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RAP1A suppresses hepatic steatosis by regulating amino acid-mediated mTORC1 activation

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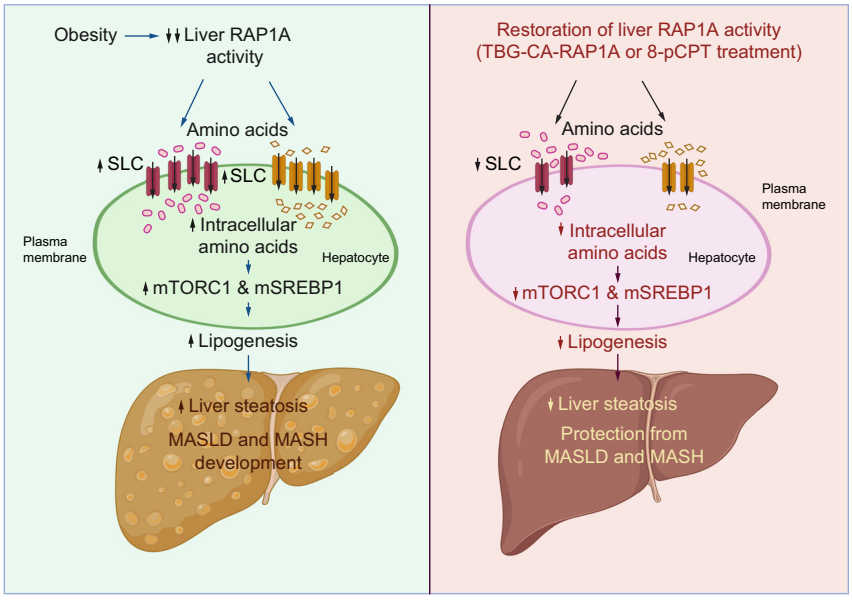
Graphical abstract



Highlights:

- Liver RAP1A activity is suppressed in obese mice with MASLD and activation of RAP1A lowers steatosis.
- RAP1A inhibits lipogenesis and SREBP1 cleavage by suppressing amino acid-mediated mTORC1 activity.
- RAP1A activation is also suppressed in humans with MASH and mice fed with a MASH-provoking diet.
- Restoring RAP1A activity lowers steatosis and fibrosis in experimental MASH.

Impact and implications:

Metabolic dysfunction-associated liver pathologies are inadequately treated with currently available therapy. Here we demonstrate that the small GTPase RAS-associated protein 1A (RAP1A) protects against liver steatosis and fibrosis development by decreasing hepatocyte amino acid levels, which results in lower mTORC1 activity and SREBP1 cleavage. The results may present new targets against metabolic dysfunction related liver diseases.

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RAP1A suppresses hepatic steatosis by regulating amino acid-mediated mTORC1 activation

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Background & Aims: Metabolic dysfunction-associated steatotic liver disease (MASLD) is characterized by triglyceride (TG) build-up in hepatocytes; however, our understanding of the underlying molecular mechanisms is limited. Here, we investigated the role of hepatic GTPase RAP1A in MASLD and its more progressive form, metabolic dysfunction-associated steatohepatitis (MASH).

Methods: RAP1A was silenced or activated by AAV8-TBG-mediated gene expression or treating mice with a small molecule RAP1 activator (n = 4–12 per group). Primary hepatocytes were used to further probe the newly elucidated pathway. Liver samples from patients with MASH and control livers were analyzed for active RAP1A levels (n = 4 per group).

Results: Activation of hepatic RAP1A is suppressed in obese mice with MASLD and restoring its activity decreases liver steatosis. RAP1A activation lowers hepatic TG accumulation through decreasing sterol regulatory element-binding protein 1 (SREBP1) cleavage by inhibiting the mechanistic target of rapamycin complex 1 (mTORC1). The mechanism linking RAP1A activation to suppression of mTORC1 involves the lowering of membrane-bound amino acid transporters, which leads to reduced hepatocyte amino acid uptake, decreased intracellular amino acid levels, and inhibition of amino acid-mediated mTORC1 activation. Furthermore, we observed that active-RAP1A levels were decreased in mice fed a MASH-provoking diet (98% lower, p <0.01) and liver extracts from patients with MASH (86% lower, p <0.05). Accordingly, restoration of RAP1A activity in mice liver lowered liver fibrotic gene expression and prevented fibrosis formation, whereas RAP1A silencing promoted the progression of MASH.

Conclusions: Activation of hepatic RAP1A lowers MASLD and MASH formation by suppressing amino acid-mediated mTORC1 activation and decreasing cleaved SREBP1. These data provide mechanistic insight into amino acid-mediated mTORC1 regulation and raise the possibility that hepatic RAP1A may serve as a mechanistic node linking obesity with MASLD and MASH.

Introduction

An increase in hepatic glucose production (HGP) is a key feature of type 2 diabetes (T2D) and an imbalance between glucagon and insulin action contributes to this process.[1] Insulin also activates the hepatic sterol regulatory element-binding protein 1 (SREBP1), which upregulates genes involved in *de novo* lipogenesis (DNL).[2] In insulin-resistant individuals, insulin fails to inhibit HGP because of defects in insulin receptor signaling; however, its ability to stimulate hepatic lipid synthesis and storage is unimpaired.[3] This selective insulin resistance results in the characteristic hyperglycemia and metabolic dysfunction-associated steatotic liver disease (MASLD) associated with T2D.[4] In this context, identification of molecular targets that uncouple the effects of insulin on hepatic lipogenesis from lowering HGP has tremendous potential to lead to novel and more specific antidiabetic drugs.

In a subgroup of people, MASLD progresses to a more severe form of liver disease referred to as metabolic dysfunction-associated steatohepatitis (MASH).[5] MASH is characterized by steatosis, hepatocyte injury, inflammation, and most importantly, fibrosis, which is the main determinant of cirrhosis and hepatocellular carcinoma (HCC).[6] Enhanced lipid flux and DNL-mediated increases in lipotoxic stress result in hepatocyte injury, which, in turn, initiates an immune and fibrogenic response driven primarily by Kupffer and hepatic stellate cells.[6] There are currently no FDA approved therapies for MASH. Therefore, investigation of molecular mechanisms involved in MASH pathogenesis could result in new therapeutic targets.

Ras-associated protein 1A (RAP1A) is a small GTPase that belongs to the RAS superfamily of proteins. RAP1A is active when it is GTP bound, which is mediated by guanine exchange factors, such as exchange factor activated by cAMP-2 (EPAC2).[7] GTPase activating proteins, including RAP1GAP stimulate the intrinsic GTPase activity of RAP1A, which results in GTP hydrolysis and inactivation of RAP1A. Although much of the previous work focused on the role of RAP1 in endothelial cell nitric oxide regulation[8] and cell adhesion,[9] recent data suggest that hepatic RAP1A is an emerging player in metabolic

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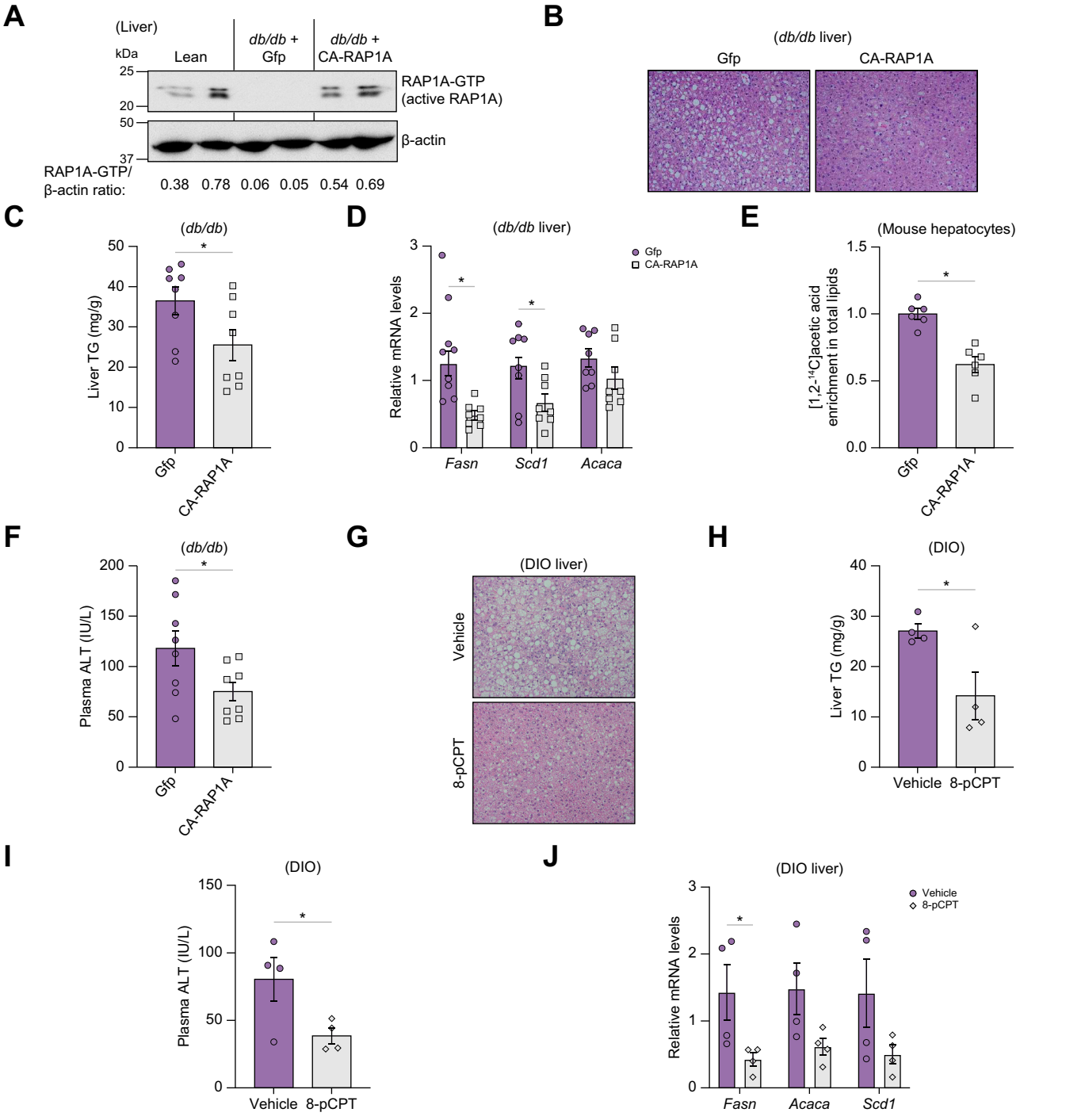

Fig. 1. Restoring hepatic RAP1A activity lowers steatosis, hepatic lipogenic gene expression, and lipogenesis in obese mice. (A) Hepatic active, GTP-RAP1A levels in lean, TBG-Gfp (Gfp) treated *db/db* (control), or TBG-CA-RAP1A (CA-RAP1A) treated *db/db* mice. Densitometric quantification of the data are shown below the immunoblots. (B–D) Representative images of liver sections from Gfp or CA-RAP1A treated *db/db* mice stained with H&E are shown (B), and TG content (C) and liver lipogenic gene expression were analyzed (D) (n = 8 mice/group; mean ± SEM, **p* <0.05 by Student's *t* test). (E) DNL assay using [1,2-14C]acetic acid was performed in primary hepatocytes isolated from Gfp or CA-RAP1A treated mice. Data are displayed as relative to Gfp (control) (n = 6/group; mean ± SEM, **p* <0.05 by Student's *t* test). (F) Plasma ALT levels of Gfp or CA-RAP1A treated *db/db* mice (n = 8 mice/group; mean ± SEM, **p* <0.05 by Student's *t* test). (G–J) DIO mice were treated with 8-pCPT for 2 weeks. Representative images of liver sections from vehicle or 8-pCPT-treated DIO mice stained with H&E are shown (G). (H–J) Hepatic TG content (H), plasma ALT levels (I), and liver lipogenic gene expression (J) were analyzed (n = 4 mice/group; mean ± SEM, **p* <0.05, by Student's *t* test or rank sum test). ALT, alanine aminotransferase; CA-RAP1A, constitutively active RAP1A; DIO, diet-induced obese; DNL, *de novo* lipogenesis; RAP1A, Ras-associated protein 1A; TBG, thyroxine-binding globulin; TG, triglyceride.

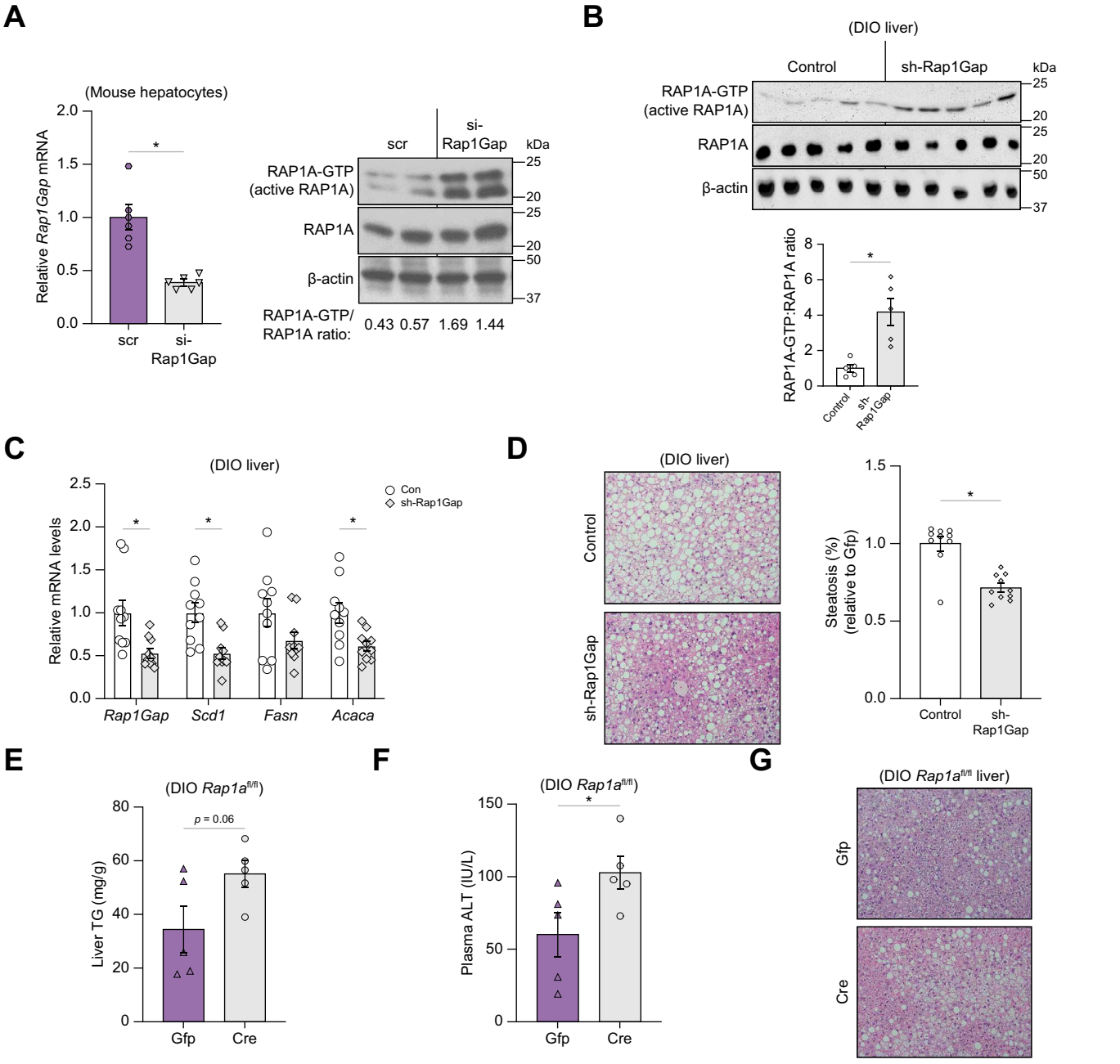

Fig. 2. Hepatic RAP1A activation via sh-Rap1Gap treatment lowers whereas RAP1A silencing induces steatosis in DIO mice. (**A**) *Rap1Gap* mRNA and active, GTP-RAP1A levels from primary mouse hepatocytes treated with scr *vs.* si-Rap1Gap were analyzed. Densitometric quantification of the data are shown below the immunoblots (n = 6; mean ± SEM, **p* <0.05 by rank sum test). (B–D) DIO mice were treated with an AAV-shRNA against RAP1GAP or control AAV. (B) Hepatic active, GTP-RAP1A levels were measured. Densitometric quantification of the immunoblot data are shown in the graphs (n = 5/group; mean ± SEM, **p* <0.05, by Student's *t* test). (C) Hepatic expression of *Rap1Gap* and lipogenic genes were analyzed (n = 10 mice/group; mean ± SEM, **p* <0.05, by Student's *t* test or rank sum test). (D) Representative images of liver sections stained with H&E. The percentage of steatosis is displayed as relative to Gfp (control) (D) (n = 10 mice/group; mean ± SEM, **p* <0.05 by rank sum test). (E–G) Liver TG content (E) and plasma ALT levels (F) were assayed in *Rap1a*$^{fl/fl}$ DIO mice treated with AAV8-TBG-Cre (Cre) or AAV8-TBG-Gfp (Gfp, control). Representative images of liver sections stained with H&E (G) (n = 5 mice/group; mean ± SEM, **p* <0.05, by Student's *t* test). AAV, adeno-associated virus; AAV8, adeno-associated virus 8; DIO, diet-induced obese; ALT, alanine aminotransferase; RAP1A, Ras-associated protein 1A; RAP1GAP, Ras-associated protein 1 GTPase activating protein; scr, scrambled; si-Rap1Gap, silencing RAP1GAP; TG, triglyceride.

control, including the regulation of proprotein convertase subtilisin/kexin type 9 (PCSK9) and low-density lipoprotein cholesterol (LDL-C) metabolism.[10] In addition, we recently reported that RAP1A activity is decreased in the livers of obese mice, which results in increased HGP and hyperglycemia via suppression of insulin-mediated AKT activation.[11,12] We also uncovered that the widely used cholesterol-lowering drugs, statins, inhibit RAP1A prenylation and activity in isolated hepatocytes and the human liver, which contributes to their hyperglycemic effects.[11] Here, we show that RAP1A has an uncommon dual metabolic role, and its inhibition also results in increased lipogenesis and hepatic triglyceride (TG)

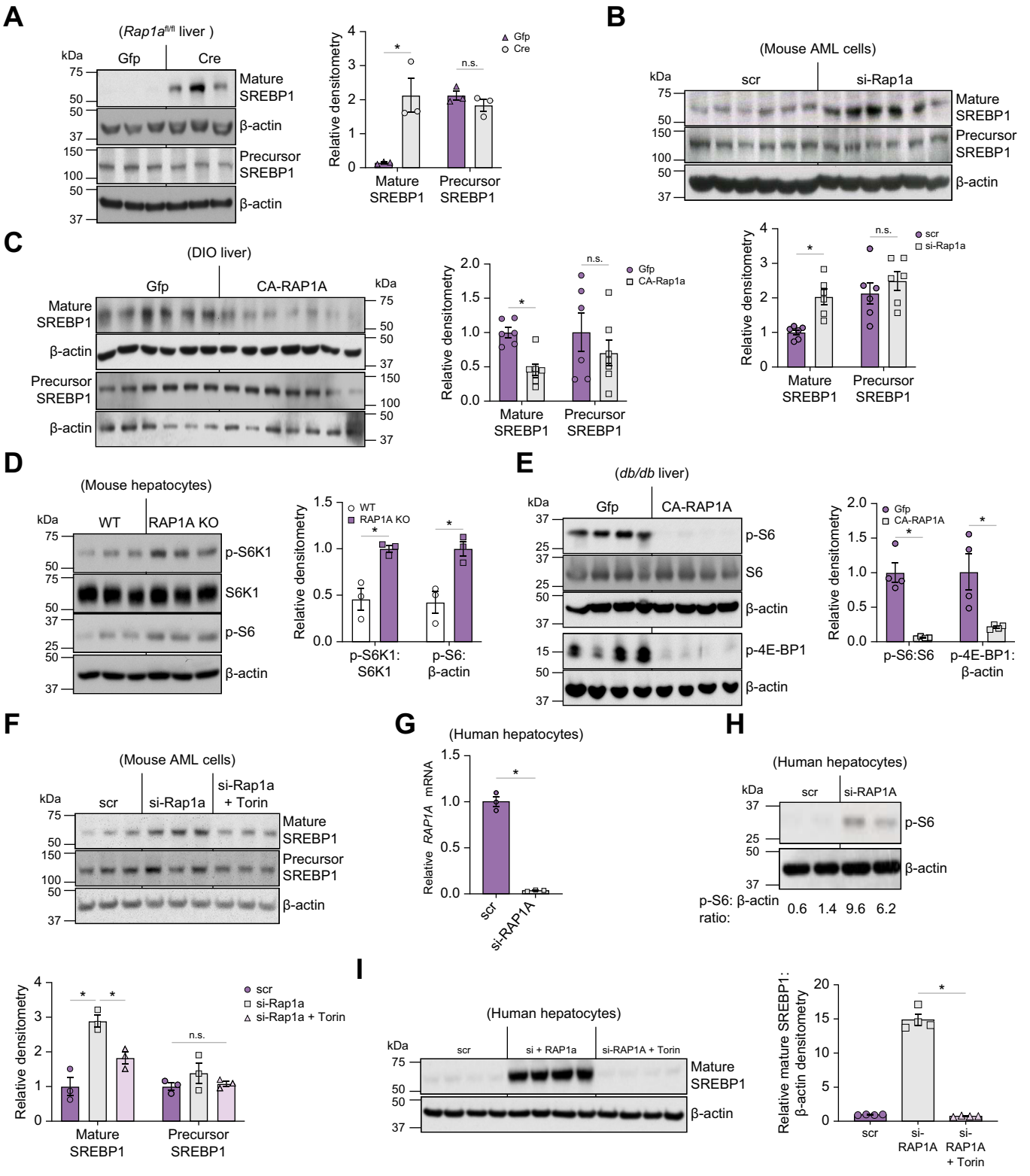

Fig. 3. Hepatic RAP1A regulates mature SREBP1 and mTORC1. (A–C) Hepatic mature and precursor SREBP1 protein levels were assayed in the livers of DIO *Rap1a*$^{fl/fl}$ mice treated with TBG-Cre (Cre) or TBG-Gfp (Gfp, control) (A), in scrambled (scr)- or si-Rap1a-treated mouse AML cells (B), or in mice treated with TBG-Gfp (Gfp) or TBG-CA-RAP1A (CA-RAP1A) (C). Densitometric quantification of the immunoblot data are shown in the graphs (n = 3–7/group; mean ± SEM, *p <0.05, n.s., not significant, by Student's t test). (D) p-S6K1 and p-S6 levels were analyzed in primary mouse hepatocytes isolated from *Rap1a*$^{fl/fl}$ mice treated with TBG-Gfp (WT) or TBG-Cre (RAP1A KO). Densitometric quantification of the immunoblot data are shown in the graphs (n = 3/group; mean ± SEM, *p <0.05, by Student's t test). (E) Gfp or CA-RAP1a-treated *db/db* mice liver were assayed for p-S6 and p-4E-BP1. Densitometric quantification of the immunoblot data are shown in the graphs (n = 3/group; mean ± SEM, *p <0.05, by Student's t test). (F) Same as in (B) except that some si-Rap1a-treated hepatocytes were also treated with the mTORC1 inhibitor, Torin. Densitometric quantification of the immunoblot data are shown in the graphs (n = 3/group; mean ± SEM, *p <0.05, n.s., not significant, by one-way ANOVA/Tukey's

accumulation, whereas RAP1A activation has the opposite effect. Mechanistically, we demonstrate that RAP1A contributes to lipogenesis homeostasis through mechanistic target of rapamycin complex 1 (mTORC1)-mediated SREBP1 regulation. By suppressing the transcription of membrane-bound amino acid transporters, RAP1A decreases amino acid uptake and reduces intracellular amino acid levels, resulting in the inhibition of amino acid-mediated mTORC1 activation. Furthermore, we report that RAP1A activity is decreased in a mouse model of MASH and in the livers of patients with MASH. Restoring hepatic RAP1A activity lowers hepatic steatosis, decreases plasma alanine aminotransferase (ALT) and mRNA levels of genes related to liver fibrosis, and reduces fibrosis. Conversely, hepatic RAP1A deficiency exacerbates steatosis and promotes liver fibrosis in MASH. These data demonstrate a key role for RAP1A in hepatic amino acid and TG homeostasis and raise the possibility of targeted RAP1A activation as a therapeutic strategy to treat MASLD and MASH.

Materials and methods

Detailed methods are provided in the Supplementary material.

Results

Restoring hepatic RAP1A activity lowers steatosis, hepatic lipogenic gene expression and lipogenesis whereas RAP1A inhibition increases them in obese mice

Our previous study revealed that hepatic RAP1A activity was decreased in obese mice liver and activation of RAP1A suppressed HGP.[11] As the rates of HGP and the extent of liver fat in patients with MASLD are closely related, we became interested in the role of RAP1A in hepatic steatosis. To gain insight into this regulation, we restored hepatic RAP1A activity in *db/db* mice by treating them with an adeno-associated virus 8 (AAV8), which expresses a constitutively active RAP1A mutant (CA-RAP1A) driven by the hepatocyte-specific thyroxine-binding globulin (TBG) promoter. CA-RAP1A possesses an amino acid substitution, Q63E, which results in constitutive GTP binding.[11] We observed that CA-RAP1A expressing *db/db* mice livers had fewer lipid droplets (Fig. 1A and B) and decreased TG content (Fig. 1C) without changes in body weight or plasma insulin levels.[11]

To determine the mechanism of protection from hepatic TG accumulation by RAP1A activation, we examined the lipid mobilization and metabolic pathways. We found that plasma TG levels were not increased, in fact they were decreased in CA-RAP1A treated obese mice (Fig. S1A). We then assessed very-low density lipoprotein (VLDL) secretion by injection of the detergent poloxamer 407 (P407); however, we observed that CA-RAP1A treatment did not increase hepatic VLDL secretion (Fig. S1B). We next measured fatty acid oxidation (FAO) and oxygen consumption rate (OCR) and found that RAP1A activation had no effect on FAO or OCR (Fig. S1C and D). Collectively, these data suggested that neither an increase in hepatic lipid output nor stimulation of lipid oxidation was responsible for the protection from steatosis induced by hepatic RAP1A activation. We next turned our attention to lipogenesis and found decreased expression of lipogenic genes, such as fatty acid synthase (*Fasn*) and stearoyl-CoA desaturase (*Scd1*) by CA-RAP1A treatment (Fig. 1D). To further assess the effect of RAP1A on lipogenesis, we performed a DNL assay using [1,2-14C]acetic acid metabolic labeling and observed that RAP1A activation by CA-RAP1A treatment significantly decreased the incorporation of [1,2-14C]acetic acid into lipids (Fig. 1E). Consistent with suppression of lipogenesis and protection from hepatic TG accumulation, CA-RAP1A-treated mice had lower plasma ALT levels, suggesting less hepatocellular injury (Fig. 1F).

We next asked whether activating RAP1A pharmacologically could have metabolic benefits and protect mice against hepatosteatosis. We used 8-pCPT-2′-O-Me-cAMP (8-pCPT), a cAMP analog, which activates RAP1A[13,14] and protects against ischemia–reperfusion injury-induced renal failure and asthmatic airway inflammation and lowers proatherogenic PCSK9 levels.[10,14–16] We previously reported that 8-pCPT treatment of diet-induced obese (DIO) mice for 2 weeks by daily injection at a dose of 1.5 mg/kg body weight robustly activated liver RAP1A.[14] Similar to RAP1A activation via CA-RAP1A, treatment of DIO mice with 8-pCPT lowered hepatic TG accumulation (Fig. 1G and H) without a change in body weight (42.3 ± 1.1 g *vs.* 41.4 ± 0.6 g). Moreover, we observed that 8-pCPT decreased plasma ALT levels and hepatic expression of lipogenic genes (Fig. 1I and J).

RAP1GAP functions as a negative endogenous regulator of RAP1A activity by facilitating GTP to GDP hydrolysis.[17] We then investigated whether silencing RAP1GAP (si-Rap1-Gap) could activate RAP1A and lower obesity-associated fatty liver formation. First, we showed that si-Rap1Gap treatment lowered *Rap1Gap* mRNA and increased RAP1A activity in primary hepatocytes (Fig. 2A). To show relevance *in vivo*, we acutely silenced liver RAP1GAP in DIO mice using AAV8-H1-sh-Rap1Gap, which specifically silences genes in hepatocytes.[18] Sh-Rap1Gap treatment increased liver RAP1A activity, lowered hepatic lipogenic gene expression and lipid droplet formation (Fig. 2B–D). Conversely, when RAP1A was deleted specifically in hepatocytes in DIO *Rap1a*$^{fl/fl}$ mice via injection with AAV-TBG-Cre (L-RAP1A knockout [KO]), the mice had increased hepatic TG and elevated plasma ALT levels compared with AAV-TBG-Gfp-injected control mice (Fig. 2E–G). Thus, hepatic RAP1A activity restoration in obese mice decreases liver TG accumulation and lipogenic gene expression and RAP1A deficiency results in the opposite effects.

Hepatic RAP1A regulates mature SREBP1 and mTORC1

Given that lipogenesis was altered upon RAP1A activation, we next determined the precursor (unprocessed) and mature (cleaved) protein levels of the master lipogenic transcription factor SREBP1. We found that L-RAP1A KO DIO mice had

post hoc analysis or ANOVA on ranks). (G) Metabolism-qualified plateable human hepatocytes were transfected with scr or si-RAP1A, and *RAP1A* mRNA levels were measured (n = 3/group; mean ± SEM, *p <0.05, by Student's t test). (H) Similar to (G) except that p-S6 levels were assayed. Densitometric quantification of the immunoblot data are shown below the immunoblots. (I) Metabolism-qualified plateable human hepatocytes transfected with scr or si-RAP1A were treated with or without Torin, and mature SREBP1 levels were assayed. Densitometric quantification of the immunoblot data are shown in the graphs (n = 4/group; mean ± SEM, *p <0.05, by ANOVA on ranks/Tukey's *post hoc* analysis). CA-RAP1A, constitutively active RAP1A; DIO, diet-induced obese; KO, knockout; mTORC1, mechanistic target of rapamycin complex 1; RAP1A, Ras-associated protein 1A; SREBP1, sterol regulatory element-binding transcription factor; WT, wildtype.

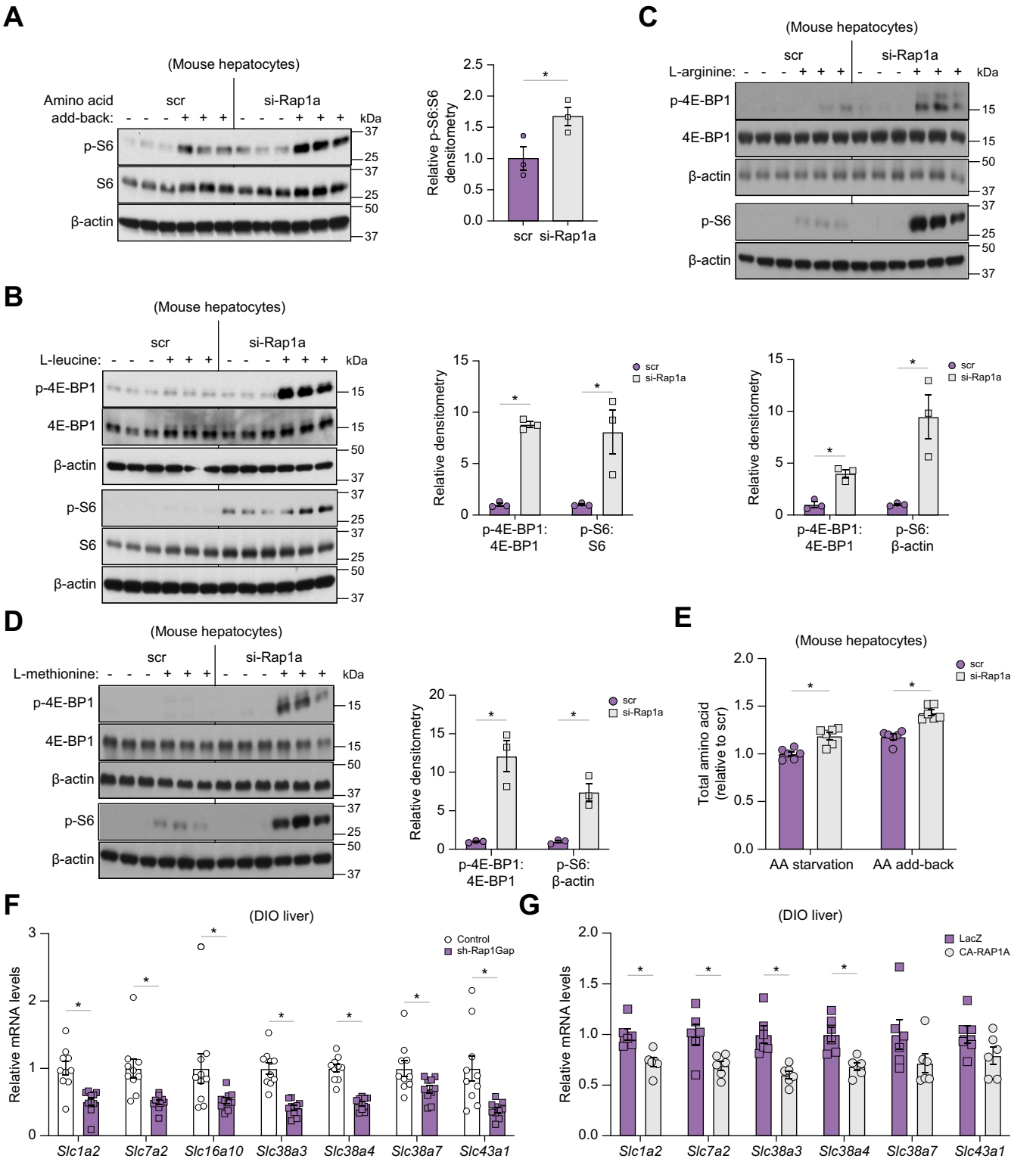

Fig. 4. RAP1A regulates amino acid-mediated mTORC1 activation. (A) Scrambled (scr) *vs.* si-Rap1a treated primary mouse hepatocytes were starved of all amino acids for 1 h and restimulated with the medium containing all the amino acids for 7.5 min. Lysates were analyzed by immunoblotting for p-S6 and S6. Densitometric quantification of the amino acid add-back immunoblot data are shown in the graph (n = 3/group; mean ± SEM, **p* <0.05, by Student's *t* test). (B) Primary mouse hepatocytes were starved of all amino acids for 1 h and restimulated with an equivalent concentration of L-leucine as the complete amino acid add-back treatment (as in A) for 30 min. Lysates were analyzed by immunoblotting for p-S6, S6, p-4E-BP1, and 4E-BP1. Densitometric quantification of the L-leucine add-back immunoblot data are shown in the graph (n = 3/group; mean ± SEM, **p* <0.05, by Student's *t* test). (C) Same as in (B) except that an equivalent concentration of L-arginine as the complete amino acid add-back treatment (as in A) was used (n = 3/group; mean ± SEM, **p* <0.05, by Student's *t* test). (D) Same as in (B) except that an equivalent concentration of L-methionine as the complete amino acid add-back treatment (as in A) was used (n = 3/group; mean ± SEM, **p* <0.05, by Student's *t* test). (E) Intracellular amino acid levels were measured in scr-*vs.* si-Rap1a-treated primary mouse hepatocytes that were starved of all amino acids (AA starvation) for 1 h

increased expression of the mature form of SREBP1 (Fig. 3A). However, the precursor form of SREBP1 was unchanged (Fig. 3A). We obtained similar results in hepatocytes treated with scrambled (scr) or siRNA against RAP1A (si-Rap1a) (Fig. 3B). In contrast, CA-RAP1A-treated DIO mice liver had decreased levels of the mature form of SREBP1, without a change in the precursor form (Fig. 3C). These data indicate that activation of RAP1A reduces obesity-associated hepatic liver TG accumulation, lipogenic gene expression, and cleavage of SREBP1, whereas RAP1A inhibition has the opposite effects.

Mice lacking hepatic insulin receptors show defects in SREBP1 expression after feeding, which is suggested to be mediated via mTORC1.[19,20] Although the role of mTORC1 in the regulation of MASLD and MASH is complex,[21–23] its ability to stimulate SREBP1 cleavage and activation has been demonstrated in multiple mouse models.[24–26] We therefore examined the phosphorylation and activation of p70 ribosomal S6 Kinase 1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which are two well-known mTORC1 targets. We observed that mTORC1 was hyper-activated upon RAP1a deficiency, as shown by increased phospho-S6K1 and its target, phospho-ribosomal *S6* protein (phospho-S6) levels in hepatocytes isolated from *Rap1a*$^{fl/fl}$ mice treated with TBG-Cre (RAP1A KO) as compared with TBG-Gfp-treated controls (wildtype [WT]) (Fig. 3D). Conversely, CA-RAP1A treated *db/db* mice livers showed lower phospho-S6 and phospho-4E-BP1 levels (Fig. 3E). To test whether increased SREBP1 seen with RAP1A deficiency was dependent on mTORC1, we treated RAP1A-silenced mouse hepatocytes with the mTORC1 inhibitor, Torin and found that this treatment lowered the mature SREBP1 levels in si-Rap1A-treated hepatocytes without significant changes in precursor SREBP1 levels (Fig. 3F). Moreover, to determine the human relevance of our mouse hepatocyte results, we investigated the effect of RAP1A silencing in metabolism-qualified human hepatocytes and found that p-S6 levels were increased upon RAP1A silencing and Torin treatment decreased mature SREBP1 levels in si-RAP1a treated cells (Fig. 3G–I). Altogether, these results are consistent with a recent study showing that RAP1A-deficient cells have constitutive mTORC1 activity, irrespective of insulin signaling.[27]

RAP1A regulates amino acid-mediated mTORC1 activation

To study how RAP1A suppresses mTORC1, we first examined protein levels of the mTORC1 complex components. However, we observed no change in the levels of mTOR, RAPTOR, or PRAS40 in scr *vs.* si-Rap1a-treated cells (Fig. S2A). Therefore, we became interested in established upstream stimuli or pathways that are crucial for mTORC1 activity. The insulin–PI3K–AKT pathway is known to activate mTORC1 signaling by phosphorylating and inhibiting tuberous sclerosis complex protein 2 (TSC2), a suppressor of TOR kinase. As RAP1A inhibition activates mTORC1, it might be predicted that RAP1A silencing enhances insulin signaling. However, our previous study in hepatocytes showed that RAP1A silencing perturbs insulin signaling, as evidenced by lower AKT phosphorylation upon insulin stimulation, which cannot explain the increased mTORC1 activity in RAP1A-silenced cells.[11] Moreover, AMP-activated serine/threonine protein kinase (AMPK), which is activated in response to an increase in AMP and energy stress, inhibits mTORC1 by activating the mTORC1 inhibitor TSC2 and inhibiting the mTORC1 binding subunit, RAPTOR. However, AMPK activity was comparable between scr (control) and RAP1A-silenced cells (Fig. S2B). We then focused on other nutrient inputs that regulate mTORC1. An increase in intracellular cholesterol has been shown to activate mTORC1;[28] however, S6 phosphorylation in response to LDL loading was comparable between scr *vs.* si-Rap1a-treated cells (Fig. S2C), suggesting that cholesterol signaling to mTORC1 was not changed upon RAP1A inhibition. Amino acids are critical regulators of mTORC1 and permit further modulation of mTORC1 activity by other inputs.[29] When cytosolic and lysosomal amino acid levels are sufficient, the mTORC1 complex is activated, whereas depletion of amino acids switches off mTORC1. Thus, we next tested whether RAP1A affected amino acid-mediated mTORC1 activation. In response to amino acid starvation followed by total amino acid stimulation, S6 phosphorylation was further increased in RAP1A silenced cells (Fig. 4A). Leucine, arginine, and methionine are the major amino acids that are known to potently activatemTORC1. We then individually added equivalent concentrations of leucine, arginine, or methionine as the total amino acid add-back stimulation and found that these treatments also further increased S6 and 4E-BP1 phosphorylation in RAP1A silenced cells after amino acid starvation.Of note, leucine, arginine, or methionine treatment of RAP1A silenced cells resulted in greater increases in p-S6 and p-4E-BP1 levels than total amino acid add-back treatment, suggesting that RAP1A may be selectively implicated in the leucine-, arginine-, or methionine-induced mTORC1 activation (Fig. 4B–D).

To test whether a change in intracellular amino acid levels was responsible for the increased mTORC1 activity upon RAP1A silencing, we measured amino acid concentrations and found that RAP1A silenced cells had significantly higher total intracellular amino acid levels (Fig. 4E). Consistently, when we assayed the concentrations of individual amino acids using HPLC–mass spectrometry (HPLC–MS), all amino acids except glycine were increased in RAP1A-silenced cells (Table S1). Two major mechanisms maintain intracellular amino acid concentrations: regulation of protein breakdown or synthesis, and influx of amino acids from the extracellular environment via transporters. In this regard, we first evaluated autophagy, which is a key process that increases intracellular amino acid levels. Although the inhibitory serine 757 phosphorylation of the autophagy-initiating kinase ULK1 was decreased in CA-RAP1A treated *db/db* liver, there were no changes in LC3-I to LC3-II ratios or p62 levels (Fig. S2D). These data suggested that

followed by complete amino acid add-back (AA add-back) treatment for 30 min (n = 6/group; mean ± SEM, *p <0.05, by two-way ANOVA/Sidak's *post hoc* analysis). (F) mRNA levels of *Slc* genes in control *vs.* sh-Rap1Gap-treated DIO mice liver (n = 10 mice/group; mean ± SEM, *p <0.05, by Student's t test or rank sum test). (G) mRNA levels of *Slc* genes in TBG-Gfp (Gfp) *vs.* TBG-CA-RAP1A (CA-RAP1A) treated DIO mice liver (n = 6 mice/group; mean ± SEM, *p <0.05, by Student's t test or rank sum test). CA-RAP1A, constitutively active RAP1A; DIO, diet-induced obese; mTORC1, mechanistic target of rapamycin complex 1; RAP1A, Ras-associated protein 1A.

autophagy was not decreased in CA-RAP1A treatment, which cannot explain the decreased intracellular amino acid levels upon RAP1A activation. Furthermore, because intracellular amino acid levels can also be affected by protein synthesis, we measured newly synthesized proteins using a puromycin incorporation assay. Puromycin is a structural analog of tyrosyl-tRNA and is incorporated into nascent peptides, which can be determined by immunoblotting using an anti*puromycin* antibody.[30] However, analysis of puromycin incorporation into newly synthesized proteins revealed no significant differences between scr- and si-Rap1a-treated cells (Fig. S2E). Amino acid transporters mediate the transport of amino acids across the plasma membrane or lysosomes and are encoded by solute carrier (SLC) transporter genes. Various pathologies have been linked to alterations in SLC proteins, and recent studies suggested that they are one of the major regulators of intracellular amino acid levels.[31] To determine whether RAP1A regulates intracellular amino acid concentrations by altering amino acid uptake, the mRNA levels of major amino acid transporter genes, including *Slc7a2*, *Slc38a3*, and *Slc38a4* were assayed. We observed significantly lower expression of most of the amino acid transporter genes in the livers of mice in which RAP1A was activated via sh-Rap1Gap or CA-RAP1A treatments (Fig. 4F and G), whereas hepatocyte RAP1A silencing increased them (Fig. S3A). Most importantly, treatment of control and RAP1A-silenced cells with the cell-permeable, dimeric leucine methyl ester derivative, leucyl-leucine-*O*-methyl-ester (LLOME) resulted in comparable S6 phosphorylation, further suggesting that RAP1A regulates mTORC1 by preventing transporter-mediated amino acid uptake (Fig. S3B).[32] We then asked if there is a feedback mechanism between RAP1A and mTORC1 that could contribute to the regulation of amino acid transporters. To this end, we treated RAP1A-silenced hepatocytes with Torin and found that mRNA expression of most of the amino acid transporters except for *Slc7a2* were similar between vehicle-*vs.* Torin-treated cells, suggesting that the effect of RAP1A on *Slc* gene expression was not dependent on mTORC1 (Fig. S3C).

The liver is a major site for regulating amino acid metabolism and contributes to systemic amino acid exposure.[31] Consistent with a decrease in hepatic expression of amino acid transporters, plasma levels of most of the amino acids, including methionine and arginine were increased in CA-RAP1A- or sh-Rap1Gap-expressing mice as compared with the control (Tables 1 and 2). Altogether, these data support the theory that RAP1A silencing enhances amino acid-mediated mTORC1 activation by upregulating the amino acid transporters, and activation of RAP1A has the opposite effect.

Table 1. Plasma amino acid levels.

Amino acid	Control	sh-Rap1Gap
Lysine	232.30 ± 11.96	343.68 ± 19.39*
Proline	70.11 ± 4.43	73.05 ± 7.15
Valine	176.75 ± 8.76	207.12 ± 7.68*
Cystine	3.86 ± 0.3	3.62 ± 0.34
Leucine	124.03 ± 6.54	130.37 ± 3.77
Isoleucine	75.18 ± 3.26	80.87 ± 2.57
Methionine	45.66 ± 1.37	59.73 ± 2.13*
Phenylalanine	69.53 ± 2.15	67.00 ± 1.37
Tyrosine	101.08 ± 6.17	108.29 ± 10.96
Tryptophan	120.87 ± 3.48	119.76 ± 5.26
Glycine	230.01 ± 14.05	253.71 ± 23.43
Alanine	395.47 ± 28.69	372.93 ± 26.43
Serine	133.1 ± 4.07	178.26 ± 4.50*
Threonine	132.77 ± 6.86	190.24 ± 7.53*
Asparagine	50.35 ± 3.76	63.46 ± 3.38*
Aspartic acid	3.58 ± 0.25	4.35 ± 0.45
Glutamine	717.91 ± 19.19	843.12 ± 21.15*
Glutamic acid	23.05 ± 1.73	23.61 ± 2.74
Histidine	78.36 ± 7.55	90.79 ± 9.39
Arginine	42.29 ± 5.08	72.13 ± 9.03*

Plasma concentrations of individual amino acids were measured in diet-induced obese mice treated with control or sh-Rap1Gap (n = 10 mice/group; *p <0.05, by Student's t test or Rank Sum test).

Table 2. Plasma amino acid levels.

Amino acid	Gfp	CA-RAP1A
Lysine	233.25 ± 8.41	341.44 ± 15.72*
Proline	70.52 ± 2.07	90.23 ± 4.53*
Valine	229.79 ± 5.71	261.40 ± 15.57
Cysteine	151.73 ± 11.11	192.95 ± 21.64
Leucine	193.94 ± 3.46	214.547 ± 19.58
Isoleucine	116.97 ± 3.31	124.52 ± 12.54
Methionine	46.04 ± 1.57	55.760± 1.58*
Phenylalanine	83.54± 3.77	94.04 ± 6.32
Tyrosine	87.48 ± 4.48	99.74 ± 8.66
Tryptophan	100.09 ± 7.51	114.10 ± 10.17
Glycine	209.02 ± 12.67	252.23 ± 18.57
Alanine	483.46 ± 43.33	473.41 ± 23.25
Serine	120.30 ± 6.07	130.24 ± 6.34
Threonine	141.73 ± 7.64	182.48 ± 5.29*
Asparagine	48.18 ± 2.69	58.53 ± 4.40
Aspartic acid	4.16 ± 1.03	3.94 ± 0.68
Glutamine	808.92 ± 48.90	848.76 ± 64.56
Glutamic acid	19.09 ± 0.78	22.60 ± 3.50
Histidine	82.85 ± 12.67	87.41 ± 20.95
Arginine	58.84 ± 4.06	80.90 ± 4.59*

Plasma concentrations of individual amino acids were measured in diet-induced obese mice treated with TBG-Gfp (Gfp) or TBG-CA-RAP1A (constitutively active-RAP1A) (n = 5 mice/group; *p <0.05, by Student's t test).

RAP1A activity is suppressed in MASH and RAP1a deficiency exacerbates MASH-induced liver fibrosis

Steatosis is an important contributor to MASH pathogenesis, and 20% of individuals with MASLD progress to MASH.[5] Thus, we next investigated whether a causal relationship exists between hepatic RAP1A and MASH. We obtained human liver specimens of individuals with normal or MASH histology from the National Institutes of Health-sponsored Liver Tissue Cell Distribution System and found that RAP1A activity was significantly lower in liver extracts from individuals with MASH *vs.* controls, as shown by lower GTP-bound (active) RAP1A levels (Fig. 5A and Table S2).

In preparation for causation studies, we next explored RAP1A activity in mice fed a MASH-provoking high fructose, palmitate, and cholesterol-rich (FPC) diet. Mice fed the FPC diet develop liver steatosis, inflammation, and fibrosis by 16 weeks of feeding, and FPC-fed mouse livers show human-like MASH pathologic and molecular features.[33,34] Similar to human data, we found lower hepatic RAP1A activity in 16-week FPC-diet-fed mice than in chow-fed mice (Fig. 5B). To study the mechanism of this regulation, we measured the levels of EPAC2, which stimulates GTP loading and activation of RAP1A, and RAP1GAP, which inactivates RAP1A. We found that RAP1GAP levels were significantly increased whereas EPAC2 was decreased in livers of FPC-fed mice as compared with control livers, which could explain the lowering of RAP1A activity (Fig. S4A). We then tested the

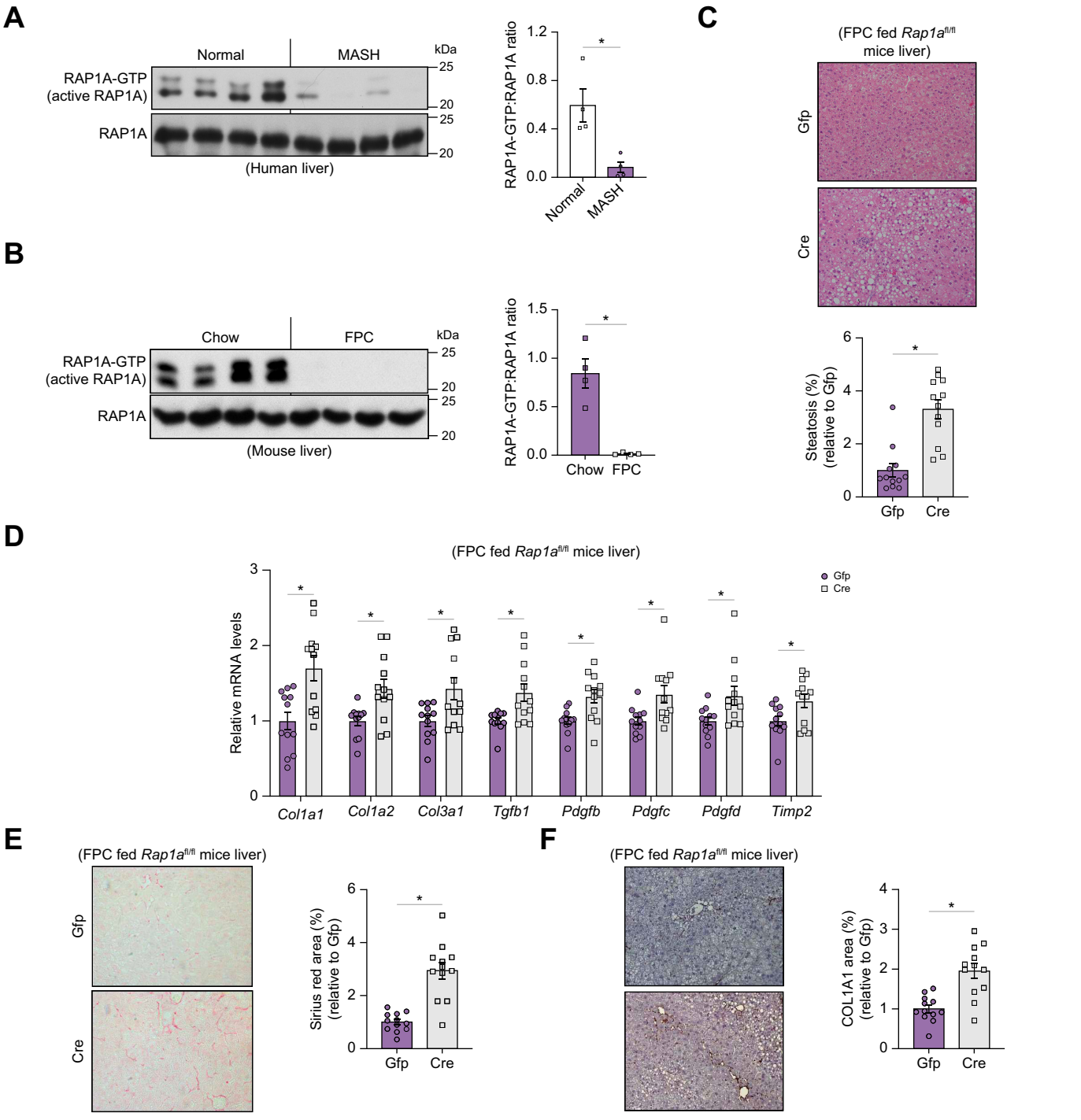

Fig. 5. RAP1A activity is suppressed in MASH and RAP1A deficiency exacerbates liver fibrosis in MASH. (A, B) Active, RAP1A-GTP levels were assayed in normal or MASH human liver (A) and chow-*vs.* FPC-diet-fed mice liver (B). Densitometric quantification of the immunoblot data are shown in the graphs (n = 4/group; mean ± SEM, **p* <0.05, by Student's *t* test). (C–F) TBG-Gfp (Gfp) or TBG-Cre (Cre)-treated *Rap1a*$^{fl/fl}$ mice were fed with FPC diet for 10 weeks. (C) Representative images of liver sections stained with H&E. The percentage of steatosis is displayed as relative to Gfp (n = 12 mice/group; mean ± SEM, **p* <0.05, by Student's *t* test). (D) Hepatic expression of mRNAs related to liver fibrosis was assayed (n = 12 mice/group; mean ± SEM, **p* <0.05, by Student's *t* test or rank sum test). (E) Staining and quantification of Picrosirius Red-positive and collagen 1A1 (COL1A1)-positive area (F) (n = 12 mice/group; mean ± SEM, **p* <0.05, by Student's *t* test). MASH, metabolic dysfunction-associated steatohepatitis; RAP1A, Ras-associated protein 1A.

functional importance of RAP1A in MASH and acutely deleted RAP1A in adult mice by injecting AAV8-TBG-Cre into *Rap1a*$^{fl/fl}$ mice. The mice were fed with the FPC diet for 10 weeks, which did not induce MASH-like features in WT mice. Interestingly, in Cre-injected *Rap1a*$^{fl/fl}$ mice, a 10-week FPC diet was sufficient to induce steatosis (Fig. 5C) and increase mRNAs related to liver fibrosis (Fig. 5D), without a change in body weight (35.5 ± 0.5 g *vs.* 35 ± 0.5 g). Consistent with this, RAP1A deficiency increased liver

fibrosis, as measured by staining with both Picrosirius Red and anti-collagen 1A1 (anti-COL1A1) (Fig. 5E and F).

Restoring RAP1A activity suppresses steatosis and MASH progression

We next investigated whether restoring hepatic RAP1A activity in FPC-fed mice using CA-RAP1A injection could protect against liver fibrosis. To test this, we first fed mice the FPC diet for 8 weeks and then injected them with AAV8-TBG-Gfp or AAV8-TBG-CA-RAP1A. After 10 more weeks on the FPC diet, body weight was similar between the two groups (37 ± 1.2 g *vs.* 36.9 ± 1.1 g); however, the CA-RAP1A-expressing group developed less liver steatosis than the Gfp-injected controls (Fig. 6A). Correspondingly, RAP1A restoration lowered mRNAs related to liver fibrosis, reduced fibrosis, as evidenced by decreased Picrosirius Red and anti-COL1A1 staining (Fig. 6B and D), and decreased plasma ALT levels (Fig. 6E).

Yes-associated protein (YAP)/transcriptional co-activator with PDZ-binding motif (TAZ) transcription factors were shown to upregulate *Slc* gene expression in endothelial cells, which results in increased import of amino acids and activation of mTORC1.[35] In this regard, we found lower levels of both TAZ and YAP in CA-RAP1A treated mice liver as compared with controls (Fig. S4B). In contrast, RAP1A silencing resulted in small but significant increases in TAZ and YAP levels in isolated hepatocytes (Fig. S4C). These data suggest that hepatocyte RAP1A suppresses YAP and TAZ, which could explain the decrease in *Slc* gene expression and mTORC1 activity upon RAP1A activation in mice. Interestingly, hepatocyte TAZ promotes MASH pathogenesis and silencing it lowers liver fibrosis in mice fed with MASH-inducing diets.[33] However, more work is needed to establish the role of lower TAZ levels in RAP1A-mediated protection from MASLD and MASH.

Discussion

In insulin resistance, insulin fails to adequately suppress HGP and augments hepatic lipogenesis and TG accumulation; however, the underlying molecular mechanisms remain unclear. Here, we demonstrate that activation of the small GTPase RAP1A results in decreased lipogenesis and hepatic TG accumulation by suppressing SREBP1 cleavage. The mechanism involves mTORC1

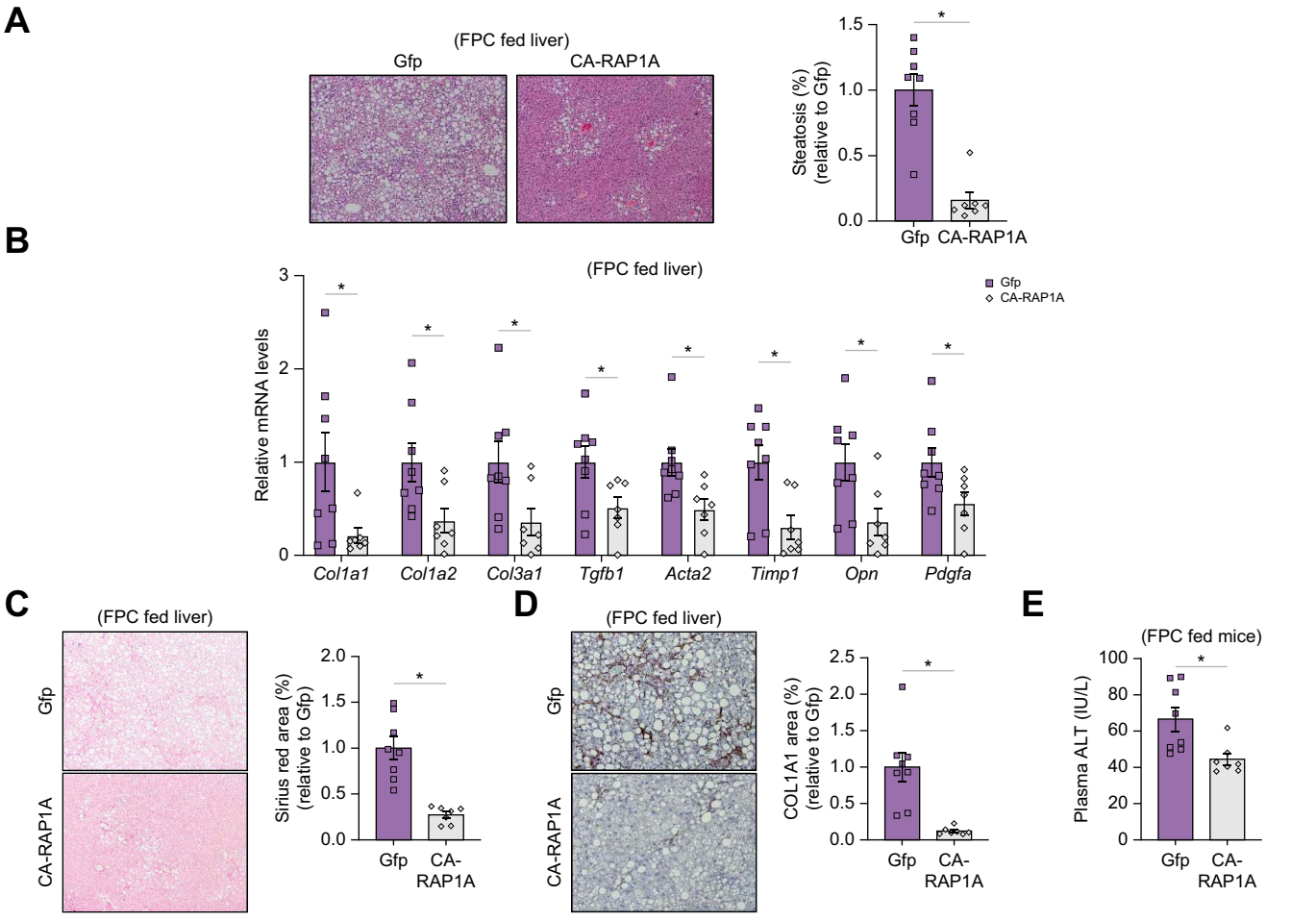

Fig. 6. Restoring RAP1A activity suppresses steatosis and MASH progression. WT mice were fed the FPC diet for 8 weeks and treated with TBG-Gfp (Gfp) or TBG-CA-RAP1A (CA-RAP1A). Mice were then fed the FPC diet for 10 additional weeks. (A) Representative images of liver sections stained with H&E. The percentage of steatosis is displayed as relative to Gfp (n = 7–8 mice/group; mean ± SEM, *p <0.05, by Student's t test). (B) Hepatic expression of mRNAs related to liver fibrosis was assayed (n = 7–8 mice/group; mean ± SEM, *p < 0.05, by Student's t test). (C and D) Staining and quantification of Picrosirius Red-positive (C) and collagen 1A1 (COL1A1)-positive area (D) (n = 7–8 mice/group; mean ± SEM, *p <0.05, by Student's t test or rank sum test). (E) Plasma ALT levels were analyzed (n = 7–8 mice/group; mean ± SEM, *p < 0.05, by Student's t test). ALT, alanine aminotransferase; CA-RAP1A, constitutively active RAP1A; FPC diet, fructose, palmitate, and cholesterol-rich diet; MASH, metabolic dysfunction-associated steatohepatitis; RAP1A, Ras-associated protein 1A; WT, wildtype.

inhibition via lowering intracellular amino acid levels. Activation of hepatic RAP1A also suppresses HGP and improves glucose tolerance through inhibiting the gluconeogenic transcription factor, FOXO1.[11] Lowering of hepatocyte amino acid levels by RAP1A activation described here likely contributes to HGP suppression and decreases circulating glucose. As glucose and lipid metabolism are linked and plasma glucose levels stimulate lipogenesis, it is possible that lower glucose levels upon RAP1A activation also provide protection against liver fat storage. Altogether, these results suggest that activation of RAP1A may serve as a signaling molecule that suppresses both HGP and hepatic steatosis, and inhibition of its activity in obese liver may underlie the pathogenesis of selective insulin resistance.

Most circulating amino acids, except glycine, are increased in patents with MASLD, which correlates with the severity of liver disease.[36–38] However, it remains unclear whether the hepatic levels of amino acids have a similar trend in MASLD and MASH. Moreover, accumulating evidence suggests that plasma levels of branched chain amino acids, leucine, isoleucine, and valine correlate with cardiovascular diseases, including heart failure and atherosclerosis.[39] In this regard, we show that when liver RAP1A is activated, hepatic amino acid levels decrease, which underlies the protection from MASLD and MASH in CA-RAP1A and sh-Rap1Gap-treated mice liver. However, as the liver contributes to the regulation of circulating amino acid levels, certain amino acids, such as arginine and methionine, were increased in plasma upon RAP1A activation. Although the levels of leucine and isoleucine levels were similar to control mice, valine was slightly increased in sh-Rap1Gap-treated mice plasma (Table 1). Therefore, further research will be required to determine whether RAP1A activation in the liver impacts heart and cardiovascular health.

Cardiovascular disease is a major cause of death in patients with MASLD, especially those with MASH.[40,41] Thus, the discovery of new metabolic pathways that contribute to both MASLD/MASH and cardiovascular disease pathogenesis may provide novel opportunities for pharmacological interventions. The results of this study and our previous work thus far suggest that activation of RAP1A in the liver is beneficial in obesity-associated cardiometabolic diseases as it (i) lowers proatherogenic PCSK9 and LDL-C, (ii) improves glucose intolerance, and (iii) protects against MASLD and MASH.[10,11,42] In this regard, treatment of mice with the small molecule activator of RAP1, 8-pCPT, protected mice from developing MASLD (Fig. 1G–J) and also lowered plasma PCSK9 levels.[14] However, as 8-pCPT was administered systemically, more work is needed to thoroughly investigate it in appropriate mouse models for possible off-target and potential side effects. Alternatively, therapeutically activating liver RAP1A by treatment with N-acetylgalactosamine (GalNAc)-siRNA targeting the hepatocyte RAP1GAP could be considered. Finally, our results also describe a critical role for RAP1A in regulating hepatic mTORC1 activity, which aligns with previous findings[27] and could have important implications for a broad range of diseases, including some types of cancer. This is particularly important in HCC, which is one of the major complications of MASH. However, it should be noted that the role of RAP1A in promoting initiation or progression of HCC is unclear.[43] Thus, a topic for future study will be to examine if activating RAP1A could confer protection against MASH-induced HCC.

Affiliations

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Abbreviations

4E-BP1, 4E-binding protein 1; 8-pCPT, 8-pCPT-2′-O-Me-cAMP; AAV8, adeno-associated virus 8; ALT, alanine aminotransferase; AMPK, AMP-activated serine/threonine protein kinase; anti-COL1A1, anti-collagen 1A1; CA-RAP1A, constitutively active RAP1A; DIO, diet-induced obese; DNL, *de novo* lipogenesis; EPAC2, exchange factor activated by cAMP-2; FAO, fatty acid oxidation; Fasn, fatty acid synthase; FPC diet, fructose, palmitate, and cholesterol-rich diet; HCC, hepatocellular carcinoma; HGP, hepatic glucose production; HPLC–MS, HPLC–mass spectrometry; KO, knockout; LLOME, leucyl-leucine-*O*-methyl-ester; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; mTORC1, mechanistic target of rapamycin complex 1; OCR, oxygen consumption rate; P407, poloxamer 407; PCSK9, proprotein convertase subtilisin/kexin type 9; RAP1A, Ras-associated protein 1A; RAP1GAP, Ras-associated protein 1 GTPase activating protein; Scd1, stearoyl-CoA desaturase; scr, scrambled; si-Rap1a, siRNA against RAP1A; si-Rap1Gap, silencing RAP1GAP; SLC, solute carrier; SREBP-1sterol regulatory element-binding protein ; T2D, type 2 diabetes; TBG, thyroxine-binding globulin; TG, triglyceride; TSC2, tuberous sclerosis complex protein 2; WT, wildtype.

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Conflicts of interest

The authors have declared that no conflict of interest exists.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceived and supervised the project: LO. Designed and planned the experiments: HA, YW, XW, LO. Designed and performed the experiments: HA, YW, BT, LO. Analyzed the data: HA, YW, BT, XW, LO. Interpreted the results: HA, YW, BT, XW, LO. Wrote the manuscript: HA, LO. Edited the manuscript: HA, YW, XW, LO. Revised the manuscript: LO.

Data availability statement

Data are available from the corresponding author upon reasonable request.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2024.101303.

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Author names in bold designate shared co-first authorship

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