

Tripartite motif 46 aggravates MASLD progression by suppressing SGPL1- mediated hepatic lipophagy

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Abstract

Metabolic dysfunction-associated steatotic liver disease (MASLD) is an important pathogenic factor in end-stage liver diseases such as cirrhosis and liver cancer, and the central basis of MASLD pathogenesis is the dysregulation of hepatic lipid metabolism. Ubiquitination modification plays a vital role in regulating hepatic lipid metabolic homeostasis and the pathogenesis of MASLD. Understanding the regulatory factors and mechanisms of ubiquitination in MASLD progression could aid in developing precise therapeutic strategies and targets. In this study, we identified a positive correlation between E3 ubiquitin ligases TRIM46 expression and MASLD progression through comprehensive multi-level screening of RNA-Seq data from mouse models and publicly available MASLD patient databases. Subsequently, we demonstrated that overexpression of TRIM46 exacerbated hepatic lipid dysmetabolism, inflammatory responses and hepatic fibrosis in a mouse model, whereas depletion of TRIM46 attenuated these pathological changes. Mechanically, multiomics analysis showed that hepatocyte TRIM46 directly binds to SGPL1 and facilitates degradation of SGPL1 via K48-linked polyubiquitination at the K107 and K142 sites, which subsequently suppresses the activation of downstream lipophagy signaling cascades in MASLD progression. Our findings identify TRIM46 as a novel accelerator of pathological MASLD and indicating that targeting the TRIM46-SGPL1 axis and represents a promising therapeutic strategy for MASLD in the clinic.

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as non-alcoholic fatty liver disease (NAFLD), has emerged as the most prevalent chronic liver disease globally ¹⁻³. MASLD is not only a major reason for end-stage liver conditions, including liver failure and HCC, but also a significant pathogenic contributor to cardiovascular events, chronic kidney disease, hepatic and extrahepatic malignancies ^{4,5}. However, current therapeutic strategies predominantly focus on lifestyle and dietary modifications, with weight reduction being a primary objective ^{3,6}. Only two pharmacological drugs, resmetirom and semaglutide, have received approval from the United States Food and Drug Administration (FDA) for the treatment of MASLD, both of which demonstrate limited efficacy and are associated with significant side effects ^{7,8}. Consequently, there is an urgent medical need to identify target-based therapeutic strategies for MASLD.

MASLD is characterized by aberrant deposition of triglycerides and other neutral lipids in intracellular organelles known as lipid droplets (LDs) ⁹. Extended accumulation of intrahepatic triacylglycerol (TAG) may lead to hepatic insulin resistance and damage via lipid peroxidation, which could potentially initiate the development of metabolic-associated steatohepatitis (MASH), a condition marked by inflammation and fibrosis ¹⁰. Autophagy has been recognized as a pivotal mechanism in facilitating lipid catabolism and alleviating hepatic steatosis ^{11, 12}. Concurrently, SGPL1, a key enzyme within the autophagy pathway, degrades S1P, thereby reducing mTOR activation and relieving autophagy inhibition. This process also generates phosphoethanolamine, which enhances phosphatidylethanolamine (PE) synthesis and LC3

lipidation, crucial for autophagosome formation^{13,14}. Lipophagy, a selective autophagic process responsible for the degradation of LDs in the lysosomes, provides free fatty acids (FFAs) for energy production in hepatocytes via β -oxidation¹⁵. Accumulating evidence shows that lipophagy as a central regulator of hepatic lipid homeostasis^{16,17}. Impaired lipophagy disrupts fatty acid oxidation (FAO), promotes hepatic steatosis, and accelerates the progression of MASLD from simple lipid accumulation to inflammatory and fibrotic stages^{12,18,19}. As an important post-translational modification (PTM) way, the Ubiquitin system that plays a crucial role in regulating hepatic lipid metabolism dysregulation and the pathogenesis of MASLD²⁰. This system is also critical in mediating lipophagy²¹, however the key ubiquitin ligases involved in this process remain elusive.

To this end, our multi-level systemic screening followed by functional verification identified a positive correlation between E3 ubiquitin ligase TRIM46 expression and the progression of MASLD. Tripartite motif (TRIM) family is the largest family of conserved RING-type E3 ligases, containing an N-terminal RING-finger domain, one or two B-box domains, and a coiled-coil (CC) domain. RING finger structure is critical for E3 ubiquitin ligases activity^{22–24}. TRIM proteins participate in various cellular processes, including intracellular signaling, apoptosis, protein quality control, immune responses, autophagy, carcinogenesis and metabolic regulation^{25–30}. As key executors of the ubiquitin-proteasome pathway, TRIM46 promotes the malignant behaviors of various tumors^{22,25}. TRIM46 also regulates neuronal polarity and Paraneoplastic neurological syndromes through ubiquitination^{31,32}. Furthermore, TRIM46 can aggravate high glucose or influenza virus-induced inflammatory response^{33,34}. Nevertheless, the precise role and molecular mechanism of TRIM46 in MASLD remain largely unknown.

In the current study, we identified TRIM46 as a significant and fundamental driver of MASLD both in vivo and in vitro models. Mechanistically, we elucidated that the TRIM46-SGPL1 axis constitutes a crucial molecular network involved in the regulation of hepatic lipophagy, thereby contributing to the pathogenesis of MASLD: TRIM46 directly interacts with SGPL1 and facilitates ubiquitin-mediated degradation of SGPL1 through K48-linked polyubiquitination, which subsequently suppresses SGPL1-mediated hepatic lipophagy, ultimately driving MASLD progression.

Results

TRIM46 expression is up-regulated in MASLD Model

To identify the most significant E3 ligase regulating MASLD, liver tissues from mice with MASLD and normal controls, which were fed either a HFHC diet or a normal diet for 16 weeks, were selected for RNA-sequencing analysis (Fig. 1A). A substantial number of differentially expressed genes are depicted in Fig. 1B. Notably, 109 differentially expressed genes encoding E3 ubiquitin ligases were identified, with the top 10 E3 ligase genes presented in Fig. 1C. Subsequently, we analyzed the expression of these top 10 E3 ligases using publicly available MASLD patient databases (GSE213621 and GSE135251). Our analysis revealed a significant upregulation of four E3 ligases—*TRIM46*, *CBL*, *CUL1* and *CUL2*—in

individuals with MASLD compared to healthy controls (Fig. 1D). To identify the most critical genes, we examined the impact of these genes on lipid droplet accumulation under PA/OA challenge using a BODIPY staining assay. Remarkably, the overexpression of TRIM46 significantly exacerbated lipid accumulation in hepatocytes (Fig. 1E). Moreover, H&E staining revealed an increase in lipid deposition in MASLD patients; concurrently, immunohistochemistry assays confirmed a marked elevation of TRIM46 levels in liver tissue sections from MASLD patients relative to healthy individuals (Fig. 1F). These observations were further substantiated by data obtained from a HFHC diet-induced mouse model of MASLD (Fig. 1G) and PA/OA-challenged primary mouse hepatocytes (Fig. 1H). The collective data suggest that the upregulation of TRIM46 is implicated in the progression of MASLD and that TRIM46 may play a significant role in the pathogenesis of MASLD.

To further elucidate the molecular mechanisms responsible for the upregulation of TRIM46 in MASLD, the Find Individual Motif Occurrences (FIMO) tool was utilized to predict transcription factors (TFs) that bind to the TRIM46 promoter in both human and mice. This analysis identified two potential upstream TFs: cAMP responsive element binding protein 3 (CREB3) and Early Growth Response 1 (EGR1), as depicted in Fig. 1I. Subsequent dual-luciferase reporter assays demonstrated that CREB3 is capable of inducing activation of the full TRIM46 promoter (Fig. 1J). In alignment with these findings, quantitative PCR (qPCR) analysis verified an upregulation of TRIM46 in hepatocytes overexpressing CREB3, whereas CREB3 knockdown resulted in a decreased mRNA level of TRIM46 (Fig. 1K). Moreover, luciferase reporter assays combined with chromatin immunoprecipitation (Ch-IP) experiments identified an interaction between CREB3 and the region spanning – 950 to –800 base pairs of the TRIM46 promoter (Fig. 1L, 1M). Mutation of the FIMO tool-predicted binding site within this region (–843 to –831 bp) significantly inhibited CREB3's effect on TRIM46 promoter activation (Fig. 1N). Additionally, CREB3 expression was found to be upregulated in tissue slices from MASLD patients, hepatic tissue from mice on a HFHC diet, and primary mouse hepatocytes challenged with PA/OA (Fig. 1O–1Q). These results suggest that CREB3 functions as an upstream regulator of TRIM46, facilitating the upregulation of TRIM46 mRNA expression during the progression of MASLD.

TRIM46 overexpression by adeno-associated virus exacerbates HFHC-induced MASLD

In light of the pronounced upregulation of *TRIM46* in MASLD, we postulated that TRIM46 plays a pivotal role in the progression of MASLD. As depicted in Fig. 2A and 2B, we initially generated Trim46-overexpressing mice by transfecting them with an adeno-associated virus 8 (AAV8) vector carrying the Trim46 gene (AAV8-Trim46). These mice were subsequently subjected to a HFHC diet for a duration of 16 weeks. Compared to the vector control group, the *Trim46*-overexpressing mice demonstrated significant increases in body weight (Fig. 2C), liver weights, and liver weight-to-body weight ratios (Fig. 2D) following 16 weeks on the HFHC diet. Histological analysis revealed that, in contrast to the AAV8-vector control group, the *Trim46*-overexpressing group exhibited significantly greater hepatic lipid accumulation, inflammatory cell infiltration, and fibrosis (Fig. 2E). Concurrently, hepatic concentrations

of TG, TC, and NEFA were markedly elevated compared to those in the control mice after 16 weeks of HFHC feeding (Fig. 2F). Furthermore, relative to the control mice, the hepatic *Trim46*-overexpressing mice showed an exacerbation of the HFHC-induced increases in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Fig. 2G). In terms of mRNA expression, the overexpression of *Trim46* resulted in a significant suppression of genes associated with fatty acid oxidation (Fig. 2I), while it did not notably influence genes related to fatty acid absorption or lipogenesis (Fig. 2H). Corresponding with these gene expression patterns, mice overexpressing *Trim46* demonstrated a marked increase in hepatic inflammation and fibrosis relative to control mice (Fig. 2J-L). These observations strongly indicate that TRIM46 overexpression aggravates MASLD in the context of HFHC diet.

Hepatocyte-specific Trim46 deletion ameliorates HFHC-induced MASLD

To investigate the *in vivo* role of hepatic *Trim46*, male hepatocyte-specific *Trim46*-knockout (*Trim46*-HKO) mice were generated by utilizing male Rosa26 (CAG-LSL-Cas9-KI) mice in conjunction with AAV8-U6-sg *Trim46*-TBG-Cre infection. These mice were subjected to a HFHC diet for 16 weeks alongside their control counterparts (Fig. 3A). Western blot analyses confirmed the targeted deletion of *Trim46* in hepatic tissue (Fig. 3B). In comparison to control mice, *Trim46*-HKO mice exhibited marked reductions in body weight (Fig. 3C), liver weight, and liver weight-to-body weight ratio after 16 weeks on the HFHC diet (Fig. 3D). Histological examination revealed that *Trim46*-HKO mice on the HFHC diet showed a further decrease in hepatic steatosis, infiltration of inflammatory F4/80 + cells, and attenuated hepatic fibrosis (Fig. 3E). Additionally, *Trim46*-HKO mice displayed significantly lower hepatic TG, TC, and NEFA levels compared to control mice after 16 weeks on the HFHC diet (Fig. 3F). Furthermore, serum TG and TC concentrations, as well as serum markers of hepatic injury, were markedly reduced in *Trim46*-HKO mice relative to control mice following 16 weeks of HFHC diet administration (Fig. 3G). In alignment with these findings, the mRNA expression levels of genes associated with fatty acid uptake and synthesis did not exhibit any significant differences between *Trim46*-HKO mice and control mice following a 16-week HFHC diet (Fig. 3H). Conversely, the expression of genes related to fatty acid oxidation was markedly upregulated in *Trim46*-HKO mice compared to their control counterparts after the same dietary regimen (Fig. 3I). Consistent with these histological observations, *Trim46*-HKO mice demonstrated reduced levels of HFHC-induced hepatic inflammation and fibrosis (Fig. 3J-L). Collectively, these results indicate that *Trim46*-HKO mitigates HFHC-induced lipid accumulation, hepatic inflammation, fibrosis, and liver injury, thereby suggesting that TRIM46 plays a pivotal role in hepatocytes during HFHC-induced MASLD.

TRIM46 directly interacts with SGPL1

Considering the critical function of TRIM46 in the progression of MASLD, we sought to elucidate the molecular mechanism by which TRIM46 accelerates this condition. We conducted a comprehensive analysis combining immunoprecipitation mass spectrometry (IP-MS) with quantitative ubiquitinomics to

identify potential targets of TRIM46 in *TRIM46*-overexpressing hepatocytes stimulated by PA/OA (Fig. 4A). Volcano plots identified differentially ubiquitination proteins and expressed proteins regulated by TRIM46 (Fig. 4B, 4C). Through the integrated analysis of these datasets, encompassing TRIM46-binding proteins, down-regulated proteins, and proteins with increased ubiquitination, we identified five potential targets of TRIM46: SGPL1, HNRNPUL2, ITPR1, PPIA, and ALG5 (Fig. 4D). Among these candidates, SGPL1 exhibited a maximum increase in the expression in the TRIM46-overexpressing hepatocytes (Fig. 4E). Consistent with this finding, SGPL1 was further validated as the most significantly altered protein in *TRIM46*-knockdown (Sh*TRIM46*) hepatocytes, as demonstrated by western blot analysis (Fig. 4F). Given the inhibitory role of TRIM46 in modulating SGPL1-associated signaling pathways, our in vitro and in vivo findings prompted us to investigate a crucial question: the mechanism by which TRIM46 interacts with SGPL1 within the context of MASLD progression. As anticipated, the results from exogenous co-immunoprecipitation (CO-IP) assays and GST-pulldown experiments demonstrated that TRIM46 can directly bind to SGPL1 and vice versa in transfected human HepG2 cells (Fig. 4G, 4H). Furthermore, endogenous CO-IP assays and confocal microscopy corroborated the direct interaction between TRIM46 and SGPL1 (Fig. 4I, 4J). These findings strongly suggest that TRIM46 directly interacts with SGPL1. Notably, Co-IP analysis of the binding regions between SGPL1 and TRIM46, found that the PDCD domain of SGPL1 plays a critical role in its interaction with TRIM46 (Fig. 4K), and the SPRY domain of TRIM46 is essential for its interaction with SGPL1 (Fig. 4L).

TRIM46 facilitates degradation of SGPL1 via K48-linked polyubiquitination

In our study, SGPL1 was identified as a TRIM46-binding protein and a potential candidate in ubiquitinomics. Based on these findings, we hypothesized that TRIM46 may facilitate the degradation of SGPL1 via the ubiquitin-proteasome pathway. Subsequently, we examined the impact of activated TRIM46 on the stability of the SGPL1 protein. Notably, overexpression of *Trim46* significantly accelerated the degradation of Sgpl1, whereas knockdown of *Trim46* markedly inhibited this process in primary hepatocytes from mice (Fig. 5A, 5B). Furthermore, TRIM46 overexpression exacerbated the degradation of SGPL1 induced by cycloheximide (CHX) (Fig. 5C), whereas the effect was reversed by the proteasome inhibitor MG132 (Fig. 5D). Given that nearly all proteasome substrates are targeted for ubiquitination-mediated degradation, we further verified that lipotoxicity (induced by PA/OA treatment) enhanced the expression of TRIM46 protein and SGPL1 ubiquitination in hepatocytes (Fig. 5E). Additionally, we discovered that TRIM46 overexpression could significantly promote K48-type ubiquitination of SGPL1 (Fig. 5F, 5H). In contrast, hepatocytes transfected with Ad-sh*Trim46* exhibited impaired ubiquitination of SGPL1 (Fig. 5G).

To determine potential TRIM46-catalyzed lysine sites, we engineered the mutant variants of SGPL1 at the lysine sites of K107 and K342 (SGPL1^{K107R/K342R/2KR}) based on the TRIM46 ubiquitination omics analysis results (Fig. 5I). We found the combined mutation (2KR) completely abolished TRIM46-mediated the degradation and K48-type ubiquitination of SGPL1, indicating that K107 and K342 sites

were the specific residues responsible for TRIM46-catalyzed ubiquitination of SGPL1 under PA/OA treatment (Fig. 5J, 5K). In addition, we analyzed the amino acid sequences of the TRIM46 RING domain across multiple species and have highlighted two highly conserved motifs, TRIM46 (C³³XXC³⁶) and TRIM46 (C⁴⁸XXXXC⁵³), within the RING domain (Fig. 5L). Given the strong correlation between SGPL1 degradation and the catalytic activity of the TRIM46 RING domain, we sought to further confirm whether the two mutant motifs of the TRIM46 RING domain were indispensable for its inhibitory role in SGPL1 ubiquitination. To achieve this, we generated two separate TRIM46 mutants (C³³R/C³⁶R and C⁴⁸R/C⁵³R) and co-transfected them with the indicated plasmid constructs into HepG2 cells. Our study demonstrated that both TRIM46 wild type (WT) and TRIM46 mutant (C⁴⁸R/C⁵³R) were capable of promoting the degradation and K48-type ubiquitination of SGPL1, whereas TRIM46 mutant (C³³R/C³⁶R) lost this function (Fig. 5M, N), suggesting that TRIM46 (C³³XXC³⁶) motif within the RING domain was responsible for its ubiquitination activity on SGPL1. These findings were further corroborated by lipid accumulation assays in overexpression-*TRIM46* or *TRIM46* mutants HepG2 hepatocytes following PA/OA treatment (Fig. 5O). Collectively, these above findings indicate that the TRIM46-mediated ubiquitination and subsequent degradation of SGPL1 dependent on its E3 ubiquitin ligase activity.

TRIM46 inhibits hepatic lipophagy through degrading SGPL1

To further explore the underlying pathway by which TRIM46 facilitates the degradation of SGPL1 in the regulation of MASLD, we conducted a comparative proteomics analysis on hepatocytes overexpressing TRIM46 and control hepatocytes (vector) under lipotoxic conditions. Enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA) indicated that proteins downregulated by TRIM46 were significantly enriched in the top 20 predominant pathways, among which the autophagy signaling pathway possessed the highest enrichment score (Fig. 6A, 6B). Lipophagy, a specialized form of autophagy targeting LDs, involves the sequestration of LDs within autophagosomes, followed by their transport to lysosomes for degradation, thereby releasing free fatty acids for subsequent oxidation and energy production. Given SGPL1 modulates autophagy by catalyzing the irreversible degradation of sphingosine-1-phosphate (S1P) and facilitating phosphatidylethanolamine (PE) production^{14, 35}, we reasoned that TRIM46 aggravates MASLD by suppressing SGPL1-mediated lipophagy.

In subsequent analyses, we further confirmed the in vivo and in vitro experimental results. We observed a considerable reduction in autophagic flux, as evidenced by reduced expression of the autophagosome marker Lc3b-II, lysosome marker Lamp1, and Sgpl1, alongside increased accumulation of the autophagy substrate p62 and LD marker Plin2 in *Trim46*-overexpressing mice or hepatocytes compared to Vector (control) mice or hepatocytes under metabolic stress (Fig. 6C, 6E). Conversely, *TRIM46*-HKO or *TRIM46* knock-down resulted in enhanced autophagic flux (Fig. 6D, 6F). We also found that the number of autophagosomes in liver tissues of *Trim46*-overexpressing mice was significantly decreased compared to that in Vector mice under HFHC diet (**Supplementary Fig. S1A**), but was enhanced in *TRIM46*-HKO

mice (**Supplementary Fig. S1B**). These findings suggest that TRIM46 exacerbates the accumulation of LDs by inhibiting autophagic flux.

To further validate the involvement of TRIM46 in lipophagy, we conducted co-staining of primary hepatocytes from mice with antibodies against LC3B (to label autophagosomes) and Lamp1 (to label lysosomes), in conjunction with BODIPY (to label LDs). Remarkably, the results demonstrated that colocalization of LC3B and Lamp1 with LDs was decreased in hepatocytes overexpressing TRIM46 under PA/OA-treatment (Fig. 6G, 6H). In contrast, knockdown of TRIM46 significantly enhanced the colocalization of LDs with autophagosomes and lysosomes (Fig. 6I, 6J). Subsequently, we performed OCR assay to measure fatty acid β -oxidation rates. The OCR was significantly decreased by *Trim46* overexpression but increased by *Trim46* knock-down in primary hepatocytes under PA/OA treatment (Fig. 6K, 6L). And the expressions of fatty acid β -oxidation related factors were also significantly decreased by *Trim46* overexpression but inhibited by *Trim46* ablation (Fig. 2I, 3I). Furthermore, analysis of MASLD patient databases (GSE135251) showed a negative correlation between *TRIM46* expression and the fatty acid β -oxidation pathway or its key genes (*ACOX1* and *ACADM*), whereas *SGPL1* expression was positively correlated with these factors (**Supplementary Fig. S2A-S2F**).

In conclusion, our findings summarized in Fig. 6M elucidate the role of TRIM46 as a pivotal regulator of SGPL1 proteasomal degradation during the pathological progression of MASLD. Specifically, the liver-enriched E3 ubiquitin ligase TRIM46 facilitates the interaction with SGPL1, promoting its degradation through enhanced polyubiquitination. This process leads to the inactivation of downstream SGPL1-mediated lipophagy, ultimately driving hepatic lipid accumulation and exacerbating steatohepatitis. Our study highlights the TRIM46-SGPL1 axis as a promising therapeutic target for MASLD and provides a theoretical framework for the development of pharmacological interventions against this globally prevalent metabolic disorder.

SGPL1 is required for TRIM46-induced MASLD progression

To demonstrate the contribution of SGPL1 to the TRIM46-mediated regulation of MASLD progression, we utilized SGPL1 inhibitor 2-acetyl-5-tetrahydroxybutyl imidazole (THI) to inhibit its activity in *Trim46*-HKO and Control mice fed a HFHC diet (Fig. 7A). Our results indicated that SGPL1 inhibitor THI significantly reduced autophagic flux (Fig. 7B), and robustly exacerbated the diet-induced increases in liver weight, hepatic steatosis, inflammatory cell infiltration, liver fibrosis and liver injury in control mice (Fig. 7C-7F). Moreover, THI diminished hepatic fatty acid oxidation and enhanced the expression of genes related to inflammation and fibrosis, as well as the activation of their signaling pathways in control mice (Fig. 7G-7K). Remarkably, THI largely negated the improvements in autophagic flux and MASLD phenotype triggered by *Trim46*-HKO (Fig. 7B-7K). Additionally, we utilized THI to the *Trim46* knock-down primary hepatocytes under PA/OA treatment. THI abrogated the promoting effect of *Trim46* knock-down on autophagic flux, lipophagy and fatty acid oxidation, while exacerbating the accumulation of LDs in primary hepatocytes (**Supplementary Fig. S3A-S3F**). These findings further substantiate the role of TRIM46 in accelerating MASLD progression.

Discussion

MASLD involves the abnormal accumulation of various lipids in the liver (such as triglycerides, free cholesterol and saturated fatty), leads to inflammation, cellular dysfunction, and cell death and potentially initiating MASH³⁶. While the pathogenesis of MASLD remains a topic of ongoing debate, there is a broad consensus that the compromised ability of hepatocytes to metabolize lipids plays a pivotal role in the onset and progression of MASLD²⁰. And mounting evidence has confirmed that ubiquitination modification functions as a critical regulator of hepatic lipid metabolic homeostasis and the pathogenesis of MASLD. To identify crucial ubiquitin-related molecules that can regulate lipid metabolic homeostasis, we conduct comprehensive multi-omics screening analysis, finding that a positive link between TRIM46 expression and MASLD progression.

TRIM46 has been documented as a destructive molecule through E3 ligase activity. TRIM46 activates AKT/HK2 signaling by modifying PHLPP2 ubiquitylation, thereby enhancing glycolysis and chemoresistance in lung cancer cells²². Moreover, TRIM46 interacts with I κ B α to activate the NF- κ B signaling pathway, thereby enhancing cell proliferation inhibition, hyper permeability and the inflammatory response³³. In our study, we also show that TRIM46 disrupted metabolic homeostasis and exerts pro-inflammatory effects, aggravated MASLD progression. Notably, hepatocyte-specific *Trim46* overexpression significantly exacerbated liver steatosis, hepatic inflammation, and fibrosis phenotypes. Conversely, *Trim46*-HKO markedly attenuated MASLD-associated pathological alterations in the liver. Multi-omics analysis to demonstrate that TRIM46 aggravated MASLD by promoting ubiquitination-dependent degradation of SGPL1, thereby inhibiting the activation of lipophagy signaling pathway. Interestingly, the conserved TRIM46 (C³³XXC³⁶) motif within the RING domain was responsible for TRIM46-catalyzed ubiquitination of SGPL1. The result suggests that the RING domain plays a crucial role in regulating the enzymatic activity of TRIM46 on downstream SGPL1 signalling in the context of steatohepatitis, and the conserved TRIM46 (C³³XXC³⁶) motif within the RING domain could serve as a specific target for inhibiting the development of MASLD and its related metabolic syndrome produced by metabolic stress.

Lipophagy, a distinct subtype of macroautophagy, plays a crucial role in the catabolism of LDs, encompassing neutral lipids such as cholesterol esters and triglycerides³⁷. This process is characterized by the sequestration of small LDs or segments of larger LDs by autophagosomes, which subsequently transport the lipid cargo to lysosomes for degradation¹⁹. The resulting degradation products, including NEFA, can then undergo mitochondrial oxidation to generate ATP. Importantly, its dysfunction is linked to several metabolic diseases. Impaired lipophagy can lead to excessive lipid accumulation in tissues, contributing to hepatic steatosis, atherosclerosis, and relevant metabolic syndrome^{17,37}. Consequently, activating lipophagy has emerged as a promising therapeutic strategy to reduce lipid accumulation and restore liver function. In the current study, based on the comparative proteomics analysis and colocalization of LDs with autophagosomes/lysosomes, we discovered that TRIM46 inhibits hepatic lipophagy by receding autophagy signaling pathway and reducing autophagic

flux. Furthermore, we have identified that TRIM46 inhibits hepatic lipophagy through degradation of SGPL1. However, the function and molecular mechanisms of SGPL1 in the development of MASLD are still unclear.

Mounting evidences highlight the critical involvement of the bioactive sphingolipid mediator sphingosine-1-phosphate (S1P) and its rate-limiting catabolic enzyme, sphingosine phosphate lyase 1 (SGPL1), in modulating autophagic processes^{13, 14, 38}. SGPL1 catalyzes the irreversible cleavage of S1P into phosphoethanolamine and hexadecenal³⁹. Recent mechanistic studies implicate S1P in controlling selective autophagy potentially via modulation of autophagy-related proteins (ATGs) or the mTOR signaling pathway¹³. The metabolic product of SGPL1, phosphoethanolamine, participates in the biosynthesis of phosphatidylethanolamine (PE), which functions as an anchor for phagophore membranes by facilitating the attachment of LC3 and converting LC3-I to LC3-II on these membranes. Furthermore, LC3 family proteins modified with PE are capable of binding to autophagic receptors, thereby promoting the maturation of autophagosomes^{14, 40}. These findings suggest that SGPL1 plays a crucial role in regulating autophagy. In our study, we discovered that TRIM46 promoted the degradation of SGPL1, which was associated with impaired autophagic flux, as evidenced by a reduced LC3-II/LC3-I ratio and the accumulation of p62, as well as diminished lipophagy, indicated by decreased colocalization of LDs with autophagosomes/lysosomes, both in vivo and in vitro under metabolic stress conditions. Notably, the use of the SGPL1 inhibitor THI significantly mitigated the impaired lipophagy and MASLD phenotype induced by TRIM46 deficiency, while exacerbating hepatic lipid accumulation in the wild-type strain under metabolic stress. These findings suggest that TRIM46 exacerbates MASLD progression by inhibiting SGPL1-mediated hepatic lipophagy, and that SGPL1 serves as a key regulator of lipophagy signaling in MASLD.

In conclusion, our findings indicate that TRIM46 serves as a viable regulator in the elimination of SGPL1 during the pathological advancement of MASLD. The liver protein TRIM46 is transcriptionally activated by CREB3 under metabolic stress. And the TRIM46, particularly its RING domain, engages in an interaction with SGPL1 and facilitates its degradation through enhanced polyubiquitination, resulting in the inactivation of downstream SGPL1-mediated lipophagy. This inactivation ultimately leads to the deterioration of steatohepatitis diseases. Targeting the TRIM46-SGPL1 axis may represent a promising strategy for MASLD treatment, and offers theoretical insights for the development of pharmaceutical interventions for this globally prevalent condition.

Methods

Animals and treatment

All animal experiments conducted in this study received approval from the Animal Experimentation Ethics Committee of Zhengzhou University. To establish a mouse MASLD model, mice (8 weeks old) were fed a high-fat and high-cholesterol (HFHC) diet (comprising 14% protein, 42% fat, 44% carbohydrates, and 1% cholesterol; Trophic, Nantong, China) for 16 weeks. Mice fed a normal chow (NC)

diet (20.6% protein, 12.0% fat, and 67.4% carbohydrates; Trophic, Nantong, China) served as the control group. hepatocyte-overexpressed mice with *Trim46* were generated by injecting AAV8-TBG-*Trim46* virus (obtained from BiO Technology, China) through the tail vein at a dosage of 5.0×10^{11} vg per mouse. A hepatocyte-specific conditional knockout of *Trim46* (*Trim46*-HKO) mice were generated through the use of an AAV8-based sgRNA delivery system. Male Rosa26-LSL-Cas9 knock-in mice (Strain NO. T002249, Gempharmatech, China) were administered intravenously with AAV8-U6-sgRNA (*Trim46*)-TBG-Cre (purchased from BiO Technology, China) at a dosage of 5.0×10^{11} vg per mouse. The target sequences for the sgRNAs are provided in Table S1. In order to inhibit SGPL1 activity, the specific SGPL1 inhibitor THI (HY-14113, MCE, China) was administered intraperitoneally to both *Trim46*-HKO and Control mice at a dosage of 0.1 mg/kg every two days. This dosage was selected based on prior studies⁴¹.

Human liver samples

Liver specimens from adult donors diagnosed with MASLD were acquired through biopsy tissue collection or liver transplantation procedures. Corresponding control samples were sourced from individuals deemed unsuitable for liver donation, who did not have MASLD. Informed consent was obtained from all donors or their families. The study protocols involving human samples received approval from the Ethics Committee of Henan Provincial People's Hospital, and all procedures complied with the principles outlined in the Declaration of Helsinki and Declaration of Istanbul. The human liver samples utilized in this study are identical to those employed in our previous publication⁴².

Histopathology, immunohistochemistry, and immunofluorescence staining

Histopathology: liver tissue samples were embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin (H&E) and Picrosirius Red (PSR; 26357-02, Hedebio Technology, Ltd.). Lipid droplet accumulation in the liver was visualized using Oil Red O staining (0625, Sigma, CT, USA) of Tissue-Tek OCT-embedded frozen liver tissue.

Immunohistochemistry: Immunohistochemical staining was employed to examine the expression of the TRIM46 protein in human liver tissue. The liver sections underwent deparaffinization before being incubated overnight with the TRIM46 antibody (21026-1-AP, Proteintech, China) and CREB3 antibody (11275-1-AP, Proteintech, China). Subsequently, they were incubated with the appropriate secondary antibody. Visualization of the immunohistochemical staining was achieved using a diaminobenzidine (DAB) horseradish peroxidase color development kit (P0202, Byotime, China), and hematoxylin was used for nuclear counterstaining. The histological characteristics of the tissues were observed and documented using a light microscope.

Immunofluorescence staining: To analyze hepatic inflammatory and immune cell infiltration, the liver sections were incubated overnight with the F4/80 antibody (29414-1-AP, Proteintech, China), followed by incubation with the corresponding fluorescent secondary antibody. Nuclei were visualized using DAPI staining. The immunofluorescence images were captured using an immunofluorescence microscope system (Olympus BX53M, Japan).

Hepatic lipid and serum biochemistry analyses

The extraction of hepatic triglycerides (TG), total cholesterol (TC), and non-esterified free fatty acids (NEFA) was conducted as outlined by ⁴². Briefly, liver tissues were homogenized and disrupted by the addition of an appropriate volume of PBS, followed by the addition of a chloroform:methanol (2:1) solution to facilitate triglyceride extraction. Lipid concentrations were quantified using commercial kits (290-63701 for TG, 294-65801 for TC, 294-63601 for NEFA, Wako, Tokyo, Japan) in accordance with the manufacturer's protocols. The serum alanine transaminase (ALT) and aspartate transaminase (AST) levels were measured by Thermo Scientific™ Indiko™ Clinical Chemistry Analyzer.

Cells and cell treatment

Human HEK293T, HEK293A, HepG2 cells, and murine AML12 cells were procured from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HEK293T, HEK293A and HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. AML12 cells were maintained in DMEM/F12 medium, supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% ITS Liquid Media Supplement (C0341, Bytotime, China), and 40 ng/ml Dexamethasone (ST1254, Bytotime, China). Primary hepatocytes from male C57BL/6 mice aged 6–8 weeks were isolated using a non-recirculating collagenase perfusion method, as previously described with slight modifications^{43, 44}.

To establish an in vitro MASLD model, HepG2 cells, primary murine hepatocytes, and AML12 cells were exposed to 0.5 mM palmitic acid/1 mM oleic acid (PA/OA) for a duration of 24 hours. In the SGPL1 inhibition experiment, hepatocytes were treated with 2 mM 2-acetyl-5-tetrahydroxybutyl imidazole (THI, HY-14113, MCE, China) to suppress SGPL1 activation. To investigate the proteasomal degradation, cells were treated with 25 μ M MG132 (HY-13259, MCE, China), 20 μ M chloroquine (CQ, HY-17589A, MCE, China), or 20 μ g/ml cycloheximide (CHX, HY-12320, MCE, China) for specified time intervals.

Plasmids construction

Plasmids encoding full-length, mutant, or truncated forms of *TRIM46*, *CREB3*, *SGPL1*, *HNRNPUL2*, *ITPR1*, *PPIA* and *ALG5* were cloned from complementary DNA (cDNA) sequences and inserted into the pHAGE vector using the ClonExpress II One Step Cloning Kit (C112, Vazyme, China). Luciferase reporter

plasmids were constructed by incorporating TRIM46 promoter truncations or its mutant regions into the PGL4.17 vector. The TRIM46 knockdown plasmid was generated by inserting a short hairpin RNA (shRNA) into the pLKO.1 lentiviral vector. The shuttle plasmid pENTR-U6-CMV and ViraPower Adenoviral Expression System (V493-20, Invitrogen, USA) were used to generate mouse Trim46 and Sgpl1 overexpressing or knockdown adenoviral plasmids. To generate the TRIM46 and SGPL1 prokaryotic expression plasmids, TRIM46 cDNA sequence was cloned into pGEX-6p-1 vector and SGPL1 cDNA sequence was cloned into pET-21b vector. The primers used for plasmid construction are listed in Tables S2 and S3.

Virus construction and infection

To establish TRIM46-overexpressing or TRIM46-knockdown HepG2 cell lines via lentiviral infection, lentiviral vectors (pHAGE-*TRIM46*, pHAGE-*TRIM46* (C33A/C36A), pHAGE-*TRIM46* (C48A/C53A), and pLKO.1-*ShTRIM46*) were co-transfected with helper plasmids (psPAX2 and pMD2.G) into HEK293T cells using polyethyleneimine (PEI; 24765-1, Polysciences, UK) as the transfection reagent. Following a 48-hour incubation period post-transfection, viral particles were harvested and filtered through a 0.45 µm filter. These viral particles were subsequently employed to infect HepG2 cells in the presence of 10 µg/ml polybrene. Stable cell lines expressing the desired constructs were selected using hygromycin screening. For the overexpression or knockdown of mouse Trim46 and Sgpl1, adenoviral plasmids were linearized with the restriction endonuclease PacI (R0547L, NEB, USA) prior to transfection into HEK293A cells using Polyethylenimine Linear (PEI). Initial adenovirus stocks were collected after 7 days, followed by amplification, purification, and titration. These adenoviruses were then utilized to infect primary hepatocytes isolated from mice.

Dual-luciferase reporter assay

A dual-luciferase reporter assay was conducted in accordance with previously established protocols^{45, 46}. HepG2 cells were cultured in 24-well culture plates and subsequently transfected with either the luciferase reporter plasmid (pGL4.17) blank vector or the pGL4.17 plasmid containing TRIM46 promoter truncations or its mutant region, in conjunction with the pRL-TK Renilla luciferase plasmid. Following a 24-hour post-transfection period, cell lysis was performed, and luciferase activity was quantified using the Dual Luciferase Reporter Gene Assay Kit (RG027, Beyotime, China) substrate, measured with a Multi-Mode Microplate Reader (BioTek Synergy HT). The primers utilized for the construction of the TRIM46 promoter luciferase reporter plasmid were detailed in Table S2.

Chromatin immunoprecipitation assay (ChIP)

Human HepG2 cells were transfected with Flag-CREB3 plasmids, and after 24 hours, cross-linked with 1% formaldehyde at 37°C for 10 minutes. The reaction was halted using 125mM glycine in PBS for 2 minutes at room temperature. Cells were washed twice with cold PBS containing a protease inhibitor

cocktail (P1005, Beyotime, China) and lysed using SDS lysis buffer (P2078, Beyotime Biotechnology, China) with the same inhibitor. Chromatin was sheared into 200-500bp fragments by sonication (260 W, 30 cycles of 30s on/60s off) on ice using an ultrasonic cell disruption system (Diagenode, Belgium). Chromatin was precipitated using protein A/G magnetic beads (PB101, Vazyme, China) with Flag antibody (80801-2-RR, Proteintech, China) or IgG (Rabbit) control antibody. The immunoprecipitated DNA was purified with MolPure® DNA Purification Kit (19106ES50, Yeasen, China) and analyzed by qPCR. ChIP-PCR primers are listed in **Table S4**.

RNA isolation and qPCR

Total RNA was extracted from hepatocytes and liver tissues utilizing RNAiso Plus (T9108, TAKARA, Japan) in accordance with the manufacturer's instructions. Subsequently, 1 µg of RNA was reverse-transcribed into complementary DNA (cDNA) using the HiScript III RT Super Mix for qPCR kit (R323-01, Vazyme, China). Quantitative PCR assays were conducted with the ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme, China) and analyzed using the ABI QuantStudio 5 system (ABI, Carlsbad, USA). The ACTB gene was employed as the reference transcript. The primers used for qPCR in this study are detailed in **Table S5**.

Western Blot Assay

Western blot analysis was performed as previously described^{42, 43}. Briefly, tissue or cell samples were lysed using RIPA lysis buffer (P0013K, Beyotime, China) supplemented with a protease inhibitor cocktail (P1005, Beyotime, China). The total protein concentration was determined utilizing a BCA Protein Assay Kit (P0010, Beyotime, China). Proteins were resolved on 10% SDS-PAGE gels and subsequently transferred onto PVDF membranes (IPVH00010, Merck Millipore, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) for 1 hour, followed by incubation with the specified primary antibodies at 4°C overnight. Afterward, the membranes were incubated with appropriate secondary antibodies at room temperature for 1 hour. Protein signals were detected using a chemiluminescence image analyzer (Amersham ImageQuant 800, Cytiva, USA). ACTB served as the loading control. Details of the antibodies used are provided in **Table S6**.

Co-immunoprecipitation (Co-IP) and mass spectrometry (MS) assays

For co-immunoprecipitation (Co-IP) assays, cultured cells were transfected with the specified plasmids for 24 hours, followed by lysis in ice-cold immunoprecipitation buffer (composed of 20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) supplemented with a protease inhibitor cocktail (HY-K0010, MCE, China) for 10 minutes. The lysates were clarified by centrifugation at 12,000 g for 10 minutes. The resulting supernatants were then incubated with the specified antibodies and Protein G

Bestarose 4FF beads (AA0142, Bestchrom, China) at 4°C for 3 hours. Subsequently, the beads were washed three times with cold IP buffer. The samples were then subjected to analysis via western blotting.

For mass spectrometry (MS) analysis, TRIM46 and its interacting proteins obtained through immunoprecipitation were separated using SDS-PAGE. The relevant band was excised and submitted for MS analysis (APT BIO, Shanghai, China). The resulting data were analyzed using MaxQuant 1.6.0.1 software, with searches conducted in the UniProt database.

GST Pull-down assay

For the GST Pull-down assay, GST-*TRIM46* and His-*SGPL1* proteins were expressed in *Escherichia coli* (BL21) with induction by IPTG. Then GST-*TRIM46* and GST control proteins were incubated with GST Bestarose 4FF beads (AA0071, Bestchrom, China) at 4°C for 3h. The beads were then washed three times in cold PBS buffer, after which His-*SGPL1* protein was added and incubated for an additional 4 h at 4°C. Finally, the beads underwent three additional washes with cold PBS buffer and were analyzed using western blotting.

Ubiquitination assays

Cultured cells were transfected with the indicated plasmids for 24 hours. Cell lysis was performed using 100 µl of 1% SDS in PBS buffer, followed by denaturation for 10 minutes. This was then combined with 900 µl of IP lysis buffer (comprising 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing a protease inhibitor cocktail. The lysates were clarified by centrifugation at 12,000 g for 10 minutes, and the resulting supernatants were subjected to Co-IP assays using the specified antibodies, followed by western blot analysis.

RNA-seq and data processing

Total RNA was extracted from mice liver tissues as described above, and then submitted to GENE DENOVO Biotechnology Company (Guangzhou, China) for RNA-seq analysis. Briefly, cDNA libraries were constructed, and detection is carried out using the MGI DNBSEQ-T7. paired-end clean reads were mapped to the Ensembl mouse genome (mm10 or GRCm38) using HISAT2 (v2.1.0). Subsequently, the mapped reads of each sample were assembled by using StringTie (v1.3.4). For each transcription region, a FPKM (fragment per kilobase of transcript per million mapped reads) value was calculated to quantify its expression abundance and variations, using Salmon (v1.10.3) software. Differential expression analysis was conducted using DESeq2 (v1.24) for two-group comparisons. Genes/transcripts were considered significantly differentially expressed if they exhibited a fold change exceeding 1.5 and a *P*-value of less than 0.05.

Ubiquitinated proteome analysis

TRIM46-Overexpression (OE) or Vector control HepG2 cells were exposed to PA/OA (0.5 mM /1mM) for 12h and then subjected to label-free Ubiquitylation analysis (PTM BIO, China). In this process, the cells were lysed and the resultant protein samples are enzymatically digested into a mixture of peptide segments. Then, the modified peptides were then selectively enriched using high-affinity antibodies and biomaterials specific to ubiquitination modifications. Finally, they are loaded onto liquid chromatography-tandem mass spectrometry (LC-MS) for analysis and quantification. Differentially ubiquitinated peptides were screened using two criteria: (1) a fold change greater than 1.2 and (2) a corresponding adjusted P value less than 0.05.

Confocal microscopy

In the co-localization experiments, the cells underwent fixation, permeabilization, and blocking, The *TRIM46* and *SGPL1* proteins in cell were respectively labeled with the corresponding coupled fluorescent antibodies, and the nuclei were labeled with DAPI. Confocal microscopy was conducted utilizing a laser-scanning confocal microscope (TCS SP8, Leica, Germany).

For the analysis of autophagosome, lysosome, and lipid content in hepatocytes, mice primary hepatocytes were fixed, permeabilized, and blocked. Subsequently, the cells were incubated overnight at 4°C with LC3B antibody (14600-1-AP, Proteintech, China) and LAMP1 antibody (A21194, ABclonal, China). This was followed by incubation with the appropriate fluorescent secondary antibody and staining of lipid droplets using BODIPY^{493/503} (HY-D1614, MCE, China). Nuclei were visualized through DAPI staining. Images were captured using a confocal laser scanning microscope (FV4000, Olympus, Japan).

Oxygen Consumption Rate (OCR) assay

OCR assay was performed using a Seahorse XFe24 Extracellular Flux Analyzer (Agilent) as outlined in previous studies⁴⁷. Primary mouse hepatocytes were seeded at 20,000 cells per well in a 24-well Seahorse assay plate (Agilent). Cells were then incubated overnight in a substrate-limited medium to enhance exogenous fatty acid utilization. Before the assay, the medium was replaced with fatty acid oxidation (FAO) assay medium (111 mM NaCl, 4.7 mM KCl, 2 mM MgSO₄, 1.2 mM Na₂HPO₄, 2.5 mM Glucose, 0.5 mM L-Carnitine) supplemented with PA/OA (0.5 mM/1 mM). Etomoxir (40 μM) (HY-50202, MCE, china) was added 15 minutes prior to measurement to inhibit FAO. The oxygen consumption rate (OCR) was evaluated under basal conditions and after the addition of oligomycin (2 μM), FCCP (0.5 μM), rotenone (0.5 μM), and antimycin A (2.5 μM).

Pathway enrichment and validation analysis

The genes encoding the down-regulated proteins were initially subjected to KEGG pathway enrichment analysis utilizing the cluster Profiler R package (version 4.10.0). Pathways of particular interest identified through this analysis, such as "Autophagy – animal," underwent subsequent validation via Gene Set Enrichment Analysis (GSEA) using the same software package. The GSEA was conducted on a pre-ranked list of genes, organized according to differential expression metrics, employing the specific KEGG gene set associated with the target pathway.

Correlation analysis of gene expression and pathway activity.

A Pearson correlation analysis was conducted to evaluate the associations between the expression levels of key genes, including TRIM46, SGPL1, ACOX1, ACADM, and the activity score of the "Fatty acid oxidation" pathway, as well as the interrelationships among the genes themselves. Pathway activity scores were obtained through single-sample gene set enrichment analysis (ssGSEA). All analyses were performed using the R programming environment, with the results presented as scatter plots and accompanied by correlation coefficients (R) and p-values.

Statistical analysis

All data are presented as means $\pm$ standard deviation (s.d.). Statistical analyses were conducted using SPSS Statistics, version 21.0. For comparisons between two groups, a two-tailed Student's t-test was employed to assess statistical significance when the data conformed to a normal distribution. In cases where the data exhibited a skewed distribution, the nonparametric Mann-Whitney U test was utilized. For analyses involving more than two groups, a one-way analysis of variance (ANOVA) was performed to evaluate simple effects, followed by the Bonferroni post hoc test for data with equal variance, or Tamhane's T2 test for data with unequal variance. A P-value of less than 0.05 was considered indicative of statistical significance, and exact P-values are provided in each figure.

Declarations

Authorship contribution statement

Li Xiao and Dating Sun designed and performed experiments, analyzed data, wrote the manuscript, and contributed equally. Yaoyao Jin and Ziwei Zhou performed and analyzed omics assay. Yifan Qin, Jialin Zhao and Yuanhan Geng participated in the animal and pathological experiments and checked the animal data. Sumei Ren provided constructive comments on the study design and data analysis. Hui Ding, Bingyong Zhang and Zhi-Gang She provided valuable suggestions for the study design and guided the article. Yaping Guo supervised the project and revised the manuscript. Junyong Wang conceived and designed the study, analyzed data, edited the final article, and approved the submitted version. All authors have approved the final version of this article.

Competing interest statement

The authors declare no competing interests.

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Data availability Statement

The original proteomics and ubiquitinomics data of this study were deposited to PRIDE (PXD076863). GSE213621 and GSE135251 were download on public MASLD patients GEO database.

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Figures

Figure 1

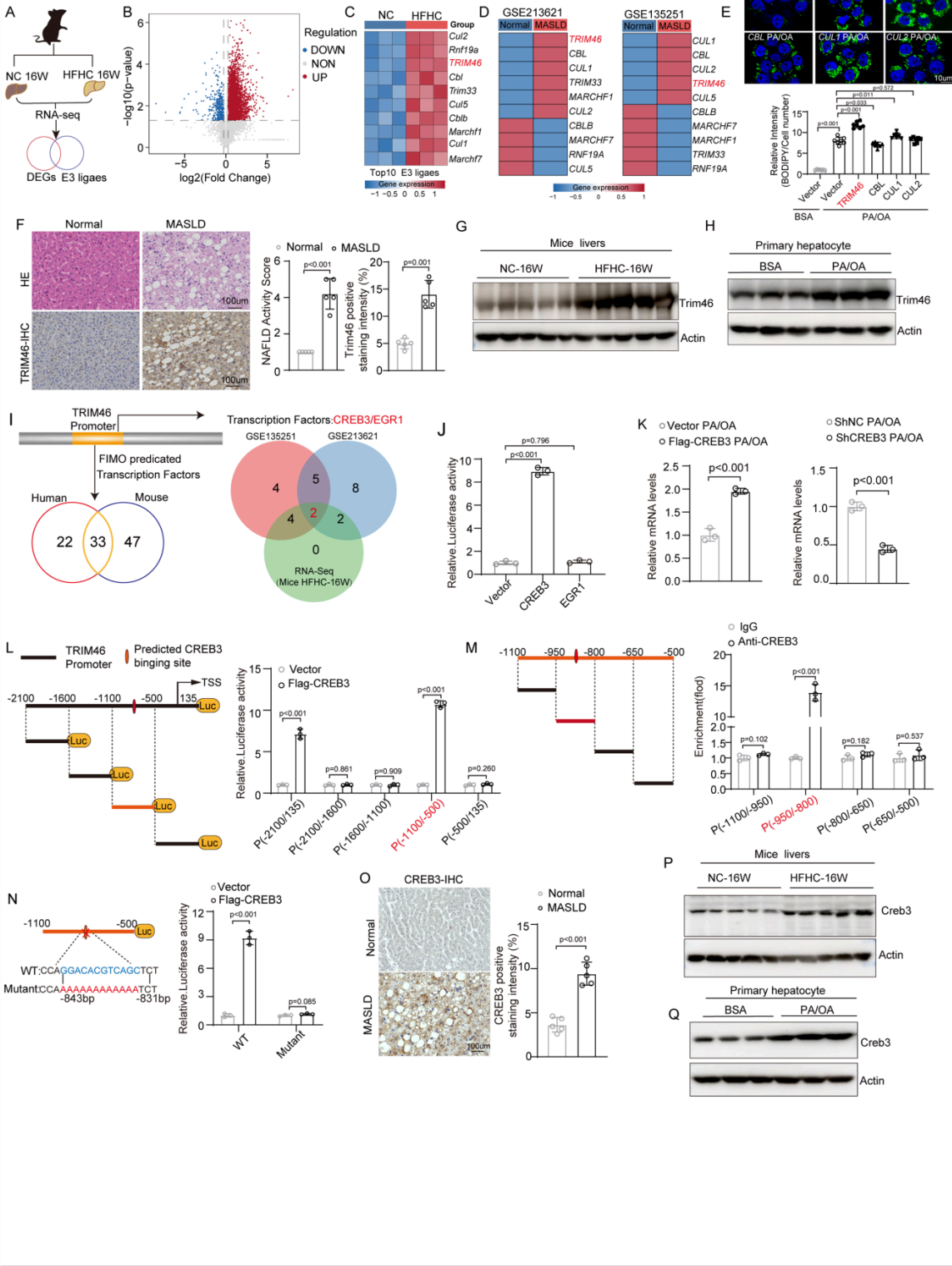


Figure 1

TRIM46 expression is UP-regulated in MASLD Model

(A) RNA-seq analysis was conducted to identify differentially expressed genes in liver tissues of mice subjected to HFHC and NC diets. (B) A volcano plot illustrated the differentially expressed genes in the liver tissues of these mice. (C) A heatmap presented the top 10 differentially expressed ubiquitin-related

genes in mice liver tissues. (D) Another heatmap displayed the top 10 E3 ligase expressions based on MASLD patient databases (GSE213621 and GSE135251). (E) Representative images of BODIPY staining demonstrated lipid accumulation following the overexpression of *TRIM46*, *CBL*, *CUL1*, and *CUL2* in HepG2 cells challenged with PA/OA(0.5mM/1mM) for 12 hours. The scale bar, 10 μ m. Quantification of relative BODIPY intensity was provided in the accompanying histograms. (F) H&E staining analyzed hepatic steatosis and Immunohistochemical staining images of TRIM46 expression in the livers of individuals with or without MASLD ($n = 5$ per group). (G) Western blot assay analyzed TRIM46 expression in NC and HFHC diet induced mice liver samples ($n = 6$ per group). β -ACTIN was used as internal control. (H) TRIM46 protein expression in primary hepatocytes in response to the PA/OA stimulus. b-ACTIN was used as internal control, $n = 3$ independent experiments. (I) The prediction of transcription factors (TFs) that bind to the TRIM46 promoter was conducted using the Find Individual Motif Occurrences (FIMO) software, version 5.5.6, in conjunction with a database screening process, applying a p-value threshold of <0.05 . (J) Results from the luciferase assay demonstrated the effects of the identified TFs on TRIM46 promoter activity, $n = 3$ independent experiments. (K) qPCR analysis revealed the impact of CREB3 on the TRIM46 mRNA levels following treatment with PA/OA for 12 hours, $n = 3$ independent experiments. (L) The relative luciferase activity of luciferase reporter plasmids containing the wild-type TRIM46 promoter (WT) and its truncated variants in HepG2 cells transfected with the plasmids overexpressing CREB3 or the Vector plasmid, $n = 3$ independent experiments. (M) Chromatin immunoprecipitation (Ch-IP) assays were performed to evaluate the enrichment of CREB3 binding across different regions of the TRIM46 promoter in HepG2 hepatocytes overexpressing either TRIM46 or a vector control, in HepG2 cells transfected with the plasmids overexpressing CREB3 or the Vector plasmid, $n = 3$ independent experiments. (N) Relative luciferase activity of luciferase reporter plasmids containing wild-type TRIM46 promoter (WT) or its mutant in HepG2 hepatocytes transfected with the plasmids overexpressing CREB3 or the control plasmid, and then treated with PA/OA for 12 h ($n = 3$ independent experiments). (O) Immunohistochemical staining images of CREB3 expression in the livers of individuals with or without MASLD ($n = 5$ per group). (P) Western blot assay analyzed CREB3 expression NC and HFHC diet induced mice liver samples ($n = 6$ per group). β -ACTIN was used as internal control. (H) CREB3 protein expression in primary hepatocytes under the PA/OA stimulus. b-ACTIN was used as internal control, $n = 3$ independent experiments. All data are shown as the mean $\pm$ s.d.

Figure 2

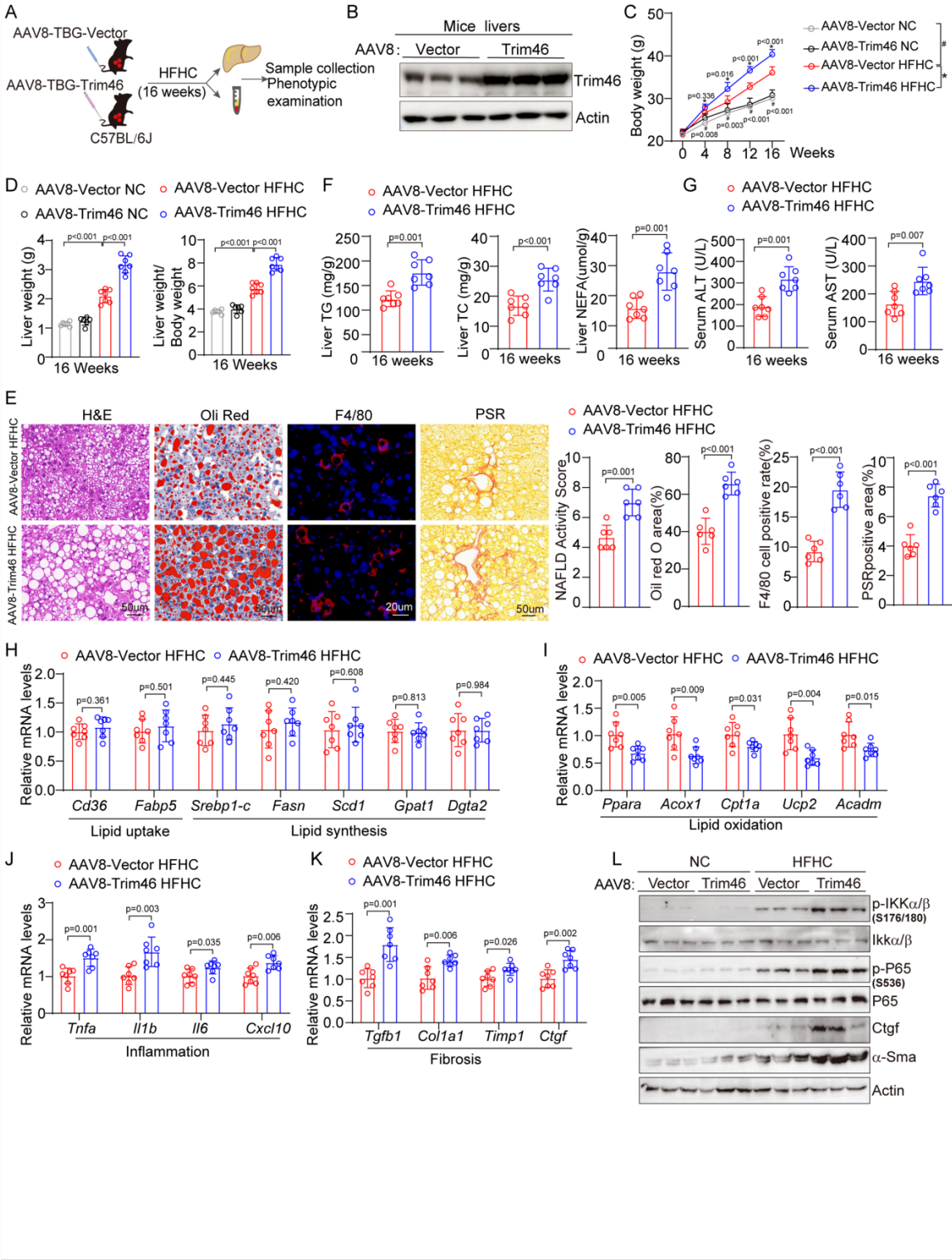


Figure 2

Trim46 overexpression by adeno-associated virus exacerbates HFHC-induced MASLD

(A) Experimental schematic to assess the effect of Trim46 function in HFHC-induced mouse MASLD model. Adeno-associated virus serotype 8 (AAV8), Thyroxine-Binding Globulin (TBG). (B) Immunoblotting and quantitation of Trim46 protein expression in AAV8-Vector and AAV8-Trim46 mice, β-Actin was used

as internal control, $n = 3$ per group. (C) Body weight in AAV8-Vector and AAV8-*Trim46* mice fed with NC ($n = 6$ mice per group) and HFHC ($n = 7$ mice per group) diet from 0 to 16 weeks. (D) Liver weight and liver weight to body weight ratio of AAV8-Vector and AAV8-*Trim46* that were fed with ($n = 6$ mice per group) and HFHC ($n = 7$ mice per group) for 16 weeks. (E) Representative images of H&E staining (Scale bar, 50um), Oil red O staining (Scale bar, 50um), F4/80-positive cell immunofluorescence staining (Scale bar, 20um) and Picric Sirius red (PSR) staining (Scale bar, 50um) of liver sections in AAV8-Vector and AAV8-*Trim46* mice after 16 weeks feeding with HFHC. $n = 6$ mice in each group (quantified in the histograms). (F) Hepatic tissue Triglyceride (TG), Total cholesterol (TC) and Non-esterified free fatty acids (NEFA) contents of AAV8-Vector and AAV8-*Trim46* at 16 weeks after HFHC treatment. $n = 7$ mice in each group. (G) Liver function index AST and ALT levels of AAV8-Vector and AAV8-*Trim46* mice after 16 weeks feeding with HFHC ($n = 7$ mice per group). (H-K) qPCR analysis the mRNA level of fatty acid uptake and synthesis genes (H), fatty acid oxidation genes (I), inflammation genes (J) and fibrosis genes (K) in liver tissues from the indicated mice ($n = 7$ mice per group). (L) Immunoblotting and quantitation of inflammation (p-IKK α /b, IKK α /b, p-P65 and P65) and fibrosis (Ctgf and α -Sma) proteins expression in liver tissues from the indicated mice, b-Actin was used as internal control, $n = 3$ mice per group. All data are shown as the mean $\pm$ s.d. * for AAV8-*Trim46* HFHC compared to AAV8-Vector HFHC. # for AAV8-Vector HFHC compared to AAV8-Vector NC.

A AAV8-TBG-Cre Control
AAV8-U6-sgTrim46-TBG-Cre Trim46-HKO
Rosa26(CAG-LSL-Cas9-KI) Trim46-HKO
HFHC (16 weeks)
Sample collection
Phenotypic examination

B Mice livers
Control Trim46-HKO
Trim46
Actin

C Body weight (g)
Control NC Trim46-HKO NC
Control HFHC Trim46-HKO HFHC
p=0.001 p=0.014
p=0.008 p=0.001
p=0.001 p=0.001
p=0.087 p=0.001
0 4 8 12 16 (Weeks)

D Liver weight (g)
Control NC Control HFHC
Trim46-HKO NC Trim46-HKO HFHC
p<0.001 p=0.005
0 1 2 3 4 5
16 Weeks

E Liver weight/Body weight
Control NC Control HFHC
Trim46-HKO NC Trim46-HKO HFHC
p=0.001 p=0.004
0 2 4 6 8 10
16 Weeks

F Liver TG (mg/g)
Control HFHC Trim46-HKO HFHC
p<0.001
0 50 100 150 200
16 Weeks

G Liver TC (mg/g)
Control HFHC Trim46-HKO HFHC
p=0.019
0 10 20 30
16 Weeks

H Liver NEFA (umol/g)
Control HFHC Trim46-HKO HFHC
p=0.006
0 10 20 30
16 Weeks

I Serum AST (U/L)
Control HFHC Trim46-HKO HFHC
p=0.018
0 100 200 300 400
16 weeks

J Serum ALT (U/L)
Control HFHC Trim46-HKO HFHC
p=0.011
0 100 200 300 400 500
16 weeks

K H&E Oli Red F4/80 PSR
Control HFHC Trim46-HKO HFHC
50um 50um 20um 50um

L NAFLD Activity Score
Control HFHC Trim46-HKO HFHC
p=0.008
0 2 4 6 8

M Oil red O area (%)
Control HFHC Trim46-HKO HFHC
p<0.001
0 20 40 60

N F4/80 cell positive rate (%)
Control HFHC Trim46-HKO HFHC
p<0.001
0 5 10 15

O PSR positive area (%)
Control HFHC Trim46-HKO HFHC
p<0.001
0 2 4 6

P Relative mRNA levels
Control HFHC Trim46-HKO HFHC
p=0.291 p=0.895 p=0.301 p=0.335 p=0.483 p=0.370 p=0.446
Cd36 Fabp5 Srebp1-c Fasn Scd1 Gpat1 Dgta2
Lipid uptake Lipid synthesis

Q Relative mRNA levels
Control HFHC Trim46-HKO HFHC
p=0.002 p=0.006 p=0.033 p=0.001 p=0.003
Ppara Acox1 Cpt1a Ucp2 Acadm
Lipid oxidation

R Relative mRNA levels
Control HFHC Trim46-HKO HFHC
p=0.010 p=0.028 p=0.011
Tnfa Il1b Il6 Cxcl10
Inflammation

S Relative mRNA levels
Control HFHC Trim46-HKO HFHC
p=0.006 p=0.003 p=0.031 p=0.006
Tgfb1 Col1a1 Timp1 Ctgf
Fibrosis

T NC HFHC
Control Trim46-HKO Control Trim46-HKO
p-IKKα/β (S176/180)
IKKα/β
p-P65 (S536)
P65
Ctgf
α-SMA
Actin

Hepatocyte-specific *Trim46* knockout (*Trim46*-HKO) ameliorates HFHC-induced MASLD

(A) The strategy for the generation of hepatocyte-specific *Trim46*-knockout (*Trim46*-HKO) mice by using Rosa26 (CAG-LSL-Cas9-KI) mice combined with AAV8-U6-sg*Trim46*-TBG-Cre Adeno-associated virus infection, and the experimental design for evaluating *Trim46*-HKO function in the HFHC diet-induced

mouse MASLD model. (B) Immunoblotting and quantitation of Trim46 protein expression in *Trim46*-HKO and Control mice, β -Actin was used as internal control, $n = 3$ mice per group. (C) Body weight in *Trim46*-HKO and Control mice after 16-week NC ($n = 5$ mice per group) or HFHC ($n = 7$ mice per group) diet treatment. (D) Body weight and liver weight of *Trim46*-HKO mice and corresponding control mice after 16-week NC ($n = 5$ mice per group) or HFHC ($n = 7$ mice per group) diet treatment. (E) Representative images of H&E staining (Scale bar, 50 μ m), Oil red O staining (Scale bar, 50 μ m), F4/80-positive cell immunofluorescence staining (Scale bar, 20 μ m) and PSR staining (Scale bar, 50 μ m) of liver sections in *Trim46*-HKO and Control mice at 16 weeks after HFHC treatment (quantified in the histograms). (F) TG, TC and NEFA concentrations in the indicated groups of mice at 16 weeks of HFHC treatment. $n = 7$ mice per group. (G) Enzyme activities of serum ALT and AST in *Trim46*-HKO and Control mice after 16 weeks of HFHC diet feeding. $n = 7$ mice per group. (H-K) qPCR analysis the mRNA level of fatty acid uptake and synthesis genes (H), fatty acid oxidation genes (I), inflammation genes (J) and fibrosis genes (K) in liver tissues from the indicated mice ($n = 7$ mice per group). (L) Immunoblotting and quantitation of inflammation (p-IKK α /b, IKK α /b, p-P65 and P65) and fibrosis (Ctgf and α -Sma) proteins expression in liver tissues from the indicated mice, β -Actin was used as internal control, $n = 3$ per group. All data are shown as the mean $\pm$ s.d. * for *Trim46*-HKO HFHC compared to Control HFHC. # for Control HFHC compared to Control NC.

Figure 4

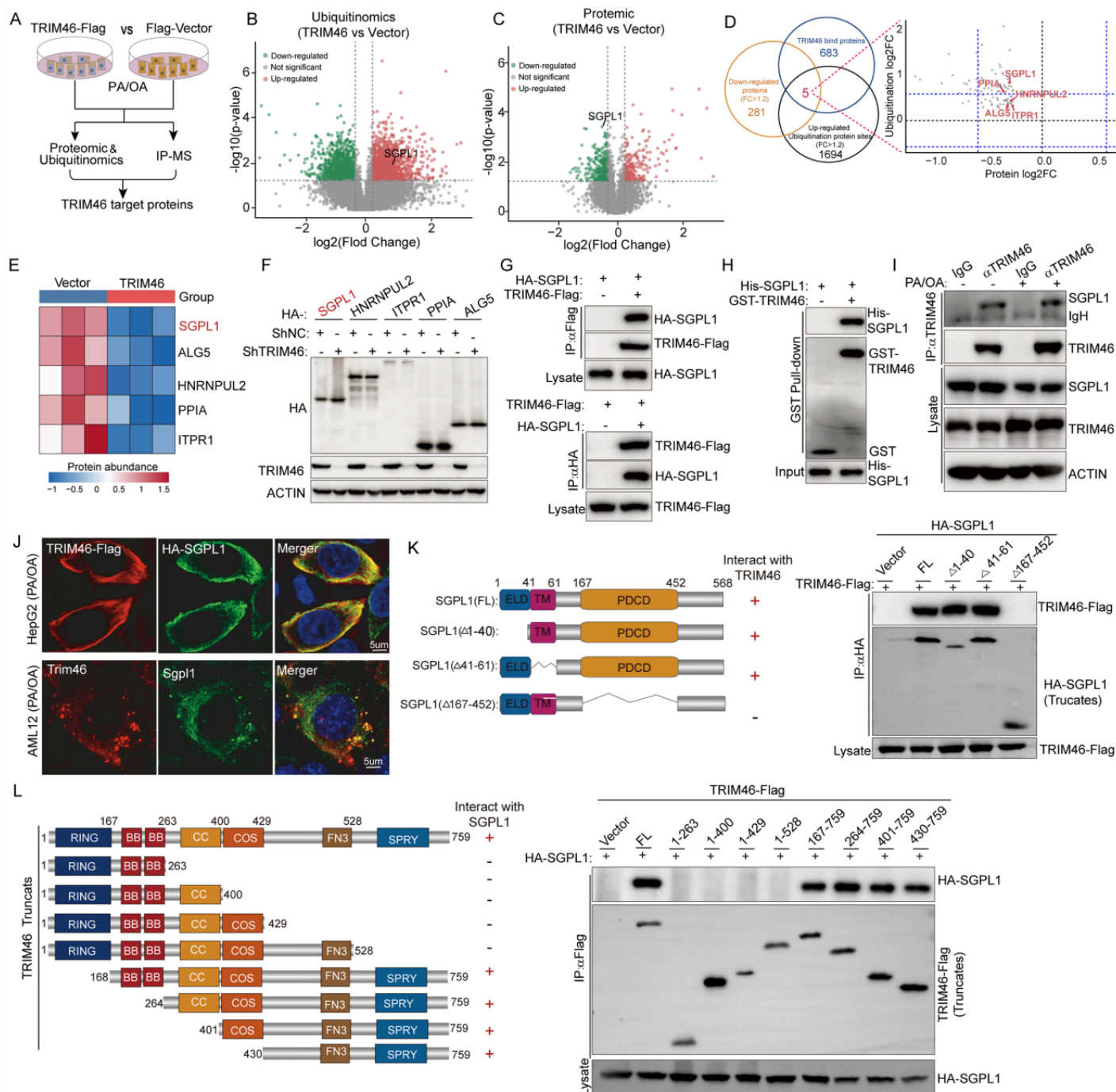


Figure 4

TRIM46 directly interacts with SGPL1

(A) Scheme showing the procedure of identifying the down-stream targets of TRIM46 by the intersection analysis of IP-MS and quantitative ubiquitinomics. (B, C) A volcano plot showing average proteins containing ubiquitylation sites (B) and proteins expression (C) ratio of 3 replicates (log2 (fold change))

between over-expression TRIM46 and control (vector) HepG2 cells treated with PA/OA (Red: upregulated ubiquitylation sites or proteins; Green: ubiquitylation sites or proteins). (D) IP-MS combined with quantitative ubiquitinomics & proteomics analysis the downstream targets of TRIM46. (E) Heatmaps showing expression of five proteins identified in (D) (labeled by red) in HepG2 cells with TRIM46-overexpression compared to vector from the proteomics datasets. (F) Western blots showing TRIM46 potential target proteins (SGPL1, HNRNPUL2, ITPR1, PPIA and ALG5) expression in *TRIM46* knock-down (Sh*TRIM46*) and control HepG2 cell-lines, b-Actin was used as internal control. (G) Interaction between TRIM46 and SGPL1 assayed by Coimmunoprecipitation (CO-IP) followed by Western blotting in 293T cells transfected with the indicated plasmids. (H) GST Pull-down assays showing the direct TRIM46 and SGPL1 binding. (I) Co-IP assays determining the endogenous interaction between TRIM46 and SGPL1 in HepG2 hepatocytes treatment with BSA or PA/OA (0.5/1mM) for 12 hours. (J) Representative confocal microscopy images showing co-localization between TRIM46 (Red) and SGPL1 (Green) in HepG2 hepatocytes were co-infected with Flag-TRIM46 and HA-SGPL1 plasmids and AML12 hepatocytes. (K) CO-IP assays analyses showing the binding domains of SGPL1 to TRIM46 in 293T cells transfected with the indicated plasmids. (L) Domain Mapping analyses showing the binding domains of TRIM46 to SGPL1 in 293T cells transfected with the indicated plasmids. For (F-L) experiments were repeated independently 3×. All data are shown as the mean ± s.d.

Trim46) (B) followed by treatment with PA/OA (0.5/1mM) for 12 hours. (C) Immunoblotting and quantitation of HA-SGPL1 protein expression in overexpression TRIM46 and control (Vector) HepG2 cells by treatment with cycloheximide (CHX) (20ug/m) was incubated for the indicated time frame. (D) Representative Western blot images and quantifications showing Sgpl1 expression in mice primary hepatocytes infected with Ad-Vector/Ad-*Trim46*-Flag with indicated treatment for 6 hours (MG132, 25μM); Chloroquine (CQ, 20μm). (E) Ubiquitination assays confirming the ubiquitination of SGPL1 in HepG2 hepatocytes treatment with or without PA/OA (0.5/1mM) for 12 hours. (F, G) Ubiquitination assays confirming the ubiquitination of Sgpl1 in mice primary hepatocytes infected with Ad-*Trim46*-Flag (F) and Ad-ShTrim46 (G) followed by treatment with PA/OA (0.5/1mM) treatment for 12 hours. (H) Results of ubiquitination assays confirming the ubiquitination of SGPL1 after overexpression TRIM46 for 24 h in HepG2 cells co-transfected with HA-SGPL1 and the indicated Myc-tagged ubiquitin plasmid constructs (WT, K48O, K48R, K63O, K63R). (I) TRIM46 structure showing the two conservative ubiquitination sites in quantitative ubiquitinomics (TRIM46-Flag vs Vector). (J) Western blots showing SGPL1(WT) or SGPL1 mutans (K107R↔K342R and 2KR) protein expression after overexpression TRIM46 for 24 hours in HepG2 cells co-transfected with indicated plasmid constructs. (K) Ubiquitination assays confirming the ubiquitination of SGPL1 or SGPL1 mutans (K107R↔K342R and 2KR) after overexpression TRIM46 for 24 h in HepG2 cells co-transfected with indicated plasmid constructs followed by treatment with MG132 (25 μM) for 6 hours. (L) TRIM46 structure showing the two conservative motifs (TRIM46(C³³XXC³⁶) and TRIM46(C⁴⁸XXXXC⁵³) of RING domain in different species. (M) Western blots showing SGPL1 protein expression after overexpression TRIM46 or TRIM46 mutans in HepG2 cells. (N) Ubiquitination assays confirming the ubiquitination of SGPL1 after overexpression of TRIM46 or TRIM46 mutans (C³³A/C³⁶A, C⁴⁸A/C⁵³A) for 24 h in HepG2 cells co-transfected with indicated plasmid constructs followed by treatment with MG132 (25μM) for 6h. (O) Representative BODIPY staining images showing the lipid accumulation after over-expression of TRIM46 or TRIM46 mutans (C³³A/C³⁶A, C⁴⁸A/C⁵³A) HepG2 cells challenged with PA/OA for 24 hours Scale bar,10um. Quantification of relative BODIPY intensity in the histograms. All experiments were repeated independently 3×. All data are shown as the mean ± s.d.

Figure 6

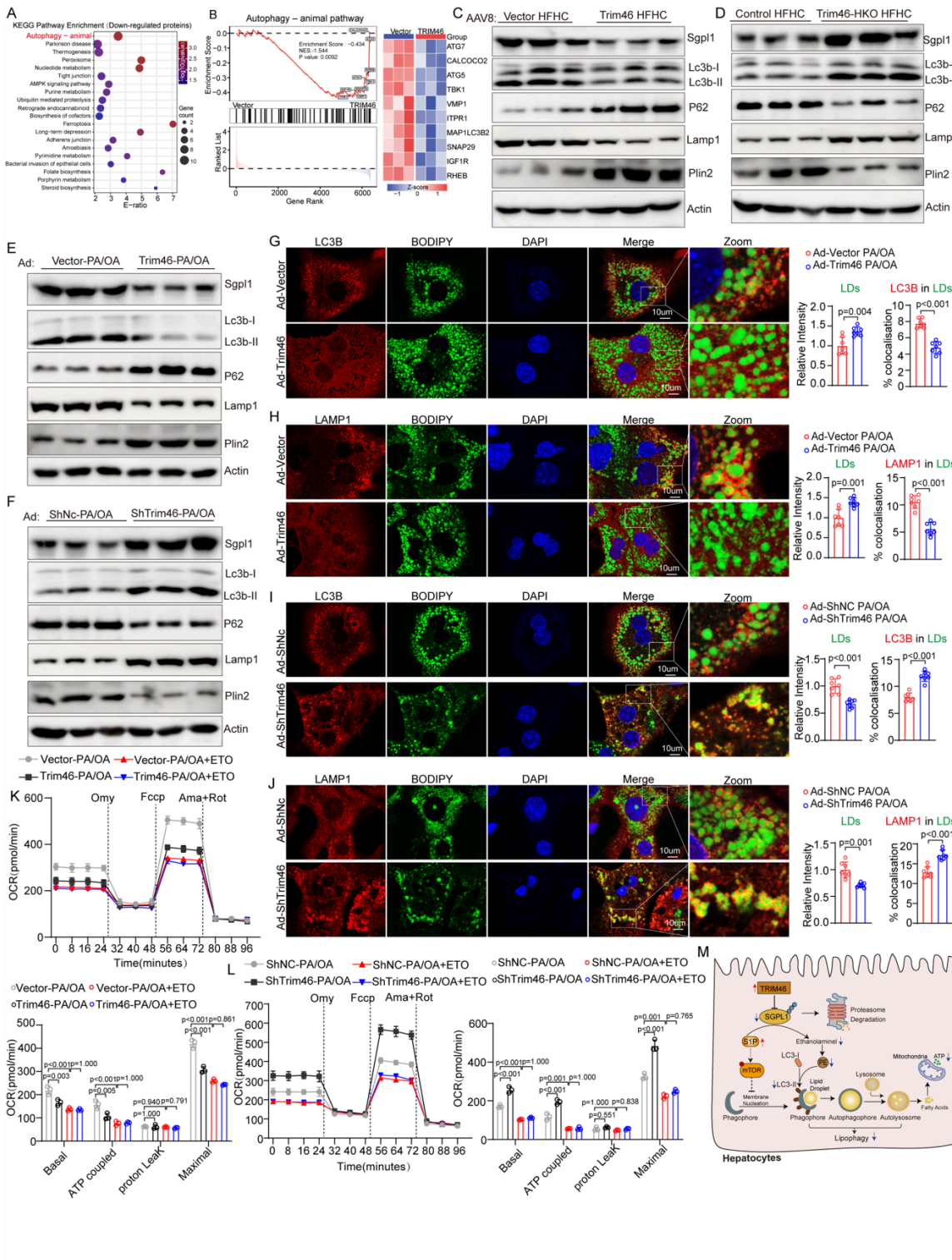


Figure 6

TRIM46 inhibits hepatic lipophagy through degrading SGPL1

(A) KEGG enrichment analysis of proteomics data (Down-regulated proteins) revealed dominant biological pathways in relation to over-expression TRIM46 under metabolic stress in HepG2 hepatocytes. (B) Enrichment score of the Autophagy-animal pathway and the top 10 leading-edge genes expressed in

over-expression TRIM46 versus Vector control group analyzed by GSEA based on the over-expression TRIM46 proteomics data. (C, D) Representative western blot images showing the protein levels of Sgpl1, Lc3b, P62, Lamp1 and Plin2 in the livers of HFHC-fed AAV8-*Trim46*/Control AAV8-Vector mice (C) or *Trim46*-HKO mice/their controls (D). $n = 3$ mice per group. (E, F) Representative western blot images showing the protein levels of Sgpl1, Lc3b, P62, Lamp1 and Pin2 in mice primary hepatocytes infected with Ad-Vector/Ad-*Trim46* (E) or Ad-Shnc/Ad-*ShTrim46* (F) followed by treatment with PA/OA (0.5/1mM) treatment for 12 hours. $n = 3$ independent experiments. (G-J) Representative confocal microscopy images of LC3B (G, I) or LAMP1 (H, J) on Lipid droplets (LDs) in mice primary hepatocytes infected with Ad-Vector/Ad-*Trim46* or Ad-Shnc/Ad-*ShTrim46*, followed by treatment with PA/OA (0.5/1mM) treatment for 24 hours. Scale bars, 10 μ m. Quantification of relative fluorescence intensity in the histograms. (K, L) Representative and statistical results of oxygen consumption rate in mice primary hepatocytes infected with Ad-Vector/Ad-*Trim46* (K) or Ad-Shnc/Ad-*ShTrim46* (L) under PA/OA (0.5/1mM) treatment with and without etomoxir (100 nM). $n = 3$ independent experiments. (M) Schematic diagram showing the molecular mechanism underlying Trim46 inhibits hepatic lipophagy through facilitating degradation of SGPL1. All data are shown as the mean $\pm$ s.d.

Figure 7

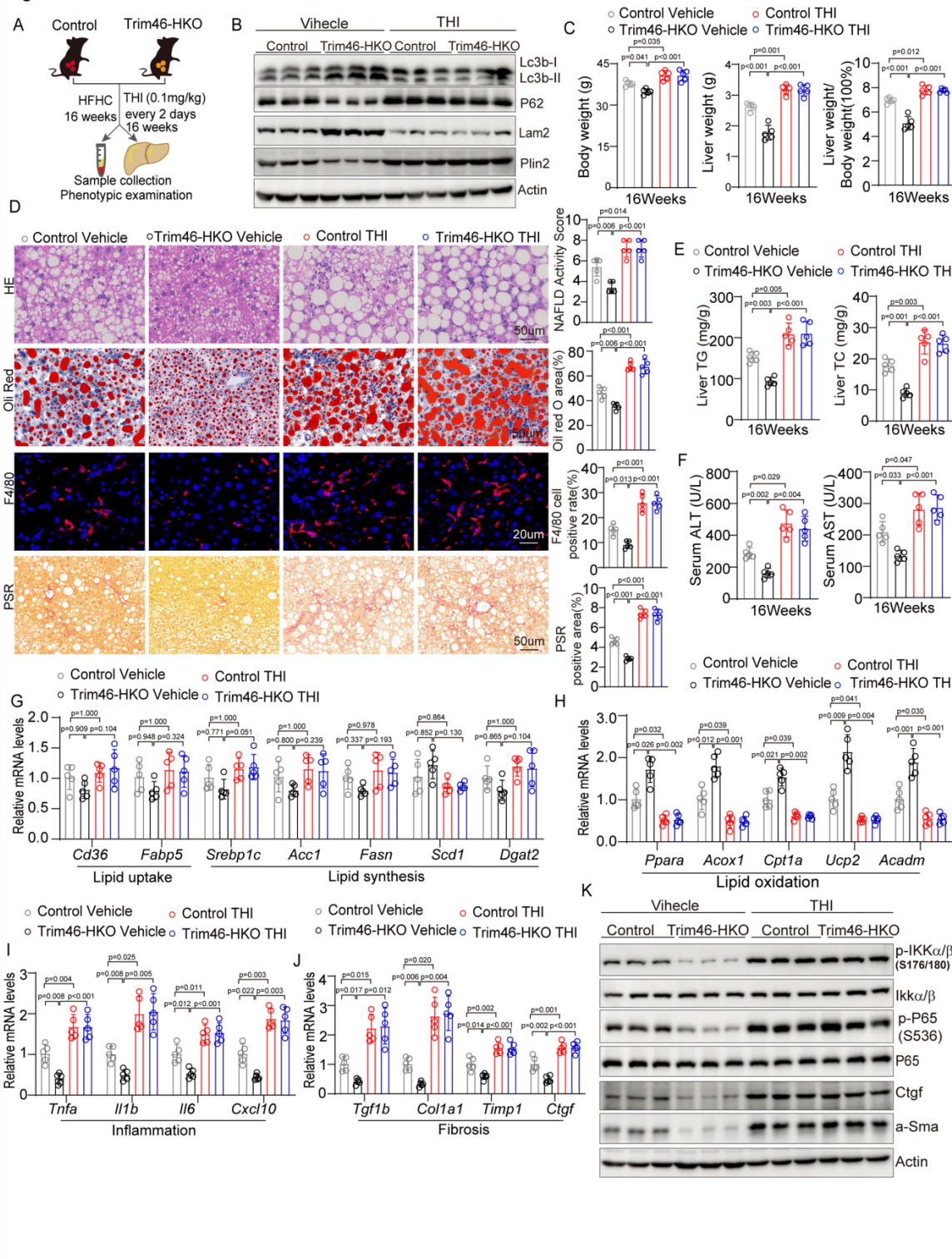


Figure 7

SGPL1 is required for TRIM46-induced MASLD progression

(A) Experimental schematic to assess SGPL1 is essential for TRIM46-induced MASLD progression by treatment with SGPL1 inhibitor (2-acetyl-5-tetrahydroxybutyl imidazole, THI) strategy on a HFHC diet-induced MASLD mouse model. (B) Representative western blot images showing the protein levels of

Sgpl1, Lc3b, P62, Lamp1 and Plin2 in the livers of HFHC-fed *Trim46*-HKO and Control mice with or without THI administration. *n* = 3 mice per group. (C) Body weight and liver weight of *Trim46*-HKO and Control mice with or without THI administration after 16-week HFHC diet treatment. *n* = 5 mice per group. (D) Representative images of H&E (Scale bar, 50um), Oil red O staining (Scale bar, 50um), F4/80-positive cell immunofluorescence staining (Scale bar, 20um) and PSR staining (Scale bar, 50um) of liver sections from mice in the indicated groups. Quantifications of their NAS scores, oil red O-positive area, F4/80-positive cell rate, and PSR-positive area are shown in the right panel. *n* = 5 mice in each group. (E) Liver triglyceride (TG), total cholesterol (TC) concentrations in the indicated groups of mice at 16 weeks of HFHC treatment. *n* = 5 mice per group. (F) Enzyme activities of serum ALT and AST in the indicated groups of mice. *n* = 5 mice per group. (G-J) qPCR analysis the mRNA level of fatty acid uptake and synthesis genes (G), fatty acid oxidation genes (H), inflammation genes (I) and fibrosis genes (J) in liver tissues from the indicated mice (*n* = 5 mice per group). (K) Immunoblotting and quantitation of inflammation (p-IKKa/b, IKKa/b, p-P65 and P65) and fibrosis (Ctgf and a-Sma) proteins expression in liver tissues from the indicated mice, b-Actin was used as internal control, *n* = 3 mice per group. All data are shown as the mean $\pm$ s.d.

Supplementary Files

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- [TRIM46sourcedata.xlsx](#)
- [SupplementaryMaterials.docx](#)