



GADD45 α alleviates acetaminophen-induced hepatotoxicity by promoting AMPK activation

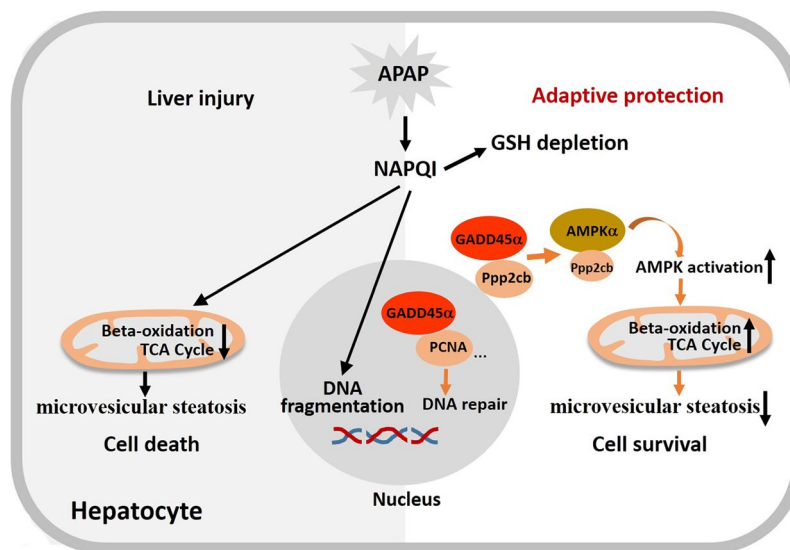
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Abstract

As an analgesic and antipyretic drug, acetaminophen (APAP) is commonly used and known to be safe at therapeutic doses. In many countries, the overuse of APAP provokes acute liver injury and even liver failure. APAP-induced liver injury (AILI) is the most used experimental model of drug-induced liver injury (DILI). Here, we have demonstrated elevated levels of growth arrest and DNA damage-inducible 45 α (GADD45 α) in the livers of patients with DILI/AILI, in APAP-injured mouse livers and in APAP-treated hepatocytes. GADD45 α exhibited a protective effect against APAP-induced liver injury and alleviated the accumulation of small lipid droplets *in vitro* and *in vivo*. We found that GADD45 α promoted the activation of AMP-activated protein kinase α and induced fatty acid beta-oxidation, tricarboxylic acid cycle (TCA) and glycogenolysis-related gene expression after APAP exposure. Liquid chromatography–mass spectrometry (LC–MS) analysis showed that GADD45 α increased the levels of TCA cycle metabolites. Co-immunoprecipitation analysis showed that Ppp2cb, a catalytic subunit of protein phosphatase 2A, could interact directly with GADD45 α . Our results indicate that hepatocyte GADD45 α might represent a therapeutic target to prevent and rescue liver injury caused by APAP.

Graphical abstract



Keywords Acetaminophen (APAP) · Drug-induced liver injury (DILI) · GADD45 α · AMPK

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Extended author information available on the last page of the article

Introduction

Drug-induced liver injury (DILI) is a significant clinical problem and a challenge to drug development worldwide [1]. Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP) is commonly used as an analgesic and antipyretic drug and is known to be safe at therapeutic doses. However, APAP overdose has been one of the most common causes of acute liver injury and even liver failure in many countries [2, 3]. APAP-induced liver injury (AILI) is the most frequent drug hepatotoxicity and the most used experimental model of DILI.

The accumulation of *N*-acetyl-*p*-benzoquinone imine (NAPQI), the reactive and toxic metabolite of APAP, is generally considered as the main cause of liver injury induced by an overdose of APAP [4]. The reaction between NAPQI and a protein containing sulfhydryl groups on its cysteine residues can trigger mitochondrial damage, oxidative stress, c-jun N-terminal kinase (JNK) activation, nuclear DNA fragmentation and cell death [5–8]. APAP-induced liver injury also exhibits microvesicular steatosis that is characterized by the accumulation of numerous small lipid droplets in the hepatocyte cytoplasm [9]. Microvesicular steatosis caused by APAP toxicity might be associated with the suppression of PPAR α -regulated pathways and the inhibition of fatty acid β -oxidation induced by APAP treatment [10].

The growth arrest and DNA damage-inducible 45 (GADD45) family genes (Gadd45 α , Gadd45 β , Gadd45 γ) are rapidly induced in response to endogenous and exogenous stress stimuli. The three small (18-kDa) proteins participate in genomic stability, cell cycle arrest, cell survival, apoptosis and DNA demethylation and repair [11–13]. One study has demonstrated that both agents that activate nuclear receptors (such as CAR and PXR) and partial hepatectomy (PH) can induce extremely high expression levels of Gadd45 [14]. Based on our own data, Gadd45 α and Gadd45 β levels rapidly increase in livers and hepatocytes that are subjected to APAP, while Gadd45 γ does not increase. Gadd45 α is the most elevated member of the Gadd45 family in response to APAP treatment.

Gadd45 α is transcriptionally regulated by mitogen-activated kinase (MAPK) signaling, p53, BRCA1, FOXO3, C/EBP and ATF4 [15–18]. Upon exposure to cellular stress stimuli, Gadd45 α carries out its functions via protein–protein interactions. Gadd45 α increases DNA repair by interacting and/or affecting various proteins, such as proliferating cell nuclear antigen (PCNA), APE and XPG [19–21]. Gadd45 α also induces cell cycle arrest at both the S phase and G2/M phase by displacing PCNA from the cyclin D1 complex, binding to CDK1 and preventing the CDK1 association with cyclin B1 [22–24]. Protein

partners also include histones, members of the p38/JNK stress-induced kinase pathway, p21, ER-1 α , Bim, Nek2, Aurora-A, members of the β -catenin pathway and members of the mTOR pathway [25–27].

During the development of DILI, many aspects of hepatic metabolism are dysregulated, which influences the progression and prognosis of DILI [28]. AMP-activated protein kinase (AMPK) functions as a sensor of the cellular energy status and a master regulator of metabolism by controlling various metabolic pathways [29]. As a heterotrimeric enzyme, AMPK comprises catalytic α (α 1 and α 2) subunits, regulatory β (β 1 and β 2) subunits and γ (γ 1, γ 2 and γ 3) subunits [30, 31]. In response to an energy deficit (low ATP/AMP), AMPK is activated and phosphorylates targets and then restores the energy balance by activating energy-producing pathways, such as glucose uptake, glycogen synthesis, glycolysis, fatty acid β -oxidation and lipolysis while inhibiting energy-consuming processes, such as the de novo biosynthesis of fatty acids and cholesterol, protein synthesis and gluconeogenesis [32–35]. A recent study reported that salicylate, the base component and the unacetylated form of aspirin, can directly activate AMPK by binding to the β 1 subunit [36]. However, until now, the effect of Gadd45 α in the process of APAP-induced acute liver injury has not been well understood, and the relationship between Gadd45 α and AMPK had not been previously explored.

In the present study, we investigated the role of Gadd45 α in APAP overdose-induced acute liver injury in vitro and in vivo. Importantly, we demonstrated that the activation of AMPK was an underlying key mechanism through which Gadd45 α ameliorated APAP-induced liver injury.

Materials and methods

Human sample collection

A total of nine DILI cases (four of them are APAP-induced DILI) were enrolled in this study to evaluate the expression of Gadd45 α . Three healthy liver tissues (used as the control group) were collected from donors whose livers were subsequently used for liver transplantation. All patients provided written informed consent, and the protocol was approved by the Shanghai Jiao Tong University Ethics Committee.

Animals

C57BL/6J male mice, aged 6–8 weeks and weighing 18–22 g at the time of the experimental procedures, were obtained from the Shanghai Experimental Animal Centre of the Chinese Academy Sciences (Shanghai, China). All mice were maintained under controlled conditions (24 ± 2 °C and 50 ± 5 % humidity) with free access to food and water under

a 12-h light/dark cycle. All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine and were approved by the National Research Council Guide for the Care and Use of Laboratory Animals.

Animal studies

Fresh acetaminophen (Sigma Aldrich, USA) solution was prepared for each experiment by dissolving acetaminophen in saline, which was warmed up to 55 °C and cooled down to 37 °C before use. Mice were fasted at 6:00 p.m. and then intraperitoneally (i.p.) injected with acetaminophen at a dose of 300 mg/kg (body weight) or with saline at 10:00 a.m. the following day. Mice were then killed after APAP intoxication, and the liver tissues were collected at the indicated time points ($n=4-6$ per time point). Some mice were injected with saline as parallel controls at the same time points ($n=4-6$ per time point). The livers were frozen at -80 °C for later analysis.

Knockdown of GADD45 α in vivo using siRNA

Cholesterol-conjugated GADD45 α siRNA for in vivo RNA interference and its negative control were obtained from RiboBio (Guangzhou, China) [37, 38]. For in vivo transfection, 6- to 8-week-old C57BJ/6J mice were intravenously injected in the tail vein with 20 nmol siRNA of GADD45 α (GCTCGGAGTCAGCGCACCA) or the control siRNA in a total volume of 100 μ l. 48 h later, mice were administered APAP (300 mg/kg) and killed after 24 h.

Histological analysis

For histological analyses, the livers were isolated and fixed in 4% paraformaldehyde solution for 24 h, or frozen in OCT embedding medium (Leica, Wetzlar, Germany). Paraffin embedded liver sections 5 μ m in size were stained with hematoxylin and eosin (HE). Frozen sections of the liver were stained with Oil Red O. Images were captured directly using Leica DMI3000B Fluorescent microscope (Wetzlar, Germany).

Cell culture

The mouse hepatocyte AML-12 cell line was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai) and cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12, Gibco-BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum, penicillin, streptomycin and dexamethasone in a humidified incubator with 5% CO₂ at 37 °C.

Isolation of hepatocytes from mice

Primary hepatocytes were purified from mice using the collagenase IV perfusion method [39, 40]. Briefly, the livers were perfused with 40 ml of EDTA solution (NaCl 137 mM, KCl 5.4 mM, NaH₂PO₄ 0.64 mM, Na₂HPO₄ 0.85 mM, HEPES 1 mM, NaHCO₃ 4.17 mM, EDTA 0.5 mM and glucose 5 mM, pH 7.4) at a flow rate of 5 ml/min and then further perfused with 25 ml of DMEM containing 1 mg/ml collagenase IV at the same rate. The digested liver sections were filtered through a 70- μ m nylon mesh. The hepatocytes were collected after centrifugation at 50 \times g for 5 min. The cells were washed twice with DMEM and then resuspended in culture medium (William's medium E, 10% FBS). Approximately, 4×10^5 cells were placed in the wells of six-well cell culture plates and incubated for 4 h with 5% CO₂ at 37 °C to permit adhesion. The plates were then washed twice to remove dead or unattached cells and further incubated in William's medium E with penicillin (100 U/ml) and streptomycin (100 mg/ml).

Infection of the adenovirus, and transfection of plasmid and siRNA in vitro

Mouse GADD45a adenovirus was obtained from Genechem Co., Ltd. (Shanghai, China). AML12 cells and primary mouse hepatocytes were infected with adenoviruses expressing GADD45a or with an adenovirus expressing EGFP used as a control for 48 h, followed by treatment with APAP for the indicated time. AML12 cells and primary mouse hepatocytes were transfected with small interfering RNA (siRNA) targeting murine Gadd45 α , AMPK α (both AMPK α 1 and AMPK α 2) and Ppp2cb or control siRNA, and the overexpression plasmid of Ppp2cb was transfected into AML-12 cells using Lipofectamine 3000 (Life Technologies, Thermo Fisher Scientific) for 48 h, followed by treatment with APAP. The siRNA sequences were designed and synthesized by RiboBio. (Guangzhou, China) The siRNA sequence against Gadd45 α was GCTCGGAGTCAGCGCACCA. The siRNA sequence against AMPK α 1 (Prkaa1) was GCACACCCTGGATGAATTA. The siRNA sequence against AMPK α 2 (Prkaa2) was CCAATTGACAGGCCATAAA. The siRNA sequence against Ppp2cb was GCAGATCACCAAGTGTAT.

Immunohistochemistry and immunofluorescence staining

Paraffin sections were dewaxed and rehydrated. Antigens were retrieved by heat-induced epitope retrieval. Endogenous peroxidase was quenched with 3% H₂O₂ in methanol. Sections were blocked and then incubated (4 °C) with anti-Gadd45 α antibody (Cell Signaling Technology, USA), according to the manufacture's guidelines. Based on the

species origin of the primary antibody, an appropriate HRP-conjugated IgG was selected as the secondary antibody. After incubation with the secondary antibody for 90 min at 37 °C, the slices were washed with PBS three times at 3-min intervals. Finally, staining was visualized with DAB, and sections were counterstained with hematoxylin. Cell slices of AML12 were incubated with rabbit anti-Gadd45 α (GENETEX, USA) followed by fluorescence staining with Cyanine CyTM3-conjugated donkey anti-rabbit IgG (H+L) (Jackson Immuno Research Laboratories, Inc., PA, USA). DAPI was applied to visualize the nuclei. Representative images were captured with a fluorescence microscope.

Co-immunoprecipitation and Western blotting

For immunoprecipitation, cells were lysed using NP-40 lysis buffer (Beyotime, China) supplemented with protease inhibitors. Lysates were incubated with appropriate monoclonal antibodies [GADD45 α (GENETEX, USA), Ppp2cb (Proteintech, USA) and AMPK α (Cell Signaling Technology, USA)] or control IgG. After an overnight incubation at 4 °C, Protein G Sepharose beads (Bio-Rad) were added into the lysate mixtures, and then the lysates were incubated for 30 min at room temperature. The Sepharose beads were washed three times with 1 ml of ice-cold buffer. The protein precipitates were separated by SDS-PAGE, and Western blot analysis was performed.

Terminal dUTP nick-end labeling (TUNEL) staining

DNA fragmentation was detected with the TUNEL method using an in situ cell detection kit (Roche) for the detection. The staining of cell or tissue sections was performed according to the manufacturer's instructions. Afterward, cells with positive nuclear staining were visualized, and images were captured with a microscope (Leica DFC310 FX).

Western blot analysis

Total protein was isolated from treated livers and cells using radioimmune precipitation assay (RIPA) lysis buffer. Equal amounts (30–50 μ g) of protein samples from liver tissues or cells were loaded onto SDS-PAGE gels and resolved, and then the protein samples were transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies against GADD45 α (GENETEX, USA), P-AMPK, T-AMPK, P-S6, T-S6, P-ACC, T-ACC (Cell Signaling Technology, USA), Ppp2cb (Proteintech, USA) or β -actin (Proteintech, USA). After the membranes were incubated with the corresponding secondary antibodies, namely, horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG, the signals were detected. The

protein bands were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences), and the membranes were then developed and quantified using an imaging system (Tanon, China).

Real-time quantitative PCR

Total RNA was isolated using TRIzol reagent (Life Technologies, Thermo Fisher Scientific), and cDNA synthesis was performed using the Prime Script RT reagent kit (Takara, Shiga, Japan) according to the manufacturer's instructions. Quantitative PCR was performed with the SYBR Green PCR kit using 36B4 as an internal standard. The primers used for the gene expression studies are listed in Table 1.

Statistical analysis

Data were expressed as the mean \pm standard error of the mean. Two-tailed Student's *t* tests were performed for two-sample comparisons. Multiple-group comparisons were examined using one-way ANOVA with Tukey's post hoc test. $P < 0.05$ was considered as statistically significant. Calculations and graphs were prepared using GraphPad Prism software version 6.0 (San Diego, CA, USA).

Results

APAP overdose triggers the upregulated expression of GADD45 α

To determine the expression level of GADD45 α in DILI, we conducted a series of immunohistological analyses to examine GADD45 α expression. As shown in Fig. 1a, our results revealed a dramatic increase in both nuclear and cytoplasmic GADD45 α accumulation in the human liver tissues of DILI, and particularly GADD45 α also presented an obvious increase in APAP-induced DILI both in the nucleus and cytoplasm. Then, we generated a model of liver injury by injecting C57BL/6J mice with acetaminophen (APAP) at a dose of 300 mg/kg. In line with the observation in Fig. 1a, the GADD45 α mRNA and protein expression levels showed dramatic increases in the liver as early as 1 h after the APAP treatment compared with after the saline treatment (Fig. 1b, c). To further examine the levels of GADD45 α during the process of APAP-induced liver injury, both AML-12 cells and primary mouse hepatocytes were treated with APAP [APAP was dissolved in DMEM/F12 or DMEM without FBS and filter-sterilized (0.22 μ m Millipore filter, Millipore,

Table 1 Primer sets used for qPCR

Gene name	Primer (5'–3')	Accession number
GADD45 α	Forward: AGAAGACCGAAAGGATGGAC Reverse: CACGGATGAGGGTGAAATG	NM_007836.1
36b4	Forward: TGAGATTCGGGATATGCTGTTGG Reverse: CGGGTCCTAGACCAGTGTCT	NM_001177352.1
PCK1	Forward: CGCTGGATGTCGGAAGAG Reverse: CACCACATAGGGCGAGTCT	NM_011044.2
PCX	Forward: AGAGGTGGTCCGCAAGAT Reverse: TAATAGGGAAGCCGTAGGTG	NM_001162946.1
Prkaa1	Forward: AGGTGGACATCTGGAGCA Reverse: AAAGGCTGATTACTGAAGGG	NM_001013367.3
Prkaa2	Forward: TCTCAACCGTTCTGTCGC Reverse: AGGGGTCTTCAGGAAATAGG	NM_178143.2
PYGM	Forward: CAAATCAGCGTTCGTGGCTTA Reverse: CCACATTGCGATCCTTGACCA	NM_011224.2
PDK1	Forward: GGACTTCGGGTCAGTGAATGC Reverse: TCCTGAGAAGATTGTCGGGGA	NM_001360002.1
CPT1 α	Forward: GGAGAGAATTTTCATCCACTTCCA Reverse: CTCCCAAAGCGGTGTGAGT	NM_013495.2
PDHB	Forward: AGTTGCCAGTATGACGGTG Reverse: TCTGAGATGGGGGTGTCGAT	NM_024221.3
PCK2	Forward: ATGGCTGCTATGTACCTCCC Reverse: GCGCCACAAAGTCTCGAAC	NM_028994.3
PDK2	Forward: AGGGGCACCCAAGTACATC Reverse: TGCCGGAGGAAAGTGAATGAC	NM_133667.2
CROT	Forward: AGAACGGACATTTTCAGTACCAGG Reverse: TTCCAGCCAGTTTCGTTTTCC	NM_023733.3
ACOX	Forward: TAACTTCCTCACTCGAAGCCA Reverse: AGTTCCATGACCCATCTCTGTC	NM_001271898.1
ACAA2	Forward: CTGCTACGAGGTGTGTTTCATC Reverse: AGCTCTGCATGACATTGCC	NM_177470.3
PPAR α	Forward: AACATCGAGTGTGCAATATGTGG Reverse: CCGAATAGTTCGCCGAAAGAA	NM_001113418.1
PKLR	Forward: GAACATTGCACGACTCAACTTC Reverse: CAGTGCGTATCTCGGGACC	NM_001099779.1

USA)]. As shown in Fig. 1d, using immunofluorescence, we found that GADD45 α was also remarkably increased after APAP administration for 6 h and that GADD45 α expression was obvious in both the nucleus and cytoplasm. Moreover, the mRNA expression of GADD45 α after APAP exposure was elevated in both the AML-12 cells and primary mouse hepatocytes (Fig. 1e, f). These results indicated that GADD45 α significantly increased when the liver was subjected to an APAP overdose.

GADD45 α plays a critical role in preventing APAP-induced hepatotoxicity

To investigate the role of GADD45 α in APAP-induced hepatotoxicity, we used primary mouse hepatocytes and AML-12 cells to examine the direct effect of a challenge with APAP in vitro. We first used Ad-GADD45a infection to induce GADD45 α overexpression and siRNA to reduce GADD45 α

expression. The expression levels of GADD45 α are shown in Fig. 1a. DNA fragmentation, which is a specific feature of APAP-induced hepatocyte death [41], was detected by TUNEL staining. As shown in Fig. 2b, the TUNEL staining of hepatocytes confirmed that GADD45 α directly protected hepatocytes against APAP-induced DNA fragmentation. The accumulation of small lipid droplets was also decreased in the hepatocytes overexpressing GADD45 α , and when GADD45 α loss of function occurred, a significant increase in small lipid droplets was evident (Fig. 2c). The PI staining which can verify the cell death when exposed to APAP treatment also demonstrated that a more serious hepatocytes' death occurred followed by the reduced expression of GADD45 α (Fig. 2d). A similar increase in small lipid droplets was observed in GADD45 α -overexpressing AML-12 cells (Fig. 2e). Taken together, these data demonstrate that GADD45 α plays a significant protective role against APAP-induced hepatotoxicity.

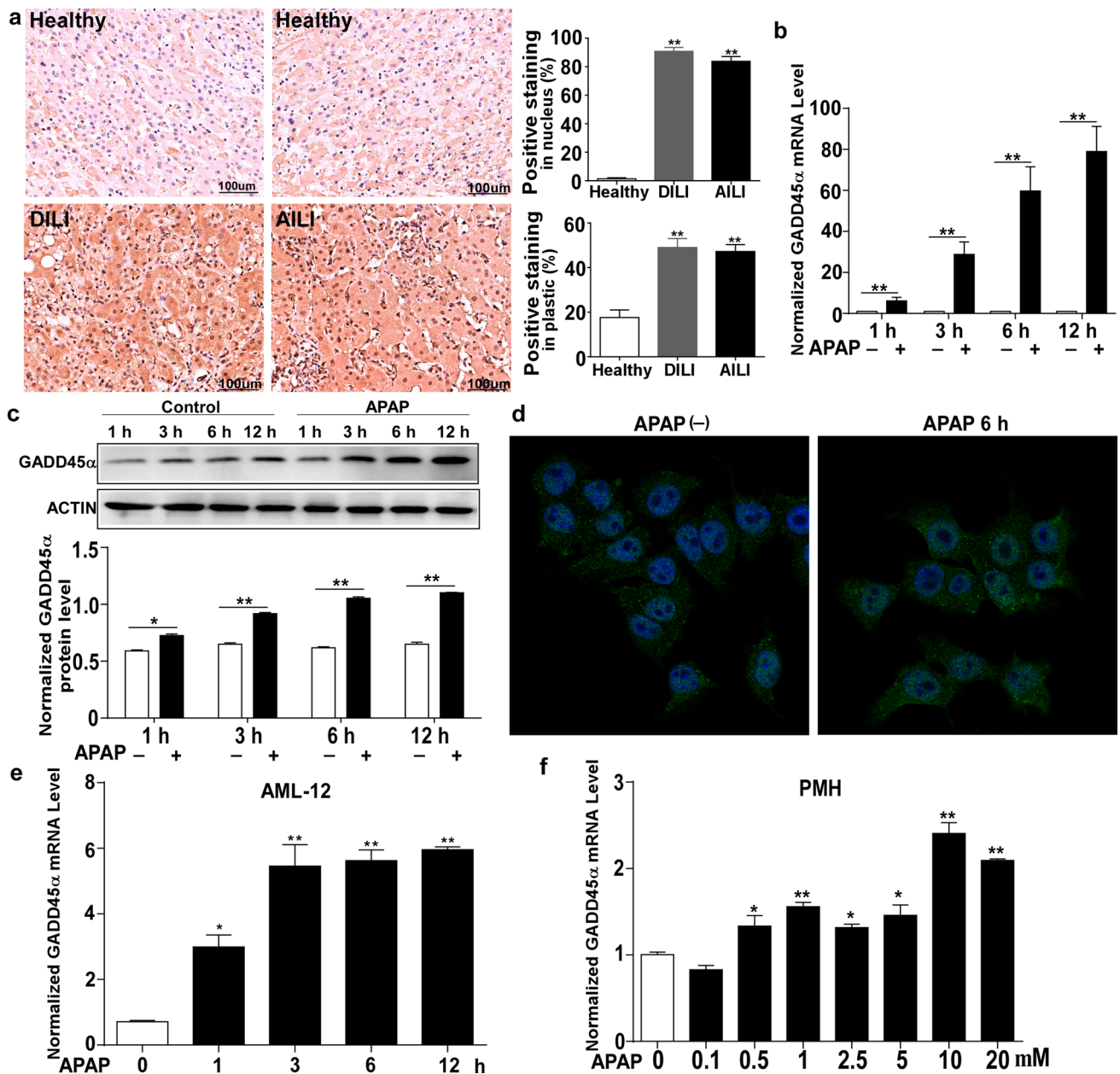


Fig. 1 GADD45 α is upregulated during the process of APAP-induced liver injury. **a** GADD45 α expression in the liver was determined by immunohistochemistry and quantified. Human samples from healthy control subjects (Healthy) ($n=3$) and from patients with drug-induced liver injury (DILI) ($n=9$, four of them APAP-induced DILI). **b**, **c** qRT-PCR analysis of the mRNA levels and Western blot analysis of the protein levels of GADD45 α in livers from C57BL/6J mice after 300 mg/kg APAP treatment ($n=5$ per time point). **d** Representative

images of immunofluorescence staining for GADD45 α in AML-12 cells. **e** qRT-PCR analysis of GADD45 α in AML-12 cells after APAP treatment at different time points. **f** qRT-PCR analysis of GADD45 α in primary mouse hepatocytes after different doses of APAP for 6 h. The mRNA levels were normalized to 36B4 and subsequently normalized to the control group. Data are expressed as the mean \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$

The AMPK pathway is activated after exposure to APAP

Using cell samples from AML-12 cells treated with APAP, global gene expression was analyzed by RNA-seq and bioinformatics analysis. As shown in Fig. 3a, 998 and 860 genes

were upregulated and downregulated, respectively, (over twofold, $P < 0.05$) in the DILI group compared with the control group. We then conducted gene ontology (GO) analysis using the default parameters of DAVID. We observed that the 998 genes upregulated in the DILI group were mainly enriched in the areas of “response to oxygen levels”, “lipid

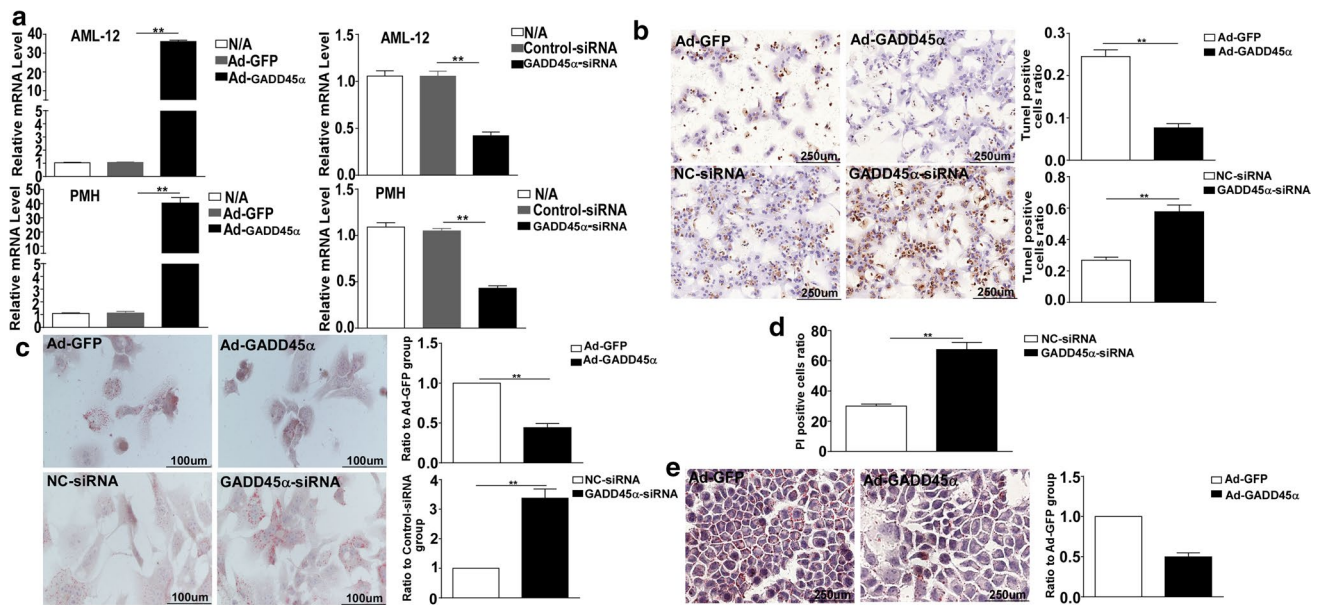


Fig. 2 GADD45 α shows an important capability of protecting against APAP-induced hepatocytes' death and decreasing the APAP-induced accumulation of small lipid droplets. **a** The mRNA expression levels of the GADD45 α after overexpression and siRNA treatment. **b** Representative images of TUNEL staining in primary mouse hepatocytes after the overexpression or knockdown of GADD45 α at 6 h after APAP treatment and the quantification of the number of TUNEL-positive cells. **c** Oil Red O staining of primary mouse hepatocytes after

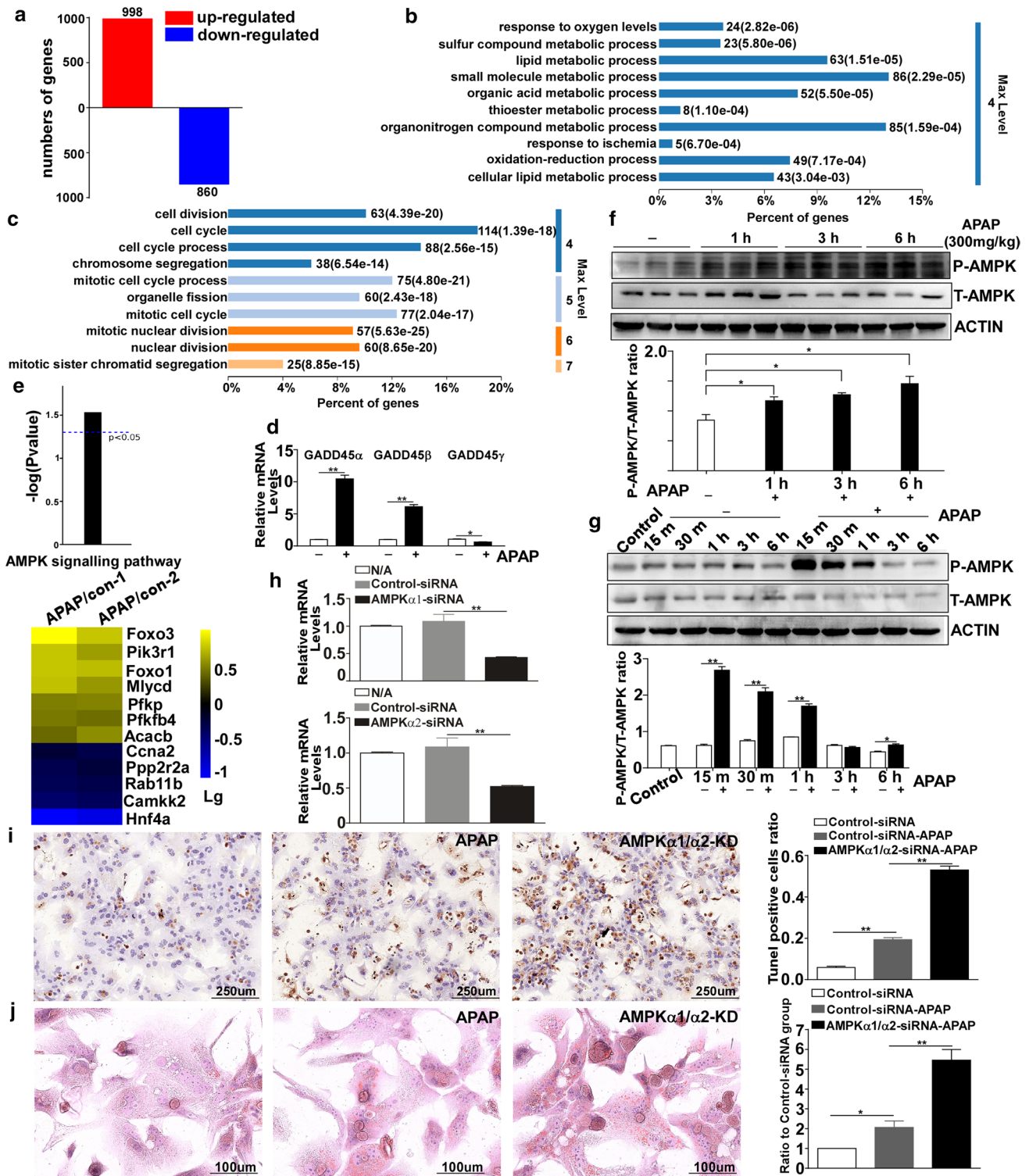
the overexpression or knockdown of GADD45 α at 6 h after APAP treatment and the quantification of the ratio relative to the control group. **d** PI staining of AML-12 cells after knockdown of GADD45 α at 24 h after APAP treatment. **e** Oil Red O staining of AML-12 cells from the overexpression of GADD45 α at 6 h after APAP treatment and the quantification of the ratio relative to the control group. Data are expressed as the mean \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$. LPF, low power field

metabolic process” and “small molecule metabolic process” (Fig. 3b), suggesting that multiple metabolic processes were affected in response to APAP overdose. However, as shown in Fig. 3c, the genes downregulated in the DILI group were all enriched in biological processes, including “cell division”, “cell cycle” and “cell cycle process”. Based on the RNA-seq data, we found that in response to APAP treatment, the expression levels of GADD45 family genes (Gadd45 α , Gadd45 β and Gadd45 γ) were altered; Gadd45 α and Gadd45 β were increased after APAP treatment compared with the control group, and Gadd45 γ was reduced. We also found that GADD45 α showed the most significant increase in response to APAP exposure (Fig. 3d). GO analysis suggested that APAP overdose upregulated the transcription of genes involved in metabolic progression. Using KEGG pathway analysis, we further found that the AMPK pathway was activated in APAP-induced hepatotoxicity (Fig. 3e), and the heat map in Fig. 3e represents the \log_{10} fold change in the expression of the AMPK pathway genes in APAP-induced hepatotoxicity compared with the control group. The protein levels of P-AMPK in APAP-injured mouse livers were analyzed by Western blotting (Fig. 3f). The results showed that P-AMPK levels were enhanced. Furthermore, P-AMPK was also activated as early as 15 min in the AML-12 cells (Fig. 3g). To investigate whether AMPK activation played

a critical role during the process of APAP-induced liver injury, we isolated primary mouse hepatocytes, which were transfected with either 100 nM murine AMPK α 1 siRNA and AMPK α 2 siRNA or control siRNA. As shown in Fig. 3h, the expression levels of AMPK α 1 and AMPK α 2 were obviously knocked down. TUNEL staining revealed that after APAP exposure, DNA fragmentation was more aggravated when AMPK α was knocked down compared to the control group (Fig. 3i). We further conducted Oil Red O staining to observe the accumulation of small lipid droplets in the hepatocytes, and as shown in Fig. 3j, when we knocked down AMPK α expression, the number of small lipid droplets was increased compared with the control siRNA group after exposure to APAP. Together, these findings suggest that APAP exposure-induced cytotoxicity promotes AMPK activation, which may play a vital role in alleviating APAP-induced acute liver injury.

GADD45 α promotes AMPK activation in APAP-induced hepatotoxicity

As evident from the above results, GADD45 α plays an important protective role against APAP-induced liver injury and can reduce the accumulation of small lipid droplets in



the hepatocyte cytoplasm. Furthermore, AMPK activation is increased during APAP-induced liver injury. We next investigated whether GADD45 α prevented APAP-induced hepatotoxicity by promoting AMPK activation. We used Ad-GADD45 α infection to induce the overexpression of GADD45 α in AML-12 cells. Cell lysates were collected at

different time points (15 min, 30 min, 1 h, 3 h, 6 h) after APAP treatment, and P-AMPK and P-ACC levels were further measured in AML-12 cells from the Ad-GADD45 α and Ad-GFP groups by Western blotting. As shown in Fig. 4a, we found that P-AMPK was increased significantly in the AML-12 cells overexpressing GADD45 α as early

Fig. 3 The AMPK pathway is activated after exposure to APAP. **a** Diagram illustrating the genes that were upregulated and down-regulated at 6 h. **b**, **c** Significantly enriched biological process category in the gene ontology (GO) analysis concerning the genes that were upregulated or downregulated at 6 h after APAP treatment. **d** The mRNA expression levels of the GADD45 family members (GADD45 α , GADD45 β and GADD45 γ). **e** Diagram displaying the activation of the AMPK signaling pathway and a heat map representing the log(10) fold change for the genes relating to the AMPK signaling pathway in the APAP treatment group compared with the control group. **f** Western blot analysis of P-AMPK and T-AMPK in the livers of C57BL/6J mice after treatment with 300 mg/kg APAP for different times. **g** The protein levels of P-AMPK and T-AMPK in AML-12 cells after APAP treatment for different times. **h** The mRNA expression levels of AMPK α 1 and AMPK α 2 in primary mouse hepatocytes after transfections with siRNA targeting AMPK α , confirming the knocking down of AMPK α . **i** TUNEL staining in primary mouse hepatocytes with AMPK α knockdown at 6 h after APAP treatment and the quantification of the number of TUNEL-positive cells. **j** Oil Red O staining of primary mouse hepatocytes with AMPK α knockdown at 6 h after APAP treatment and the quantification of the ratio relative to the control group. Data are expressed as the mean \pm standard error of the mean. * P < 0.05, ** P < 0.01

as 15 min after APAP exposure compared with the control group. Similarly, the levels of P-ACC, a downstream protein of the AMPK pathway, were higher in the GADD45 α -overexpressing group than in the control group. Moreover, the levels of P-S6K and P-S6, which are downstream proteins of the AMPK pathway, were consistent with the levels of P-AMPK. To further confirm these findings, we isolated primary mouse hepatocytes from C57BL/6J mice as described in “Materials and methods” and reduced the expression of GADD45 α using siRNA. The hepatocytes were exposed to APAP for 5 min, 10 min and 15 min, and the results showed that when GADD45 α was knocked down, the protein levels of P-AMPK and P-ACC in the hepatocytes were decreased compared with the levels in the control siRNA group, and P-S6 was dramatically increased in line with the reduced levels of P-AMPK (Fig. 4b). Collectively, these results indicate that GADD45 α can promote AMPK activation during the process of APAP-induced hepatotoxicity.

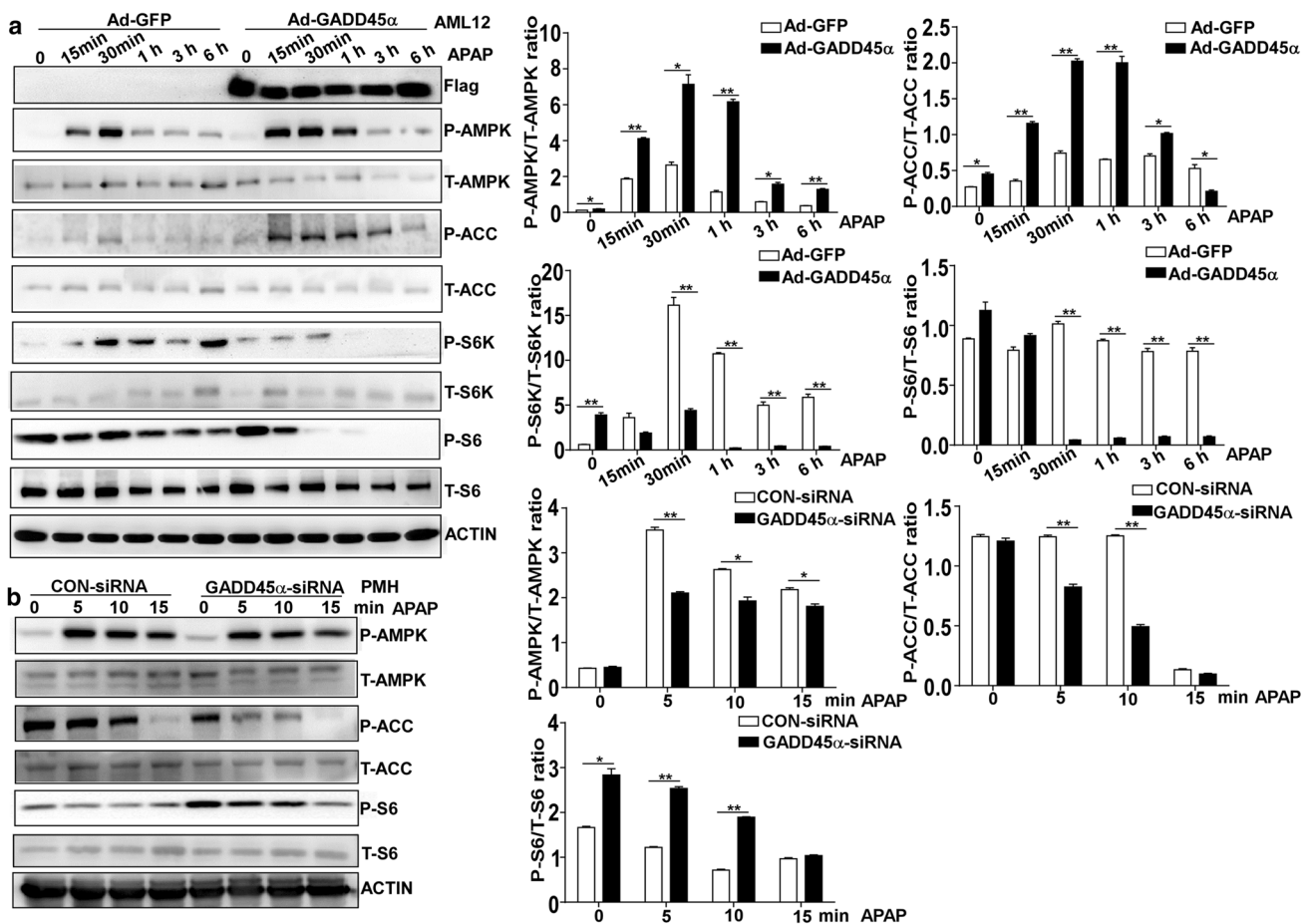


Fig. 4 GADD45 α promotes AMPK activation in APAP-induced hepatocyte injury. **a** Western blot analysis for P-AMPK, T-AMPK, P-ACC, T-ACC, P-S6K, T-S6K, P-S6 and T-S6 expression in AML-12 cells with overexpression of GADD45 α in response to APAP treatment for 0, 0.25, 0.5, 1, 3 and 6 h, followed with quantified protein levels of the above genes. **b** Western blot analysis for P-AMPK,

T-AMPK, P-ACC, T-ACC, P-S6 and T-S6 expression in primary mouse hepatocytes transfected with NC-siRNA or GADD45 α -siRNA in response to APAP treatment for 0, 5, 10 and 15 min, followed by quantified protein levels of the above genes. Data are expressed as the mean \pm standard error of the mean. * P < 0.05, ** P < 0.01

Enhanced expression of GADD45 α affects fatty acid beta-oxidation-, tricarboxylic acid cycle- and glycogenolysis-related gene expression after APAP exposure

Previous studies have shown that AMPK functions as a sensor of the cellular energy status and as a master regulator of metabolism by controlling various metabolic pathways [29]. We have already verified that GADD45 α can promote AMPK activation. To confirm whether GADD45 α could affect metabolic events by promoting AMPK activation during the process of APAP-induced liver injury, we measured the mRNA expression levels of genes from various metabolic pathways, including fatty acid beta-oxidation, the tricarboxylic acid (TCA) cycle and glycogenolysis, in AML-12 cells with stably overexpressed GADD45 α after APAP treatment. As shown in Fig. 5a, after the exposure to APAP, the cells overexpressing GADD45 α showed strikingly enhanced mRNA levels of PPAR α compared with the Ad-GFP group. Similar results were observed for the expression of CPT1 α , ACOX and ACAA2, which are all involved in fatty acid beta-oxidation. The mRNA levels of PDK2 and PDHB, which are associated with the TCA cycle, and PYGM, which is associated with glycogenolysis, also showed similar patterns (Fig. 5b, c). Moreover, when we measured the mRNA expression levels of the genes involved in the above metabolic pathways in primary mouse hepatocytes after the forced expression of GADD45 α , we found that the expression levels of genes involved in fatty acid beta-oxidation were decreased after exposure to APAP, while the forced expression of GADD45 α enhanced the fatty acid beta-oxidation pathway (Fig. 5d). The levels of genes associated with TCA cycle and gluconeogenesis were significantly increased after GADD45 α overexpression compared to the control group (Fig. 5e, f). The mRNA levels of PYGM and PKLR showed similar dynamics (Fig. 5g, h). Together, these data demonstrate that GADD45 α affects the metabolic events associated with fatty acid beta-oxidation, the TCA cycle and glycogenolysis by promoting AMPK activation after APAP exposure.

APAP-induced tricarboxylic acid (TCA) cycle metabolites are enhanced in GADD45 α -overexpressing AML-12 cells

To confirm the contribution of enhanced GADD45 α expression to these metabolic pathways during the process of APAP-induced liver injury, we applied liquid chromatography–mass spectrometry (LC–MS) to analyze the levels of several metabolites in APAP-treated AML-12 cell samples. As shown in Fig. 6a, the heat map demonstrated changes in the levels of 23 metabolites after APAP treatment. Moreover, our results showed that the levels of the metabolites

involved in the TCA cycle (such as NAD, NADH, succinate and fumarate), lactate and AMP were significantly increased after APAP treatment, with more marked increases in the cells with the forced expression of GADD45 α compared to the control group (Fig. 6b–i). Together, these data demonstrate that the overexpression of GADD45 α can increase the levels of TCA cycle metabolites after APAP treatment.

Ppp2cb interacts with GADD45 α in hepatocytes

To investigate the underlying molecular mechanism through which GADD45 α promoted AMPK activation, we collected the cell lysates of AML-12 cells with forced expression of GADD45 α after APAP treatment and then conducted co-immunoprecipitation followed by mass spectrometry analysis. As shown in Fig. 7a, a total of 11 proteins (Pcna, Dnaja3, Diras2, GADD45 α , Eif4g1, Ppp2cb, Asns, Esd, Prkacb, Aldoa and Psmc3) were identified in two independent experiments. Protein phosphatase 2A (PP2A), which comprises two regulatory subunits, namely, A and B, and one C catalytic subunit, including Ppp2ca and Ppp2cb, is an important serine/threonine phosphatase [42]. Thus, we further conducted a series of co-immunoprecipitations to determine whether GADD45 α interacted directly with Ppp2cb. As shown in Fig. 7b, GADD45 α interacted directly with Ppp2cb. We further confirmed the direct interaction between Ppp2cb and AMPK α by co-immunoprecipitation (Fig. 7c). Then, we overexpressed and knocked down the expression of Ppp2cb in AML-12 cells. As shown in Fig. 7d, the overexpression of Ppp2cb could suppress APAP-induced AMPK activation; however, when we knocked down the expression of Ppp2cb, APAP-induced AMPK activation was obviously enhanced in hepatocytes. Taken together, these results indicated that GADD45 α could interact with Ppp2cb, which might influence AMPK activation induced by APAP.

Knockdown of GADD45 α aggravated APAP-induced liver injury in vivo

Since GADD45 α performs the hepatoprotective effect in AML-12 cells and primary mouse hepatocytes, we next further confirmed the protective effect of GADD45 α on APAP-induced liver injury in vivo. As shown in Fig. 8a, we successfully reduced GADD45 α expression in the mice liver. As shown in Fig. 8b, in response to APAP treatment, liver histological observation demonstrated that there was more severe liver damage in the GADD45 α -siRNA treated mice, accompanied by a dramatic elevation of serum levels of ALT, AST and LDH (Fig. 8c) compared to the control group. The TUNEL staining showed that DNA fragmentation in the mice liver was obviously aggravated by GADD45 α knocking down (Fig. 8d). Moreover, the Oil Red O staining also showed that small lipid droplets were

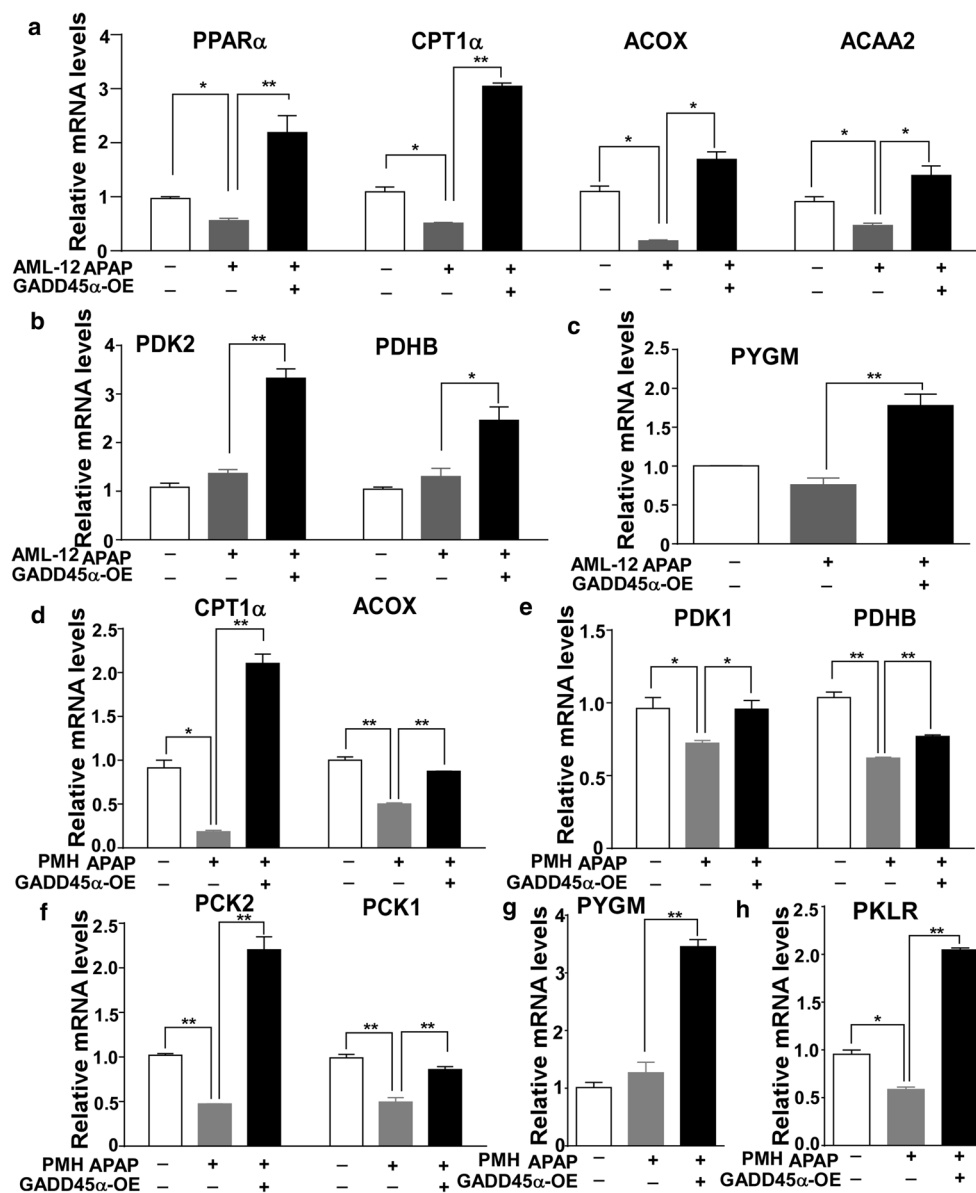


Fig. 5 Enhanced expression of GADD45 α affects fatty acid beta-oxidation-, tricarboxylic acid cycle- and glycogenolysis-related gene expression after APAP treatment. **a–c** The mRNA levels of genes involved in fatty acid beta-oxidation, tricarboxylic acid cycle and glycogenolysis in AML-12 cells with the enhanced expression of GADD45 α after APAP treatment for 6 h. **d–h** The mRNA levels of

genes involved in fatty acid beta-oxidation, tricarboxylic acid cycle and glycogenolysis in primary mouse hepatocytes with overexpression of GADD45 α after APAP treatment for 6 h. The relative mRNA levels were normalized to 36B4 and subsequently normalized to the control group. Data are expressed as the mean \pm standard error of the mean, * P < 0.05, ** P < 0.01

markedly increased after GADD45 α -siRNA treatment compared to the control group (Fig. 8e). As CK18-M65 is considered a sensitive marker of cell necrosis, we determined the serum levels of CK-18 M65 by ELISA assays. As shown in Fig. 8f, upon stimulation with APAP, the level of CK18-M65 in the GADD45 α -siRNA group was increased obviously compared with the control group. To further verify whether GADD45 α can affect the metabolite pathway via promoting AMPK activation during the process of APAP-induced liver injury in mice, we further detected the mRNA

levels of genes participating in a number of metabolic pathways, including fatty acid beta-oxidation, tricarboxylic acid cycle and gluconeogenesis. As shown in Fig. 8g, the expression levels of genes involved in fatty acid beta-oxidation, tricarboxylic acid cycle and gluconeogenesis were decreased when the expression of GADD45 α was reduced.

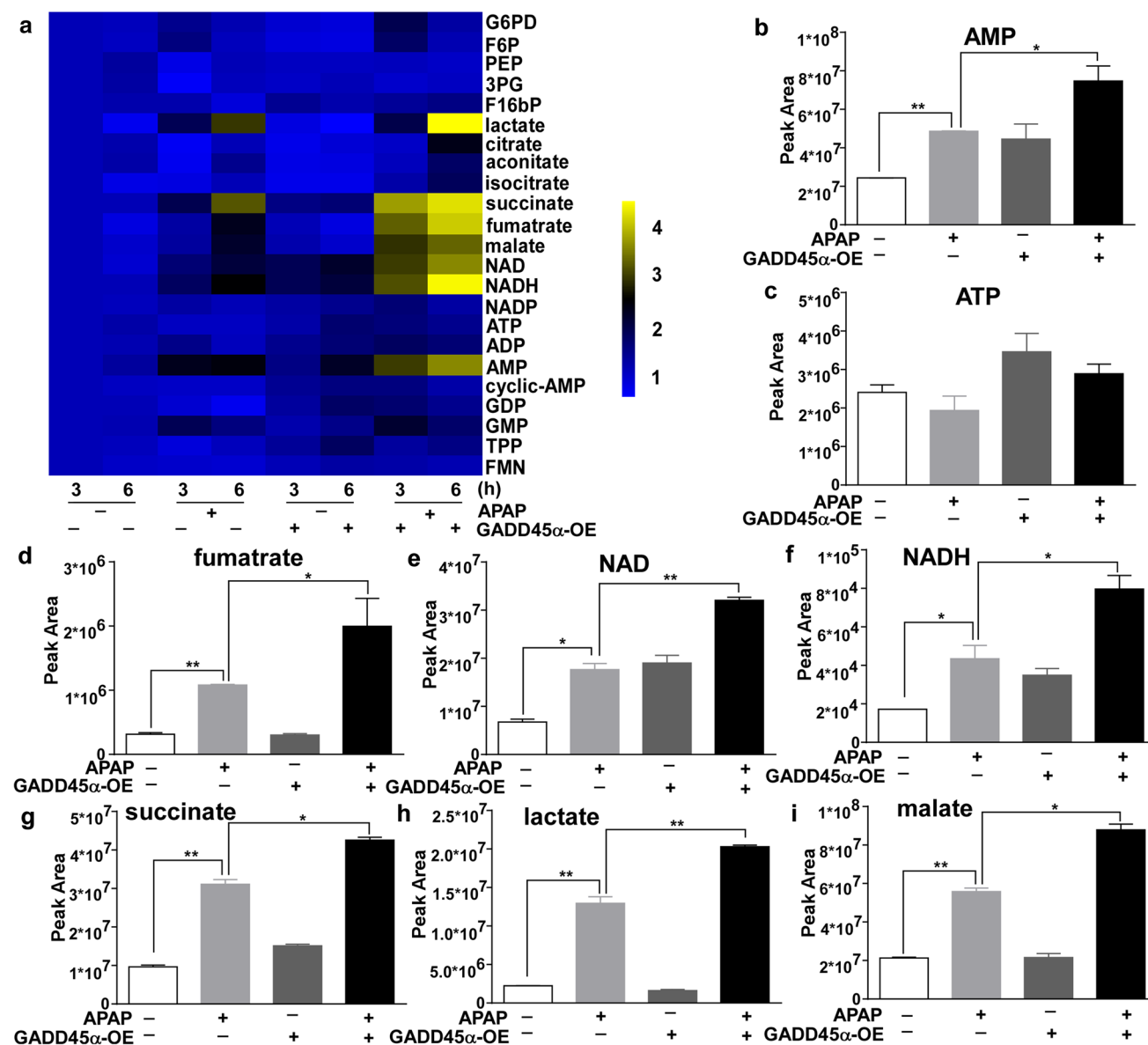


Fig. 6 Overexpressed GADD45 α can increase the levels of tricarboxylic acid (TCA) cycle metabolites after APAP treatment. **a** The heat map shows the changes in the levels of 23 metabolites after APAP

treatment. **b–i** The relative peak areas of the metabolites involved in the TCA cycle. Data are expressed as the mean \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$

Discussion

Acetaminophen overdose-induced liver injury is the most frequent cause of acute liver failure in many developed countries and threatens human health and drug development [43]. The identification of the molecular mechanisms of APAP-induced liver injury will provide promising therapeutic avenues for the treatment of APAP overdose [44, 45]. In the current study, we have demonstrated, for the first time, that hepatocyte GADD45 α is crucial for protecting against APAP-induced hepatotoxicity and functions by promoting AMPK activation.

Gadd45 proteins, a family of p53-regulated and DNA damage-inducible proteins, have been shown to regulate cell growth and apoptosis [12, 46]. Additionally, several studies have demonstrated an essential role for GADD45 α in various kinds of cancer, with GADD45 α having anti-tumor properties [47, 48]. Recently, GADD45 α was shown to play an important role in liver disease. A recent study reported that GADD45 α could counteract hepatic fibrosis by regulating the activation of HSCs via the inhibition of the TGF- β /Smad signaling pathway [49]. Another recent study revealed a protective role for GADD45 α in non-alcoholic steatohepatitis, a common liver disease worldwide [50]. A

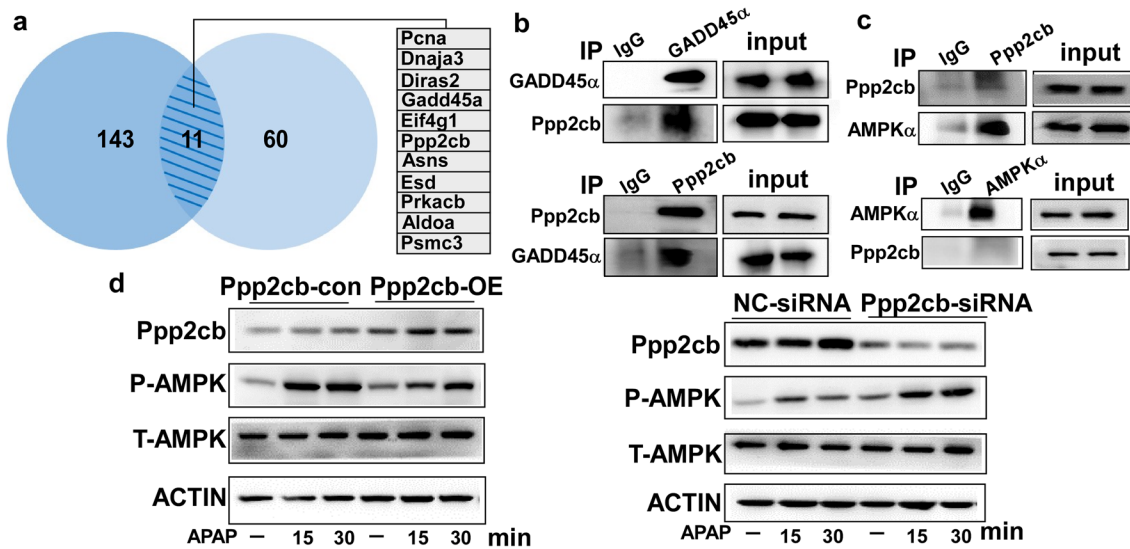


Fig. 7 Ppp2cb interacts with GADD45 α in hepatocytes. **a** The diagram represents a total of 11 proteins that were found in two independent co-immunoprecipitation experiments followed by mass spectrometry analysis. **b** GADD45 α (Ppp2cb) was immunoprecipitated from AML-12 cell lysates with enhanced GADD45 α expression. Then, the precipitate was evaluated for the presence of Ppp2cb

(GADD45 α) by Western blot. **c** AML-12 cells were treated with APAP for 6 h. Then, Ppp2cb (AMPK α) was immunoprecipitated with anti-Ppp2cb (anti-AMPK α), and the presence of AMPK α (Ppp2cb) in the precipitate was evaluated with an anti-AMPK α (anti-Ppp2cb) antibody. **d** The protein levels of Ppp2cb, P-AMPK and T-AMPK in AML-12 cells were analyzed by Western blot

recent report demonstrated that GADD45 α , as a downstream gene regulated by Egr1, played an important role in preventing APAP-induced liver injury [51]. In line with these findings, our own study demonstrated that GADD45 α had an important role in alleviating cell necrosis and accumulation of lipid droplets in APAP-induced hepatotoxicity in vitro. Importantly, when we reduced the expression of GADD45 α in mice liver using siRNA injection through the tail vein, we also observed enhanced liver injury and accumulation of lipid droplets after APAP treatment.

GADD45 α functions as a stress sensor in response to various physiological or environmental stressors, including hypoxia, ionizing radiation, UV radiation, growth factor withdrawal and medium depletion, finally resulting in cell cycle arrest, DNA repair, genomic stability and apoptosis [11, 13, 27]. However, studies related to the underlying relationship between GADD45 α and metabolic mechanisms in disease are few. Until now, no reports have shown that GADD45 α participates in some metabolic processes to protect against various physiological or environmental stressors. In our study, we have provided new insights into how GADD45 α exhibits a protective effect against APAP-induced liver injury. Our data have demonstrated that the activation of AMPK is promoted by GADD45 α in hepatocytes, following with the mRNA levels of genes associated with a number of metabolic pathways presented the consistent change in vivo and vitro. Hence, our current data indicate for the first time that GADD45 α is not only a stress sensor,

but also a hepatoprotectant protein in the context of APAP-induced liver injury by promoting AMPK activation.

AMPK is a key regulator of energy homeostasis [52, 53]. Abundant studies have confirmed that AMPK not only regulates energy homeostasis, but also carries out cytoprotective functions in hepatocytes by inhibiting apoptosis, protecting against mitochondrial injury and activating autophagy [54–56]. A recent study has shown that AMPK plays a critical role in APAP cytotoxicity by regulating autophagy [57]. Another study revealed that the pharmacological activation of AMPK can obviously ameliorate APAP-induced liver injury [58]. A large number of studies have indicated that AMPK is responsible for regulating hepatic lipid metabolic pathways, including lipid biogenesis and uptake, fatty acid β -oxidation and the TCA cycle [59, 60]. Furthermore, the enhancement of hepatic lipid metabolism involving fatty acid β -oxidation and the TCA cycle via the activation of AMPK have been shown to play an important role in alleviating liver injury. A recent study has demonstrated that demethyleberberine (DMB), a natural product that exists in Chinese herbs, can serve as an AMPK activator to regulate hepatic lipid metabolism by reducing lipid synthesis and inducing fatty acid β -oxidation, with the subsequent attenuation of non-alcoholic fatty liver disease [61]. Another report has confirmed that CYP2J2 overexpression obviously decreases lipid accumulation and increases fatty acid oxidation mostly by activating the AMPK pathway, with a subsequent amelioration of HFD-induced hyperlipidemia [62]. Similarly, in our present study, qRT-PCR and LC–MS

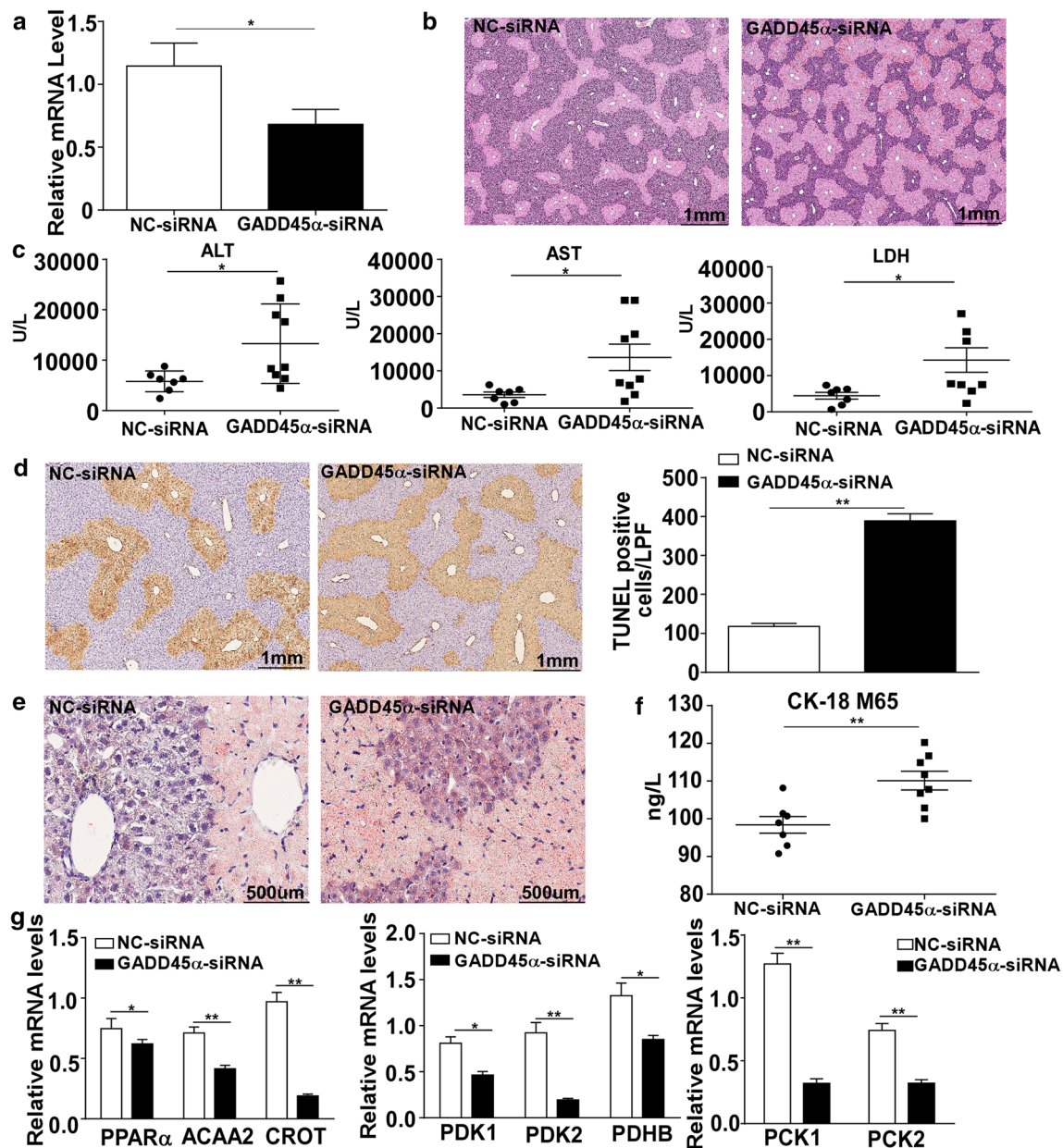


Fig. 8 Knockdown of GADD45 α aggravated APAP-induced liver injury in vivo. **a** The mRNA expression levels of the GADD45 α after GADD45 α -siRNA transfection in mice. **b** Liver histological examination with H&E; typical images were chosen from each experimental group. **c** Serum was harvested at 24 h after APAP injection for the measurement of ALT, AST and LDH in the mice of GADD45 α -siRNA and control groups. **d** Representative images of TUNEL staining after the knockdown of GADD45 α in mice at 24 h after APAP treatment and the quantification of the number of TUNEL-positive

cells. **e** Oil Red O staining after the knockdown of GADD45 α in mice at 24 h after APAP treatment. **f** Serum levels of CK18 M65, in the GADD45 α -siRNA or control groups. **g** The mRNA levels of genes involved in fatty acid beta-oxidation, tricarboxylic acid cycle and glycogenolysis in the mice with knockdown of GADD45 α after APAP treatment for 24 h. The relative mRNA levels were normalized to 36B4 and subsequently normalized to the control group. Data are expressed as the mean \pm standard error of the mean, * $P < 0.05$, ** $P < 0.01$

analysis revealed that the significantly increased fatty acid β -oxidation and TCA cycle components corresponded to the activation of the AMPK pathway promoted by the overexpression of GADD45 α .

PP2A is one of four major serine/threonine phosphatases. PP2A consists of a common heteromeric core enzyme,

which comprises two regulatory subunits, namely, A and B, and one C catalytic subunit, including Ppp2ca and Ppp2cb [42]. Several studies have shown that a group of protein phosphatases, which includes PP2A, PP1 and PP2C, can dephosphorylate AMPK in either cell culture or in vitro kinase dephosphorylation assays [63–66]. Moreover, a

previous report has shown that Ppp2ca, one of the catalytic subunits of PP2C, can interact directly with AMPK α in co-immunoprecipitation assays, leading to the dephosphorylation of AMPK. This study also showed that after the expression levels of Ppp2ca or Ppp2cb were reduced, the Thr-172 residue of AMPK showed a significant increase in phosphorylation; moreover, an elevation in Acc1 phosphorylation was evident [67]. These findings suggest that Ppp2cb plays a vital role in regulating the dephosphorylation of AMPK. In our current study, we found that overexpression of Ppp2cb could suppress APAP-induced AMPK activation, but knock-down of Ppp2cb could enhance APAP-induced AMPK activation in hepatocytes. GADD45 α could interact with Ppp2cb, which might influence AMPK activation induced by APAP. However, how GADD45 α -Ppp2cb interaction affects AMPK activation might need further investigation in future.

In conclusion, our present study demonstrates for the first time that GADD45 α has a protective effect against APAP-induced liver injury. The protective effects of GADD45 α on the APAP overdose-induced liver injury can be attributed to its activation of AMPK α . Our encouraging results indicate that hepatocyte GADD45 α may be a potential therapeutic target for APAP-induced liver injury and may be helpful in the development of a potential hepatoprotective agent for remedying APAP-induced liver injury.

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