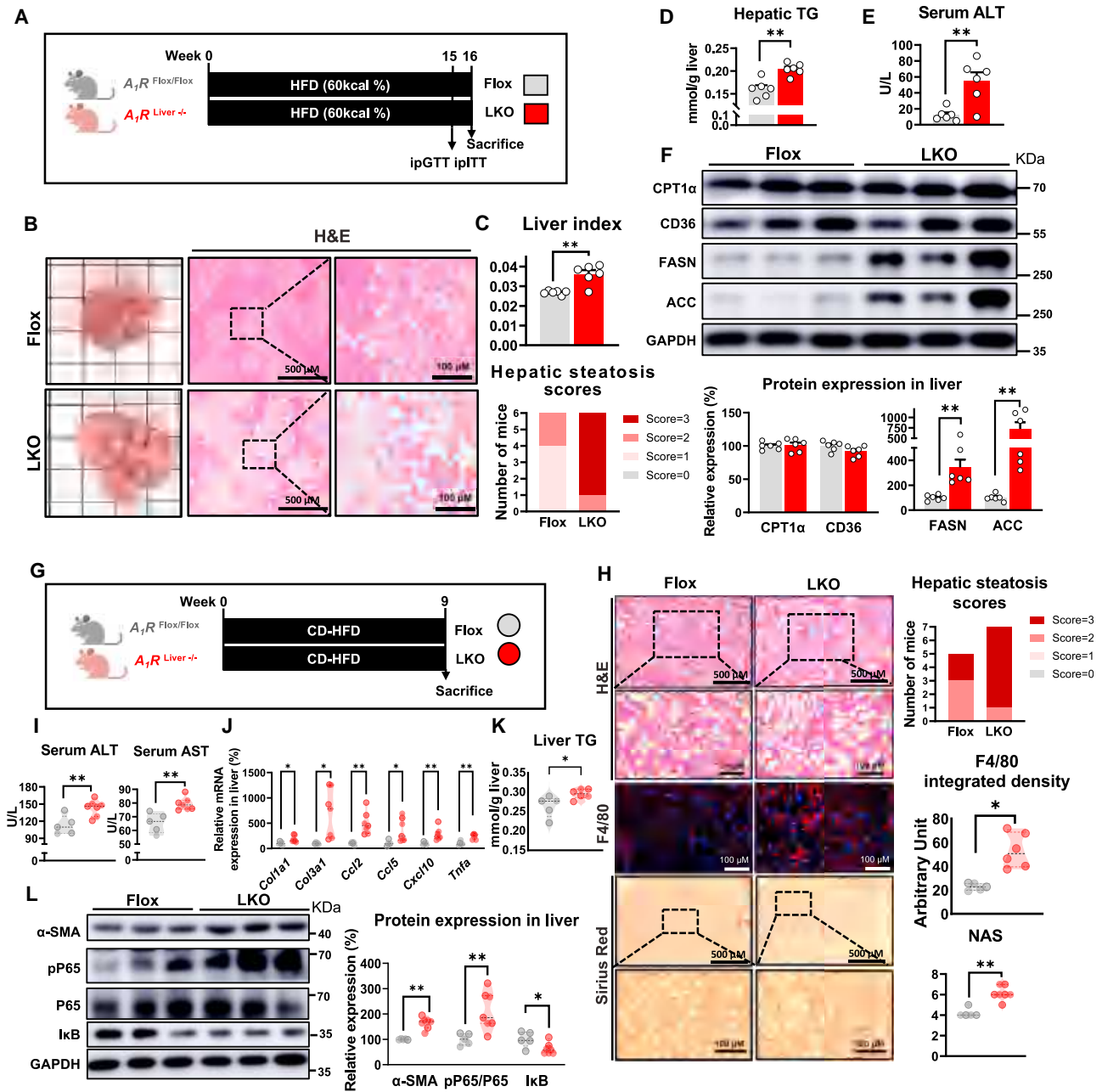


Figure 1. Liver-specific A_1R knockout exacerbates MAFL and MASH in mice

(A–F) Control mice ($A_1R^{Flox/Flox}$ without Cre, Flox) and liver-specific A_1R knockout mice ($A_1R^{Liver-/-}$, LKO) were fed an HFD (60 kcal%) for 16 weeks ($n = 6$). (A) Diagram of experimental design. (B) Representative image of liver and liver H&E staining. (C) Hepatic steatosis scores and liver index. (D) Quantification of hepatic triglycerides (TGs). (E) The level of serum alanine aminotransferase (ALT). (F) Relative protein expression of CPT1 α , CD36, FASN, and ACC. (G–K) Flox mice and LKO mice were fed a calorie-restricted HFD (CD-HFD) for 9 weeks ($n = 5\sim 7$). (G) Diagram of experimental design. (H) H&E, F4/80, and Sirius red staining of liver sections, with histological evaluation. (I) Liver injury indicators, including serum ALT and AST. (J) mRNA expression in liver. Results are normalized for 18S. (K) Quantification of hepatic triglycerides (TGs). (L) Relative protein expression of α -SMA, pP65, P65, and I κ B. GAPDH functioned as a reference protein. Results are representative of one biological replicate. Data are depicted as mean \pm SEM. Student's unpaired t test; * $p < 0.05$, ** $p < 0.01$.

was also validated in LKO mice, in which the hepatic A_1R expression was rescued by AAVs-mediated A_1R overexpression (LKO^{Res}, Figure S2J). Rescue of hepatic A_1R protected mice from HFD-induced MAFL, including improved hepatic steatosis,

and reduced hepatic TG accumulation and liver injury (Figures S2K–S2N). Beside the preventive effect, overexpression of hepatic A_1R also exerted a therapeutic effect on the established MAFL in HFD-fed mice (Figures S2O–S2R). Collectively, these

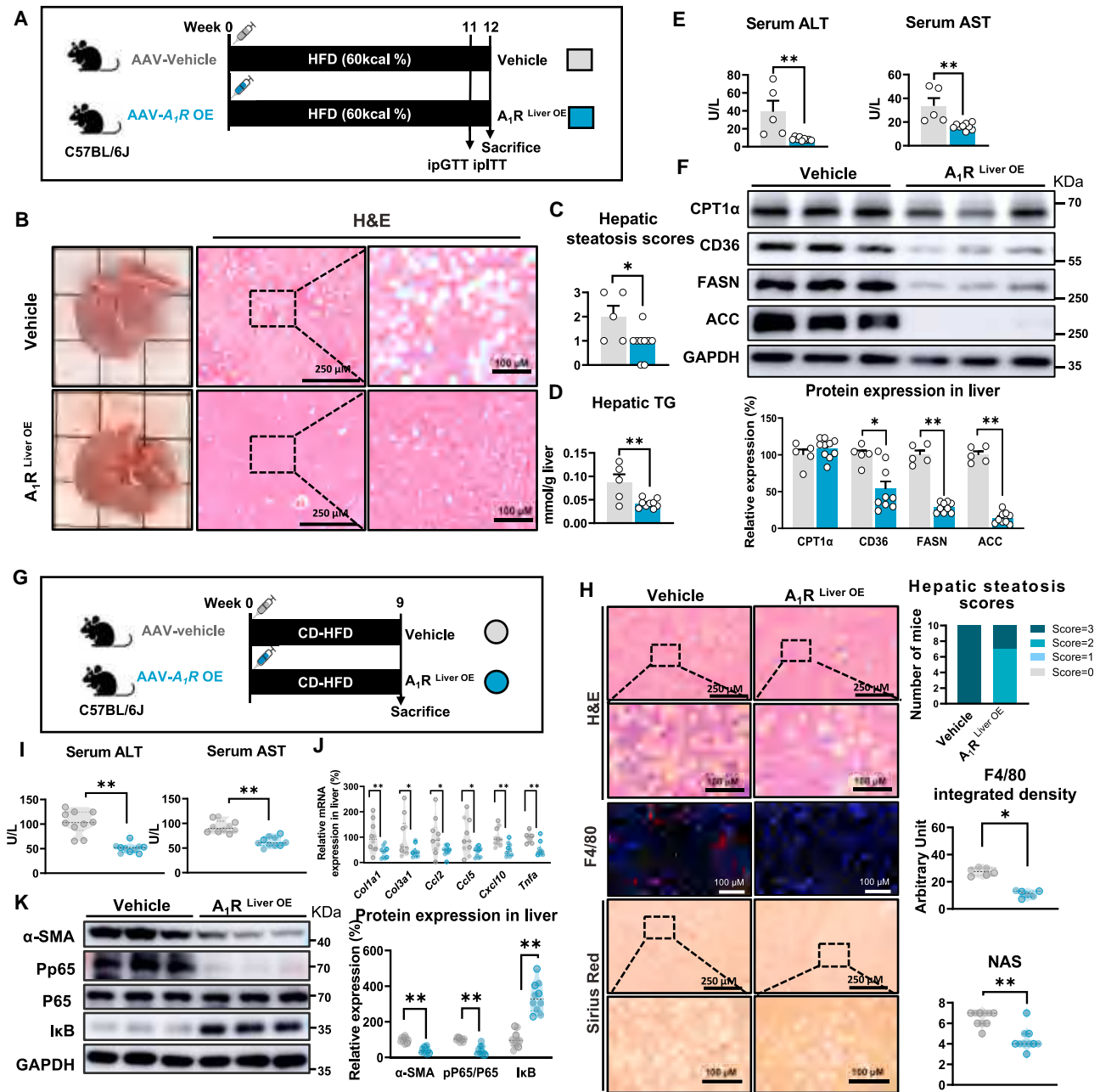


Figure 2. Liver-specific A_1R overexpression protects mice from MAFL and MASH in mice

(A–F) C57BL/6J mice were inoculated with vehicle-AAV (vehicle) or A_1R -overexpression-AAV ($A_1R^{Liver OE}$) intravenously, and then were fed an HFD for 12 weeks (n = 5–9). (A) Diagram of experimental design. (B) Representative image of liver and liver H&E staining. (C) Hepatic steatosis scores. (D) Quantification of hepatic TG. (E) Liver injury indicators, including serum ALT and AST. (F) Relative protein expression of CPT1 α , CD36, FASN, and ACC.

(G–K) Vehicle mice and $A_1R^{Liver OE}$ mice were fed a CD-HFD for 9 weeks (n = 10). (G) Diagram of experimental design. (H) H&E (n = 10), F4/80 (n = 6), and Sirius red staining (n = 10) of liver sections, with histological evaluation. (I) Liver injury indicators, including serum ALT and AST. (J) mRNA expression in liver. (K) Relative protein expression of α -SMA, pP65, P65, and I κ B. Results are representative of one biological replicate. Data are depicted as mean \pm SEM. Student's unpaired t test; *p < 0.05, **p < 0.01.

results indicated that hepatic A_1R activation attenuated diet-induced MAFL in mice.

We then tested whether overexpression of hepatic A_1R could also ameliorate MASH formation by feeding CD-HFD in mice.

Our results showed that $A_1R^{Liver OE}$ mice had reduced lipid accumulation, fibrosis, and macrophage infiltration in liver, as well as reduced serum ALT and AST levels (Figures 2G–2I, S2S, and S2T). Furthermore, hepatic A_1R activation suppressed the

mRNA expression of fibrosis markers, chemotactic markers, and inflammatory cytokines, as well as the protein expression of α -SMA and pP65/P65, but it increased I κ B expression (Figures 2J and 2K). In addition, the anti-MASH effect by activating hepatic A₁R was further confirmed in a 28-week AMLN diet-induced MASH mouse model,^{37,38} as indicated by reduced hepatic steatosis, NAS, Sirius red staining, and improved liver function (Figures S2U–S2Z). Altogether, these results demonstrated activation of hepatic A₁R protected mice from diet-induced MAFL and MASH.

A₁R controls the maturation of SREBPs via PKAc

SREBPs (mainly SREBP1c and SREBP2) are critical nuclear transcriptional factors that regulating the DNL, cholesterol, and inflammation signaling pathways in hepatocytes that are involved in MAFLD formation.³⁹ To investigate whether the hepatic A₁R regulates the MAFL and MASH development and progression through modulation on SREBPs, we measured the hepatic expression of precursor SREBPs (full-length SREBPs [flSREBPs]) in whole-cell and mature SREBPs (cleaved form of flSREBPs) in the nucleus (nSREBPs) in both LKO and A₁R^{Liver OE} mice under HFD or CD-HFD feeding. LKO mice showed reduced flSREBP1c and flSREBP2 but increased nSREBP1c and nSREBP2 in the nucleus compared to Flox mice, while A₁R^{Liver OE} mice showed the opposite trend in diet-induced MAFL or MASH mice (Figure 3A), implying A₁R negatively controlled the maturation of SREBPs in hepatocytes.

Activated A₁R modulates the intracellular signaling molecules through decreasing adenylate cyclase (AC) activity in cytomembrane,⁹ resulting in suppression of activity of cAMP/PKAc in cytoplasm controlling the downstream targets, including SREBPs. The negative control of PKAc by hepatic A₁R was confirmed in both LKO and A₁R^{Liver OE} mice as well as in AML-12 cells treated by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and CPA, specific antagonist and agonist of A₁R, respectively⁹ (Figures 3B and 3C). However, the exact modulation on SREBPs by PKAc remains unclear because both positive and negative regulation of SREBPs by cAMP/PKAc were previously reported.^{40,41} Thus, the nuclear translocation of mature SREBP1c and SREBP2 was measured in AML-12 cells under the treatment of either DPCPX or CPA alone, or in combination with H89, a selective and potent inhibitor of PKA,⁴² or dibutyryl-cAMP sodium salt (DbcAMP), a cell-permeable PKA activator by mimicking the action of endogenous cAMP.⁴³ The results showed the translocation of SREBP1c and SREBP2 to nucleus was stimulated by DPCPX but suppressed by CPA, whereas the combined treatment of H89 or DbcAMP reversed the SREBP nuclear translocation under DPCPX or CPA, as shown by consistent observations with either western blot or immunofluorescence. These results suggested the controlling of SREBP endonuclear content by A₁R was dependent on PKAc (Figures 3C–3E, S3A, and S3B).

To further evaluate the contribution of increased PKAc to MAFL formation in LKO mice, H89 was administered to either Flox or LKO mice during the 8 weeks of HFD feeding (Figure S3C). H89 treatment significantly improved hepatic steatosis and reduced ALT activity in both Flox and LKO mice

(Figures S3D–S3I). In addition, the nuclear level of mature SREBP1c (nSREBP1c) was dramatically reduced by H89 in both Flox and LKO groups (Figure S3J). In summary, hepatic A₁R inhibited the nuclear translocation of SREBPs by decreasing PKAc, resulting in the attenuation of MAFLD.

A₁R reduces the cellular content of SCAP and its anchoring at Golgi membrane

The maturation of SREBPs is tightly controlled by SCAP and insulin-induced gene 1 protein (INSIG1) in endoplasmic reticulum (ER) escorting SREBPs from ER to Golgi apparatus where SREBPs is sequentially cleaved by two proteases: S1P and S2P^{44,45} (Figure 4A). To explore how A₁R controlled the maturation of SREBPs, we tested the expression of these proteins in liver tissue of either LKO or A₁R^{Liver OE} mice. We found only SCAP and S2P protein were significantly increased in LKO mice and decreased in A₁R^{Liver OE} mice compared to their counterparts (Figures 4B; S4A).

Because the transport of SCAP from ER to Golgi apparatus and anchoring is the critical step for facilitating the maturation of SREBPs,⁴⁶ we then further explored the anchoring status of SCAP at Golgi apparatus by using 3D modeling of confocal image. As expected, inhibition of A₁R by DPCPX not only increased the cellular content of SCAP protein but also increased its anchoring at Golgi apparatus (yellow color in merged image and co-localization in 3D image), while CPA showed the opposite effect (Figure 4C), implying the negative regulation of SCAP content and function by A₁R. Given the established regulation of PKAc-SREBPs by the A₁R and SCAP-SREBPs axis,⁴⁶ we further asked whether the regulation of SCAP by A₁R was dependent on PKAc. The observations in vitro showed that the effects of CPA and DPCPX on SCAP protein content were abolished by co-treatment with H89 or DbcAMP, respectively (Figure 4D), suggesting A₁R might control the cellular content of SCAP protein through PKAc. Meanwhile, the binding between PKAc and SCAP was facilitated by DPCPX but suppressed by CPA (Figure S4B), implying A₁R inhibited the direct binding between PKAc and SCAP. Altogether, these results indicated that A₁R reduced intracellular SCAP content as well as its anchoring at Golgi membrane through PKAc.

A₁R activation accelerates SCAP protein degradation through SQSTM1 in lysosome

The cellular protein content is determined by the balance between synthesis and degradation. To test whether A₁R regulated the degradation of SCAP protein, AML-12 cells were treated with either CPA or DPCPX in the presence of cycloheximide (CHX), an inhibitor of protein biosynthesis. The results showed that CPA dramatically accelerated the degradation of SCAP, which was maintained stable by DPCPX in the presence of CHX treatment (Figure 5A), implying A₁R may control the cellular content of SCAP protein by accelerating its degradation. In line with the impact of PKAc on SCAP content, we found that the degradation of SCAP protein was accelerated by H89 but maintained by DbcAMP (Figure S4C), suggesting PKAc also controlled the degradation of SCAP. There are two major pathways regulating protein catabolism: the ubiquitin-proteasome system and the autophagy-lysosomal system.⁴⁷ To determine how A₁R controlled the SCAP

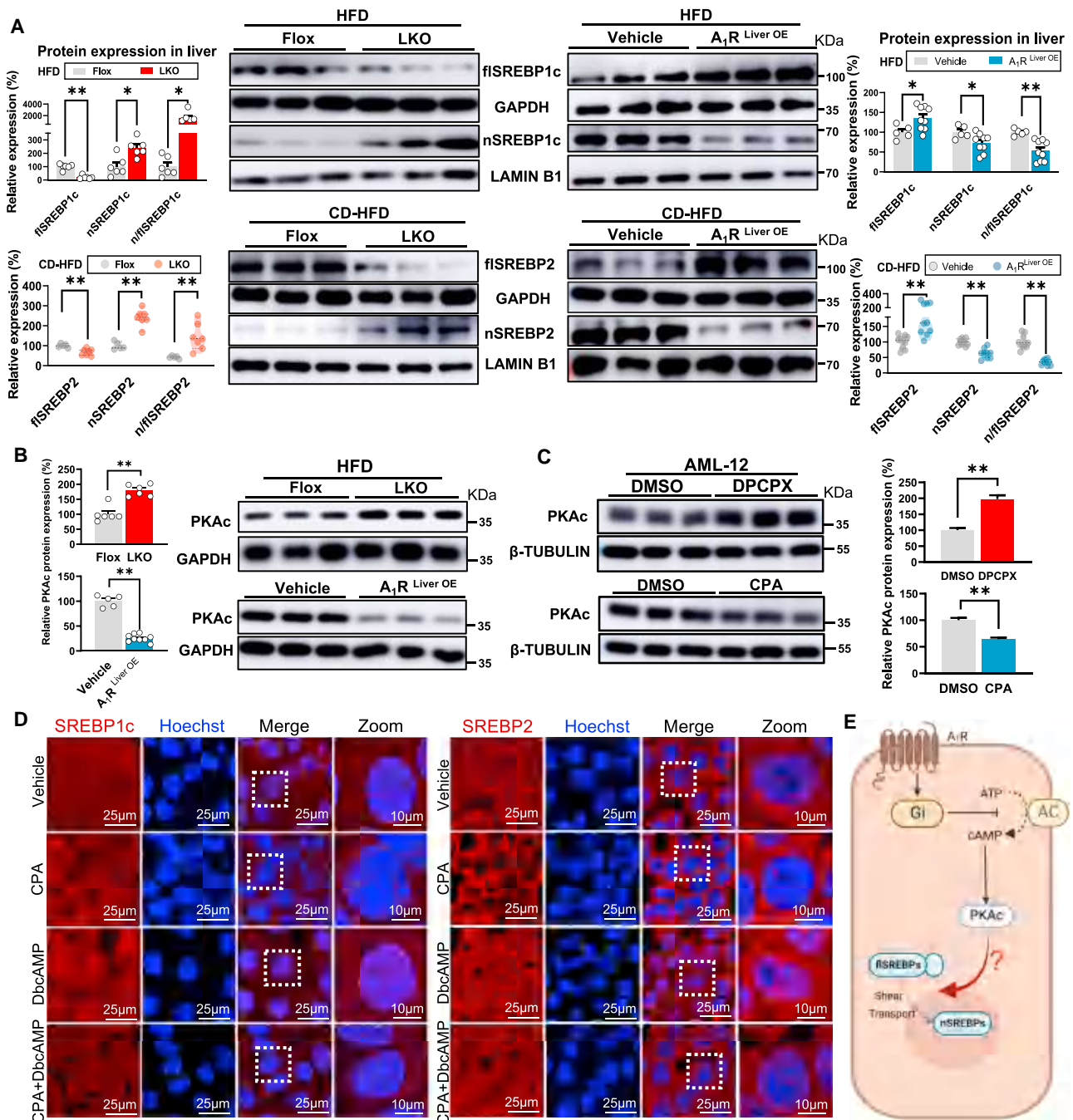


Figure 3. A₁R controls the maturation of SREBPs via PKAc

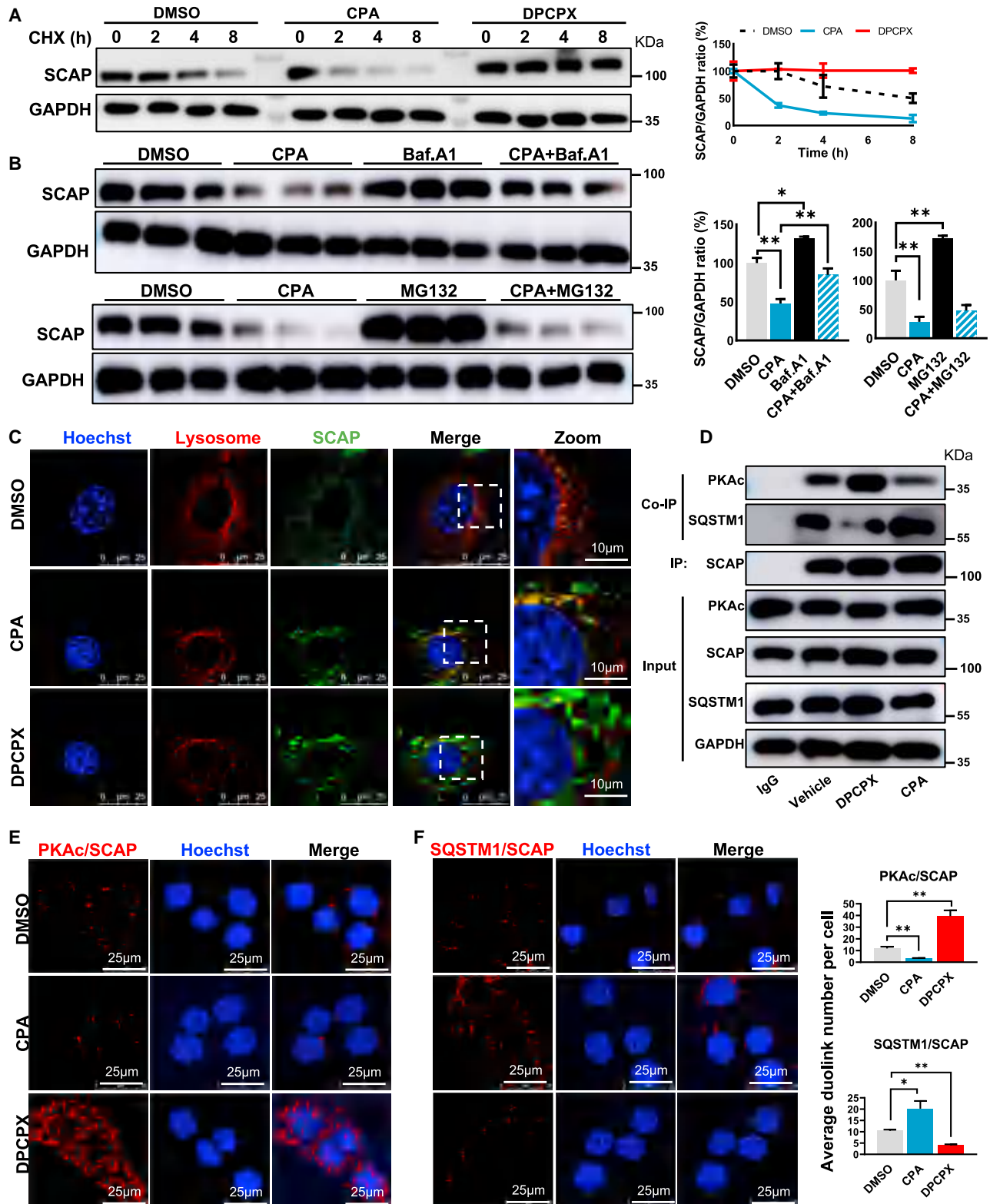
(A) Relative hepatic protein expression of fSREBP1c and nSREBP1c from LKO mice and A₁R^{Liver OE} mice on HFD and CD-HFD.

(B) Hepatic PKAc protein expression in LKO and A₁R^{Liver OE} mice.

(C) PKAc protein expression in AML-12 cells treated with CPA (A₁R activator, 1 mM) and DPCPX (A₁R inhibitor, 1 mM) for 48 h. GAPDH or tubulin was used as total protein control, and lamin B1 was used as nuclear protein control.

(D) AML-12 cells were treated with CPA (1 mM, 48 h), DbcAMP (PKA activator, 200 mM, 12 h), or co-treated with CPA and DbcAMP, respectively. Fluorescent staining of SREBP1c or SREBP2 (red) in each group was performed. The nuclei were stained by Hoechst (blue).

(E) Overview of A₁R intracellular signaling pathways. Activated A₁R decreases adenylate cyclase (AC) activity to suppress the activity of cAMP/PKAc in cytoplasm, resulting in modulation of the intracellular signaling molecules. However, the exact modulation on SREBPs by PKAc remains unclear. Image created using BioRender. Results are representative of one biological replicate. Data are depicted as mean ± SEM. Student's unpaired t test; *p < 0.05, **p < 0.01.



(legend on next page)

degradation, bafilomycin A1 (Baf.A1), blocking autophagosome-lysosome fusion and inhibiting protein degradation in lysosomes, or MG132, a potent proteasome and calpain inhibitor,⁴⁸ was co-treated with CPA in AML-12 cells, respectively. Results showed that coculture of Baf.A1, but not MG132, with CPA prevented the accelerated SCAP degradation induced by CPA (Figure 5B), suggesting that CPA may mainly accelerate SCAP degradation in lysosome. Further evidence was consistently observed by immunofluorescence, in which CPA increased the co-localization of SCAP with lysosome (yellow color in merged image), but not in DPCPX-treated cells (Figure 5C). These results indicated activated A₁R promoted the degradation of SCAP in lysosome.

We next investigated how A₁R controlled SCAP degradation. Recently, Zheng et al. revealed that SCAP was captured by SQSTM1 after it exited from ER and underwent a novel SQSTM1-mediated autophagy-independent lysosomal degradation.⁴⁸ Given the role of PKAc in maintaining cellular content of SCAP (Figures 4D and S4C) and the physical binding with SCAP directly (Figure S4B), we hypothesized that the increased degradation of SCAP by activated A₁R was due to the competitive binding with SCAP between PKAc and SQSTM1. The co-immunoprecipitation (Co-IP) test showed that CPA stimulated the formation of SCAP-SQSTM1 and reduced SCAP-PKAc complex, whereas the opposite effect was observed in DPCPX-treated cells (Figure 5D). Moreover, in agreement with the coIP observation, proximity ligation assay (PLA) revealed that CPA reduced the direct interaction between PKAc and SCAP but increased SQSTM1 and SCAP interaction, which was oppositely regulated by DPCPX (Figures 5E and 5F). Therefore, the results indicated that activated A₁R promoted the competitive binding of SCAP with SQSTM1 rather than PKAc, leading to SCAP degradation in lysosome.

Pharmacological hepatic A₁R activation attenuates MAFL and MASH in mice

Since the exact status of hepatic A₁R in MAFLD remains unclear, we examined the expression of A₁R protein in liver specimens from clinical patients. A total of 30 liver samples from a group of patients who underwent hepatectomy were retrospectively studied with immunohistology, in which 22 of them were diagnosed with the presence of hepatic steatosis, and eight of them were used as control without hepatic steatosis. The patient information is described in the supplementary methods (Table S1). The results showed universal positive expression of A₁R protein in hepatocytes in patients with hepatic steatosis but not in control patients (Figures 6A–6C). To further characterize the status of hepatic A₁R in MASH patients, the expression of hepatic A₁R was evalu-

ated by using immunofluorescence in specimens from nine patients with MASH and four controls who underwent liver biopsy (Table S2). In agreement with the observation in patients with hepatic steatosis retrospectively, hepatic A₁R expression was obviously triggered in patients diagnosed with MASH but not in controls (Figures 6D–6F). Consistently, public clinical data also revealed the increased mRNA expression of A₁R in liver of patients with MAFLD^{49,50} (Figure S4D, GSE89632, GSE135251). In agreement with these clinical findings, elevated hepatic A₁R protein expression was also present in HFD-fed mice time dependently or CD-HFD-fed mice compared to their chow diet controls (Figures S4E and S4F). As a result, we hypothesized that the elevated expression of hepatic A₁R in MAFLD patients or diet-induced MAFLD mice might be an adaptively protective response against MAFLD, but it is not sufficient.

To determine whether activation of A₁R signaling could protect against MAFLD, specific A₁R agonist CCPA (intraperitoneal [i.p.], 1 mg/kg once per day) was administered in both HFD-induced MAFL and CD-HFD-induced MASH mice (Figures S5A and S5L). The results showed CCPA treatment substantially improved the diet-induced MAFL and MASH in mice, including attenuation in hepatic steatosis and fibrosis in histology, and improvement in dyslipidemia, glucose intolerance (fasting blood glucose, insulin, and IGTT), liver function (serum ALT, AST, or alkaline phosphatase [ALP]), and inflammation (levels of serum IL-1b, IL-6 and TNF- α , and gene expression of chemokines), without affecting energy intake (Figures S5B–S5J and S5M–S5O). Moreover, CCPA treatment also suppressed the expression of FASN, ACC, PKAc, and SCAP protein; reduced maturation of SREBP1c/2 in MAFL mice; inhibited α -SMA; and enhanced I κ B proteins in MASH mice (Figures S5K and S5Q). Collectively, these results demonstrated that pharmacological activation of hepatic A₁R was effective for MAFL and MASH therapy. To further test whether adenosine administration could also attenuate MAFLD in mice, we treated HFD-induced MAFLD mice with either adenosine (10 mg/kg, i.p.) or CCPA (1 mg/kg, i.p.) in parallel. As shown in Figures S6A–S6F, we found that even high doses of adenosine did not attenuate the extent of MAFLD, while specific A₁R agonist, CCPA, was effective in reducing lipid accumulation in hepatocytes and serum ALT/AST levels. These results suggested that the anti-MAFLD effect was due to specific activation of A₁R but not in non-specific ligand of ARs.

Hepatic A₁R is an applicable target for screening MAFLD therapy compounds

Based on our current observations, we further asked whether hepatic A₁R was applicable for screening potential MAFLD

Figure 5. A₁R activation accelerates SCAP protein degradation through SQSTM1 in lysosome

(A) Relative protein expression of SCAP in the AML-12 cells treated with CPA or DPCPX in the presence of cycloheximide (CHX, 50 mM) for 0, 2, 4, and 8 h. (B) Relative protein expression of SCAP in the AML-12 cells treated or co-treated with CPA and bafilomycin A1 (Baf.A1, lysosomal inhibitor, 1 mM, 8 h) or MG132 (proteasome inhibitor, 10 mM, 8 h). (C) SCAP colocalizes with the lysosome. AML-12 cells were treated with DPCPX or CPA for 48 h. Fluorescent staining of nuclei (Hoechst, blue), lysosome (red), and SCAP (green) was performed. (D) Co-immunoprecipitation (coIP) of PKAc or SQSTM1 with SCAP in the AML-12 cells treated with DPCPX or CPA for 12 h. (E and F) Representative results and quantification of the proximity ligation assay (PLA) analysis of (E) PKAc or (F) SQSTM1 with SCAP in the AML-12 cells treated with CPA or DPCPX. Results are representative of one biological replicate. Cell experiments performed n = 3. Data are depicted as mean \pm SEM. Student's unpaired t test; *p < 0.05, **p < 0.01.

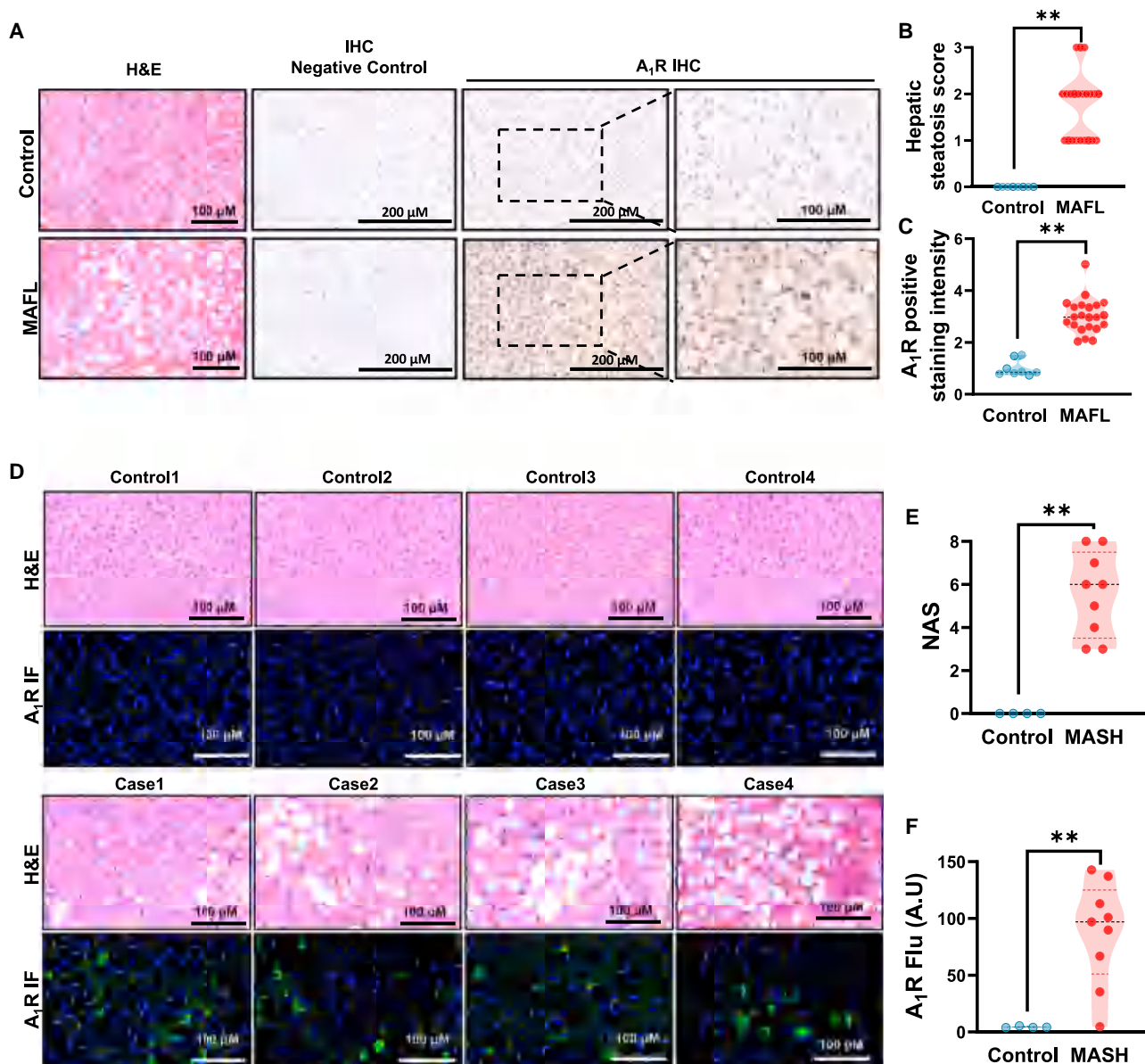


Figure 6. Hepatic A₁R negatively correlates with the advance of MAFLD

(A) Representative images showing H&E staining and A₁R immunohistochemical (IHC) staining of liver tissue from individuals with or without hepatic steatosis (n = 8–22).

(B) Hepatic steatosis score.

(C) A₁R IHC-positive staining intensity.

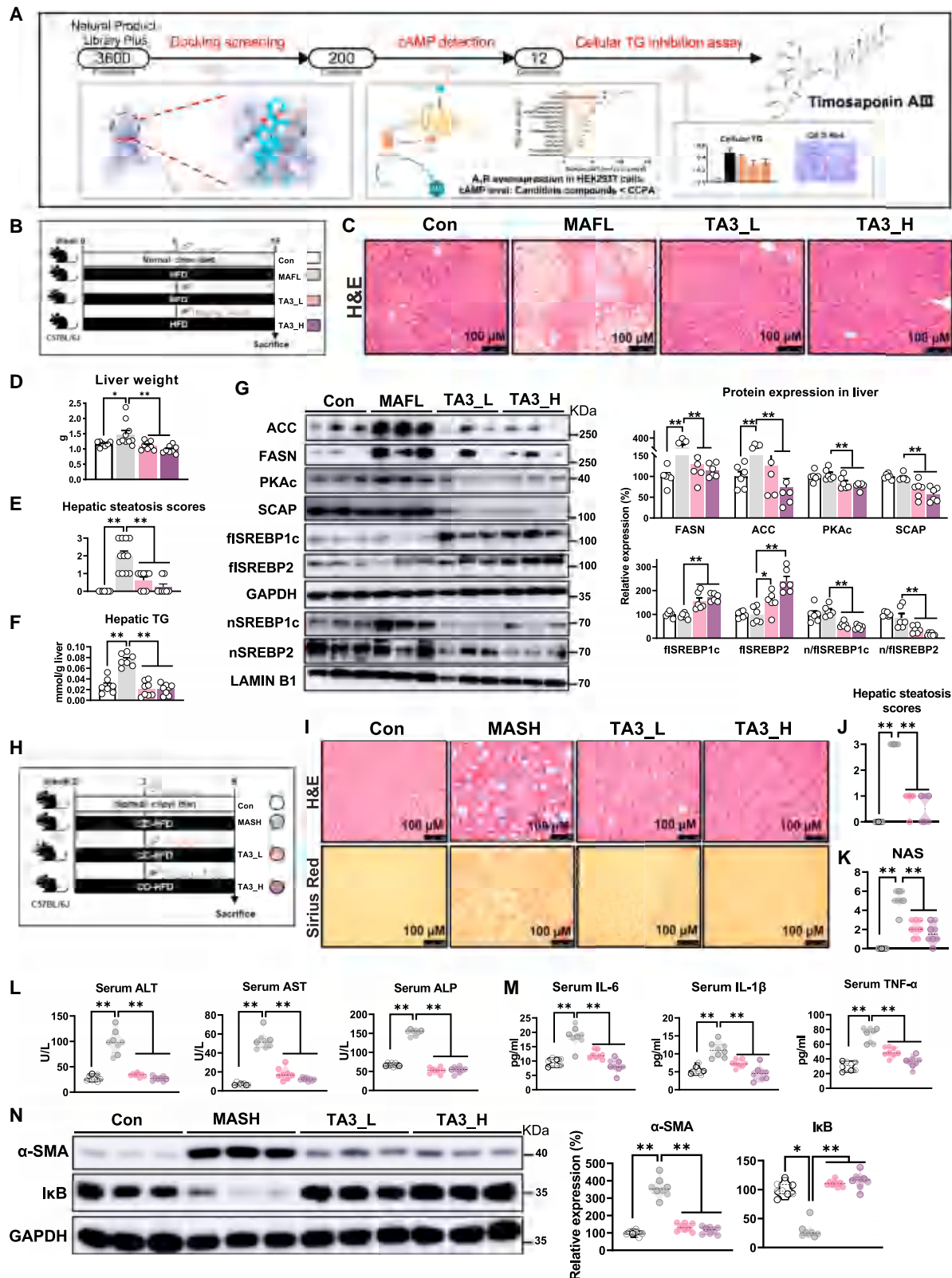
(D) Representative images showing H&E staining and A₁R immunofluorescence staining of livers from healthy control or MASH individuals (n = 9).

(E) NAFLD activity score (NAS).

(F) A₁R fluorescence-positive staining intensity. Results are representative of one biological replicate. Data are depicted as mean ± SEM. Student's unpaired test; *p < 0.05, **p < 0.01.

therapy compounds. For this purpose, we then screened natural compounds with activating capacity on A₁R among 3,600⁺ natural compounds library by using computer-aid molecular docking and followed by in vitro test on inhibition of cAMP production and TG accumulation. A total of 200 compounds stood out based on their binding free energy with A₁R, then 12 of them were selected with stronger activating

capacity on A₁R than CCPA in A₁R-overexpressed HEK293T cells by measuring production of cAMP. At last, a natural component, timosaponin AIII (TA3), was identified based on its potent activating A₁R capacity, inhibition of intracellular TG production in primary hepatocytes from WT mice, and AML-12 and HepG2 cells dose dependently but not in primary hepatocytes from LKO mice (Figure 7A, S6G–J). TA3 also



(legend on next page)

dose-dependently inhibited the expression of PKAc, FASN, ACC, and nuclear localization of nSREBP1c/2 in AML-12 cells, which were consistent with those of CCPA treatment or A₁R^{Liver OE} mice (Figures S6K, S5K, 3A, and 3B). Additionally, ligand activation assay indicated that TA3 was strongest in reducing intracellular cAMP content in AML-12, HEK293T or A₁R-overexpressed HEK293T cells compared to CCPA or adenosine, endogenous ligand for ARs (Figure S6L). Moreover, we observed that adenosine had no effect on cAMP content, intracellular TG accumulation, or protein expression of ACC and FASN in AML-12 or HEK293T cells, while reduced cAMP content was only observed in A₁R-overexpressed HEK293T cells (Figures S6L–S6N), implying selectivity on A₁R was necessary for suppressing TG accumulation.

To evaluate the potential of TA3 as a novel MAFLD therapeutic candidate, the anti-MAFLD effect of TA3 was evaluated in HFD-induced MAFL and CD-HFD-induced MASH mice (5 or 10 mg/kg, i.p., once per day) (Figures 7B and 7H). As expected, TA3 treatment obviously attenuated HFD-induced MAFL, as shown by improved hepatic steatosis in histology, liver TG, and serum total cholesterol (TC), ALT, and AST (Figures 7C–7F and S7A–S7D). In line with the effects of CCPA (Figure S5K), TA3 suppressed the expression of PKAc, SCAP, ACC, and FASN as well as the maturation of SREBP1c/2, resulting in inhibition of DNL (Figure 7G). In parallel, TA3 treatment also effectively ameliorated CD-HFD-induced MASH, as shown by reduced hepatic steatosis, liver injury, and fibrosis based on histology; reduced serum inflammation cytokines; as well as related gene and protein expressions (Figures 7I–7N, S7E, and S7F). Taken together, these results indicated that the screened TA3 by targeting A₁R was effective for MAFL and MASH therapy, highlighting the potential of hepatic A₁R as MAFLD therapy target.

To further determine whether hepatic A₁R was the key therapeutic target for MAFLD, the anti-MASH effect of either CCPA (1 mg/kg, i.p., once per day) or TA3 (10 mg/kg, i.p., once per day) was tested in LKO mice that were fed with CD-HFD and treated with an identical dosage to that in WT mice (Figure S7G). To our expectation, both CCPA and TA3 treatment did not improve the histological change or the scores of hepatic steatosis and NAS, and they had minor impacts on serum parameters in LKO mice (Figures S7G–S7J), in contrast to their significant effects in WT mice (Figures 7 and S5). Consistently, neither CCPA nor TA3 reversed the expression of serum inflammation cytokines or hepatic α -SMA and I κ B in CD-HFD-fed LKO mice (Figures S7K and S7L).

Altogether, these results revealed a novel role of hepatic A₁R as an anti-MAFLD therapeutic target, especially for MASH therapy.

DISCUSSION

We report here that hepatic A₁R activation protected mice from diet-induced MAFL and MASH. Activated hepatic A₁R reduced the cellular content of SCAP protein and its anchoring at Golgi apparatus due to increased lysosomal degradation by forming SCAP-SQSTM1 complex rather than SCAP-PKAc. The reduced cellular SCAP resulted in less formation of mature SREBP1c/2 and nuclear SREBP1c/2 translocation, leading to reduced DNL and inflammation. The anti-MAFLD role of hepatic A₁R was further confirmed by specific A₁R agonist CCPA and screened natural compound TA3 with potent activating capacity on A₁R activity, which implied that the observed increase of A₁R expression in liver of MAFL/MASH patients or MAFLD mice might be an adaptive response against MAFLD development and progression.

The four subtypes of ARs are widely distributed with diversified functions and involved in various pathophysiological processes upon activation by extracellular adenosine.⁹ Extracellular adenosine can be released from intracellular stores, and is predominantly derived from the metabolism of precursor nucleotides ATP/ADP by CD39 and CD73 catalyzed, especially in pathological conditions such as inflammation and hypoxia.^{11,51} The adenosine signaling could be terminated by adenosine uptake from the extracellular compartment into cytoplasm through equilibrative nucleoside transporter 1 (ENT1) and equilibrative nucleoside transporter 2 (ENT2) where adenosine is converted to AMP through adenosine kinase or to inosine through the adenosine deaminase.^{52–54} A₁R is mainly expressed in central nervous system and some peripheral organs where extracellular adenosine performs different functions in a cell-type-dependent way.^{9,55,56} Blocking A₁R-mediated signaling could abolish heart-rate-slowing effects of intravascular adenosine. Since the four subtypes of ARs are present in hepatocytes, or partly expressed in stellate cells, Kupffer cells, and sinusoidal endothelial cells,^{9,57} previous study suggested that the number and affinity of A₁R and A₃R did not change in cirrhotic and fatty livers but the numbers of A_{2A}R and A_{2B}R increase in cirrhotic and fatty livers.³¹ In agreement with the observation that disrupted A_{2A}R exacerbated MAFLD development in A_{2A}R-deficient mice,⁵⁸ our findings showed a higher ratio of hepatocytes with positive A₁R

Figure 7. Timosaponin AIII, an identified potent A₁R activator, inhibits diet-induced MAFL and MASH in mice

(A) Schematic of the drug screen.

(B–G) C57BL/6J mice were fed with HFD for 16 weeks and injected intraperitoneally with 0.9% NaCl solution (MAFL) or 5 or 10 mg/kg timosaponin AIII (TA3) daily from ninth week (n = 7–10). (B) Diagram of experimental design. (C) Representative image of liver H&E staining. (D) Liver weight. (E) Hepatic steatosis scores. (F) Quantification of hepatic TG. (G) Relative protein expression of PKAc, SCAP, SREBPs (fSREBP1c, fSREBP2, nSREBP1c, nSREBP2), and SREBP1c regulated proteins (FASN, ACC) (n = 6).

(H–N) C57BL/6J mice were fed a normal chow diet (NCD, Con) or CD-HFD for 9 weeks; during the process, CD-HFD mice were divided into three subgroups and injected intraperitoneally with 0.9% NaCl solution (CD-HFD) or 5 or 10 mg/kg TA3 daily from fourth week. (H) Diagram of experimental design.

(I–N) C57BL/6J mice were fed with CD-HFD for 9 weeks and injected intraperitoneally with 0.9% NaCl solution (MASH) or 5 or 10 mg/kg timosaponin AIII (TA3) daily from fourth week (n = 8). (I) H&E and Sirius red staining of liver sections. (J and K) Histological evaluation. (L) Serum ALT, AST, and ALP. (M) Serum inflammatory cytokines. (N) Relative protein expression of α -SMA and I κ B. GAPDH was used as total protein control; lamin B1 was used as nuclear protein control. Results are representative of one biological replicate. Data are depicted as mean \pm SEM. One-way ANOVA analysis; *p < 0.05, **p < 0.01.

protein expression in liver of patients with MAFL/MASH or MAFLD mice than their healthy controls. In addition, we observed the expression of these four ARs in mouse liver, in which A₁R was the highest among them in both male and female (Figure S4G). We therefore questioned whether the increased adenosine and hepatic A₁R signaling contributed to the development of MAFLD or was an adaptive response for combating MAFLD development instead. To answer this question, genetic deletion of hepatic A₁R in LKO mice served as an ideal model, rather than global A₁R knockout mice, which makes it hard to exclude the impacts derived from A₁R deletion in other organs. To our surprise, LKO mice were more prone to diet-induced MAFL and MASH than Flox mice, which was also reversely validated in A₁R^{Liver^{OE}} mice, and, moreover, rescue of hepatic A₁R attenuated hepatic steatosis in LKO mice. In contrast to the report by Peng et al. that global knockout of either A₁R or A_{2B}R protected mice against ethanol-induced fatty liver disease,³¹ our results demonstrated that hepatic A₁R activation would attenuate diet-induced MAFL/MASH based on liver-specific A₁R knockout or overexpression mice. Meanwhile, the other three subtypes of ARs in liver of mice were not altered by the deletion or overexpression of hepatic A₁R. Therefore, our current findings revealed a previously unreported function of hepatic A₁R against MAFLD development, implying that increased expression of A₁R in liver in MAFLD patients or mice might be an adaptive response instead of causative for MAFLD development.

Sterol-regulatory element binding proteins (mainly SREBP1c and SREBP2) are essential transcription factors activating the expression of genes in DNL and cholesterol synthesis^{59,60} and promoting inflammation, and they are crucial for MAFL and MASH formation.⁶¹ In agreement with the phenotypes in LKO or A₁R^{Liver^{OE}} mice, the maturation and nuclear translocation process of SREBPs was stimulated by A₁R deletion and inhibited by A₁R overexpression, as well as consistent observation by A₁R antagonist DPCPX or agonist CPA in vitro. The premature SREBPs are synthesized as ER membrane-bound proteins that are activated upon sterol through the escorting from ER to Golgi apparatus by SCAP.^{46,61,62} Consistent with the changes of nSREBPs upon A₁R activation or inhibition either in vivo or in vitro, cellular content of SCAP protein was increased by A₁R deletion and decreased by A₁R activation, as well as its anchoring at Golgi apparatus. Previous studies showed liver-specific deletion of SCAP caused low SREBPs protein and mRNA expression, resulting in marked reduction of both the precursor and nSREBPs.^{63–65} In contrast, our results suggested that activated A₁R blocked the maturation and translocation to nucleus of SREBPs by reducing cellular SCAP content, instead of the suppression of SREBP generation because of the observed accumulation of fSREBPs in cytoplasm. This discrepancy between ours and a previous report might be due to the altered cellular SCAP content controlled by A₁R in our model, which was different from the direct impact on SREBPs by liver-specific Scap knockout.

There are several other signal pathways that are tightly controlled by A₁R, including cAMP/PKA, PLCb-IP3-PKC, and MAPK family such as ERK, p38, and JNK.^{66,67} Nevertheless, the role of cAMP/PKA in A₁R-controlled TG accumulation was

also validated by intervention of A₁R agonist CCPA in the presence or absence of PKA agonist or inhibitors of PKC, ERK, p38, and JNK in vitro. These results showed that only PKA agonist abolished the inhibitory effect of cellular TG accumulation upon A₁R activation, while inhibitors of PKC, ERK, p38, and JNK did not (data not shown). So, it was concluded that cAMP/PKA might mediate the subsequent intracellular signal upon A₁R activation. cAMP/PKA is known to coordinate with the SCAP-SREBPs pathway through controlling SCAP phosphorylation.⁶⁸ In our study, although we observed that specific PKA inhibitor, H89, attenuated hepatic steatosis and nSREBP1c content in liver of LKO mice, our phosphoproteomic analyses of PKA-dependent phosphorylated targets excluded the possibility of SCAP phosphorylation in response to A₁R suppression (Figure S4H) but facilitated the co-localization of SCAP protein in lysosome instead. PKA is a heterotetrametric holoenzyme in which two catalytic subunits from two major families, Ca/b, combine with homodimers formed by any of four R-subunits (R1a, R1b, R11a, R11b) to form a number of R2:C2 holoenzymes.^{69–72} Canonically, PKA is activated when cAMP binds to the regulatory subunits, triggering release of the catalytic subunit PKAc.⁷³ We observed that the catalytic subunit PKAc was reduced upon A₁R activation or increased by A₁R inhibition, which was consistently accompanied by SCAP-SREBP alteration. Since we did not observe the evidence of SCAP phosphorylation by PKA upon A₁R activity inhibition, we then postulated that the SCAP content might be modulated by A₁R through direct binding with PKAc, which was shown by the following coIP test.

The cellular content of protein is tightly controlled by the balance between biosynthesis and degradation. We demonstrated that activation of A₁R by CPA increased the degradation of SCAP protein in the context of inhibiting protein biosynthesis with CHX. Moreover, CPA increased SCAP co-localization and degradation in lysosome, instead of the ubiquitin-proteasome system, which are the two major pathways regulating protein catabolism.⁴⁷ In agreement with our current finding, Zheng et al. revealed an autophagy-independent SCAP degradation in lysosome through binding with SQSTM1 induced by the small molecule lycorine.⁴⁸ It is unclear whether A₁R controlled the lysosomal SCAP degradation via SQSTM1. We observed that A₁R activation resulted in increased binding of SCAP with SQSTM1 or promoted the formation of SCAP-PKAc complex upon A₁R inhibition, as shown by both co-IP and PLA tests. These results implicated an interestingly competitive binding of SCAP with SQSTM1 rather than PKAc upon A₁R activation, facilitating SCAP degradation in lysosome and then reducing maturation of SREBPs. This is a new mechanism underlying the process of A₁R controlling the PKAc-SCAP-SREBPs pathway.

Finally, in agreement with the protection of hepatic A₁R against MAFLD development based on liver-specific knockout or overexpression of A₁R mice, pharmacological activation of A₁R by specific agonist CCPA, a lead compound for improving lung function, ischemia reperfusion, neuro-protection, and anticonvulsant activity,^{74–77} also effectively attenuated diet-induced MAFL and MASH in mice. Moreover, the potential of A₁R as an anti-MAFLD target was validated by the screened natural compound TA3 based on its potent ability for A₁R activation. TA3 is a well-established lead compound with multiple-pharmacological activities, including

anti-cancer, anti-neuronal disorders, anti-inflammation, and anti-coagulant,⁷⁸ while our results demonstrated its anti-MAFLD effect by activating A₁R. Notably, both CCPA and TA3 were effective in treating diet-induced MAFL and MASH in WT mice, but not in LKO mice, implicating that hepatic A₁R is the target of pharmacological agonists for MAFLD therapy instead of other tissues.

In summary, our current study reveals a previously undetermined role of hepatic A₁R as anti-MAFLD drug target and elucidates the underlying mechanism by controlling the PKAc-SCAP-SREBPs pathway. The increased expression of hepatic A₁R in MAFLD patients might be an adaptive response to combating MAFLD development, but it is not sufficient. Pharmacological activation of hepatic A₁R is practical for MAFLD treatment, in particular MASH.

Limitations of the study

In the current study, we demonstrated that genetic or pharmacological activation of hepatic A₁R inhibited MAFL/MASH development. The underlying mechanism was that activated A₁R promoted the competitive binding of SCAP with SQSTM1 rather than PKAc, leading to SCAP degradation in lysosome, which reduced SREBP maturation and its regulated DNL and inflammation. It should be noted that the main limitations of the study were summarized follows.

First, although our results demonstrated the control by A₁R on SCAP degradation through PKAc, the mechanistic insight into how A₁R regulates the competitive binding between PKAc and SQSTM1 with SCAP remains unclear; for example, the key binding site of SCAP with PKAc or SQSTM1 needs to be fully characterized.

Second, A₁R agonists of CCPA and TA3 were effective in treating MAFL/MASH; however, CCPA inhibited locomotor activity in mice but TA3 did not. Because both CCPA and TA3 could be detected in brain, we postulated that these two agonists might activate the A₁R in different ways, such as different binding structural domains of A₁R. Currently, the binding characteristics of these two agonists on A₁R were under in-depth exploration in cooperation with experts in the structural biology group. We believe the elucidation of the binding characteristics by agonists of CCPA or TA3 would accelerate the research and development of anti-MASH drugs by targeting activation of A₁R with more safety and fewer side effect in inhibition of central nervous system.

Third, although our results demonstrated that liver-specific deletion A₁R exacerbated the severity of MAFL/MASH, which was relieved by its liver-specific overexpression in vivo and in vitro, we could not completely rule out whether the observed effects might be partly attributed to the endogenous adenosine action on other subtypes of ARs, which are also implicated in metabolic diseases such as MAFLD.⁵⁸

Fourth, the underlying mechanism of the elevated expression of hepatic A₁R both in MAFLD/MASH patients and mouse model was not addressed in our current report. Further investigation is warranted to uncover the exact mechanism that regulates the expression of hepatic A₁R during the development of MAFLD/MASH.

Finally, Stagg et al. recently demonstrated that A_{2A}R protected against MASH-HCC development,¹² so it would be of interest to

explore whether A₁R is also involved in the development of MASH-HCC in the future. Since TA3 has been reported to have anti-tumor effects on various tumor cells, including HCC,⁷⁹ we speculated that hepatic A₁R may be involved in the development of MASH-HCC. It should be noted that the presence and correlation of hepatic A₁R with pathological stages of MASH was only evaluated on nine liver specimens from MASH patients with liver biopsy in our current study. Further study is urgently needed by including a clinical cohort with large-scale MASH patients, which is of vital significance to translate current findings to the clinic.

STAR+METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xcrm.2024.101477>.

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AUTHOR CONTRIBUTIONS

W.Z. and Y.H. performed all experiments, data analysis, and figure arrangement and took part in manuscript writing. Z.T., X.Z., X.W., and Z.T. were responsible for clinical sample and patient information collection. P.S. was responsible for pathological diagnosis of MAFLD patients. X.H., Y.L., H.W., X.G., W.H., Z.L., Y.B., J.M., and N.Z. were involved in most of the animal experiments and data analysis. C.X. guided the mechanistic study and helped in the manuscript writing. X.K. guided the PLA experiment and data analysis. W.Z. assisted in screening and identification of TA3 compound from dozens of candidates. W.J. helped in the study design. J.Z. coordinated the clinical study of the project, including sample and patient information collection and A₁R analysis of clinical samples. L.S. took part in the study design, data analysis, and manuscript writing. M.L. participated in manuscript revision and data analysis. H.L. organized the studies, supervised the project, and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR+METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
A ₁ R antibody	Abcam, UK	ab151523
A ₁ R antibody	Abcam, UK	ab82477, RRID: AB_2049141
SREBP1c antibody	Abcam, UK	ab28481, RRID: AB_778069
SREBP2 antibody	Abclonal, China	A13049, RRID, AB_2759897
FASN antibody	Cell signaling, USA	#3180, RRID: AB_2100796
ACC antibody	Cell signaling, USA	#3676, RRID: AB_2219397
CPT1 antibody	Proteintech, USA	ag7202
CD36 antibody	Abcam, UK	ab133625, RRID, AB_2716564
F4/80 antibody	Servicebio, China	GB113373, RRID, AB_2938980
CD11B antibody	Boster, China	BM3925, RRID: AB_2832991
TGF- β antibody	Abclonal, China	A2124, RRID: AB_2764143
Smad2 antibody	Affinity, China	AF6449, RRID: AB_2835272
pSmad2 antibody	Affinity, China	AF3449, RRID: AB_2834844
PKAc antibody	Cell signaling, USA	#4782, RRID: AB_2170170
SCAP antibody	Cell signaling, USA	#13102, RRID: AB_2798121
SCAP antibody	Abmart, China	TD13713
SCAP antibody	SantaCruz, USA	SC-13553, RRID: AB_628237
S2P antibody	Abcam, UK	ab140594
S2P antibody	Affinity, USA	DF13911
S1P antibody	Abcam, UK	ab140592, RRID: AB_2811283
Insig1 antibody	Abcam, UK	ab70784, RRID: AB_1269181
SQSTM1 antibody	Abcam, UK	ab109012, RRID: AB_2810880
NF- κ B p65 antibody	Proteintech, USA	66535-1, RRID: AB_2881898
Phospho-NF- κ B p65 antibody	Cell signaling, USA	#3033s, RRID: AB_331284
α -SMA antibody	Cell signaling, USA	#19245, RRID: AB_2734835
I κ B α antibody	Cell signaling, USA	#4814s, RRID: AB_390781
LAMIN B1 antibody	Cell signaling, USA	#13435, RRID: AB_2737428
β -TUBULIN antibody	Cell signaling, USA	#2146, RRID: AB_2210545
GAPDH antibody	Proteintech, USA	60004-1, RRID: AB_2107436
ATP1A1 antibody	PTM Bio, China	PTM-6271
Bacterial and virus strains		
AAV8-TBG-A ₁ R	This paper	N/A
AAV8-TBG-GFP	This paper	N/A
Biological samples		
Human liver paraffin blocks	This paper	N/A
Mouse liver paraffin blocks	This paper	N/A
Chemicals, peptides, and recombinant proteins		
CCPA	Aladdin, China	C170001
DPCPX	Sigma, USA	C101
CPA	Sigma, USA	C8301
H89	Selleck, China	S1582
DbcAMP	Selleck, China	S7858
Cycloheximide	Selleck, China	S7418

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
MG132	MCE, USA	HY-13259
Bafilomycin A1	MCE, USA	HY-100558
Timosaponin AIII	MCE, USA	HY-N0810
PMSF	Beyotime, China	ST506

Critical commercial assays

IL-1b ELISA Kit	Mlbio, China	ml301814
IL-6 ELISA Kit	Mlbio, China	ml063159
TNFa ELISA Kit	Mlbio, China	ml002095
Insulin ELISA Kit	Mlbio, China	ml001983V
PKA kinase activity kit	Enzo Life Sciences, USA	ADI-EKS-390A
cAMP Gi Kit	Cisbio, France	62a.m.9PEB
Hoechst	Beyotime, China	P0133
LysoTracker red	Beyotime, China	C1046
Golgi Staining Kit	Abcam, UK	ab139483
Mouse genotyping kit	Vazyme, China	PD101-01
FastKing gDNA Dispelling RT SuperMix	TIANGEN, China	KR118
qPCR SYBR Green Master Mix	Vazyme, China	Q131
RIPA Lysis Buffer	Beyotime, China	P0013B
Nuclear and Cytoplasmic Protein Extraction Kit	Beyotime, China	P0028
Triglycerides Assay Kit	Njcbio, China	A110-1-1
Total cholesterol assay kit	Njcbio, China	A111-1-1
Alanine aminotransferase Assay Kit	Njcbio, China	C009-2-1
Aspartate aminotransferase Assay Kit	Njcbio, China	C010-2-1
Alkaline phosphatase assay kit	Njcbio, China	A059-2-2
Low-density lipoprotein cholesterol assay kit	Njcbio, China	A113-1-1
High-density lipoprotein cholesterol assay kit	Njcbio, China	A112-1-1
Nonesterified Free fatty acids assay kit	Njcbio, China	A042-2-1
Oil Red O Stain Kit	Njcbio, China	D027-1-2
Duolink In Situ Wash Buffers, Fluorescence	Sigma, USA	DUO82049
Duolink In Situ Detection Reagents Red	Sigma, USA	DUO92008
Duolink In Situ PLA Probe Anti-Rabbit PLUS	Sigma, USA	DUO92002
Duolink In Situ PLA Probe Anti-Mouse MINUS	Sigma, USA	DUO92004
Immunoprecipitation Kit	Invitrogen, USA	10007D
Membrane and Cytosol Protein Extraction Kit	Beyotime, China	P0033
EZ-press RNA purification kit	EZBio, USA	B0004DP
DMEM	Meilunbio, China	MA0212
DMEM/F-12	Meilunbio, China	MA0214

Deposited data

Human NAFLD expression profiling data	GEO portal	GSE89632
Human NAFLD transcriptomic data	GEO portal	GSE135251

Experimental models: Cell lines

AML12 cells	ATCC	CRL-2254
HepG2 cells	ATCC	HB-8065

Experimental models: Organisms/strains

C57BL/6 Adora1 ^{liver-/-} mice	Shanghai Model Organisms Center Inc.	N/A
Hepatic ADORA1-OE C57BL/6 mice	This paper	N/A
C57BL/6 mice	Shanghai SLAC Laboratory Animal Co., Ltd	N/A

(Continued on next page)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Continued		
Oligonucleotides		
Primes for mouse see Table S3	This paper	N/A
Software and algorithms		
Prism, version 10	GraphPad	https://www.graphpad.com/
LAS X, version 3.5.7	Leica	https://www.leica.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Houkai Li (hk_li@shutcm.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the [key resources table](#). All data reported in this paper will be shared by the [lead contact](#) upon reasonable request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human samples and approval

The patients' paraffin sections for A₁R IHC staining were collected from Huzhou Central Hospital (Zhejiang, China). The study protocol was approved by Medical Ethics Committee of Huzhou Central Hospital (No.20190401-02). The human liver samples for A₁R immunofluorescence that patients with biopsy-proven metabolic (dysfunction)-associate steatohepatitis (MASH) were recruited from Huzhou Central Hospital. This study procedures were approved by Medical Ethics Committee of Huzhou Central Hospital (No.20201201-02). All tissue samples were collected in compliance with informed consent policy. Clinical information is summarized in [Tables S1](#) and [S2](#). Each specimen was evaluated by experienced pathologists without knowledge of the clinical findings. The NAS consists of liver steatosis (scale of 0–3), lobular inflammation (scale of 0–3), hepatocellular ballooning (scale of 0–2).

Mice study and approval

Male C57BL/6J mice were housed under a 12:12-h light/dark cycle at controlled temperature conditions in specific-pathogen-free (SPF) condition. All animals were purchased from the Shanghai Model Organisms Center Inc, Shanghai SLAC Laboratory Animal Co., Ltd, and bred at the experimental animal center, Shanghai University of Traditional Chinese Medicine. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine. All experiments were conducted in strict accordance with the Guidelines for the Investigation of Pain in Conscious Animal Experiments in order to minimize animal suffering and improve animal welfare (Ethical Approval Number: PZSHUTCM220221003, PZSHUTCM201030006, PZSHUTCM190426007, PZSHUTCM2304190012, PZSHUTCM2304190013).

HFD/CD-HFD diet feeding, AAV8 injection, and pharmacological treatment

Diet intervention started at 6-week-old. For diet induced MAFLD, mice were fed with high fat diet (HFD, 60% kcal from fat; D12492; Research Diets, New Brunswick, NJ, USA) for 12 or 16 weeks. To establish the NASH model, mice were fed with CD-HFD diet (A06071302, Research Diets, New Brunswick, NJ, USA) for 9 weeks or AMLN diet (Research Diet, Cat. D09100301, consisting of 40% fat, 20% fructose and 2% cholesterol) for 28 weeks.³⁷

Hepatic A₁R overexpression mice (A₁R^{Liver OE}) were conducted via AAV8 mediated A₁R overexpression purchased from Shandong Vigene, vehicle-injected mice (pAV-TBG-P2A-GFP, Vehicle) were used as controls.

For PKA inhibition *in vivo*, PKA inhibitor H89 (1 mg/kg body weight, in 0.9% NaCl solution) was intraperitoneally injected into Flox and LKO mice daily with HFD feeding.⁴¹ For CCPA treatment *in vivo*, CCPA (1 mg/kg body weight, in 0.9% NaCl solution) was injected into mice daily from 9th week of the 16-week HFD feeding, or from 4th week of the 9-week CD-HFD feeding, or from 13th week of the 28-week AMLN diet feeding. For adenosine treatment *in vivo*, adenosine (10 mg/kg, ip) or CCPA (1 mg/kg, ip) were treated from 13th week to 18th. For timosaponin AIII (TA3) treatment *in vivo*, TA3 (5, 10 mg/kg body weight, in 0.9% NaCl solution) was injected into mice daily from 4th week of the 9-week CD-HFD feeding.

All mice were fasted 12 h before the collection of blood and tissues. Serum was distributed into several centrifuge tubes for further analysis after being centrifuged at 4000 rpm for 15 min at 4°C. Mice tissue samples were used instantly or snap-frozen in liquid nitrogen and stored at –80°C after collection and being weighted.

METHOD DETAILS

Generation of genetically modified mice

Hepatic A₁R knockout mice (A₁R^{liver -/-}, LKO) was generated using the CRISPR/Cas9 system and Cre-loxP-mediated recombination technology. First, two single guide RNAs (sgRNA1 and sgRNA2) were used to target a fragment of A₁R exon3. Cas9 mRNA and gRNA were obtained by in vitro transcription. The donor vector was constructed by In-Fusion cloning, which contained a 3.0kb 5' homology arm, a 1.0kb flox region and a 3.0kb 3' homology arm. Microinjection of Cas9 mRNA, gRNA, and donor vector into zygotes of C57BL/6J mice. We selected one founder mouse and crossed it with a C57BL/6J mouse to generate A₁R-Flox mice. Finally, the A₁R LKO mice were generated by crossing albumin-Cre recombinase (Alb-Cre) transgenic mice with A₁R^{flox/flox} mice. Littermates Alb-Cre negative, A₁R^{flox/flox} mice (Flox) were used as controls. Mice were housed in barrier facility with free access to drinking water and fed standard chow diet.

To generate hepatic A₁R over expression mice (A₁R^{liver OE}), AAV8-TBG-A₁R adenoviruses were injected intravenously (mousetail) at 10¹² vg per mice, single injection. The gene ID number is 11539, and transcript is NM_001008533.3 from *Mus musculus*. And control mice were injected with AAV8-TBG-GFP adenoviruses (Vehicle).

Cell lines

HepG2 cells were purchased from the Chinese Academy of Science (Shanghai, China), and were cultured in DMEM medium (Gibco, USA) supplemented with 10% FBS (BI, Israel), 100 U/ml penicillin and 100 mg/mL streptomycin (Meilunbio, China). AML-12 cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% FBS (BI, Israel), 100 U/ml penicillin and 100 mg/mL streptomycin (Gibco, USA), 1% Insulin-Transferrin-Selenium-Sodium pyruvate (ITS-A) (Gibco, USA), 40 ng/mg dexamethasone (Sigma, USA).

For the stable A₁R over expression cell lines, AAV8-A₁R was purchased from Vigene Biosciences (Shandong). A₁R was over expressed in AML-12 cell line using pAV-TBG-P2A-GFP, packaged in Adeno-associated virus. AAV infection was carried out in 6-well plates by mixing 10 mL virus supernatant plus 10 mL complete medium with a final concentration of 10¹¹ vg/ml. Then, the selection via fluorescence-activated cell sorter was performed 48 h after infection, and then infected cells were further grown for about 2 weeks in the medium. Identification of over expression was carried out by RT-qPCR and western blotting.

Mice primary hepatocytes were isolated from 8 to 12-week-old Flox or LKO mice by using collagenase perfusion and gradient centrifugation, as previously described.⁸⁰ Briefly, the mice liver was perfused with reperfusion buffer (135 mmol/L NaCl, 5.3 mmol/L KCl, 28.2 mmol/L NaHCO₃, 0.12 mmol/L Na₂HPO₄, 0.56 mmol/L NaH₂PO₄, 0.5 mmol/L EGTA, 9.0 g/L glucose, and 0.06 g/L Phenol red, PH 7.4) and enzyme buffer (135 mmol/L NaCl, 5.3 mmol/L KCl, 28.2 mmol/L NaHCO₃, 0.12 mmol/L Na₂HPO₄, 0.56 mmol/L NaH₂PO₄, 0.5 mmol/L EGTA, 10 mmol/L HEPES, and 0.06 g/L phenol red, 200 mg/L collagenase D and 3.8 mmol/L CaCl₂, PH7.4). The isolated primary hepatocytes were cultured in DMEM medium (Gibco, USA) supplemented 10% FBS (BI, Israel). Cells were grown at 37°C in a 5% CO₂ and 95% air atmosphere.

In vitro, cells were treated or co-treated with DPCPX (1 mM) for 48 h, CPA (1 mM) for 48 h, H89 (20 mM) for 4 h, DbcAMP (200 mM) for 12 h, and TA3 (0.5–10 mM) for 48 h, respectively. For mechanistic insight, cells were treated with cycloheximide (CHX, 50 mM) for 0, 2, 4, 8 h, bafilomycin A1 (Baf.A1, 1 mM) for 8 h, or MG132 (10 mM) for 8 h, in the presence of drugs.⁴⁸

Intraperitoneal glucose tolerance test (ipGTT) and insulin tolerance test (ipITT)

For ipGTT, mice were fasted for 15 h overnight with free access to water, and then were injected intraperitoneally with glucose solution (1 g/kg body weight).⁸¹ Fasting glucose levels were first measured as time 0, then blood glucose was examined at 15th, 30th, 60th, 90th, and 120th min after glucose injection. ipITT was performed by intraperitoneal injection with human insulin (0.75 units/kg body weight, Novo Nordisk) without fasting.⁸²

Serum insulin level test and HOMA-IR

Homeostatic model assessment of insulin resistance (HOMA-IR) was employed to measure the fasting insulin levels in serum samples using a mouse Insulin ELISA kit. The following equation was used to determine the homeostasis model assessment of insulin resistance (HOMA-IR): HOMA-IR=(fasting glucose 3 fasting insulin)/22.5.⁸³

Serum biochemistry index test

Serum cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were tested according to the instruction manual. List of reagents (including catalog numbers) can be found in "Critical Commercial Assays" list.

Inflammatory cytokines test

For liver samples, the tissue was rinsed with pre-cold PBS to remove residual blood. Twenty mg of liver tissue was added to 200 mL PBS containing protease inhibitor PMSF, and further ground by tissue grinding instrument at 4°C. The homogenate was centrifuged at 5000 g for 10 min and the supernatant was collected for assay. The concentrations of proinflammatory factors (TNF α , IL-6, and IL-1 β) from serum or liver samples were measured using ELISA according to the manufacturer's instructions.

Liver triglycerides examination

For the hepatic lipid extraction, each 12 mg of liver tissue was added to 500 mL extraction solvent mixed with chloroform: methanol (v: v = 2:1), homogenized by tissue grinding instrument, then fully shaken for 1 min, centrifuged at 4000 rpm at 4°C for 10 min, 10 mL supernatant were transferred into a clean centrifugal tube and dried at room temperature. Then, 10 mL pure water was added to the precipitates for redissolution. After adding with 200 mL working solution supplied by the TG test kit, the reaction mixture was incubated at 37°C for 10 min. After briefly vortexing, the absorbance at 510 nm was measured in 96-well plate. The concentration of TG was calculated according to the standard curve, and the results were presented as mmol/g liver weight.⁸⁴

Tissues staining

Tissues were fixed with 10% formalin for over 24 h, embedded in paraffin, and then stained with hematoxylin-eosin staining (H&E) and Sirius Red using a standard protocol. Hematoxylin staining was used for nuclear counterstaining (blue), and eosin was used to stain the cytoplasm red. Sirius Red was used for collagen fibers staining (red). For Oil red O staining, fresh large lobes of the liver were embedded in OCT and flash-frozen in liquid nitrogen, then stored at -80°C. The embedded tissues were sectioned into 8 mm pieces after equilibrated at -20°C, and then stained with Oil red O to determine the extent of hepatic steatosis.

Histopathological evaluation

NAFLD activity score (NAS, 0 to 8) including separate scores for steatosis (0–3), lobular inflammation (0–3) and hepatocellular ballooning scale (0–2). The criteria for hepatic steatosis scoring include grade 0, steatosis involved <5%; grade 1, steatosis involved between 5 and 33%; grade 2, steatosis involved between 34 and 66%; and grade 3, steatosis involved >66%. Hepatic lobular inflammation was grade based on overall assessment of all inflammatory foci per 200 field, including grade 0, no foci; grade 1, <2 foci per 200 field; grade 2, 2–4 foci per 200 field; grade 3, >4 foci per 200 field. Ballooning degeneration was scored by the severity based on numbers of hepatocytes: 0 points assigned as none, 1 point as mild, 2 points as marked.⁸⁵

Liver F4/80 and CD11b immunofluorescence staining

Tissue paraffin sections were permeabilized in PBS supplemented with 0.4% Triton X-100 (Sigma-Aldrich) and 3% BSA (Sigma-Aldrich) for 1 h and preincubated in blocking buffer (PBS supplemented with 3% BSA) for 1 h. Tissues were then labeled with anti-F4/80 or anti-CD11b primary antibody and the corresponding secondary antibody for 2 h at room temperature. Then, immunofluorescence imaging was performed.

Quantification of ¹³C incorporated fatty acids

Flox and LKO mice (n = 6) were fed an AMLN diet for 7 days, fasted from 9 a.m. to 7 p.m., refed for 2 h, and force-fed ¹³C-fructose. The mice were fed overnight and killed the next morning. LC-MS was performed to examine the amount of ¹³C label incorporation into hepatic fatty acids.

Oil red O staining for cells

Cells were gently washed 2 times with PBS. 10% formalin was added to each well and incubated for 30 min. Then formalin was removed, and cells were gently washed 2 times with ddH₂O. Each well was added 60% isopropanol and incubated for 5 min. After removing isopropanol, Oil Red O working solution was added to cover the cells completely and evenly and incubated for 10–20 min, then cells were washed 2–5 times with ddH₂O as needed. Hematoxylin was added to the cells and incubated for 1 min, and washed 2–5 times as needed. Cells was viewed under microscope. Lipid droplets appear red and nucleal appear blue.⁸⁶

Immunohistochemical staining and quantification

After deparaffinization and dehydration, antigen retrieval was performed using a microwave for 5 min, before peroxidase quenching with 3% H₂O₂ in phosphate-buffered saline (PBS) was done for 15 min. Subsequently, the sections were blocked with 5% bovine serum albumin (BSA) for 30 min and then incubated with a primary antibody at a dilution ratio of 1:500 in PBS overnight at 4°C. The negative control was treated in the same way, as were the sample groups, except that primary antibody were omitted in the incubation steps. The percentage of the area of the section occupied by positive staining was determined using ImageJ 1.53 software.

Immunofluorescence

For multiple immunofluorescence, Firstly, after washing twice with PBS, the cells were fixed in 4% paraformaldehyde in PBS for 15 min and then washed three times in PBS with Tween 20 (PBST). After removing supernatant, the PBS solution containing 0.5% Triton X-100 was used for permeation for 15–20 min. The permeation solution was discarded, PBS solution containing 5%

donkey serum was added to seal the cells at room temperature for 1 h. After removing the blocking solution, a primary antibody diluted with 5% BSA and 0.1% Triton X-100 PBS solution (1:150 dilution) was added and incubated overnight at 4°C. The next day, cells were washed 3 times with PBST. Fluorescent secondary antibody was added (diluted with PBS solution containing 5% BSA, 1:200), and incubated for 1 h at room temperature in dark. After washing with PBST, the sealing solution containing Hoechst dye was added under dark conditions and stored at 4°C. Images were detected and captured by Fluorescent Confocal Microscopy (Leica, TCS SP-8, Germany). To generate three-dimensional images, images were recorded as vertical z stacks and processed using Leica LAS X 3.5.7 software.

Co-immunoprecipitation (Co-IP)

AML-12 cells were lysed with IP lysis buffer (P0013, Beyotime, China) containing an inhibitor cocktail. After centrifugation, the supernatant containing proteins was subjected to IP with protein G agarose beads (10007D, invitrogen, USA) and the indicated antibodies overnight at 4°C. The beads were washed with NaCl buffer and boiled with 23SDS loading buffer prior to analysis by Western blotting.

Proximity ligation assay (PLA)

AML-12 cells were seeded on coverslips and allowed to attach overnight. After fixation in 4% paraformaldehyde, PLA was performed using Duolink In Situ Proximity Ligation Assay reagent (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, blocking was performed with blocking solution for 30 min at 37°C, and primary antibodies: anti-SCAP(1:100 dilution), anti-SQSTM1(1:100 dilution), and anti-PKAc(1:100 dilution) were incubated overnight at 4°C. The PLA Probe anti-mouse MINUS and PLA Probe anti-rabbit PLUS were incubated for 1 h at 37°C. Ligation and amplification were performed using Detection Reagents Red. Duolink Mounting Medium with DAPI was used for nuclear staining and mounting. The average PLA signal intensity of each cell was calculated by ImageJ software.⁸⁷ List of reagents (including catalog numbers) can be found in "Critical Commercial Assays" list. List of antibodies can be found in "Antibodies" list.

Natural compound database screening

Natural compounds screening with activating capacity on A₁R activity was performed based on a commercial Natural Product Library Plus (website: <https://www.medchemexpress.cn/screening/natural-product-library-plus.html>) by using computer-aid molecular docking. The Virtual Screening Workflow module was used to perform virtual screening by importing the prepared compounds and using the Glide module to perform molecular docking, i.e., docking between receptor and ligand molecules by geometric and energy matching. The 3600 prepared small molecules in the Natural Product Library Plus were first screened using the High Throughput Screening (HTVS) mode in the Glide module, and the top 20% of the scored small molecules were selected for a second round of screening using the Standard (SP) mode; then the top 20% of the scored compounds were selected for a third round of screening using the High Precision (XP) mode to obtain the ranking of the small molecules. The top 200 compounds screened from Natural Product Library Plus, was further analyzed using cAMP test *in vitro*.

Protein isolation and western blot analyses

Tissue/Cells were lysed in RIPA Lysis Buffer (Beyotime, China) supplemented with PMSF (Beyotime, China) and protease inhibitors and phosphatase inhibitors (Sigma, USA). Nuclear or membrane extracts were prepared with a nuclear and cytoplasmic protein extraction kit (Beyotime, China) according to the manufacturer's instructions. Protein concentrations were measured with the BCA reagent (YEASEN, China) by using a Microplate Reader Spark System (TECAN, Switzerland). Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with different antibodies as described previously. The immunoblots were detected using an Image 600 ECL System (GE, USA).

Extraction of total RNA and real-time quantitative PCR

Total RNA was isolated using an RNA purification kit (EZBioscience, USA), and then used to synthesize cDNA with the FastKing gDNA Dispelling RT SuperMix (TIANGEN, China). Real-time qPCR was performed using SYBR Green (Vazyme, China), 96-well plates and the CFX connect Real-Time System. Each well was loaded with a total of 20 μL containing 2 μL of cDNA, 0.5 μL of target primers, 7.5 μL of water and 10 μL of SYBR Select Master Mix. Real-time qPCR was performed for 40 cycles, with each cycle consisting of denaturation for 15 s at 94°C, annealing for 30 s at 60°C and elongation for 30 s at 72°C. Relative quantification was done using the 2^{-DDCT} method. Expression was normalized against 18s. Mean expression levels of chow-fed mice were set as 100%. The primers used are shown below.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data representation and statistical analysis

Data are presented as mean ± SEM (Standard Error of Mean). p values were obtained using unpaired Student's t test (between two groups) or One-way ANOVA analysis (during over two groups) for continuous variables via prism 9 (GraphPad Software Inc., La Jolla, CA). Significant differences among groups are indicated as *p < 0.05, **p < 0.01.