

Activation of hepatic USP5 as a novel strategy to ameliorate metabolic dysfunction-associated steatotic liver disease by deubiquitinating CPT-1A

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Abstract

Ubiquitin-specific protease 5 (USP5) is a key regulator in cancer, pain, and inflammatory disorders, yet its role in metabolic dysfunction-associated steatotic liver disease (MASLD) remains largely unexplored. This study aims to elucidate the functional significance and therapeutic potential of USP5 in MASLD. Hepatic USP5 expression was markedly reduced in liver specimens from MASLD patients, as well as in relevant *in vivo* and *in vitro* models. Hepatocyte-specific genetic ablation of USP5 exacerbated MASLD-associated hepatic steatosis and inflammation, whereas USP5 overexpression alleviated steatohepatitis. Mechanistically, co-immunoprecipitation coupled with mass spectrometry (Co-IP-MS), surface plasmon resonance (SPR), and ubiquitination assays revealed that USP5 directly interacts with carnitine palmitoyltransferase 1A (CPT-1A) and selectively cleaves K6-linked ubiquitin chains, thereby stabilizing CPT-1A *via* deubiquitination. In the context of MASLD, reduced USP5 expression disrupts this deubiquitination process, accelerating proteasomal degradation of CPT-1A. Consequently, USP5 deficiency impairs mitochondrial fatty acid oxidation (FAO) and promotes hepatic lipid accumulation. Notably, hepatocyte-specific restoration of CPT-1A substantially rescued the exacerbated lipid deposition induced by USP5 deficiency. Furthermore, docking-based virtual screening coupled with *in vivo* validation identified ligustroflavone (LF) as a potent USP5 activator. Animal studies clearly demonstrated that LF robustly enhanced USP5-mediated CPT-1A stabilization and significantly ameliorated lipid accumulation and inflammation in MASLD mice. Collectively, our findings uncover a previously unrecognized ubiquitination regulatory axis centered on USP5 and CPT-1A, and establish hepatic USP5 as an emerging therapeutic target for restoring mitochondrial FAO in MASLD.

Highlights

- Hepatocyte-specific ablation exacerbates MASLD, whereas its overexpression alleviates this disorder.
- USP5 stabilizes CPT-1A by cleaving K6-linked ubiquitin chains, USP5 deficiency impairs CPT-1A-mediated FAO.
- Ligustroflavone is identified as a novel USP5 activator that stabilizes CPT-1A deubiquitination.
- Ligustroflavone ameliorates hepatic lipid accumulation and inflammation in MASLD mice.

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) affects 30% to 55% of the global population, and its incidence is rising in parallel with the growing prevalence of obesity and diabetes ¹. Without effective intervention, MASLD can progress to metabolic dysfunction-associated steatohepatitis (MASH), a more aggressive stage characterized pathologically by hepatocyte injury with or without concurrent fibrosis ². Mortality associated with MASLD/MASH is primarily attributable to hepatic fibrosis and the subsequent development of cirrhosis. The pathogenesis is multifactorial, involving metabolic dysregulation, genetic susceptibility, inflammatory, and gut microbiota dysbiosis ³. Lifestyle modification

confer only modest benefits, and although the FDA recently approved two anti-MASH drugs (*resmetirom* and *semaglutide*), effective pharmacotherapies remain extremely limited ^{4,5}. Therefore, deciphering the molecular mechanisms driving MASLD and identifying new therapeutic targets is of both scientific and clinical urgency.

Protein ubiquitination is a dynamic and multifaceted post-translational modification that regulates core eukaryotic cellular processes, including apoptosis, cell division, and immune responses ⁶. Ubiquitination can direct substrates to proteasomal degradation. Alternatively, through distinct ubiquitin chain linkages, it modulates protein-protein interactions and function ⁷. Emerging evidence implicates ubiquitination dysregulation in MASLD pathogenesis ^{8,9}. Several E3 ubiquitin ligases have been shown to modify key metabolic pathways in the diseased liver. For example, TRIM56 binds and promotes its ubiquitin-dependent degradation of fatty acid synthase (FASN), thereby attenuating MASH progression ¹⁰. Similarly, TRIM26 interacts with CCAAT/enhancer-binding protein delta (C/EBP δ) and catalyzes its ubiquitination and degradation. Hepatocyte TRIM26 deficiency disrupts lipid homeostasis, leading to aggravated steatosis, inflammation and fibrosis ¹¹. These findings underscore that precisely targeting ubiquitin ligases and deubiquitinating enzymes (DUBs) in MASLD could yield novel therapeutic strategies for MASLD.

The reversibility of ubiquitination is orchestrated by DUBs. Among all identified DUBs, ubiquitin-specific protease 5 (USP5) is a key member of the DUB superfamily and is first identified by Wilkinson et al. ¹². Mammalian USP5 is highly conserved, sharing 98.7% amino acid identity between humans and mice, and is widely expressed in various organs, including the liver ^{13,14}. USP5 regulates diverse cellular processes such as cell cycle regulation, apoptosis, DNA double-strand break repair, methylation, and the heat shock response through interactions with distinct substrate proteins ¹⁵. Importantly, USP5 has been demonstrated to play significant roles in various pathological conditions, including cancer, pain, and inflammatory diseases ¹⁶. Despite its established involvement in these diseases, the specific function of USP5 in MASLD remains unclear⁸.

This study aimed to investigate how USP5 regulates the development of MASLD. Firstly, USP5 was identified decreased in the liver of both patients and mice with hepatic steatosis. To this end, hepatocyte-specific USP5 deficiency and overexpression mouse models were generated and subjected to diet-induced MASLD, which evaluated the effects of USP5 on hepatic steatosis and disease progression. Mechanistically, co-immunoprecipitation, surface plasmon resonance (SPR), ubiquitination assays and seahorse were employed to determine that USP5 directly interacts with carnitine palmitoyltransferase 1A (CPT-1A) and enhances its stability by specifically removing K6-linked ubiquitination, thereby regulating fatty acid oxidation (FAO) and hepatocellular lipid metabolism. Furthermore, rescue of CPT-1A block the effects of USP5 deficiency. Next, utilizing docking-based virtual screening combined with cell-based TG-lowering assays, ligustroflavone (LF) was identified as a novel USP5 activator. LF potentiated USP5-mediated stabilization of CPT-1A and effectively ameliorated

MASLD. Collectively, this study aims to define the USP5/CPT-1A regulatory axis in hepatic lipid homeostasis and to explore its potential as a therapeutic strategy for MASLD.

Results

1. USP5 was identified as a key regulator of MASLD

To explore whether DUBs contribute to MASLD, a published transcriptomic dataset (GSE126848) of human liver biopsies from individuals with or without MASLD was interrogated. This analysis aimed to identify DUBs whose expression is altered in MASLD. Among all DUBs, the mRNA levels of *Usp5* were significantly decreased in MASLD samples compared to controls (Figure 1A). Next, immunohistochemical (IHC) analysis of liver biopsies from 20 MASLD patients and 20 normal individuals confirmed a marked reduction in the USP5-positive staining area in MASLD livers (Figure 1B-D). Importantly, lower hepatic USP5 levels were associated with higher NAS score, more severe hepatic steatosis, and elevated serum triglycerides (TG) levels (Figure 1E-G). Thus, these associations indicated that USP5 protein progressively declines as MASLD advances.

To extend these clinical findings, we next examined USP5 protein expression in well-established murine models of MASLD, including mice fed a high-fat/high-cholesterol (HFHC) diet or a methionine-choline-deficient (MCD) diet. Consistently, western blotting (WB) analysis showed that USP5 protein levels were also significantly reduced in the livers of mice fed the HFHC or MCD diet (Figure 1H-J). We further investigated USP5 expression in different liver cell types under relevant stress conditions: specifically, in hepatocytes exposed to free fatty acids (FFAs) (Figure 1K), in Kupffer cells stimulated with lipopolysaccharide (LPS), and in hepatic stellate cells (HSCs) treated with transforming growth factor-beta (TGF- β). Notably, USP5 was markedly downregulated in both mouse primary hepatocytes (Figure S1D) and AML-12 cells upon FFAs exposure (Figure 1L-M). In contrast, no significant changes were observed in LPS-stimulated Kupffer cells or TGF- β 1-treated HSCs (Figure S1E-F). These findings suggest that downregulation of USP5 expression is closely associated with hepatocyte lipid accumulation and MASLD progression.

2. Hepatocyte-specific *Usp5* knockout exaggerates hepatic lipid accumulation in MASLD mice

To delineate the functional role of USP5 on MASLD *in vivo*, hepatocyte-specific *Usp5* knockout (*Usp5*^{Hep^{KO}}) mice and littermate controls were generated (Figure 2A, knockout efficiency confirmed by WB analysis, Figure S2A). Under normal chow conditions, *Usp5*^{Hep^{KO}} mice displayed no obvious phenotype change. However, after 9-weeks of feeding HFHC diet, *Usp5*^{Hep^{KO}} mice exhibited significantly higher body weight, liver weight, and liver-to-body weight ratio compared to littermate controls (Figure S2B-D). Moreover, hepatic TG and non-esterified fatty acids (NEFA), as well as serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST) activity, and hepatic hydroxyproline (HYP) content, were all significantly elevated (Figure 2B-F). Consistent with these metabolic disturbances, histopathological examination further revealed more severe hepatic steatosis in *Usp5*^{Hep^{KO}} mice (Figure 2G-H), which was

accompanied by upregulation of the lipid synthesis genes (*Srebf1*, *Scd1*) and downregulation of lipid consumption genes (*Fabp1*) (Figure S2H). Importantly, the detriment of USP5 loss extended beyond steatosis. Sirius red staining showed that hepatic collagen deposition was significantly exacerbated in *Usp5*^{Hep KO} mice. In addition, immunohistochemical (IHC) staining revealed increased areas positive for the macrophage marker F4/80 and myofibroblast marker α -SMA (Figure S2 E-G). These pathological changes were further supported by a significant elevation in mRNA levels of the pro-inflammatory gene *Ccl2* and the pro-fibrotic gene *Col3a1* (Figure S2H).

To test whether hepatocyte-specific USP5 ablation also aggravated MASH under a more aggressive dietary challenge, mice were fed a choline-deficient, L-amino acid-defined high-fat diet (CDA-HFD) (Figure 2I), a common model to induce MASH over a relatively short period. Strikingly, *Usp5*^{Hep KO} mice again exhibited aggravated hepatic steatosis and inflammation, along with the elevated serum ALT and AST levels (Figure 2J-N).

USP5 knockdown was also performed in THLE-2 cells, followed by treatment with or without FFAs (Figure 2O, knockdown efficiency was confirmed by GFP fluorescence and WB, Figure S2I-J). Upon FFAs challenge, USP5 knockdown significantly increased lipid droplet accumulation and intracellular TG content compared to control cells (Figure 2P-R). These observations clearly demonstrate that hepatocyte-specific USP5 knockout exacerbates MASLD progression by promoting steatosis, inflammation, and fibrosis.

3. Hepatocyte-specific USP5 overexpression alleviates hepatic lipid accumulation in MASLD mice

To further complement the functional role of hepatocyte USP5 in MASLD, USP5 was overexpressed in hepatocytes using AAV2/8 vectors (AAV_UsP5^{Hep OE}) via tail vein injection (Figure 3A). Successful hepatic USP5 overexpression was confirmed by Western blot analysis (Figure S3A). After 12 weeks of HFHC diet feeding, AAV_USP5^{Hep OE} mice exhibited significantly reduced body weight, liver weight, and liver-to-body weight ratio compared to those in the AAV_Vehicle group (Figure 3B-D). Moreover, the HFHC diet induced elevations in hepatic TG and NEFA levels, as well as serum ALT activity levels were also reversed by USP5 overexpression (Figure 3E-I). Furthermore, hepatocyte USP5 overexpression markedly ameliorated HFHC diet-induced insulin resistance and lowered serum levels of TG, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) (Figure S3B-E).

Consistent with these metabolic improvements, histopathological staining further demonstrated that hepatocyte USP5 overexpression markedly attenuated hepatic lipid accumulation (Figure 3J-K), accompanied by decreased *Srebf1*, *Scd1* and *Fabp1* mRNA levels (Figure S3I). In addition, IHC staining revealed a reduced F4/80-positive staining area following USP5 overexpression, whereas no significant change was observed in α -SMA staining (Figure S3F-H). Concomitantly, mRNA levels of *Ccl2* and *Col3a1* were also reduced upon USP5 overexpression (Figure S3I).

To extend these *in vivo* findings, USP5 overexpression were next performed in THLE-2 cells, followed by treatment with or without FFAs (Figure 3L). The efficiency of USP5 overexpression was confirmed by

GFP fluorescence and WB (Figure S3J-K). Remarkably, USP5 overexpression strongly suppressed FFAs-induced lipid droplet accumulation and reduced intracellular TG content (Figure 3M-O). Taken together, these results indicate a protective function of USP5 against MASLD.

4. CPT-1A is identified as a USP5 substrate

To elucidate potential regulatory mechanisms of USP5 in MASLD, co-immunoprecipitation (co-IP) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using an anti-USP5 antibody in L02 cell lysates. A total 1,016 putative USP5-binding proteins were identified based on unique peptide sequences. To narrow down functionally relevant candidates, we intersected these hits with proteins previously implicated in MASLD pathogenesis, which yielded 10 high-probability candidates: CPT-1A, HSD17B4, NF- κ B2, ACOX1, SQSTM1, ACAA2, I κ B α , FGF2, TFAM, and FXN (Figure 4A-B).

Given that USP5 is a ubiquitin-specific protease that stabilizes its binding substrates by counteracting proteasomal degradation, it was reasoned that USP5 expression should correlate positively with the protein levels of its genuine interaction partners. To test the hypothesis, the protein expression levels of the ten candidates were examined in both THLE2 cells with USP5 knockdown or overexpression, as well as in liver tissues from *Usp5*^{Hep KO} or AAV-*Usp5*^{Hep OE} mice. In USP5-knockdown THLE2 cells, significant reductions were observed in the levels of NF- κ B2, CPT-1A, ACOX1, I κ B α , and FXN (Figure 4C, E). In parallel, liver of *Usp5*^{Hep KO} mice showed markedly decreased expression of CPT-1A, HSD17B4, SQSTM1, and FXN (Figure 4D, E). Conversely, USP5 overexpression in THLE2 cells upregulated NF- κ B2, CPT-1A, HSD17B4, SQSTM1, FGF2, TFAM, and FXN (Figure 4F, H). In liver of AAV-*Usp5*^{Hep OE} mice, only CPT-1A and I κ B α protein levels were found to be enhanced (Figure 4G, H). Collectively, these findings identified CPT-1A and I κ B α as the most promising downstream binding protein of USP5.

To determine whether USP5 directly interact with these candidates, co-IP and SPR analysis were performed. The results showed that USP5 could directly interact with CPT-1A with high binding-affinity ($K_a=3.955E+4(1/Ms)$) (Figure 5A-C), but not I κ B α (Figure S4A). Therefore, CPT-1A was selected as the key USP5-binding partner in MASLD.

5. USP5 directly interacts with CPT-1A and stabilizes this rate-limiting enzyme in FAO

As a key rate-limiting enzyme in FAO, CPT-1A plays a pivotal role in linking lipid and energy metabolism¹⁷. Based on the above findings, it was hypothesized that USP5 interacts with CPT-1A and prevents its degradation. To characterize this action, co-IP was performed to determine that both endogenous and exogenous USP5 could interact with CPT-1A (Figure 5A-B). Surface plasmon resonance (SPR) analysis further supported the finding that USP5 directly interacted with CPT-1A ($K_a=3.955E+4(1/Ms)$, $K_d=0.005009(1/s)$) (Figure 5C). Structurally, USP5 comprises several functional domains, including the Cryptic ZnF, ZnF, C-box, UBA, and H-box domains¹⁸ (Figure 5D). To map the specific region responsible for CPT-1A binding, a series of truncated USP5 constructs were generated. It was confirmed that the

USP5 segments spanning amino acids ubiquitin binding domain (UBA) (aa625-749) and His-box (H-box) (aa749–835) were critical for mediating this interaction (Figure 5E).

To further investigate the molecular mechanisms underlying USP5-mediated regulation of CPT-1A degradation, the protein biosynthesis inhibitor cycloheximide (CHX) was used to treat THLE-2 cells with USP5 knockdown or overexpressing. The results showed that USP5 knockdown significantly accelerated CPT-1A degradation, whereas USP5 overexpression markedly stabilized CPT-1A protein (Figure 5F-G). Additionally, proteasome-dependent protein degradation inhibitor MG132 treatment substantially rescued CPT-1A degradation induced in USP5 knockdown cells, suggesting the involvement of the proteasomal degradation pathway (Figure 5H-I). Next, the ubiquitination status of CPT-1A was examined. Knockdown of USP5 enhanced CPT-1A polyubiquitination, while USP5 overexpression significantly reduced CPT-1A polyubiquitination (Figure 5J). To determine the specific linkage type, we examined various ubiquitin mutants and found that K6-linked polyubiquitination predominates in CPT-1A ubiquitination. In cells expressing K6O ubiquitin mutant (which permits only K6 linkages), USP5 overexpression markedly reduced CPT-1A polyubiquitination. However, in cells expressing the K6R ubiquitin mutant (which cannot form K6 linkages), USP5 failed to decrease CPT-1A ubiquitination levels (Figure 5K-L). Collectively, these evidences indicate that USP5 binds directly to CPT-1A and attenuates its proteasomal degradation by promoting K6-linked polyubiquitination (Figure 5M).

6. CPT-1A overexpression rescues USP5 deficiency impairs FAO and MASLD

Reduced hepatic CPT-1A expression impairs mitochondrial FAO, leading to diminished breakdown of long-chain fatty acids into Acetyl-CoA (acetyl-CoA), decreased ATP production, and exacerbated hepatic lipid deposition (Figure 6A) ¹⁷. Consistent with this notion, a significant decrease in CPT-1A protein levels was confirmed in livers of HFHC-fed *Usp5*^{Hep KO} mice (Figure 6B). Transmission electron microscopy (TEM) revealed exacerbated mitochondrial structural damage in hepatocytes of those mice (Figure 6C). Furthermore, USP5 knockdown in THLE2 cells significantly reduced the oxygen consumption rate (OCR) upon FFAs treatment, indicating a diminished FAO capacity (Figure 6D). In line with this, the basal respiration, maximal respiration (Figure S5A), and ATP levels (Figure 6E) were all significantly decreased. Conversely, when hepatocyte USP5 was overexpressed, CPT-1A protein levels were increased in livers of HFHC-fed mice (Figure 6F). Simultaneously, OCR was enhanced and mitochondrial FAO was promoted in steatosis cells (Figure 6G). Correspondingly, basal respiration, maximal respiration (Figure S5B), and ATP levels (Figure 6H) were significantly increased.

To test whether CPT-1A serves as the critical downstream mediator, CPT-1A was manipulated through pharmacological modulation using the activator C75 or the inhibitor etomoxir, as well as via AAV-mediated overexpression. In THLE2 cells treated with FFAs, C75 efficiently reduced TG content following USP5 knockdown, whereas etomoxir attenuated the TG-lowering effect observed upon USP5 overexpression (Figure 6I-J). Moreover, CPT-1A overexpression significantly reversed the increase in TG content and intracellular lipid droplet accumulation caused by USP5 knockdown *in vitro* (Figure 6K-L). Based on these *in vitro* findings, the *in vivo* effects of hepatocyte CPT-1A restoration were further

evaluated (Figure 6M). Under HFHC diet conditions, AAV_CPT-1A^{Hep OE} significantly reversed the increases in body weight and liver weight in mice caused by USP5 knockout (Figure S5C-D). Meanwhile, reduced hepatic TG content, lower serum ALT and AST levels, and decreased HOMA-IR values were observed (Figure 6N-O). Histopathological examination further revealed CPT-1A restoration ameliorated hepatic steatosis and inflammation (Figure 6P). These results indicate that the USP5-CPT-1A axis is functionally essential for maintaining hepatic lipid homeostasis.

7. Ligustroflavone is identified as an USP5 activator

Given that USP5 deficiency drives MASLD and USP5 overexpression is protective, we next investigated whether pharmacological activation of USP5 can serve as a novel anti-MASLD strategy. In this work, docking-based virtual screening and cell-based lipid-lowering phenotypic assays were combined and used to identify potential USP5 activators as novel anti-MASLD agents. Firstly, natural compound libraries including 2,800 compounds, were screened to discover small molecules that tightly bind to the allosteric site of USP5 (Asp103/Asp104/Try62) (Figure S6A). Intersecting the hits from both screens yielded 16 candidate compounds. Subsequent cell-based TG lowering assays in FFAs-induced AML-12 cells identified compounds with strong triglyceride-reducing activity (Figure S6B). Among these candidates, ligustroflavone (LF), a naturally occurring flavonoid, was selected for further investigations owing to its potent USP5-activating capacity and the strongest triglyceride-reducing activity (Figure 7A). The drug affinity responsive target stability (DARTS) and cellular thermal shift assay (CETSA) revealed that LF markedly regulated the stability of USP5 (Figure 7B-C). Furthermore, SPR assay confirmed the direct binding between LF and USP5 ($KD=8.215 \times 10^{-5}$ M) (Figure 7D-E). The deubiquitination activity of USP5 upon addition of LF were also investigated using ubiquitin-AMC (Ub-AMC) as the substrate. As shown in Figure 7F, LF significantly activated USP5-catalyzed ubiquitin-AMC deubiquitination, with the EC_{50} value of 7.797×10^{-1} nM. Since USP5 reduces CPT-1A protein degradation, we next assessed whether LF reduces USP5-dependent CPT-1A ubiquitination. As shown in Figure 7G-H, USP5-mediated CPT-1A polyubiquitination was substantially decreased by LF in steatotic AML-12 cells.

Given the critical importance of safety for any anti-MASLD agent, the *in vivo* toxicity of LF was rigorously evaluated. Strikingly, mice receiving a high dose of 200 mg/kg for 14 consecutive days exhibited neither mortality nor any sign of organ damage, indicating an excellent safety profile (Figure 7I-K). Moreover, at 12 hours after the final dose, the parent compound was readily detected in liver, kidneys and other organs (Figure 7L), confirming therapeutic relevant hepatic exposure.

8. LF significantly alleviates lipid accumulation and inflammation in MASLD mice

The therapeutic efficacy of LF against MASLD was next evaluated *in vivo*. A 13 week-HFHC mouse model was established and then orally administered LF (20 or 40 mg/kg/day) under continued HFHC feeding for an additional 6 weeks (Figure 8A). Notably, LF treatment dose-dependently reduced body, liver weights, hepatic TG content and serum ALT activity (Figure 8B-F). Histologically, photomicrographs of H&E and Oil Red O stained liver sections revealed a notable reduction in micro- and macrovesicular

steatosis, as well as hepatocellular ballooning, following LF treatment. In addition, SR staining further indicated that LF significantly reduced hepatic collagen deposition in HFHC-fed mice (Figure 8G). In parallel, LF treatment also effectively ameliorated CDA-HFD-induced MASLD, as evidenced by reduced liver weight, lower serum ALT and AST activity (Figure 8H-M), and marked improvement in hepatic steatosis, inflammation and fibrosis based on histology (Figure 8N). These results clearly suggest that hepatocyte USP5 represents a promising therapeutic target for MASLD, while LF emerges as a novel USP5 activator for mitigating MASLD *via* modulating the USP5-CPT-1A axis.

Discussion

This study established hepatocyte USP5 as a key protective mediator against MASLD, operating as follows. First, integrated analyses of published transcriptomic datasets and our experimental evidence consistently demonstrate reduced USP5 levels in both MASLD patients and preclinical models. Second, hepatocyte-specific *Usp5* knockout exacerbated MASLD-induced hepatic injury in mice, whereas *Usp5* overexpression significantly attenuated hepatic steatosis and dysfunction. Third, mechanistically, *Usp5* directly interacts with CPT-1A via its structural domain (aa625-835) and catalyzes K6-linked deubiquitination, thereby stabilizing CPT-1A, enhancing enzymatic activity, and reducing intracellular lipid accumulation. Critically, CPT-1A overexpression substantially rescue hepatic injury induced by *Usp5* ablation, confirming the functional centrality of this axis. Furthermore, we identified LF as a novel USP5 agonist that promotes CPT-1A stabilization and significantly ameliorates MASLD pathology *in vivo*. Collectively, these findings validate USP5 as a promising therapeutic target and support pharmacologic activation of the USP5/CPT-1A axis as a viable strategy for MASLD treatment.

Currently, over 107 distinct DUBs have been identified in humans. These enzymes participate in diverse biological and pathophysiological processes by modulating the stability or function of specific substrate proteins. Emerging evidence implicates specific DUBs in MASLD pathogenesis^{19–21}. As a member of the DUB family, USP5 specializes in the recognition of unanchored polyubiquitin chains and the catalytic cleavage of ubiquitin modifications from target substrates²². This activity not only confers protection against proteasomal degradation but also critically maintains cellular ubiquitin homeostasis. Predominantly localized in the cytoplasm and nucleoplasm, USP5 exhibits increasingly diverse regulatory functions across cellular processes. Although elevated USP5 expression is generally associated with pro-tumorigenic effects, its function in inflammatory contexts, exhibiting both pro- and anti-inflammatory activities properties by substrate specificity. While USP5 is often implicated in promoting disease progression, emerging evidence also suggests protective roles under specific pathological conditions^{23–28}. To resolve this functional dichotomy and expand its known pathophysiological repertoire. Here, we reposition USP5 as a protective regulator in hepatic lipid metabolism. Our study establishes hepatocyte-expressed USP5 as a protective mediator against MASLD. Integrated analysis of published datasets and our experimental data consistently demonstrate reduced USP5 levels in both human MASLD patients and experimental models. Utilizing multiple genetically engineered mouse models across distinct MASLD paradigms, we demonstrated that USP5

deficiency significantly impairs hepatic lipid homeostasis, exacerbates steatosis, and concurrently upregulates genes involved in compensatory lipid utilization pathways—collectively identifying USP5 as a negative regulator of hepatic steatosis pathogenesis. Specifically, hepatocyte-specific *Usp5* knockout exacerbated MASLD-induced hepatic injury in mice, whereas *Usp5* overexpression attenuated hepatic dysfunction.

Crucially, we identified CPT-1A as the direct substrate through which USP5 exerts its protective effects. Combining LC-MS/MS interaction profiling and genetic editing approaches in both cellular and murine models, we confirmed robust binding between USP5 and CPT-1A, further revealing via its UBA and H-box amino acid domain. The UBA domain is a three-helix bundle that is 45 amino acids long, with a hydrophobic surface centered on the conserved (M/L)G(F/Y) motif, which recognizes ubiquitin. UBA1 and UBA2 bind to the third and fourth ubiquitins on linear ubiquitin chains, thereby exerting regulatory functions²⁹. The H-box is a catalytic core of UBP domain that can hydrolyze isopeptide bonds, thereby exerting deubiquitination activity^{18,30}. Studies have revealed USP5 could bind to c-Myc protein via UBA domain²⁷, while bound with IMPDH2 by UBP domain³¹. Specifically, our work revealed that USP5 directly stabilize CPT-1A via K6-linked deubiquitination. The CPT-1A has been proved ubiquitination by USP10 or RNF5^{32,33}. The K6-linked chain was recognized as “atypical” chains, which are less abundant, but first roles are emerging³⁴. For example, K6-linked chains were shown to increase after DNA damage³⁵, and K6 chains assembled by the RBR E3 Ub ligase Parkin were also shown to be important for mitophagy^{36,37}, which is antagonized by the K6-selective, mitochondrial DUB USP30³⁸. This modification specifically impedes proteasomal degradation of CPT-1A, thereby enhancing its stability and enzymatic activity. Critically, CPT-1A overexpression fully rescued hepatic injury induced by *Usp5* ablation, confirming the functional centrality of this regulatory axis and highlighting its therapeutic potential for metabolic liver disorders.

CPT-1A serves as the rate-limiting enzyme in FAO, mediating mitochondrial import of FFA for acetyl-CoA generation¹⁷. This metabolically essential process involves coordinated enzymatic actions: FFAs transported across the plasma membrane is activated to acyl-CoA by long-chain acyl-CoA synthetases (e.g., ACSL1), then converted to acylcarnitine by CPT-1 for β -oxidation-driven ATP production via $\text{FADH}_2/\text{NADH}$ ^{39,40}. Mitochondrial dysfunction disrupts FAO, inducing lipid metabolic dysfunction and propagating lipotoxicity through peroxidative damage and oxidative stress. Previous studies validate targeting CPT-1A for MASLD therapy⁴¹. For instance, hepatic expression of a malonyl-CoA-insensitive CPT-1A mutant improves insulin resistance, and flavonoid-mediated CPT-1A activation ameliorates diet-induced obesity and metabolic disorders by enhancing hepatic FAO⁴². Our study positions hepatocyte USP5 as a key enhancer of CPT-1A-mediated FAO, through the following mechanistic evidence: (i) it accelerates lipid catabolism; (ii) it attenuates steatosis-induced mitochondrial stress and improves ultrastructural integrity; (iii) CPT-1A overexpression rescues both FAO-related gene dysregulation and MASLD phenotypes aggravated by USP5 knockout. Thus, we delineate a USP5/CPT-1A regulatory axis in which USP5 post-translationally stabilizes CPT-1A to activate FAO and mitigate MASLD pathogenesis.

This work further identifies a previously uncharacterized layer of post-transcriptional control over CPT-1A in functional hepatocytes and MASLD pathology.

Beyond mitigating steatosis, hepatocyte USP5 protects against inflammation in MASLD. In support of this, USP5 deficiency upregulated *Ccl2* mRNA (a key chemokine driving macrophage recruitment), suggesting USP5 may modulate chemokine signaling, a possibility meriting further investigation. Regarding fibrogenesis, hepatocyte-specific USP5 KO exacerbated collagen deposition, potentially through early upregulation of *Col3a1*, a biomarker of HSC activation. Alternatively, USP5 may modulate hepatocyte-HSC crosstalk to suppress fibrotic responses. Conversely, hepatic USP5 overexpression consistently attenuated both inflammation, concomitant with ameliorated liver injury. Collectively, USP5 downregulation drives MASLD progression across multiple pathological axes, and its therapeutic restoration may hold promise for delaying or reversing MASLD deterioration by disrupting the pathological feedback loop.

The translational relevance of USP5 targeting for MASLD is strongly supported by our identification of LF, a USP5 activator discovered through structure-guided screening, that functions via dual mechanisms: primarily enhancing USP5 enzyme activity and concomitantly promoting protein stabilization. This compound potentiates USP5-CPT-1A interaction and activates hepatic CPT-1A. Crucially, LF demonstrates oral bioavailability⁴³, a favorable safety profiles, and dose-dependent amelioration of MASLD histopathology-robustly validating USP5 as a druggable target.

In summary, USP5 is identified as a central guardian of maintaining hepatic lipid metabolism in MASLD, while the hepatoprotective role of USP5 is mediated by CPT-1A. Our findings clearly demonstrate that targeted activating of USP5/CPT-1A axis represents a clinically translatable strategy for counteracting systemically deregulated lipid metabolism in MASLD.

Limitations of the study

First, the mechanism by which USP5 stabilizes CPT-1A may exhibit cell type- or tissue-specificity and could be influenced by contextual factors such as cellular differentiation status. While key causative experiments were validated in an HFHC or CDA-HFD induced MASLD model, further validation across additional experimental systems is necessary to comprehensively evaluate the generality of this regulatory mechanism. Second, although confirming the direct physical interaction between the compound and USP5, the precise conformational details of the binding site remain unresolved. Future studies should employ high-resolution structural techniques such as cryo-electron microscopy (cryo-EM) or X-ray crystallography to facilitate structure-based drug optimization.

Materials and methods

Part of the materials and methods are described in the supplementary file.

Clinical liver biopsy specimens

The patients' paraffin sections for USP5 IHC staining were collected from NingBo No.2 Hospital (Zhejiang, China). The study protocol was approved by Medical Ethics Committee of NingBo No.2 Hospital (No.PJ-NBEY-KY-2026-070-01). The human liver samples for USP5 immunofluorescence that patients with biopsy-proven MASLD were recruited from NingBo No.2 Hospital. All tissue samples were collected in compliance with informed consent policy. Clinical information is summarized in Data S1. Each specimen was evaluated by experienced pathologists without knowledge of the clinical findings.

Mice

Male C57BL/6J mice (6–8 weeks old) were purchased from Shanghai Sippr-Bikai Laboratory Animal Co., LTD. For hepatocyte-specific USP5 knockout, the gRNA to mouse *Usp5* gene, the donor vector containing loxP sites, and Cas9 mRNA were co-injected into fertilized mouse eggs to generate targeted conditional knockout offspring. F0 founder animals were identified by PCR followed by sequence analysis, which were bred to wildtype mice to test germline transmission and F1 animal generation. Breed a F1 targeted mouse with a tissue specific Alb-cre delete mouse to generate F2. Breed heterozygous, Cre⁺ mice with homozygous mice. All operations are entrusted to Cyagen Biosciences (Suzhou) Inc.

All mice were housed in the specific pathogen-free facility of Experimental Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine with a temperature of $25 \pm 2^{\circ}\text{C}$ and a relative humidity of $50 \pm 5\%$. A 12-h on/off lighting cycle was implemented using incandescent lamps. All animal studies were approved by the Animal Research Ethics Committee of Shanghai University of Traditional Chinese Medicine.

Animal models

Mouse MASLD model were established by methionine-choline-deficient (MCD) diet (A02082002BR, Research diets, New Brunseick, USA) for 6 weeks, or HFHC (Rodent Diet with 60 kcal% Fat, 20 kcal% protein, 20 kcal% carbohydrate, D12492i, Research diets, New Brunseick, USA; Sugar water: 42 g/L, fructose : sucrose = 11:9, Fructose and sucrose (F0001/S0001) were purchased from Trophic Animal Feed High-Tech Co., Ltd., China.) for 30 weeks feeding in male C57BL/6J mice (6–8 weeks old). Control mice were administered MCS (A02082002BR, Research diets, New Brunseick, USA) or chow diet (D12450B, Research diets, New Brunseick, USA).

Usp5^{Hep KO} and *Usp5*^{Flox} mice were grouped at the age of 6–8 weeks and fed with HFHC diet for 9 weeks or with CDA-HFD diet for 3 weeks.

For hepatocyte-specific USP5 overexpression, AAV2/8-Vector/AAV2/8-*Usp5* (1×10^{11} genome copies/mouse; Genechem Biotechnology, Shanghai, China) was injected via tail vein to the male mice at the age of 6–8 weeks. The mice were then followed with 12-week HFHC feeding.

For hepatocyte-specific CPT-1A overexpression, AAV2/8-Vector/AAV2/8-*Cpt-1a* (1×10^{11} genome copies/mouse; Genechem Biotechnology, Shanghai, China) was injected via tail vein to the *Usp5*^{Hep KO} and *Usp5*^{Flox} mice at the age of 6–8 weeks. The mice were then followed with 9-week HFHC feeding.

To investigate the effects of LF in established MASLD, 1) mice were fed with HFHC diet for 13 weeks. Thereafter, mice were orally administrated with vehicle or LF (20/40 mg/kg/day, in water) for an additional 6 weeks concurrently with HFHC feeding. 2) mice were fed with CDA-HFD diet for 2 weeks. Thereafter, mice were orally administrated with vehicle or LF (5/20 mg/kg/day, in water) for an additional 4 weeks concurrently with CDA-HFD feeding.

The mouse numbers in each experiment are indicated in figure legends, and the exact mouse numbers are found in the scatter plots within each figure.

Declarations

Competing interests:

The authors disclose no conflicts of interest.

Data, code, and materials availability:

All materials used are available from the corresponding author on request. All data and code needed to evaluate and reproduce the results in the paper are present in the paper and/or the Supplementary Materials. This study did not generate any new materials.

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Author contributions:

Q.F., P.L., GB.G. and QM.S. designed the study. QM.S. performed the experiments and analyzed the data. GH.Z., ST.G., YD.S. and YS.Z. participated in part of the experiments. HS.L. and F.L. provided the human samples. X.X. and RT.L. analyzed part of the RNA-seq data. QM.S., Q.F., and JM.C. wrote the manuscript. Q.F., P.L., YY.H., and C.H. supervised the study.

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Figures

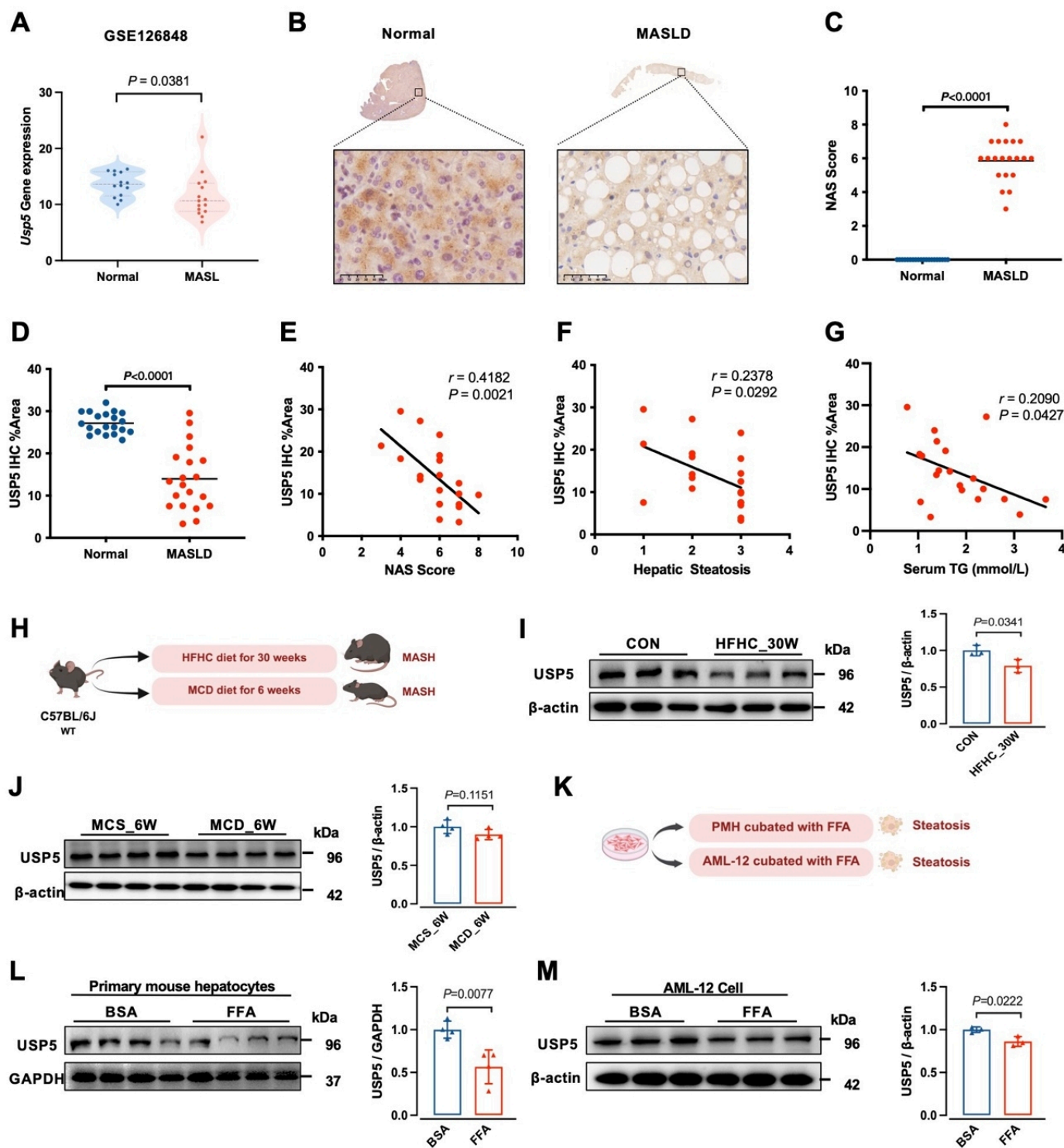


Figure 1

Identification of USP5 as a key regulator of MASLD.

(A) USP5 expression in GEO datasets. (B-D) Representative NAS score, USP5 IHC staining and quantitation in the liver biopsies; scale bar, 50 μm . (E-G) Correlation of USP5 expression with clinical indices, including NAS score, hepatic steatosis, and serum TG. (H) Schematic illustration of MASLD

mouse models. (I-J) Immunoblots of hepatic USP5 expression ($n = 3-4/\text{group}$). (K) Schematic illustration of steatosis cell models. (L-M) Immunoblots of hepatic USP5 expression ($n = 3-4/\text{group}$). Data are mean $\pm$ SD. Unpaired Student's test between two groups. One-way ANOVA between multiple groups. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. See also Figure S1.

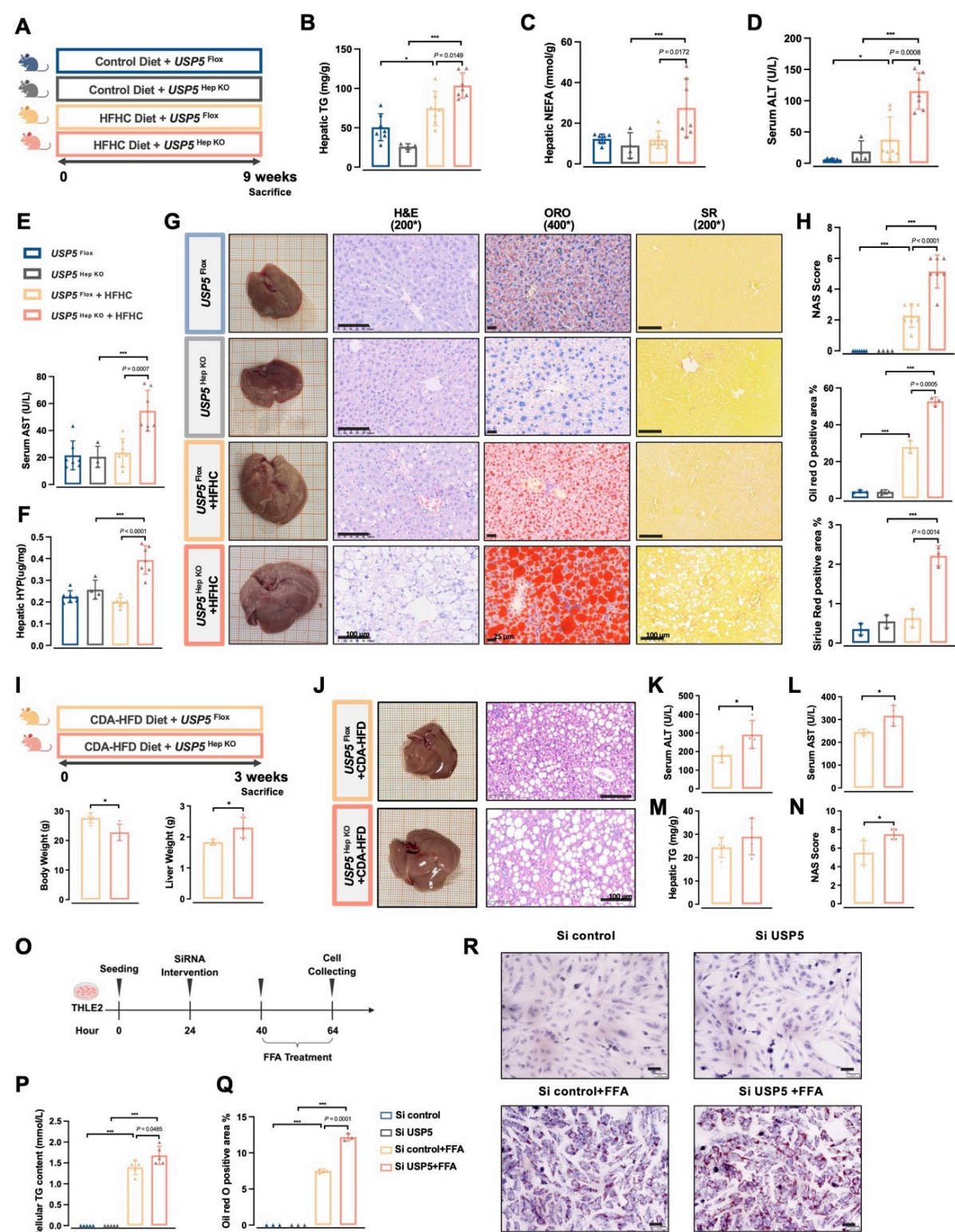


Figure 2

Hepatocyte-specific USP5 knockout exaggerates MASLD in mice.

(A) Schematic illustration ($n = 4-7/\text{group}$). (B) Hepatic TG content. (C) Hepatic NEFA content. (D) Serum ALT activity. (E) Serum AST activity. (F) Hepatic HYP content. (G) Representative H&E staining, oil red O, and Sirius red staining in the liver sections; scale bar, 25/100 μm . (H) NAS score and qualification of oil red O, and Sirius red staining. (I) Schematic illustration, Body weights and Liver weights ($n = 4/\text{group}$). (J) Representative H&E staining in the liver sections; scale bar, 100 μm . (K) Serum ALT activity. (L) Serum AST activity. (M) Hepatic TG content. (N) NAS score. (O) Schematic illustration of SiRNA lentivirus infecting and steatosis cell models constructing. (P) Intracellular TG levels were determined ($n = 5/\text{group}$). (Q-R) Representative oil red O staining in the cells; scale bar, 50 μm . Data are mean $\pm$ SD. Unpaired Student's test between two groups. One-way ANOVA between multiple groups. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. See also Figure S2.

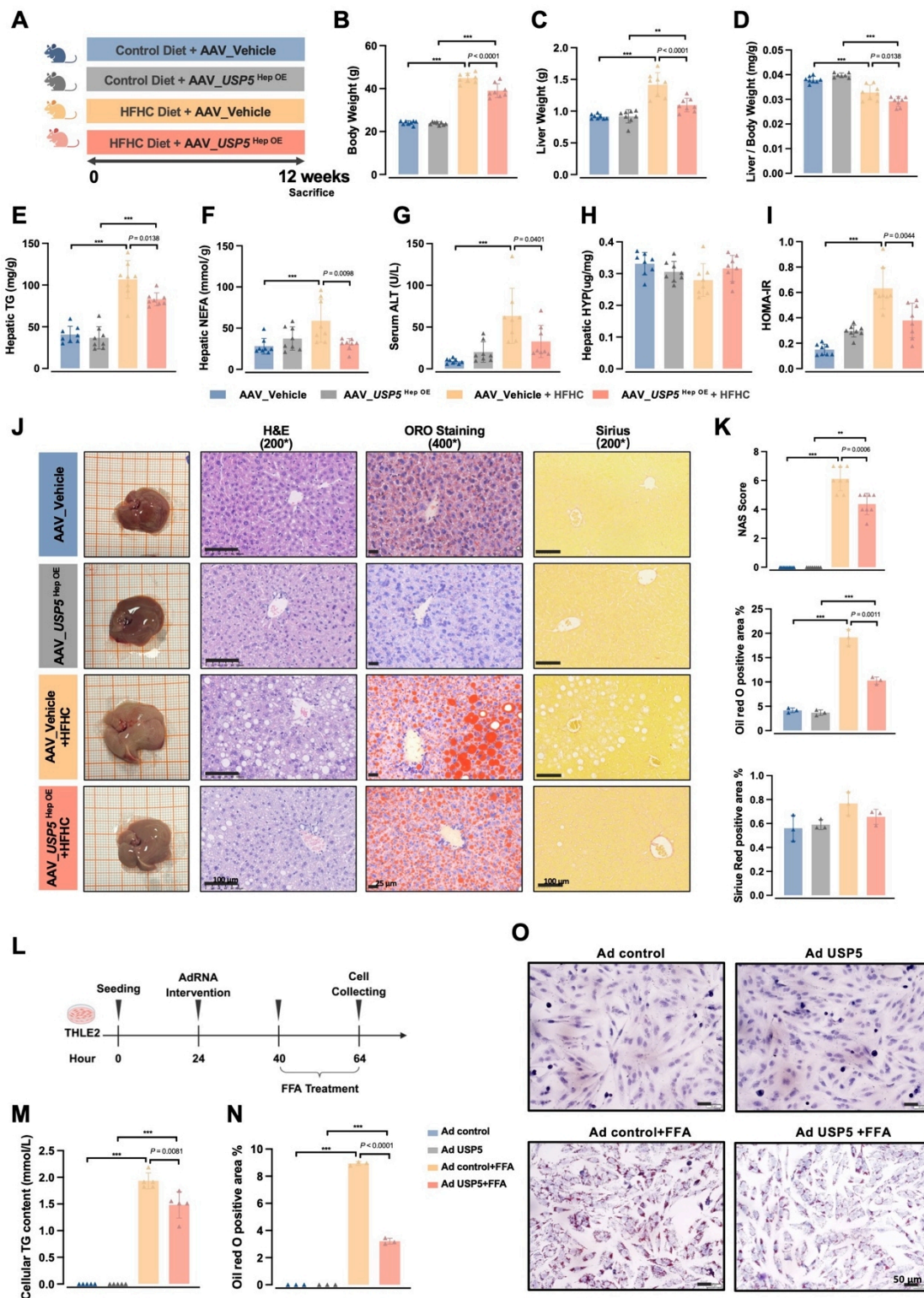


Figure 3

Hepatocyte-specific USP5 overexpression alleviates hepatic lipid accumulation and inflammation in MASLD mice.

(A) Schematic illustration ($n = 8/\text{group}$). (B) Body weights. (C) Liver weights. (D) Liver/Body weights. (E) Hepatic TG content. (F) Hepatic NEFA content. (G) Serum ALT activity. (H) Hepatic HYP content. (I)

HOMA-IR index. (J) Representative H&E staining, oil red O, and Sirius red staining in the liver sections; scale bar, 25/100 μm . (K) NAS score and qualification of oil red O, and Sirius red staining. (L) Schematic illustration of SiRNA lentivirus infecting and steatosis cell models constructing. (M) Intracellular TG levels were determined ($n = 5/\text{group}$). (N-O) Representative oil red O staining in the cells; scale bar, 50 μm . Data are mean $\pm$ SD. Unpaired Student's test between two groups. One-way ANOVA between multiple groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. See also Figure S3.

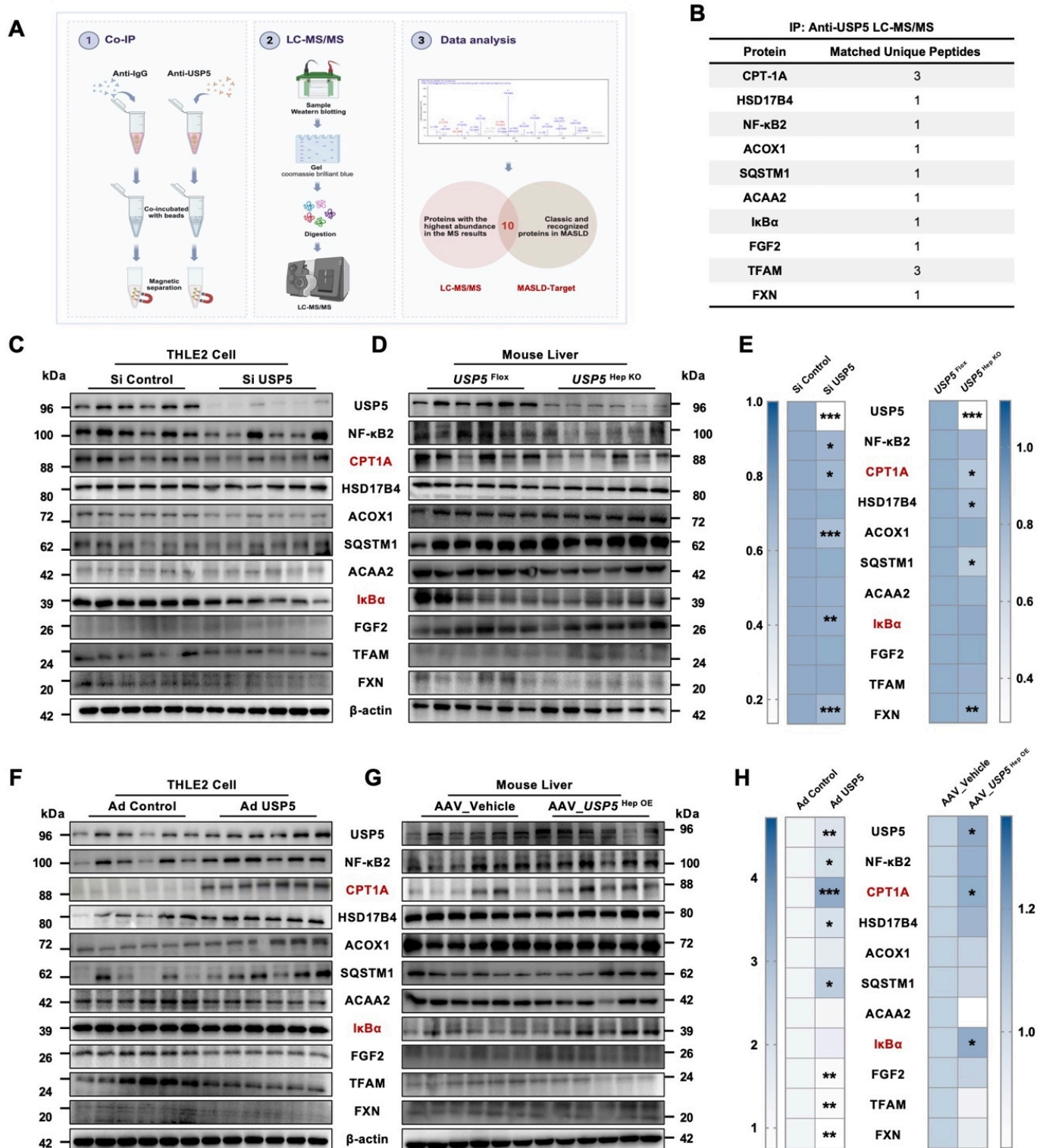


Figure 4

(A) Schematic illustration showing the procedure of identifying the downstream targets of USP5 by the intersection analysis of IP-MS. (B) The 10 most possible proteins. (C-E) Immunoblots and relative protein levels of 10 proteins expression in Si USP5 THLE2 cells and liver of *Usp5*^{Hep KO} mice ($n = 6/\text{group}$). (F-H) Immunoblots and relative protein levels of 10 proteins expression in Ad USP5 THLE2 cells and liver of *Usp5*^{Hep OE} mice ($n = 6/\text{group}$). Data are mean $\pm$ SD. Unpaired Student's test between two groups. One-way ANOVA between multiple groups. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Identification of CPT-1A as a USP5 bonding partner.

domain deletion constructs. (E) Molecular characterization of CPT-1A interaction with different truncated fragments of USP5 in THLE2 cells ($n = 3$). (F) The protein expression of CPT-1A in Si USP5 THLE2 cells treated with protein synthesis inhibitor cycloheximide (CHX; 25 $\mu\text{g}/\text{mL}$) ($n = 3/\text{group}$). (G) The protein expression of CPT-1A in Ad USP5 THLE2 cells treated with CHX (25 $\mu\text{g}/\text{mL}$) ($n = 3/\text{group}$). (H-I) The protein expression of CPT-1A in Si USP5/Ad USP5 THLE2 cells treated with or without MG132 (50 $\mu\text{g}/\text{mL}$) ($n = 3/\text{group}$). (J) Ubiquitination assay of CPT-1A in Si USP5/Ad USP5 THLE2 cells treated with MG132 (50 $\mu\text{g}/\text{mL}$) ($n = 3/\text{group}$). (K) HEK293T cells were transfected with His-CPT-1A and Myc-UB in the presence or absence of FLAG-USP5 and HA-tagged site-specific ubiquitin mutants. Then, IP was performed ($n = 3/\text{group}$). (L) HEK293T cells were transfected with His-CPT-1A and Myc-UB with or without FLAG-USP5 and HA-tagged inactive K6-linked ubiquitin (K6R) mutant. Then, IP was performed ($n = 3/\text{group}$). (M) Schematic illustration of USP5 deubiquitination CPT-1A. Data are mean $\pm$ SD. Unpaired Student's test between two groups. One-way ANOVA between multiple groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

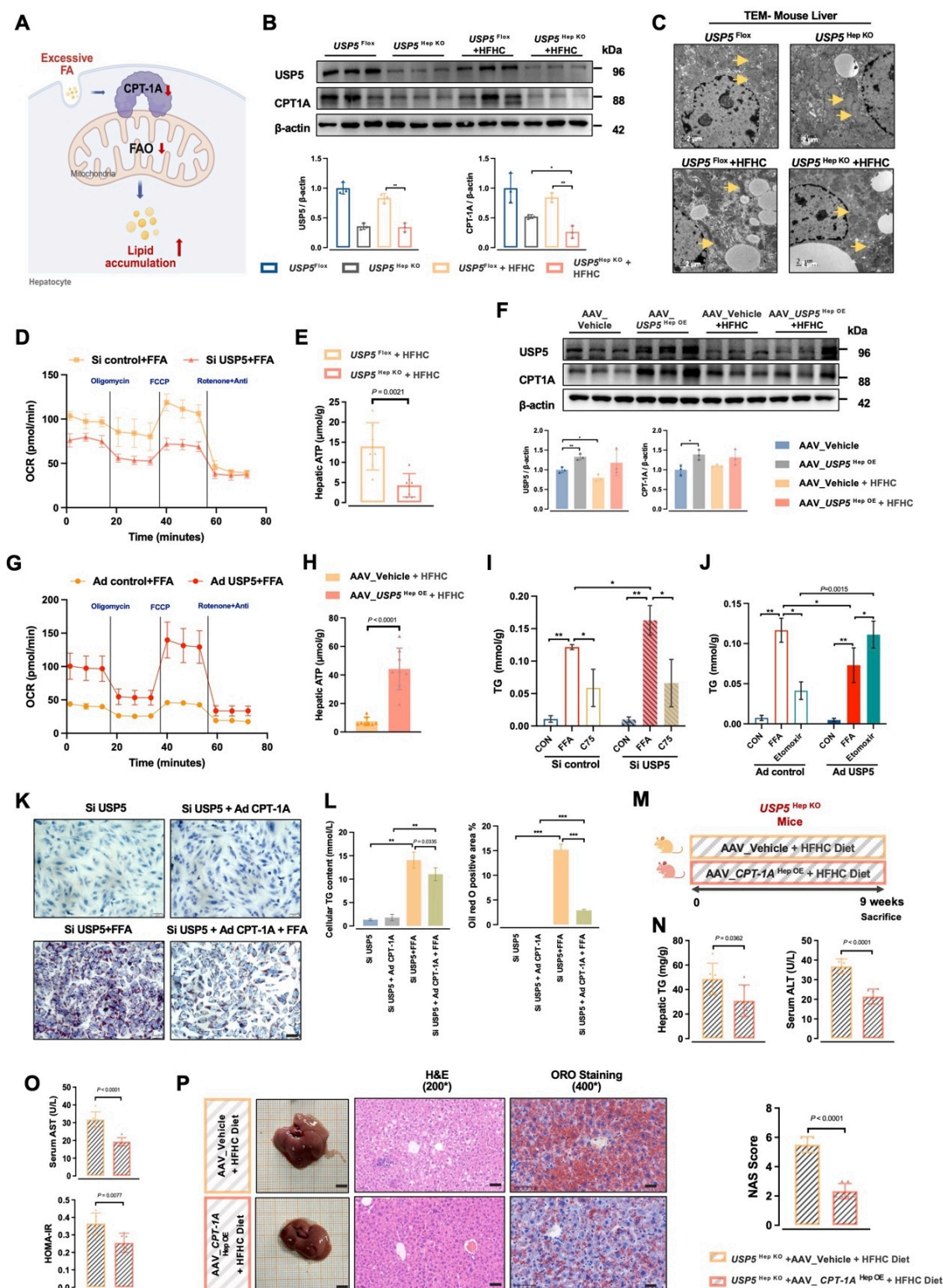


Figure 6

USP5 deficiency in hepatocytes impairs CPT-1A-mediated FAO, and CPT-1A overexpression blocked the effect of USP5-ablation on MASLD.

(A) Schematic illustration showing the excessive FA leading downregulation of CPT-1A-mediated FAO in hepatocyte. (B) Immunoblots protein levels of CPT-1A in liver of 9-weeks-HFHC-feeding *Usp5*^{Hep KO} mice

($n = 3/\text{group}$). (C) TEM of mitochondria in liver of 9-weeks-HFHC-feeding *Usp5*^{Hep KO} mice. (D) OCR analysis of Si USP5 THLE2 cells treated with or without FFA ($n = 5/\text{group}$). (E) ATP level in liver of 9-weeks-HFHC-feeding *USP5*^{Hep KO} mice ($n = 7/\text{group}$). (F) Immunoblots protein levels of CPT-1A in liver of 12-weeks-HFHC-feeding *USP5*^{Hep OE} mice ($n = 3/\text{group}$). (G) OCR analysis of Ad USP5 THLE2 cells treated with or without FFA ($n = 5/\text{group}$). (H) ATP level in liver of 12-weeks-HFHC-feeding *USP5*^{Hep OE} mice ($n = 8/\text{group}$). (I) Intracellular TG levels were determined in FFA-induced Si USP5 THLE2 cells treated with Ad C75 (activator of CPT-1A) ($n = 6/\text{group}$). (J) Intracellular TG levels were determined in FFA-induced Ad USP5 THLE2 cells treated with Ad Etomoxir (inhibitor of CPT-1A) ($n = 6/\text{group}$). (K-L) Intracellular TG levels and oil red O staining were determined in FFA-induced Si USP5 THLE2 cells trans-infected with Ad CPT-1A ($n = 6/\text{group}$). (M) Schematic illustration ($n = 6/\text{group}$). (N) Hepatic TG content and serum ALT activity. (O) Serum AST activity and HOMA-IR index. (P) NAS score and Representative liver and H&E staining; scale bar, 50 μm . Data are mean $\pm$ SD. Unpaired Student's test between two groups. One-way ANOVA between multiple groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. See also Figure S5.

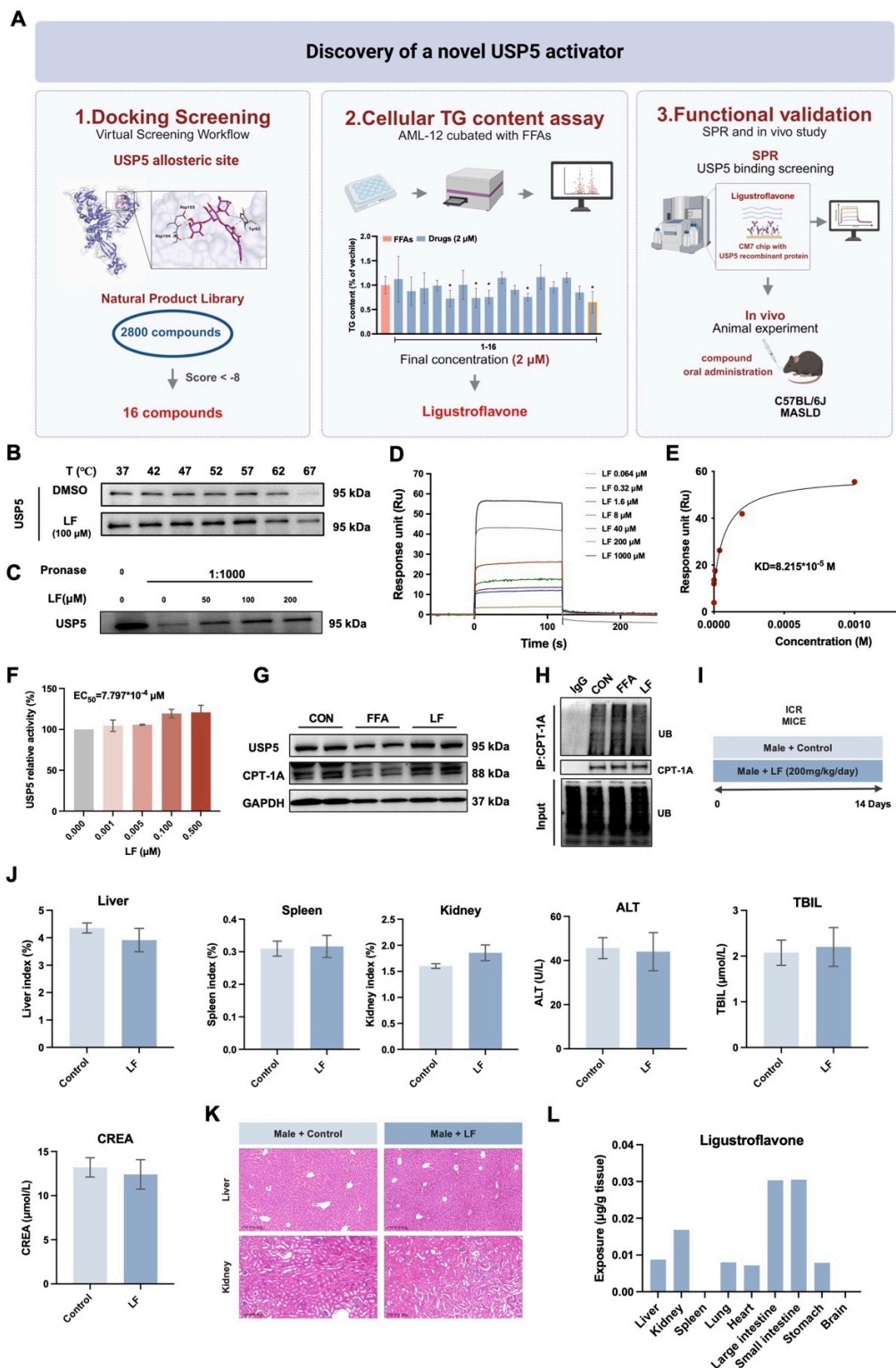


Figure 7

Ligustroflavone was identified as an activator of USP5.

(A) High-throughput virtual screening workflow for lead compound identification and pharmacological validation. (B) DARTS assay of LF on USP5. (C) CESTA assay of LF on USP5. (D-E) SPR analysis was used to determine the direct interaction between LF and USP5. (F) The relative activity of LF on USP5.

(G) Immunoblots protein levels of USP5 and CPT-1A in FFA-induced AML-12 cells treated with LF. (H) Ubiquitination assay of CPT-1A in FFA-induced AML-12 cells treated with LF. (I) Schematic illustration ($n = 4/\text{group}$). (J) Liver, spleen, kidney index and serum ALT, TBIL, CREA level. (K) Representative H&E staining in the liver and kidney sections; scale bar, 200 μm . (L) The exposure of LF in different organs. Data are mean $\pm$ SD. Unpaired Student's test between two groups. One-way ANOVA between multiple groups. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. See also Figure S6.

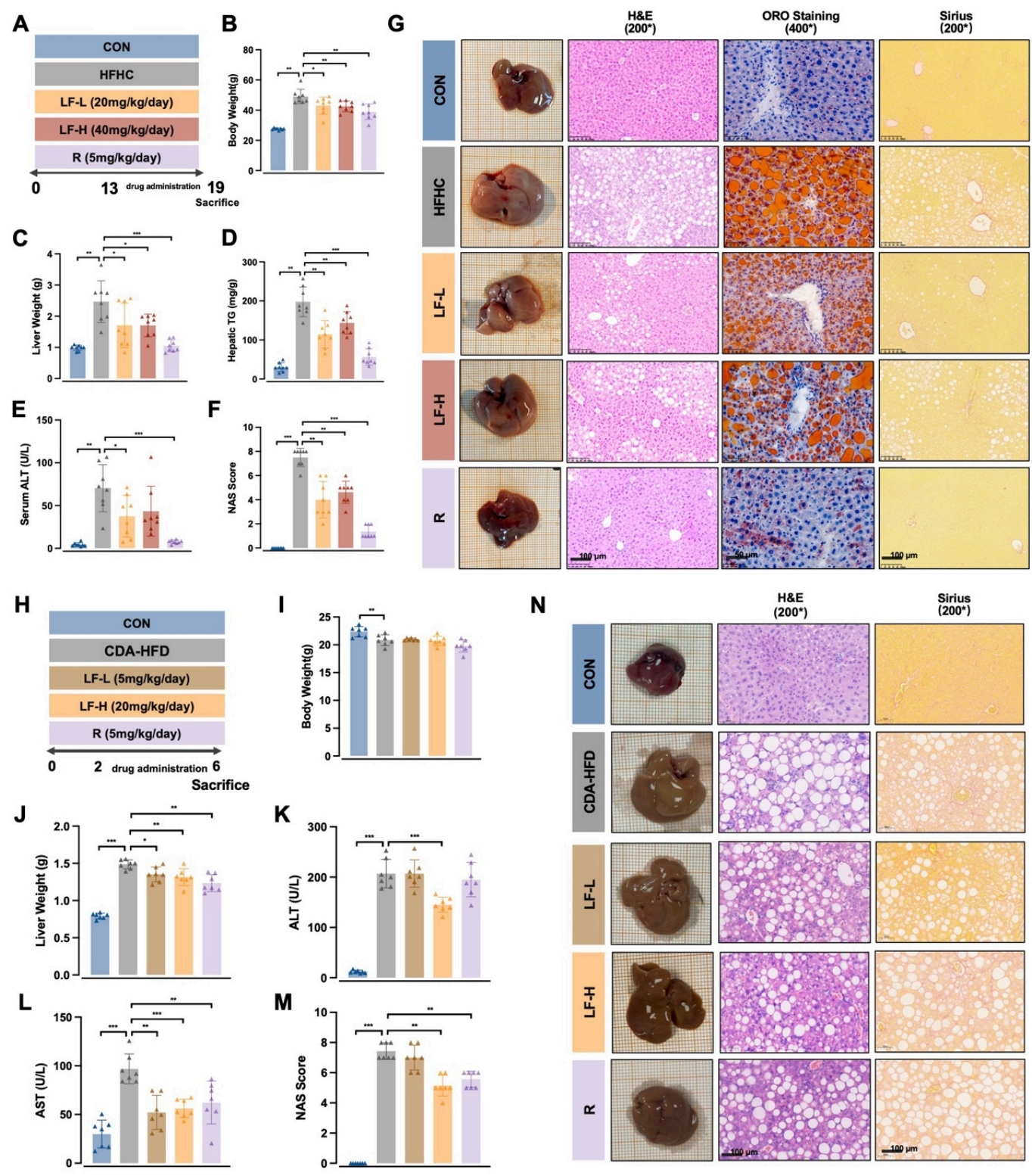


Figure 8

Ligustroflavone significantly alleviates lipid accumulation and inflammation in MASLD mice.

(A) Schematic illustration ($n = 8/\text{group}$). (B) Body weights. (C) Liver weights. (D) Hepatic TG content. (E) Serum ALT activity. (F-G) NAS score and representative H&E staining, oil red O, and Sirius red staining in the liver sections; scale bar, 50/100 μm . (H) Schematic illustration ($n = 7/\text{group}$). (I) Body weights. (J) Liver weights. (K) Serum ALT activity. (L) Serum AST activity. (M-N) NAS score and representative H&E staining and Sirius red staining in the liver sections; scale bar, 100 μm . Data are mean $\pm$ SD. Unpaired Student's test between two groups. One-way ANOVA between multiple groups. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. See also Figure S6.

Supplementary Files

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- [Supplementarymaterials.docx](#)
- [Onlinefloatimage1.png](#)