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## Targeting MDM2 for its ubiquitin-proteasome degradation: Curcumenol from turmeric ameliorates NAFLD through p53-AMPK-PGC-1 $\alpha$ -activated mitochondrial biogenesis

Shu-Ying Li <sup>a,#</sup>, Xu Gong <sup>c,#</sup>, Zhi-Xuan Li <sup>c</sup>, Dong-Li Li <sup>c</sup>, Jing-Wei Jin <sup>c</sup>, Ju-Cai Xu <sup>c</sup>, Ri-Hui Wu <sup>c</sup>,  
Li-Gen Lin <sup>d,\*</sup>, Xiao Yuan <sup>b,\*</sup>, Li-She Gan <sup>a,\*</sup>

<sup>a</sup> School of Pharmaceutical Sciences, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 311402, China

<sup>b</sup> Department of Endocrinology, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310006, China

<sup>c</sup> School of Pharmacy and Food Engineering, International Healthcare Innovation Institute, Wuyi University, Jiangmen 529020, China

<sup>d</sup> State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau 999078, China

**ABSTRACT:** Non-alcoholic fatty liver disease (NAFLD) is a growing global health concern with no approved pharmacotherapy. Curcumenol (Cul), a bioactive sesquiterpenoid derived from the well-known functional food turmeric (*Curcuma longa*), shows potential therapeutic effects in treating metabolic diseases. In this study, we demonstrated that Cul significantly ameliorated high-fat diet-induced NAFLD accompanied by obesity and hyperlipidemia in mice via promoting lipid oxidative metabolism and inhibiting *de novo* lipogenesis. In palmitic acid/oleic acid (PA/OA)-treated AML12 hepatocytes, Cul markedly reduced intracellular lipid accumulation, oxidative stress, and inflammation. Through an integrative approach combining network pharmacology, molecular docking, molecular dynamics simulation, cellular thermal shift assay (CETSA), surface plasmon resonance (SPR), immunoprecipitation (IP), and plasmid-based overexpression techniques, we demonstrated that Cul directly interacted with MDM2 protein, inducing its degradation via the ubiquitin-proteasome system, which led to p53 stabilization and subsequent activation of the AMPK-PGC-1 $\alpha$  signaling axis. This cascade enhanced mitochondrial biogenesis and fatty acid  $\beta$ -oxidation, thereby ameliorating hepatic steatosis in PA/OA-induced AML12 cells. Additionally, Cul suppressed *de novo* lipogenesis, contributing to its anti-NAFLD effects. Collectively, this study innovatively unveils MDM2 as a novel therapeutic target for NAFLD and demonstrates that Cul is the first sesquiterpenoid component capable of inducing MDM2 degradation via the ubiquitin-proteasomal pathway, representing a new mechanistic strategy for metabolic disease treatment.

**Keywords:** Curcumenol; Non-alcoholic fatty liver disease; MDM2; Ubiquitin-proteasomal degradation; Mitochondrial biogenesis;  $\beta$ -Oxidation

### 1. Introduction

Characterized by an accumulation of fat in hepatocytes exceeding 5% excluding secondary causes (e.g., alcohol/drug use), non-alcoholic fatty liver disease (NAFLD) is a chronic metabolic disorder linked to genetic

<sup>#</sup> These authors contributed equally to this work.

\*Corresponding author

lsgan@zcmu.edu.cn (Dr. Li-She Gan), ligenl@um.edu.mo (Dr. Li-Gen Lin), yuanxiao1218@163.com (Dr. Xiao Yuan)

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predisposition and insulin resistance [1,2]. The development of NAFLD is primarily driven by an imbalance between energy intake and expenditure, where increased energy intake and/or decreased energy expenditure lead to ectopic lipid accumulation outside white adipose tissue, resulting in liver steatosis [3,4].

NAFLD can progress to non-alcoholic steatohepatitis (NASH), further advancing to liver fibrosis, cirrhosis, and ultimately heightening the risk for hepatocellular carcinoma (HCC) [5,6]. The prevalence of NAFLD has increased rapidly in the past 20 years, emerging as a severe health threat to approximately 25% of the global population [7,8]. Currently, there is no approved clinical treatment for NAFLD [9,10], highlighting the urgent need for the development of safe and effective therapeutic strategies.

Mitochondria play a fundamental role in energy metabolism, and their dysfunction is recognized as a key factor in the initiation and progression of NAFLD [11,12]. As the primary site of  $\beta$ -oxidation, mitochondria are responsible for the catabolic task of gradually breaking down fatty acids into smaller molecules. Given the pivotal role of mitochondrial  $\beta$ -oxidation in lipid metabolism, strategies aiming to improve mitochondrial function, promote mitochondrial biogenesis, and minimize mitochondrial damage have gained attention as potential therapeutic approaches for NAFLD.

Curcumenol (Cul) is a bioactive sesquiterpenoid isolated from the edible rhizomes of *Curcuma* plants [13,14]. The molecular structure of Cul is shown in Figure 1A. Studies increasingly indicate that Cul possesses anti-inflammatory properties and is commonly used in the treatment of synovitis in patients with knee osteoarthritis [14,15]. Additionally, it exhibits antioxidant [16], antibacterial [17,18], and hepatoprotective activities [19]. Previously, we characterized its tautomeric properties, and discovered its ability to enhance mitochondrial content and function in C2C12 cells to promote skeletal muscle regeneration [20]. Thus, we hypothesized that Cul might exhibit favorable anti-NAFLD activity through promoting mitochondrial biogenesis.

Herein, a high-fat diet (HFD) mouse model and a palmitic acid (PA) and oleic acid (OA)-treated AML12 cell model were established to evaluate the impact of Cul on NAFLD development and progression. Furthermore, network pharmacology, molecular docking techniques, and molecular dynamics simulation were employed to predict the targets of Cul, which were subsequently verified by Cellular Thermal Shift Assay (CETSA) and Surface Plasmon Resonance (SPR). Additionally, systematic investigation of the associated signaling pathways elucidated the underlying mechanisms.

## 2. Materials and Methods

### 2.1 Materials and reagents

Cul (CAS 19431-84-6) was extracted and purified from *Curcuma wenyujin* as we described previously (purity  $\geq 98\%$  by HPLC) [21]. Compound C (dorsomorphin, CAS 866405-64-3) was purchased from MedChemExpress Technology Co. (New Jersey, USA). Assay kits for Superoxide Dismutase (SOD), Malondialdehyde (MDA), Reduced Glutathione (GSH), Triglyceride (TG; determined by GPO-PAP method), and Total Cholesterol (TC; determined by GPO-PAP method) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). 4% paraformaldehyde was purchased from Biosharp (Hefei,

China). Oil red O reagent was purchased from Sigma (St. Louis, MO, USA). RNAiso Plus was purchased from Takara Bio., Ltd (Kusatsu, Japan). ReverTra Ace™ qPCR RT Master Mix and SYBR Green qPCR SuperMix were purchased from Toyobo Co., Ltd (Osaka, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Cell Signaling (Danvers, MA, USA). Total-AMPK and phospho-AMPK rabbit monoclonal antibodies, secondary antibodies, BeyoECL Plus, and Mito-Tracker Green were purchased from Shanghai Beyotime Biotechnology Co., Ltd (Shanghai, China). The monoclonal antibodies against MDM2, fatty acid synthase (FAS), sterol regulatory element-binding protein-1c (SREBP-1c), acetyl-CoA carboxylase (ACC), phosphorylated ACC (p-ACC), peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), nuclear factor erythroid 2-related factor 2 (Nrf2), transcription factor A, mitochondrial (Tfam), peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), recombinant human murine MDM2, ubiquitin polyclonal antibody, and MG132 were purchased from Proteintech Group, Inc. (Wuhan, China). Mouse IL-1 $\beta$  and TNF- $\alpha$  ELISA kits were purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China).

## 2.2 Animal experimental procedure

Six-week-old male C57BL/6J mice were obtained from the Guangdong Medical Laboratory Animal Center (Foshan, China). Animals were housed under standard conditions ( $25 \pm 2^\circ\text{C}$ , 12 h light/dark cycle) in accordance with protocols approved by the Animal Care and Ethics Committee of Wuyi University (Jiangmen, China; Approval Code: CN2023022). Following a 2-week acclimatization period, mice were randomized into four groups ( $n=6$  per group). This sample size was determined based on a power analysis ( $\alpha=0.05$ , power=0.8) conducted from our pilot study detecting a 30% difference in TG levels with a standard deviation of 15%. Two groups received a standard chow diet (11% fat, 23% protein, 65% carbohydrate-derived calories), and orally administered with Cul (150 mg/kg) and the same volume of edible peanut oil, respectively, once a day for 6 weeks. The remaining two groups received a high-fat diet (HFD; 60% fat, 20% protein, 20% carbohydrate calories), and orally administered with Cul (150 mg/kg) and the same volume of edible peanut oil, respectively, once a day for 6 weeks.

## 2.3 Biochemical analysis

After 12 hours of fasting, all mice were euthanized under ether anesthesia, and blood was collected via orbital bleeding. Levels of triglycerides (TG), total cholesterol (TC), non-esterified fatty acids (NEFA), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured using a FAITH-1000 Automatic Biochemistry Analyzer.

## 2.4 Determination of lipid content in the liver

Hepatic lipid extraction was performed as previously described [22]. Briefly, liver tissues were lysed in 0.5 M KOH solution, homogenized, and incubated. Following centrifugation at  $3,000 \times g$  for 15 minutes, the supernatant was collected. TG and TC contents in the liver were measured according to the manufacturer's protocols for the respective assay kits.

## 2.5 Histological analysis

Liver tissues were fixed in 4% paraformaldehyde for 24 h, dehydrated, and paraffin-embedded. Sections (5  $\mu$ m) were prepared using a Leica RM22559 microtome, dewaxed, hydrated, and stained with H&E. The NAFLD Activity Score (NAS) system was used to assess hepatic steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning (0-2). For Oil Red O staining, cells were fixed with 4% paraformaldehyde, washed with PBS, stained with 0.3% Oil Red O, and rinsed. All images were acquired using an Olympus BX41 microscope, and the Oil Red O-stained areas were quantified using ImageJ software.

## 2.6 Ubiquitination assay

Cells were treated with 10  $\mu$ M MG132 for 4 h prior to lysis. Whole-cell lysates were prepared by incubating cells on ice for 20 minutes in NP-40 lysis buffer containing 1 mM PMSF. Clarified lysates were incubated overnight at 4°C with target-specific antibodies. Protein A/G magnetic beads were then added to the immune complexes and incubated for an additional 2-4 h at 4°C. The bead-bound complexes were washed three times with ice-cold lysis buffer to remove non-specific interactions. The immunoprecipitates were eluted from the beads and solubilized in lysis buffer. The resulting lysate was centrifuged, and the supernatant containing the immunoprecipitated complexes was subjected to Western blot analysis.

## 2.7 RT-qPCR

Hepatic and cellular gene expression was assessed via RT-qPCR. Total RNA was extracted, dissolved in RNase-free water, and quantified (NanoDrop One). After thermal denaturation (65°C, 5 min) and immediate chilling, cDNA was synthesized with ReverTra Ace™ qPCR RT Master Mix under the following conditions: 37°C (15 min)  $\rightarrow$  50°C (5 min)  $\rightarrow$  98°C (5 min)  $\rightarrow$  4°C hold. Target genes (*FAS*, *ACC*, *PGC-1 $\alpha$* , *Tfam*, *PPAR $\alpha$* ) were amplified using SYBR Green qPCR SuperMix on a LightCycler® 96 system with three-step annealing. Relative expression was normalized to *GAPDH*.

**Table 1** Primer sequences for RT-PCR analysis

| Primer                          | Forward (5'-3')         | Reverse (5'-3')          |
|---------------------------------|-------------------------|--------------------------|
| <i>SREBP-1</i>                  | CGACTACATCCGCTTCTTGACG  | CCTCCATAGACACATCTGTGCC   |
| <i>PGC-1<math>\alpha</math></i> | GAATCAAGCCACTACAGACACCG | CATCCCTCTTGAGCCTTTCGTG   |
| <i>Tfam</i>                     | GAGGCAAAGGATGATTCGGCTC  | CGAATCCTATCATCTTTAGCAAGC |
| <i>CPT1</i>                     | TCCAGTTGGCTTATCGTGCTG   | TCCAGAGTCCGATTGATTTTTGC  |
| <i>PPAR<math>\alpha</math></i>  | ATGGTGGACACGAAAGCC      | CGATGGATTGCGAAATCTCTTGG  |
| <i>Acc</i>                      | GTTCTGTTGGACAACGCCTTCAC | GGAGTCACAGAAGCAGCCCATT   |
| <i>GAPDH</i>                    | CATCACTGCCACCCAGAAGACTG | ATGCCAGTGAGCTTCCCGTTCAG  |

## 2.8 Western blot

Total protein from liver/cells was extracted and quantified. Samples in Laemmli buffer (20  $\mu$ L) were separated on 10% SDS-PAGE gels and transferred to PVDF membranes (90 V, 90 min). Membranes were incubated with primary antibodies (4°C, 12 h) followed by HRP-conjugated secondary antibodies (1:3000, 2 h). Proteins were detected using BeyoECL Plus, imaged with a GeIView 1500 system (Guangzhou Biolight Biotechnology Co., Ltd., Guangzhou, China), and quantified via Image Lab 6.1 with normalization to GAPDH.

## 2.9 Target prediction of Cul

The SMILES ID of Cul was retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and subsequently utilized in the SwissTargetPrediction platform ([www.swisstargetprediction.ch/](http://www.swisstargetprediction.ch/)) to predict its potential target genes, with the species specified as *Homo sapiens*.

## 2.10 Construction of the NAFLD target database

To identify targets associated with NAFLD, potential targets were retrieved from the GeneCards (<http://www.genecards.org/>) and OMIM ([www.omim.org/](http://www.omim.org/)) databases using the keyword "nonalcoholic fatty liver disease". A relevance score threshold of  $\geq 5$  was applied to filter the results. Subsequently, the predicted targets of Cul were intersected with these NAFLD-related targets to identify common entities.

## 2.11 PPI analysis

The overlapping targets of Cul for NAFLD treatment were imported into the STRING database (<http://cn.string-db.org/>), with the organism set to *Homo sapiens*, to construct a protein-protein interaction (PPI) network. Key targets were further screened using topological analysis performed with Cytoscape software versions 3.7.1 and 3.9.1. Three topological parameters—degree, closeness, and betweenness—were referenced to identify central and potentially crucial therapeutic targets in the PPI network.

## 2.12 Molecular docking

The two-dimensional structure of Cul was retrieved from the PubChem database and converted into an SDF file format. Subsequently, the structure was optimized using Chem3D software and saved in mol2 format. Crystal structures of the core target proteins were obtained from the PDB database (<https://www.rcsb.org/>). Protein-ligand docking was conducted using AutoDock Vina 1.1.2 to evaluate binding affinity and interaction mode between Cul and its target protein. Finally, the docking result was analyzed and visualized using PyMOL Version 3.0.

## 2.13 Molecular dynamics simulation

To systematically investigate the binding stability and interaction mechanisms between candidate compounds and proteins, conventional molecular dynamics (MD) simulations were performed using GROMACS software (version 2024.03). The topological parameters for ligands were generated through the Antechamber Python Parser Interface (ACPYPE) tool based on Generalized Amber Force Field (GAFF). The analysis of the MD trajectories included: root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), secondary structural changes and the binding free energy calculations of the ligand-protein complexes.

## 2.14 Surface Plasmon Resonance (SPR)

The SPR experiment was performed utilizing a BIAcore 1K device (Cytiva, Marlborough, MA, USA) equipped with a CM5 chip. In short, the MDM2 protein was immobilized on a CM5 sensor chip. Cul was then diluted into a series of concentrations and flowed over the chip from the lowest to the highest concentration to interact with the immobilized MDM2 protein. Flow rate: 30  $\mu\text{L}/\text{min}$ ; Interaction time: 150 s. The binding data

were globally fitted to a 1:1 Langmuir binding model using Biacore Insight Evaluation Software to calculate equilibrium dissociation constants ( $K_D$ ). Data visualization and curve fitting were completed with GraphPad Prism 8.0.1.

### 2.15 Statistical Analysis

Data are expressed as means  $\pm$  SD. Statistical analyses and graphical representations were created with GraphPad Prism 8.0.1. The comparison between the two groups was conducted using Student's  $t$  test, and the comparison between multiple groups was conducted using one-way ANOVA.  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1 Cul reduces lipid storage in HFD-induced obese mice

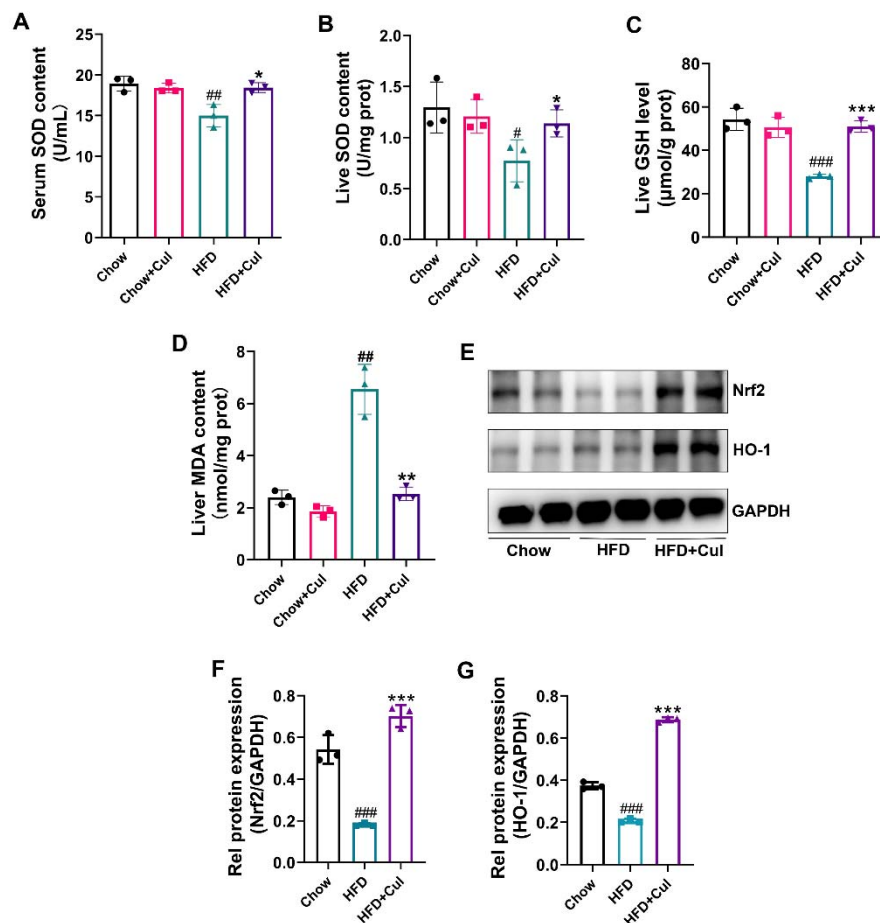
To investigate the therapeutic potential of Cul on NAFLD, HFD-induced obese mice were treated with 150 mg/kg Cul for 6 weeks. Although HFD group exhibited lower food intake compared to the normal diet (Chow) group, their body weight increased significantly. Moreover, despite having food intake comparable to the HFD group, mice that received Cul intervention (HFD+Cul) exhibited a significant reduction in body weight (Figure 1C-D). Cul intervention also significantly decreased fasting blood glucose levels in HFD-fed mice (Figure 1E). The HFD-induced hepatomegaly was notably attenuated by Cul treatment (Figure 1F). Consistent with these findings, HFD-fed mice exhibited significantly increased weights of epididymal, inguinal, and perirenal adipose tissues compared to Chow controls, with Cul administration substantially reversing these effects (Figure 1G-I). As shown in Figure 1J-O, HFD feeding markedly elevated serum lipid profiles (TG, TC, NEFA, LDL-C), while Cul treatment effectively normalized these lipid parameters. A parallel improvement was observed in hepatic TG and TC accumulation. Oil Red O staining confirmed excessive fat storage in HFD-mice, which was dramatically reduced in Cul-treated mice (Fig. 1R-S).

The impact of Cul on liver function in NAFLD mice was further investigated. The serum levels of AST and ALT, significantly elevated in HFD-fed mice, were reduced by Cul intervention (Figure 1P-Q). Histological examination via H&E staining revealed characteristic NAFLD pathologies in HFD-fed mice, including hepatocyte ballooning and inflammatory infiltration, with significantly elevated NAFLD activity scores. Notably, Cul treatment markedly reversed these HFD-induced alterations (Figure 1T-U). These collective findings demonstrated that Cul effectively mitigated HFD-induced hepatic steatosis, metabolic dysregulation, and liver injury in NAFLD progression.



### 3.2 Cul alleviates oxidation in HFD-fed mice

As shown in Figure 2A-C, the levels of SOD in serum and both SOD and GSH in the liver of the HFD-fed mice were notably lower than those in the Chow group. However, Cul treatment greatly enhanced the levels of SOD in serum and both SOD and GSH in the liver of HFD-fed mice. Additionally, the level of MDA in the liver of HFD group mice was significantly elevated, but this increase was reversed by Cul treatment (Figure 2D). Next, hepatic Nrf2 and HO-1 protein expression was quantified. HFD feeding reduced both proteins, while Cul treatment reversed the changes (Figure 2E-G). These findings indicated that Cul alleviated hepatic oxidative stress in HFD-mice via Nrf2/HO-1 pathway activation.



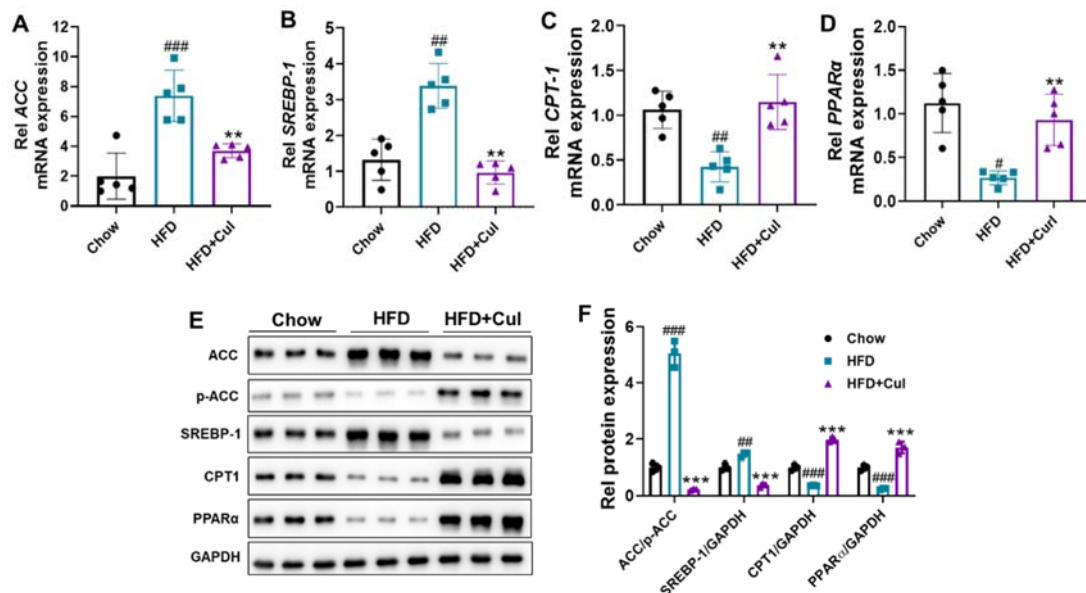
**Fig. 2.** Cul alleviates oxidative stress levels in HFD-fed mice ( $n = 3$ ). (A) Serum SOD levels. (B) Liver SOD levels. (C) Liver GSH levels. (D) Liver MDA levels. (E) Representative bands of Nrf2 and HO-1 protein expression in mouse liver tissue. (F) Nrf2/GAPDH protein expression levels in mouse liver tissues. (G) HO-1/GAPDH protein expression levels in mouse liver tissue.  $^{\#}P < 0.05$ ,  $^{\#\#}P < 0.01$ ,  $^{\#\#\#}P < 0.001$ , compared with Chow group;  $^*P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ , compared with HFD group.

### 3.3 Cul inhibits hepatic lipid synthesis and promotes lipid oxidative metabolism

Next, RT-qPCR analyses were performed to assess mRNA expression levels related to hepatic lipid metabolism, including lipid synthesis, lipolysis, and fatty acid oxidation. Acetyl-CoA carboxylase (ACC), the fatty acid synthesis gatekeeper, is widely implicated in metabolic disorders including obesity, diabetes, and hyperuricemia [23]. Sterol regulatory element-binding protein-1c (SREBP-1c) drives lipogenic gene transcription, promoting hepatic steatosis progression [24]. As shown in Figure 3A-B, hepatic mRNA levels of *ACC* and *SREBP-1c* exhibited significant increases in the HFD group compared to the Chow group, and Cul

treatment effectively reversed the increases. Furthermore, Cul treatment significantly reduced ACC/p-ACC and SREBP-1c protein expression in the liver tissue of HFD-fed mice (Figure 3E-F).

Peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) transcriptionally regulates lipid-metabolizing genes, such as lipoprotein lipase (LPL), carnitine palmitoyl transferase 1a (CPT1a), fatty acid transport protein (FATP), and acyl-CoA oxidase 1 (ACOX1), thereby promoting lipid catabolism and fatty acid oxidation. CPT1, a key regulatory enzyme in mitochondrial fatty acid oxidation, aids in the movement of fatty acids across the inner mitochondrial membrane to undergo oxidation [25, 26]. As shown in Figure 3C-F, both the mRNA and protein expression levels of CPT1 and PPAR $\alpha$  in the liver of HFD group mice were significantly reduced compared to the Chow group, while Cul treatment notably increased the mRNA and protein expression levels of CPT1 and PPAR $\alpha$ , indicating that Cul effectively promoted lipid oxidation. These results demonstrated that Cul effectively ameliorated hepatic lipid accumulation in NAFLD mice by suppressing fatty acid synthesis and promoting lipid oxidative metabolism.



**Fig. 3.** Cul inhibits fatty acid synthesis and promotes lipid oxidation in HFD-fed mice (n = 5). (A-D) mRNA expression levels of ACC, SREBP-1, CPT1 and PPAR $\alpha$  in mouse liver tissue. (E) Representative protein bands of ACC, p-ACC, SREBP-1, CPT1 and PPAR $\alpha$  in mouse liver tissue. (F) ACC/p-ACC, SREBP-1/GAPDH, CPT1/GAPDH and PPAR $\alpha$ /GAPDH protein expression levels in mouse liver tissues. <sup>#</sup>*P* < 0.05, <sup>##</sup>*P* < 0.01, <sup>###</sup>*P* < 0.001, compared with Chow group; <sup>\*</sup>*P* < 0.01, <sup>\*\*\*</sup>*P* < 0.001, compared with HFD group.

### 3.4 Cul reduces lipid content, inflammation, and oxidative stress in AML12 cells

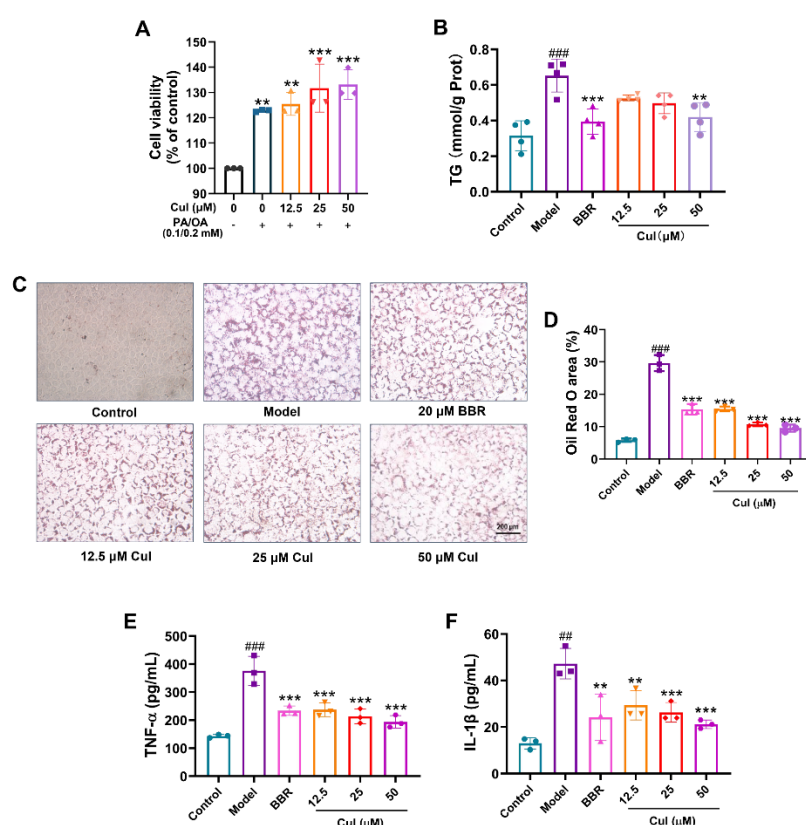
Next, a lipotoxic cellular model mimicking NAFLD was established by exposing AML12 hepatic cells to a combination of palmitate (PA) and oleate (OA). Prior to modeling, the cytotoxicity of Cul on AML12 cells was assessed via MTT assay. As illustrated in Figure S1A, Cul exhibited no cytotoxicity and even enhanced AML12 cells viability in a concentration-dependent manner. To establish the NAFLD model, AML12 cells were exposed to PA/OA mixtures. Initial MTT assays confirmed that PA/OA concentrations ranging from 0.05/0.1 mM to 0.5/1.0 mM did not induce significant cytotoxicity (Figure S1B). Subsequently, cells were treated with graded PA/OA concentrations (0.05/0.1 to 0.5/1.0 mM) for 24 h to determine the optimal dose for inducing intracellular TG accumulation. Based on TG quantification (Figure S2), 0.1/0.2 mM PA/OA was

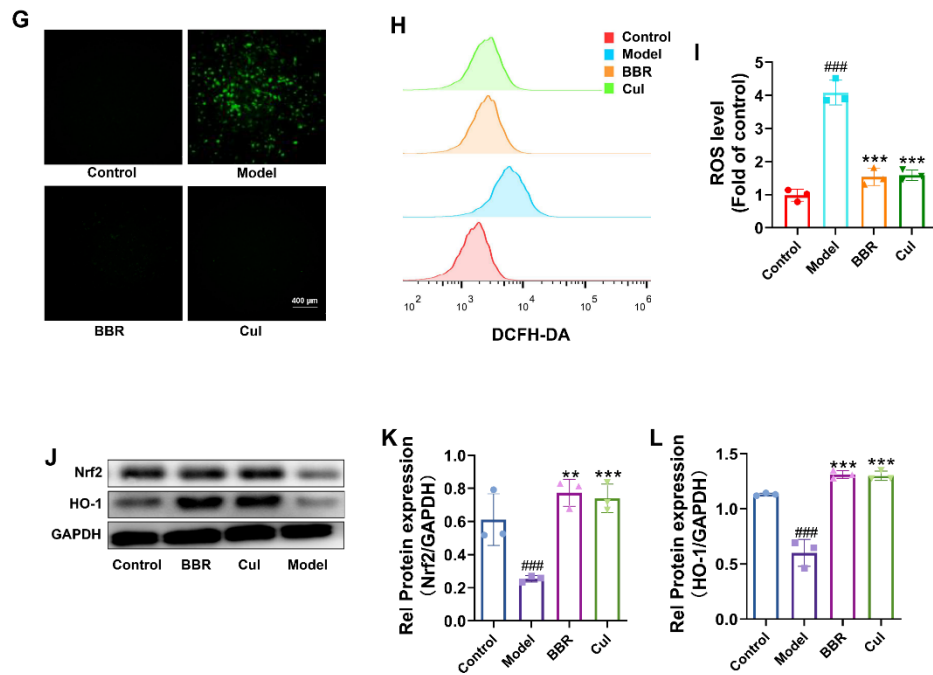
selected as the optimal dose for subsequent NAFLD modeling. Importantly, Cul (up to 50  $\mu\text{M}$ ) showed no cytotoxicity when co-administered with 0.1/0.2 mM PA/OA (Figure 4A).

As shown in Figure 4B, 0.1/0.2 mM PA/OA treatment significantly increased intracellular TG content, whereas Cul dose-dependently attenuated this effect, with berberine (BBR, 20  $\mu\text{M}$ ) serving as a positive control. Consistent with TG quantification, Oil Red O staining demonstrated that exposure to PA/OA markedly elevated both the number and size of lipid droplets in AML12 cells, while Cul treatment reduced these parameters (Figure 4C-D). These findings collectively demonstrated Cul's efficacy in alleviating lipid accumulation in the NAFLD cellular model.

Furthermore, the assessment of inflammatory markers revealed that PA/OA treatment notably up-regulated the levels of TNF- $\alpha$  and IL-1 $\beta$ . Both Cul and the positive control BBR significantly reduced the expression of these pro-inflammatory cytokines, with 50  $\mu\text{M}$  Cul exhibiting the most potent effect (Figure 4E-F).

To further explore the role of Cul in mitigating oxidative stress in PA/OA-induced NAFLD AML12 cells, cells were treated with the optimal concentration of Cul (50  $\mu\text{M}$ ), and intracellular ROS levels along with the activity of the Nrf2/HO-1 pathway were analyzed. As shown in Figure 4G-I, PA/OA treatment (0.1/0.2 mM) significantly elevated intracellular ROS levels compared to the control group, whereas Cul intervention reduced ROS accumulation. Furthermore, Western blot analysis revealed that PA/OA exposure attenuated the protein abundance of both Nrf2 and its downstream effector HO-1 in AML12 cells. Importantly, Cul treatment markedly upregulated the expression of these antioxidant proteins (Figure 4J-L), suggesting that Cul alleviated oxidative stress triggered by PA/OA exposure by activating the Nrf2/HO-1 antioxidant signaling cascade.



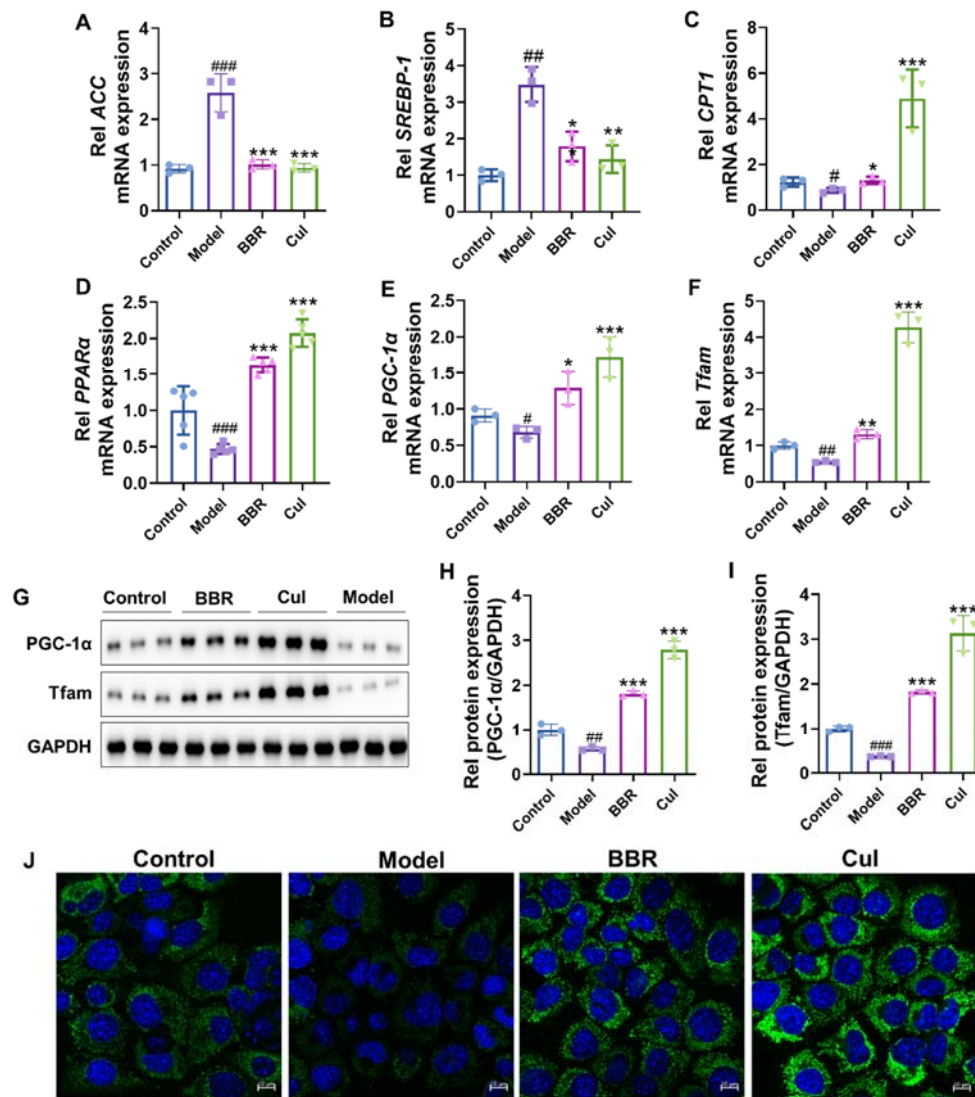


**Fig. 4.** Cul reduces lipid content, inflammation, and oxidative stress in AML12 cells. (A) Effect of 0.1/0.2 mM PA/OA with different concentrations of Cul on the viability of AML12 cells. (B) The effects of different concentrations of Cul on TG levels in the NAFLD AML12 cell model. (C) Representative images of oil red O staining of AML12 cells. (D) Quantitative analysis of Oil Red O positive area. (E-F) The expression levels of TNF- $\alpha$  (E) and IL-1 $\beta$  (F) measured using ELISA detection kits. (G) Intracellular ROS levels detected by fluorescence microscopy. (H) Intracellular ROS levels measured by flow cytometry. (I) Quantitative analysis of intracellular ROS levels. (J) Representative bands of Nrf2 and HO-1 protein expression. (K-L) Nrf2/GAPDH and HO-1/GAPDH protein expression in the NAFLD AML12 cell model. ### $P < 0.001$ , compared with control group; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with model group.

### 3.5 Cul regulates lipid metabolic homeostasis and promotes mitochondrial biogenesis in AML12 cells

The results showed that, the mRNA levels of *ACC* and *SREBP-1* in the PA/OA-treated AML12 cells were significantly up-regulated relative to controls, while 50  $\mu$ M Cul notably reversed the elevated mRNA expression levels of *ACC* and *SREBP-1*, indicating that Cul inhibited *de novo* fat synthesis (Figure 5A-B). Additionally, PA/OA-treated AML12 cells showed significantly downregulated mRNA levels of *CPT1* and *PPAR $\alpha$* , while 50  $\mu$ M Cul effectively counteracted this reduction, suggesting that Cul enhanced fatty acid oxidation (Figure 5C-D).

Fatty acid breakdown in the body primarily occurs through  $\beta$ -oxidation in mitochondria [27]. Mitochondrial biogenesis is a core driving force in maintaining overall mitochondrial function and health [28]. Therefore, mitochondrial biogenesis-associated genes (*PGC-1 $\alpha$* , *Tfam*) were quantified in AML12 cells. PA/OA induction significantly suppressed the mRNA expression levels of *Tfam* and *PGC-1 $\alpha$*  in AML12 cells compared to the control group, while 50  $\mu$ M Cul treatment markedly increased their expression levels (Figure 5E-F). Western blot results revealed similar trends at the protein level (Figure 5G-I). Furthermore, mitochondrial staining with Mito-Tracker Green in AML12 cells revealed that PA/OA-induced cells displayed a significant reduction in green fluorescence intensity relative to the untreated control group, whereas treatment with 50  $\mu$ M Cul resulted in a notable recovery of fluorescence intensity (Figure 5J). The above results indicated that Cul enhanced mitochondrial biogenesis in PA/OA-treated AML12 cells.



**Fig. 5.** Cul regulates lipid metabolic homeostasis and promotes mitochondrial biogenesis in AML12 cells. Effect of Cul on *ACC* (A), *SREBP-1* (B), *CPT1* (C), *PPARα* (D), *PGC-1α* (E), and *Tfam* (F) mRNA expression in AML12 cells. (G) Representative bands of PGC-1α and Tfam protein expression. (H-I) PGC-1α/GAPDH and Tfam/GAPDH protein expression level in AML12 cells. (J) Mitochondrial staining of AML12 cells conducted using Mito-Tracker Green. Results are expressed as the means  $\pm$  SD ( $n = 3$ ),  $^{\#}P < 0.05$ ,  $^{###}P < 0.01$ ,  $^{####}P < 0.001$ , compared with control group;  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ , compared with model group.

### 3.6 Cul directly interacts with MDM2 and promotes MDM2 ubiquitination

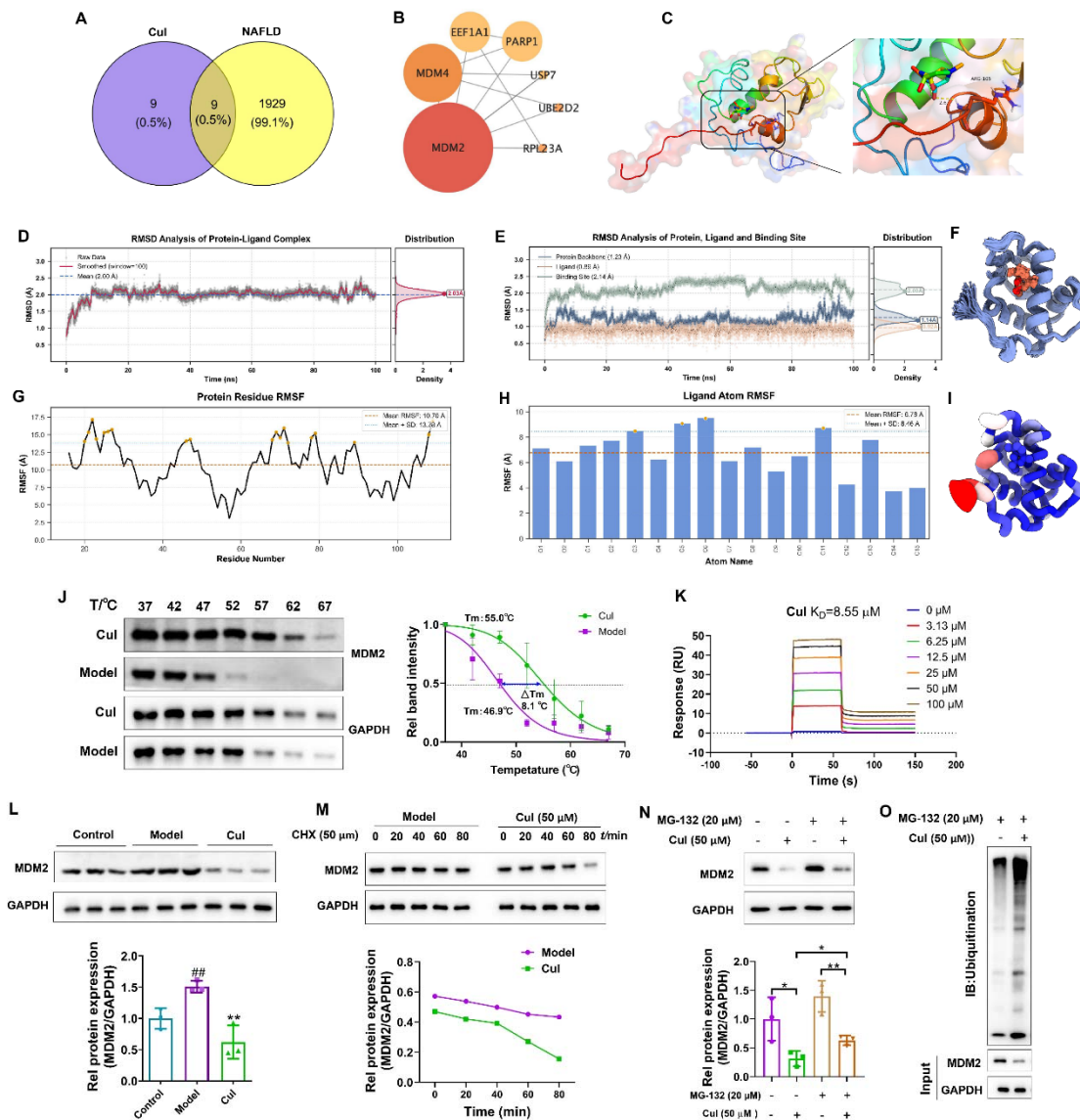
Through the SwissTargetPrediction database, 18 potential targets for Cul were successfully identified. Concurrently, 1,938 NAFLD-related target genes were retrieved from GeneCards and OMIM databases. A Venn diagram analysis revealed nine overlapping targets between Cul and NAFLD-related targets (Figure 6A). A PPI network for these intersecting proteins was constructed and visualized using Cytoscape 3.7.1. Based on degree value analysis, core targets were identified, including MDM2 (Murine Double Minute 2), MDM4, EEF1A1 (Eukaryotic Elongation Factor 1 Alpha 1), USP7 (Ubiquitin Specific Peptidase 7), PARP1 (Poly ADP-ribose Polymerase 1), UBE2D2 (Ubiquitin-conjugating enzyme E2 D2), and RPL23A (Ribosomal protein L23a) (Figure 6B). As a top candidate among these proteins, MDM2 is an E3 ubiquitin ligase, primarily responsible for ubiquitinating p53 protein and ultimately leading to its degradation [29]. Ubch5B/C,

as a physiological E2 enzyme of MDM2, collaborates with MDM2 to promote the ubiquitination and degradation of p53 [30].

Molecular docking analysis was performed between Cul and the optimal target MDM2. The results showed a strong interaction between Cul and MDM2 with a binding energy of -6.5 kcal/mol. Specifically, Cul formed a hydrogen bond with MDM2 through the amino acid residue Arg105 (Figure 6C). Molecular dynamics simulations were further performed to systematically investigate the binding stability and interaction mechanisms between Cul and MDM2. As shown in Figure 6D, the MDM2-Cul complex exhibited RMSD values fluctuating around 2.00 Å throughout the simulation, indicating robust structural stability. In Figure 6E, the RMSD values of the protein backbone, ligand, and binding site all remained within low fluctuation ranges (below 1.50 Å), further demonstrating strong conformational stability of the complex. RMSF analysis revealed an elevated flexibility in specific MDM2 residues, including SER22, VAL109, and GLN71, with RMSF values exceeding 15 Å. Similarly, ligand atoms C6, C5, and C11 displayed pronounced fluctuations (RMSF > 8 Å), suggesting potential dynamic effects on ligand-binding stability (Figure 6G-I). Rg analysis demonstrated minimal fluctuations in the overall compactness of MDM2 and Cul, with Rg components along the X-, Y-, and Z-axes converging closely, confirming well-equilibrated conformational sampling (Figure S3A-D). Additionally, the protein maintained high secondary structure stability during the simulation, as evidenced by consistent occupancy levels of  $\alpha$ -helices and  $\beta$ -strands (Figure S4A-C). MM-GBSA results indicated that the interactions between the protein and ligand were thermodynamically stable, with van der Waals forces and solvation effects serving as the primary binding driving forces (Figure S5A-C).

CETSA assays (Figure 6J) demonstrated that Cul induced a rightward shift of the melting curve and increased the melting temperature ( $T_m$ ) of MDM2 protein by 8.1°C, suggesting that Cul significantly stabilized the conformation of MDM2 protein. Additionally, the SPR analysis demonstrated dose-dependent binding between Cul and MDM2, with a dissociation constant ( $K_d$ ) of 8.55  $\mu$ M, confirming their strong binding affinity (Figure 6K).

Western blot analysis demonstrated that PA/OA treatment significantly increased MDM2 protein levels in AML12 cells, whereas Cul treatment markedly attenuated this upregulation (Figure 6L). To investigate the mechanism underlying Cul-mediated MDM2 suppression, we examined the ubiquitination-dependent degradation pathway. Cycloheximide (CHX, 50  $\mu$ M) chase experiments demonstrated that Cul treatment accelerated MDM2 protein decay within 80 min, suggesting enhanced degradation (Figure 6M). Notably, the proteasome inhibitor MG132 (20  $\mu$ M) substantially reversed Cul-induced MDM2 reduction (Figure 6N). Furthermore, compared with the control group, Cul intervention robustly upregulated MDM2 protein ubiquitination (Figure 6O). Together, these findings definitively demonstrated that Cul promoted MDM2 ubiquitination and degradation through the ubiquitin-proteasome pathway.

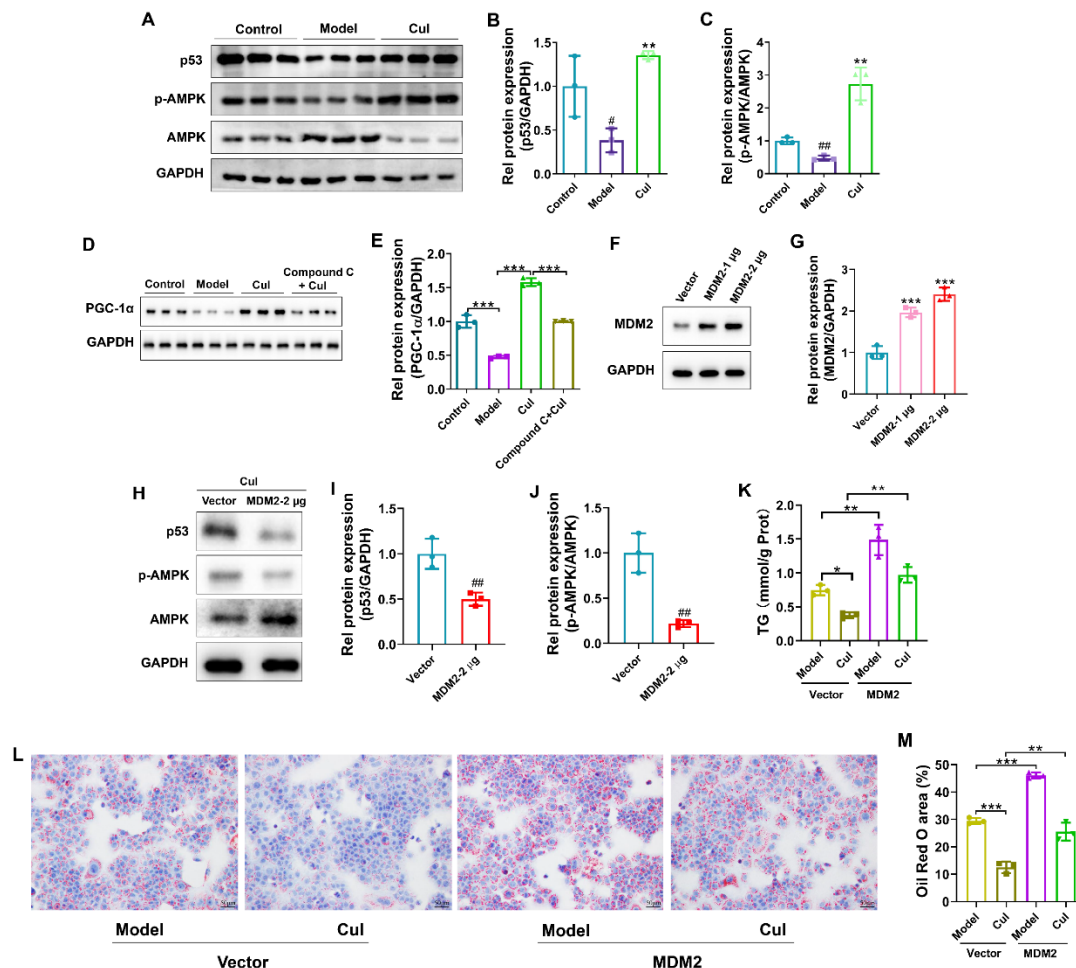


**Fig. 6.** Cul directly interacts with MDM2 and promotes MDM2 ubiquitination. (A) Venn diagram of Cul and NAFLD-related target genes. (B) PPI network diagram of core targets. (C) Molecular docking results of Cul and MDM2. (D) RMSD analysis of the MDM2-Cul complex throughout the 100 ns molecular dynamics simulation. (E) RMSD analysis of the MDM2 backbone, Cul, and binding site. (F) Structural overlay of the MDM2-Cul complex from the final 10-ns trajectory segment. (G) RMSF analysis results of MDM2 residues. (H) RMSF analysis results of Cul atoms. (I) B-factor map of the MDM2-Cul complex over the last 100 ns. Red: regions with larger B-factors (greater flexibility); blue: regions with smaller B-factors (less flexibility). (J) Thermal stability of the MDM2 protein assessed by CETSA. (K) The  $K_D$  value between Cul and MDM2 determined by SPR assay. (L) The effect of Cul on MDM2 protein expression in PA/OA-treated AML12 cells was detected by Western blot assay. (M) The effect of Cul administration on MDM2 expression in CHX-treated NAFLD model AML12 cells was detected by Western blot assay. (N) The effect of Cul administration on MDM2 expression in MG-132-treated NAFLD model AML12 cells for 6 h was detected by Western blot assay. (O) The effect of Cul on MDM2 and ubiquitinated protein expression in MG-132-treated NAFLD model AML12 cells was detected by Western blot assay. Results are expressed as the means  $\pm$  SD ( $n = 3$ ),  $^{##}P < 0.01$ , compared with control group;  $^{**}P < 0.01$ , compared with model group.

### 3.7 Cul enhances mitochondrial biogenesis by triggering MDM2 protein degradation and activating the p53-AMPK-PGC-1 $\alpha$ signaling pathway

MDM2, functioning as an E3 ubiquitin ligase, plays a crucial role in this process by recognizing and binding to the p53 protein, thereby initiating its ubiquitination. The ubiquitinated p53 protein is then efficiently degraded by the proteasome [31]. Compared to the control group, p53 protein expression level was markedly decreased in PA/OA-treated cells, and Cul treatment caused a notable increase in p53 protein

(Figure 7A-C). It has been reported that p53 regulates the activity of AMP-activated protein kinase (AMPK), which subsequently upregulates the expression level of PGC-1 $\alpha$  to control the process of mitochondrial biogenesis [32-34]. As expected, the protein expression of p-AMPK and PGC-1 $\alpha$  was reduced in PA/OA-treated cells compared to the control group; however, Cul treatment significantly increased the protein expression of p-AMPK and PGC-1 $\alpha$  (Figure 7C-E). Further research revealed that when an AMPK pathway inhibitor Compound C was used for intervention, the high expression of PGC-1 $\alpha$  induced by Cul was effectively inhibited. Then a MDM2-overexpressing AML12 cell model was established using plasmid transfection (Figure 7F-G). Notably, MDM2 overexpression effectively attenuated Cul-stimulated upregulation of both p53 and p-AMPK protein levels (Figure 7H-J). Moreover, MDM2 overexpression completely antagonized the Cul-induced reduction in TG content (Figure 7K). Furthermore, Oil Red O staining analysis further revealed that MDM2 overexpression significantly attenuated the Cul-induced decrease in lipid droplet number and size in AML12 cells (Figure 7L-M).



**Fig. 7.** Cul reduces lipid accumulation in AML12 cells by downregulating MDM2 to activate the p53-AMPK-PGC-1 $\alpha$  pathway. (A-C) The effect of Cul on the expression of p53 and p-AMPK proteins in PA/OA-treated AML12 cells was detected by Western blot assay. (D-E) Following pretreatment with AMPK inhibitor (Compound C, 20  $\mu$ M), the expression of PGC-1 $\alpha$  protein in PA/OA-treated AML12 cells was detected by Western blot assay. (F-G) The overexpression of MDM2 was detected by Western blot assay after DNA plasmid transfection into AML12 cells. (H-J) The effect of MDM2 overexpression on the expression of p53 and p-AMPK proteins in Cul-treated AML12 cells was detected by Western blot assay. (K) The effect of MDM2 overexpression on TG content in Cul-treated AML12 cells. (L) Representative images of Oil Red O staining in AML12 cells. (M) Quantitative analysis of Oil Red O positive area. Results are expressed as the means  $\pm$  SD ( $n = 3$ ), <sup>#</sup> $P < 0.05$ , <sup>###</sup> $P < 0.001$ , compared with control group; <sup>\*\*</sup> $P < 0.01$ , <sup>\*\*\*</sup> $P < 0.001$ , compared with model group.

The above results indicated that Cul directly bound to the MDM2 protein, triggering its ubiquitin-proteasome degradation cascade and thereby relieving MDM2-mediated suppression of p53. The stabilized p53 protein subsequently activated the AMPK-PGC-1 $\alpha$  energy metabolic axis, which enhanced mitochondrial biogenesis. This regulatory network ultimately promoted fatty acid  $\beta$ -oxidation metabolism, effectively reversing PA/OA-induced abnormal accumulation of intracellular TG in hepatocytes.

#### 4. Discussion

NAFLD is a significant manifestation of the metabolic syndrome, characterized by aberrant triglyceride accumulation in the liver, resulting in steatosis. This condition is often triggered by long-term overnutrition, which disrupts the balance between energy intake and expenditure. Consequently, NAFLD is closely associated with various metabolic disorders, such as hyperlipidemia, obesity, and type 2 diabetes. As a pressing public health issue worldwide, NAFLD poses significant challenges due to its complex pathogenesis, making the development of effective treatments a pressing priority [7]. During the pathological process of NAFLD, mitochondria exhibit structural and functional abnormalities, including electron transport chain dysfunction, decreased fatty acid  $\beta$ -oxidation capacity, increased ROS production, and intensified lipid peroxidation. Studies have suggested that mitochondria may be a key intervention point for NAFLD treatment, and protecting mitochondrial function can help restore hepatic bioenergetic balance [35].

Mitochondria play a crucial role in ROS production. In the electron transport chain, electron leakage reacts with oxygen to generate superoxide ( $O_2^{\cdot-}$ ). Under basal conditions, approximately 2% of oxygen is converted into superoxide, and superoxide production increases when mitochondrial respiratory rates rise. Although mitochondria contain antioxidant enzymes such as GSH and SOD, excessive ROS can overwhelm these defenses, causing molecular damage, intracellular imbalance, and mitochondrial dysfunction. Nrf2 is a key antioxidant regulatory factor involved in the regulation of multiple biological processes such as oxidative stress, inflammatory responses, apoptosis, and mitochondrial energy supply disorders. Nrf2 controls the expression of various antioxidant genes and serves as one of the main factors in combating increased mitochondrial ROS. Nrf2 enhances the expression of GSH biosynthetic enzymes and their regenerating enzyme glutathione reductase, maintaining mitochondrial GSH levels and participating in mitochondrial regulation through various intermolecular interactions, including directly upregulating mitochondrial transcription factors and activating mitochondrial autophagy for mitochondrial quality control [36,37]. Our research revealed that Cul markedly reduced the MDA levels and simultaneously elevated the levels of GSH, SOD, and the proteins of Nrf2 and HO-1 in the liver tissues of HFD-fed mice. This finding suggested that Cul alleviated the oxidative stress damage in the liver tissues of HFD-induced mice through activation of the Nrf2/HO-1 antioxidant defense pathway.

Mitochondria are also involved in tasks such as the tricarboxylic acid (TCA) cycle, fatty acid and amino acid catabolism, steroid synthesis, calcium homeostasis, and apoptosis. Glucose and lipid metabolism depend on mitochondria, and improving mitochondrial function can alleviate insulin resistance and glucose-lipid metabolism [38]. Our study found that Cul upregulated hepatic PPAR $\alpha$  and CPT1 expression at

mRNA/protein levels, indicating that Cul promoted fatty acid oxidation. Mechanistically, Cul directly targeted MDM2, an E3 ubiquitin ligase, inducing its ubiquitination and subsequent degradation, thereby stabilizing p53 protein. The accumulated p53 upregulated AMPK expression, a key energy sensor, and the activated AMPK further promoted PGC-1 $\alpha$  expression. Enhanced PGC-1 $\alpha$  levels stimulated mitochondrial biogenesis and fatty acid  $\beta$ -oxidation, presenting a highly promising NAFLD therapeutic strategy.

MDM2 has long been recognized as a key therapeutic target in oncology [39]. Current MDM2-targeting strategies primarily focus on disrupting its interaction with p53 or developing direct MDM2 inhibitors, though clinical efficacy has been limited to date [40]. Alternative approaches to MDM2 regulation include suppressing its protein expression, inhibiting its enzymatic activity, or promoting its degradation. While several small-molecule MDM2 inhibitors have been discovered, including the indole derivative SP141, the quaternary ammonium salt MA242, and the pyridone structure CPI-7c [41–43], none have yet gained FDA approval. Research has found that MDM2 not only plays a crucial role in oncogenesis, but also exhibits significantly elevated expression levels in hepatic tissues of mice with metabolic fatty liver disease (MFLD) [44]. Our study further confirmed similar MDM2 upregulation in cellular models of NAFLD. Importantly, we identified Cul, a sesquiterpenoid that specifically induced MDM2 degradation in NAFLD cells. To our knowledge, Cul represents the first sesquiterpene compound capable of targeting MDM2 for its ubiquitination and proteasomal degradation. Given this novel negative regulatory mechanism, we hypothesize that Cul may have dual therapeutic potential against both metabolic and neoplastic diseases.

Several PPAR agonists (e.g., fenofibrate and rosiglitazone) are currently used to treat hyperlipidemia. However, these synthetic drugs are associated with adverse effects such as hepatotoxicity and cardiovascular risks [45]. In the U.S. and Europe, five SGLT2 inhibitors—empagliflozin, canagliflozin, dapagliflozin, ertugliflozin, and sotagliflozin—are widely prescribed for diabetes management. Nevertheless, SGLT2 inhibitors primarily reduce hepatic fat accumulation indirectly through glucose-lowering effects, exhibiting relatively weak direct regulatory effects on lipid metabolism [46]. In contrast, the naturally derived compound Cul mechanistically avoids the toxicities linked to PPAR $\alpha$  agonists. More importantly, its therapeutic efficacy does not depend on diabetic status, making it effective for non-diabetic patients with NAFLD. This characteristic overcomes a major limitation of SGLT2 inhibitors, positioning Cul as a promising therapeutic agent for a broader patient population, including those without diabetes.

In summary, this study establishes curcumenol (Cul) as a novel therapeutic agent for NAFLD through its unique mechanism of directly binding and promoting the ubiquitination and proteasomal degradation of MDM2, representing the first sesquiterpenoid compound identified with this specific molecular action. By targeting MDM2 for degradation, Cul stabilizes p53 and activates the AMPK-PGC-1 $\alpha$  axis, leading to enhanced mitochondrial biogenesis and fatty acid  $\beta$ -oxidation while simultaneously suppressing *de novo* lipogenesis. Furthermore, Cul activates the Nrf2/HO-1 antioxidant pathway, providing comprehensive protection against metabolic dysregulation. Our integrated approach combining structural analyses, biophysical validation, and functional assays demonstrates Cul's ability to specifically induce MDM2

ubiquitination and degradation, offering a distinct therapeutic strategy compared to conventional MDM2 inhibitors. These findings not only reveal Cul's dual benefits in improving lipid metabolism and oxidative homeostasis but also highlight its potential as a promising lead compound for developing innovative treatments for NAFLD and related metabolic disorders, with possible broader applications in other MDM2-associated pathologies.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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