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Menin prevents liver steatosis through co-activation of peroxisome proliferator-activated receptor alpha

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ABSTRACT

Fatty liver is strongly associated with metabolic syndrome. Here, we show that the impaired hepatic expression of menin, the product of the MEN1 (multiple endocrine neoplasia type 1) tumor suppressor gene, represents a common feature of several fatty liver mouse models. The liver specific ablation of MEN1 gene expression in healthy mice induced hepatic steatosis under high-fat dietary conditions. Moreover, overexpression of menin in livers of steatotic db/db mice reduced liver triglyceride accumulation. At the molecular level, we found that menin acts synergistically with the nuclear receptor PPARα to control gene expression of fatty acid oxidation. Collectively, these data suggest a crucial role for menin as an integrator of the complex transcriptional network controlling hepatic steatosis.

Structured summary of protein interactions:
Menin physically interacts with **PPAR alpha** by anti tag coimmunoprecipitation (View Interaction: 1, 2).

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has emerged as an important public health problem because of its high prevalence, potential progression to severe liver disease and association with serious metabolic syndrome, including type 2 diabetes mellitus, atherosclerosis and coronary heart disease [1,2]. Although it has been connected with numerous impairments of energy homeostasis, the molecular determinants of fatty liver development remain largely unexplored.

There is an intense interest in the roles of transcription factors in the pathogenesis of NAFLD, particularly the peroxisome proliferator activated receptor alpha (PPARalpha). When activation, PPARalpha formes a heterodimer with the nuclear receptor RXR and binds to specific DNA-response elements in the promoter of target genes [3]. The genomic sequence recognized by PPARalpha consists of a direct repeat of the consensus hexameric motif AGGTCA interspaced by a single nucleotide [4]. PPARalpha is highly expressed in liver where it stimulates fatty acid uptake and activation, mitochondrial β-oxidation, peroxisomal fatty acid oxidation, ketogenesis and fatty acid elongation and desaturation [5–7]. In addition, transcriptional cofactor complexes have been identified as critical checkpoints in the coordination of metabolic programs through activation or repression of PPARalpha, exemplified by peroxisome proliferator-activated receptor coactivator 1 (PGC-1alpha), SIRT1, or the steroid hormone receptor coactivators [8–10]. In this respect, we hypothesized that the dysregulated gene expression of distinct transcriptional cofactor of PPARalpha under fatty liver conditions directly mirrors alterations of hepatic lipid homeostasis and may causally contribute to the fatty liver phenotype.

In this study, we identify the inhibition of menin gene expression as a conserved and functionally important checkpoint in the manifestation of liver steatosis. Menin, encoded by the MEN1 gene, was identified as the pathogenic gene for beta cell tumor formation in patients with multiple endocrine neoplasia type 1 [11,12]. Subsequently, it has been considered as a negative regulator in pancreatic beta cell proliferation [13,14]. Menin has been shown to be a histone methyltransferase protein complex containing MLL, which promotes trimethylation of histone H3 on lysine 4, a epigenetic mark associated with transcriptionally active chromation [15,16]. However, the biological and in particular, liver specific functions of menin remained largely unknown.

2. Materials and methods

2.1. Animal experiments

Male 10- to 12-week-old C57BL/6 and db/db mice were obtained from Shanghai Laboratory Animal Company (SLAC,

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Shanghai, China) and maintained on a 12-h light/12-h dark cycle with access to water and diet. Normal diet and high fat diet were purchased from SLAC, which contained 10% kcal and 60% kcal calories, respectively. For adenovirus injections, 2×10^9 plaque-forming units (pfu) per recombinant virus were administrated via tail vein injection. Mice (including mice treated with viruses or high fat diet food) were sacrificed for further analysis after fasting for 10 h. For intralipid infusions, mice were fasted for 12 h, and 200 μl of a 20% (v/v) intra-lipid emulsion in saline was administrated intravenously. Mice were sacrificed and liver tissues were collected 12 h after infusion.

2.2. Lipids and glucose analysis

Trunk blood was harvested in EDTA tubes on ice and plasma was stored at −70 °C. Lipids were extracted from liver in chloroform/methanol (2:1, v/v) and washed in 50 mM NaCl. Samples were redissolved in 50 ml of 1:1 chloroform/Triton X-100. All samples were measured using commercial kits (Thermo, USA), according to the manufacturer's instructions. Blood glucose levels were determined using an automatic glucosemonitor (One Touch, Lifescan).

2.3. RNA extraction and real-time PCR analysis

Total RNAs were extracted from tissues or cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Five hundred nanograms of total RNA was used for cDNA synthesis using oligo (dT) primers (Promega, USA). Real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) in an Applied Biosystems 7900 Machine. Following an initial denaturation at 95 °C for 30 s, 40 cycles of PCR amplification were performed at 95 °C for 5 s and 60 °C for 30 s. Standard curves were generated and the relative amount of target gene mRNA was normalized to GAPDH. The specificity was verified by melt curve analysis and agarose gel electrophoresis. The sequences of all used primers are listed below:

Mouse menin
Forward: 5′-CGATCTTCACACTGACTCTTTGG-3′,
Reverse: 5′-AGGTCTGCCAAGTTCCCTAGC-3′;
Mouse CPT1beta
Forward: 5′-AGGCACTTCTCAGCATGGTC-3′,
Reverse: 5′-GCTTCAGGGTTTGTCGGAAGA-3;
mouse MCAD
Forward: 5′-AGTACCCGTTCCCTCTCATCA-3′,
Reverse: 5′-TACACCCATACGCCAACTCTT-3′;
Mouse Acox1
Forward: 5′-GATTCTGAACGATCCAGACTTCC-3′,
Reverse: 5′-CACCTCGTAACGCTGGCTT-3′;
Mouse GAPDH
Forward: 5′-GTATGACTCCACTCACGGCAA-3′,
Reverse: 5′-CTTCCCATTCTCGGCCTTG-3′.

2.4. Cell culture and luciferase assay

HeLa and HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (1:1) medium (Invitrogen, USA). Adenoviruses expressing menin-specific shRNA were obtained from Santa Cruz Company (sc-35923) and constructed under control of the U6 promoter. Viruses were purified by the cesium chloride method and dialyzed against phosphate-buffered-saline buffer. Primary mouse hepatocytes were transiently transfected with menin-specific or non-specific shRNA for 36 h before harvest. For luciferase assays, cells were seeded into 24-well plates and transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Two hundred nanograms of GalTK-luciferase construct and 200 ng of the expression plasmid for Gal4-PPARalpha (AF2) were co-transfected into HepG2 cells. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, USA) and repeated for five times.

2.5. Immunoprecipitation

HeLa cells were transfected with HA-Menin or His-PPARalpha for 48 h. Cells were harvested and lysed in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, Roche complete EDTA-free protease inhibitor cocktail). Lysates were cleared by centrifugation and supernatants were subjected to immunoprecipitation (IP) with immobilized anti-HA (Sigma) resin overnight. After extensive washing, 2× SDS loading buffer was added into the resin and boiled. The samples were loaded and separated by SDS–PAGE and immunoblotted with a His-Tag antibody.

2.6. Statistics

Values were reported as mean ± SEM. Statistics analyses were performed using a Student's t test. Statistical significance is displayed as $^{*}(P < 0.05)$, $^{**}(P < 0.01)$ or $^{***}(P < 0.001)$.

3.Results

3.1. Hepatic menin expression is down-regulated in fatty liver mouse models

If menin is involved in the regulation of lipid homeostasis, it would be anticipated that its expression is altered in mice models of fatty liver. We first examined a mouse model of severe genetic obesity resulting from leptin receptor deficiency (also known as db/db mice). Quantitative real-time reverse-transcription polymerase chain reaction (Real-time PCR) and western immunoblotting analyses showed a dramatic decrease in the messenger RNA (mRNA) and protein expression levels of menin in the livers of db/db mice, in comparison with wild-type littermates (Fig. 1A and B). In contrast, comparable menin expression levels were detected in the white adipose tissue (data not shown). To test whether the hepatic inhibition of menin represents a more common feature of obesity-related dyslipidemia, we studied high-fat-diet-fed (HFD) mice as an independent standard model for nutrition-induced obesity. Indeed, menin mRNA and protein levels were found to be reduced in livers of HFD mice as compared with controls (Fig. 1C and D).

To identify potential triggers of Menin gene inhibition under these conditions, we next used an in vivo lipid infusion system, which acutely increases circulating free fatty acids (FFA) and causes insulin resistance. In this setting, there was also a dramatic decrease in menin expression in mice following lipid exposure (Fig. 1E). Moreover, exposure of HepG2 cells to palmitate significantly inhibited menin mRNA levels (Fig. 1F), further suggesting that fatty acids can impair menin expression in obesity related conditions.

3.2. Liver specific menin deficiency promotes hepatic steatosis

To address the liver-specific function of menin, we disrupted its expression in livers of lean C57BL/6 mice by delivering an adenovirus expressing menin-specific or non-specific control shRNA via tail vein injection. Menin shRNA treatment significantly reduced hepatic menin mRNA and protein levels as compared with control shRNA-injected littermates (Fig. 2A and B). Acute knockdown of

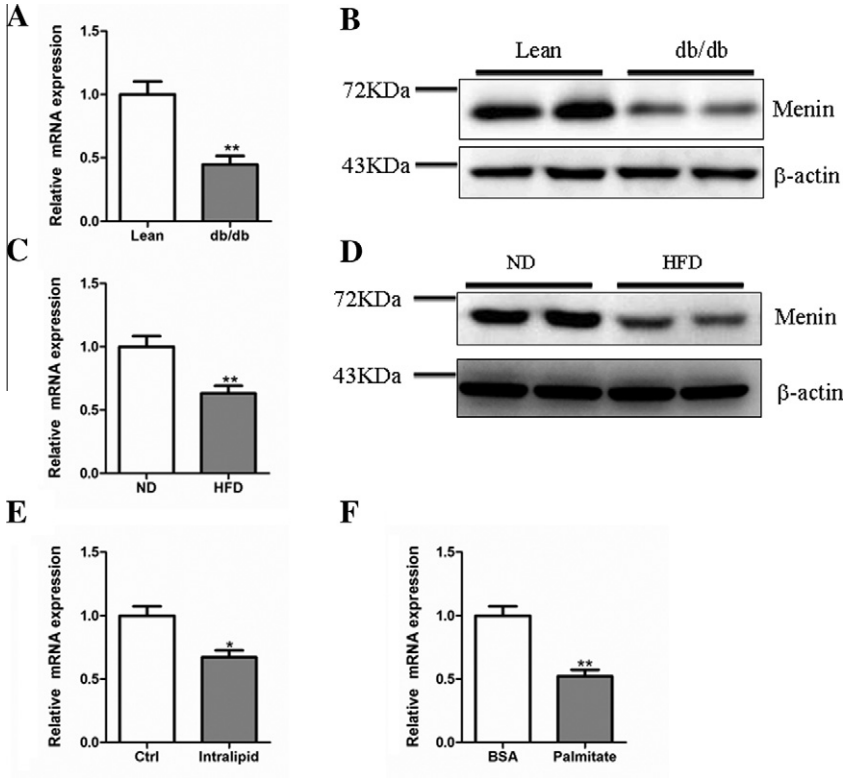

Fig. 1. Hepatic expression of menin is reduced in fatty livers. (A and B): mRNA (A) and protein (B) analysis of liver extracts from wild-type lean mice or db/db mice, $n = 6$. (C and D): Menin mRNA (C) and protein (D) levels in livers of mice fed a normal diet (ND) or high fat diet (HFD), $n = 7$. (E) Menin mRNA levels in livers of C57BL/6 mice injected with intralipid emulsion for 12 h, $n = 5$. (F) Menin mRNA levels in HepG2 cells treated with 0.5 mM palmitate or 0.5% BSA for 24 h. Bars correspond to mean ± SEM from four experiments.

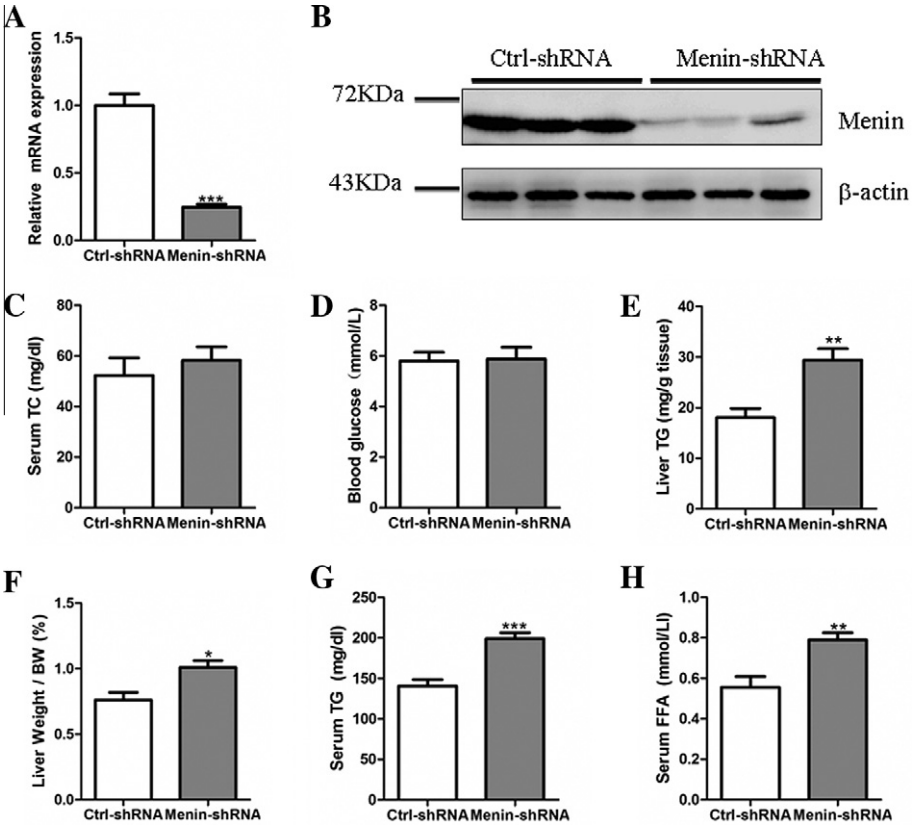

Fig. 2. Inhibition of hepatic menin results in liver steatosis. (A and B) mRNA (A) and protein (B) levels of hepatic menin from mice injected with menin-shRNA or control-shRNA viruses, $n = 8$. (C–H) Serum cholesterol (C), blood glucose (D), liver triglyceride (E), liver weight (F), serum triglyceride (G) and serum fatty acid (H) levels in the same mice as in (A), $n = 8$.

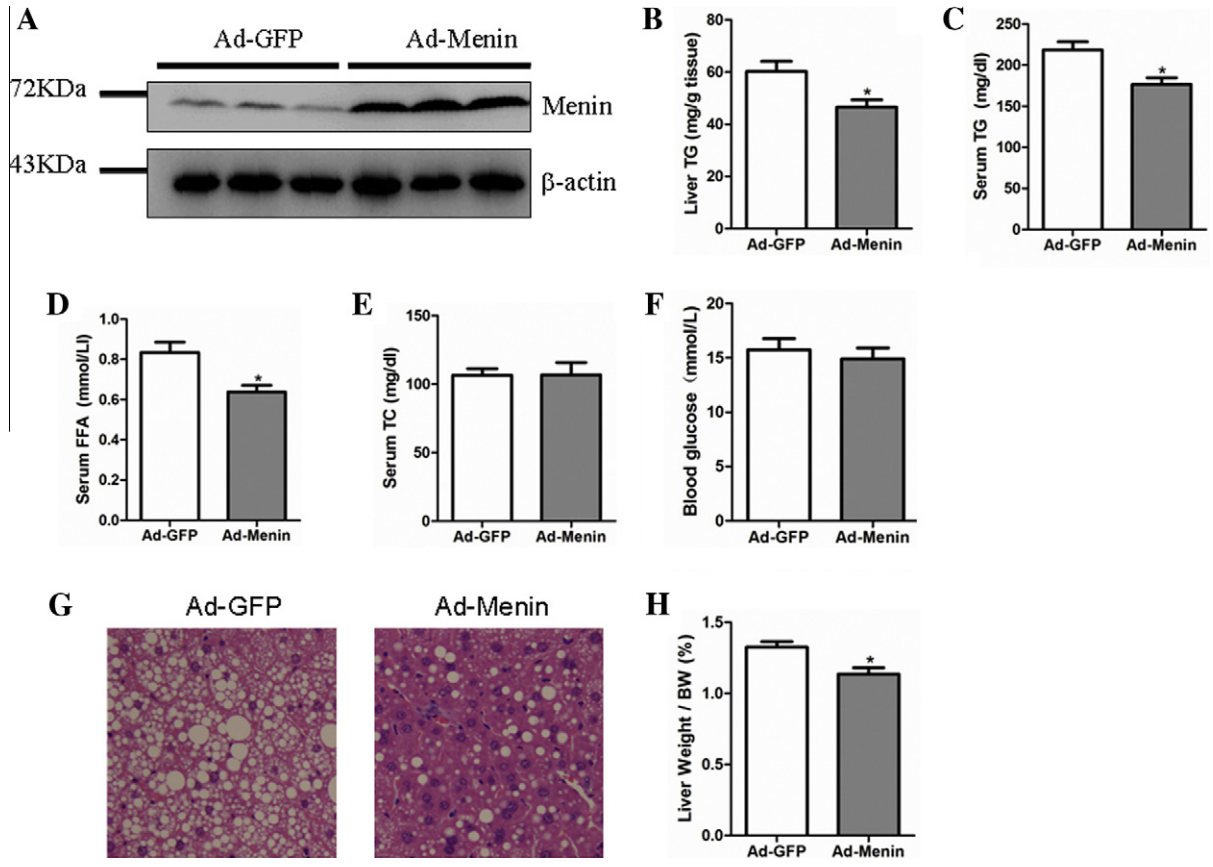

Fig. 3. Restoration of hepatic menin expression improved fatty livers in db/db Mice. (A) Menin expression in livers of db/db mice following adenoviral expression of menin (Ad-Menin) or control vector (Ad-GFP), $n = 6$. (B–F) Liver triglyceride contents (B), serum triglyceride (C), serum fatty acid (D), serum cholesterol (E) and blood glucose levels (F) were determined in mice as indicated in (A), $n = 6$. (G) Representative hematoxylin and eosin staining of liver sections from mice as indicated in (A). (H) Liver weight from two groups of mice were determined.

menin caused no major alterations in serum cholesterol levels, blood glucose levels (Fig. 2C and D), body weight, food intake and total body fat content (data not shown). In contrast, loss of hepatic menin resulted in a significant increase in triglyceride content and liver weight (Fig. 2E and F), indicating that menin predominantly prevents hepatic TG accumulation in wild-type animals. Moreover, abrogation of hepatic menin resulted in an increase in serum triglyceride and fatty acid levels (Fig. 2G and H).

3.3. Restoration of hepatic menin in db/db mice prevents liver steatosis

Because menin is dramatically down-regulated in livers of db/db mice, we reasoned that reconstitution of menin expression might be an effective way to prevent the progression of liver steatosis. Therefore, we expressed menin using an adenoviral system in the liver tissue of db/db animals to examine its metabolic impact. After adenoviral delivery, we verified that expression of menin was significantly elevated (~4-fold) in the liver tissue of obese mice, compared to vector controls (Fig. 3A). Indeed, this increase in the expression of menin in db/db mice led to decreased hepatic TG content (Fig. 3B) and serum TG, FFA levels (Fig. 3C and D), while serum cholesterol, and blood glucose levels remained unaffected (Fig. 3E and F). Results from histological examination on hepatic sections also confirmed our observations (Fig. 3G), showing an improved triglyceride accumulation in mice over-expressed menin. Besides, menin overexpression markedly alleviated the hepatomegaly, as shown by a significant decrease in the liver weight (Fig. 3H).

3.4. Menin cooperates with nuclear receptor PPARalpha to improve liver steatosis

To determine whether changes in the fatty acid oxidation pathway contribute to the observed phenotype in menin-depleted animals, we measured the circulating levels of total ketone bodies and found that mice deficient in hepatic menin showed decreased levels of ketone bodies in the serum (Fig. 4A), indicating reduced hepatic oxidation of fatty acids. Consistently, real-time PCR analysis revealed particularly decreased expression of genes in the mitochondrial and peroxisomal fatty acid oxidation pathways (Fig. 4B), suggesting that disruption of menin expression promotes liver steatosis by specifically blunting fatty acid combustion.

The nuclear receptor PPARalpha has been identified as a master regulator of fatty acid oxidation in the liver, prompting us to test whether menin might serve as a physiological coactivator for PPARalpha. Indeed, we found a ligand independent interaction between menin and PPARalpha (Fig. 4C and D). To further study the mechanism of co-activation of PPARalpha by menin, we performed luciferase reporter assays. To this end, HepG2 cells were transiently transfected with increasing amounts of menin together with the expression vector for a fusion protein containing the AF2 domain of PPARalpha fused to the DNA binding domain of the yeast Gal4 activator. We found that menin could enhance this luciferase reporter in a dose dependent manner (Fig. 4E). Moreover, inhibition of menin expression in primary mouse hepatocytes reduced the induction of prototypic PPARalpha target genes (Fig. 4F and G), demonstrating that menin is indeed required for

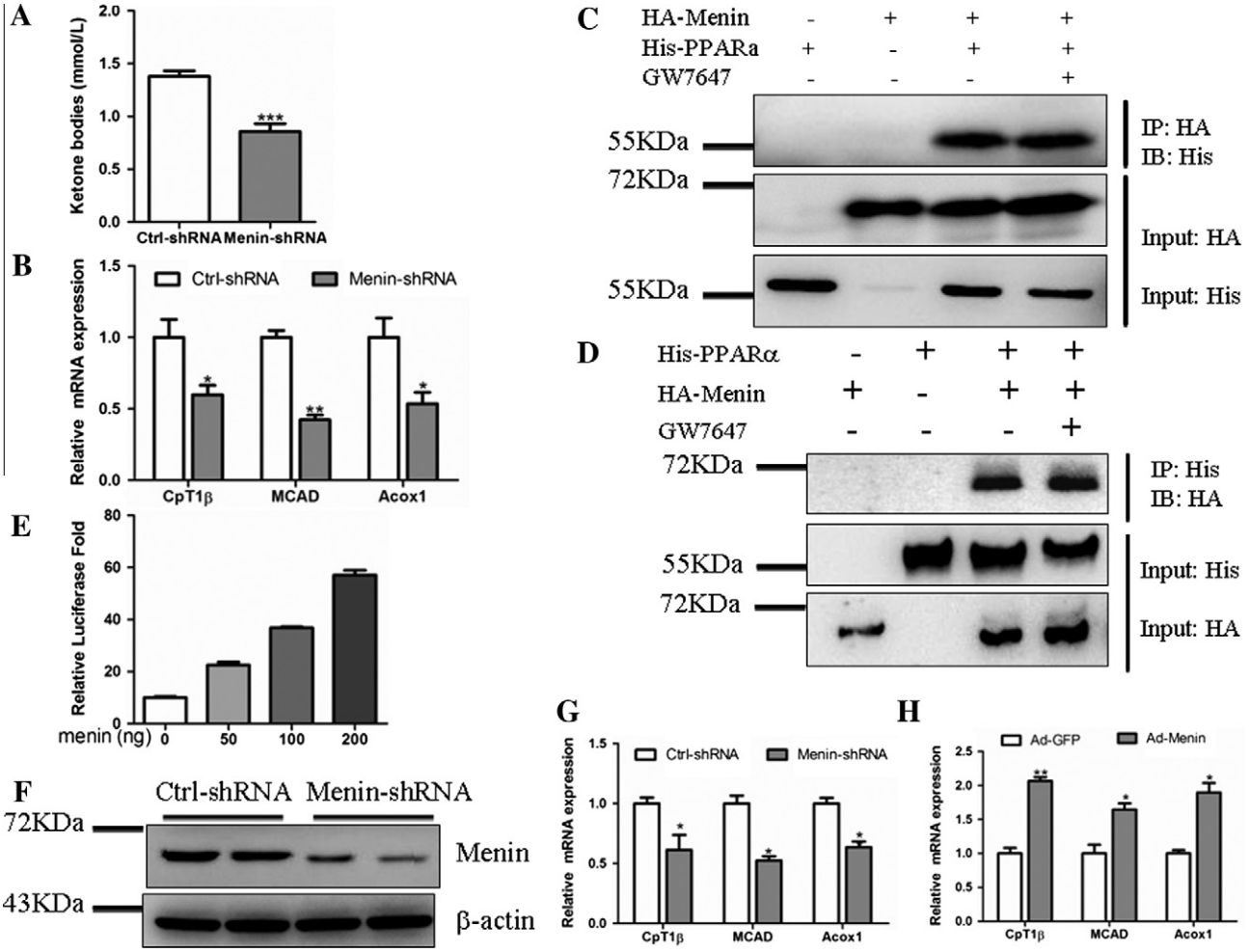

Fig. 4. Menin cooperates with nuclear receptor PPARalpha. (A) Total serum ketone body levels were determined in mice injected with menin or control shRNA viruses, $n = 8$. (B) mRNA levels of fatty acid oxidation in livers of the same mice as in (A), $n = 8$. (C and D) Co-immunoprecipitation assays performed with HA-menin and His-PPARalpha in HeLa cells. (E) Menin promoted PPARalpha transcriptional activity in a dose dependent manner in a luciferase reporter assay. (F) Western blot analysis of menin expression levels in primary mouse hepatocytes transfected with menin-specific or control shRNA. (G) mRNA levels of genes involved in fatty acid oxidation in primary hepatocytes transfected with menin or control shRNA viruses, $n = 8$. (H) Restoration of hepatic menin expression promoted genes expression in livers from obese db/db mice, $n = 6$.

PPARalpha transcriptional activity in a cell-autonomous manner. In agreement, overexpression of menin in db/db mice activated the expression of PPARalpha target genes (Fig. 4H).

4. Discussion

Menin is the product of the MEN1 tumor suppressor gene, which participates in many cellular processes, including transcription regulation, apoptosis and DNA repair [17]. As a transcriptional regulator, menin is able to co-activate estrogen receptor alpha (ERalpha) and PPARgamma mediated transcription [18,19]. Activation of transcription by menin also involves histone modifications. It is shown that menin acts as a key component of MLL1 (KMT2A) or MLL2 (KMT2B) protein complexes which have histone methyltransferase (HMT) activity specific for lysine 4 of histone H3 (H3K4) [16].

Here our data provide novel insights into liver-specific functions of menin with further implications for the pathogenesis of obesity-related fatty liver. The results of our studies are consistent with mouse models in which hyperlipidemic conditions are associated with the down-regulation of hepatic menin expression. An increased exposure of the liver to fatty acids might at least in part explain the menin inhibition under these conditions, although other additional stress signals such as hyperinsulinemia might also contribute to the regulation of menin gene expression. Furthermore, menin inhibition is sufficient to promote liver triglycerides accumulation, thereby establishing a key role for it in metabolic disorders.

PPARalpha agonists, such as fibrates, are commonly prescribed as a hypolipidemic drugs for NAFLD treatment [20]. PPARalpha lowers TG accumulation by enhancing fatty acid oxidation and stimulating clearance of TG-rich lipoproteins. Mice lack of PPARalpha is reported to be prone to liver steatosis and hypertriglyceridemia [5]. Here, our data indicate that menin act as a co-activator to regulate its transcriptional activity. We studied the interaction between menin and PPARalpha and found that this interaction was not dependent on the presence of ligand. This is in contrast to the interactions of menin with ERalpha and RXR, which are ligand dependent and represent classical NR coactivator interactions.

Together, our studies demonstrate that menin plays a critical regulatory checkpoint in the prevention of liver steatosis. Menin is a direct coactivator for PPARalpha mediated transcription by recruiting other co-regulators to PPARalpha target genes.

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