

Acetaminophen-induced reduction of NIMA-related kinase 7 expression exacerbates acute liver injury

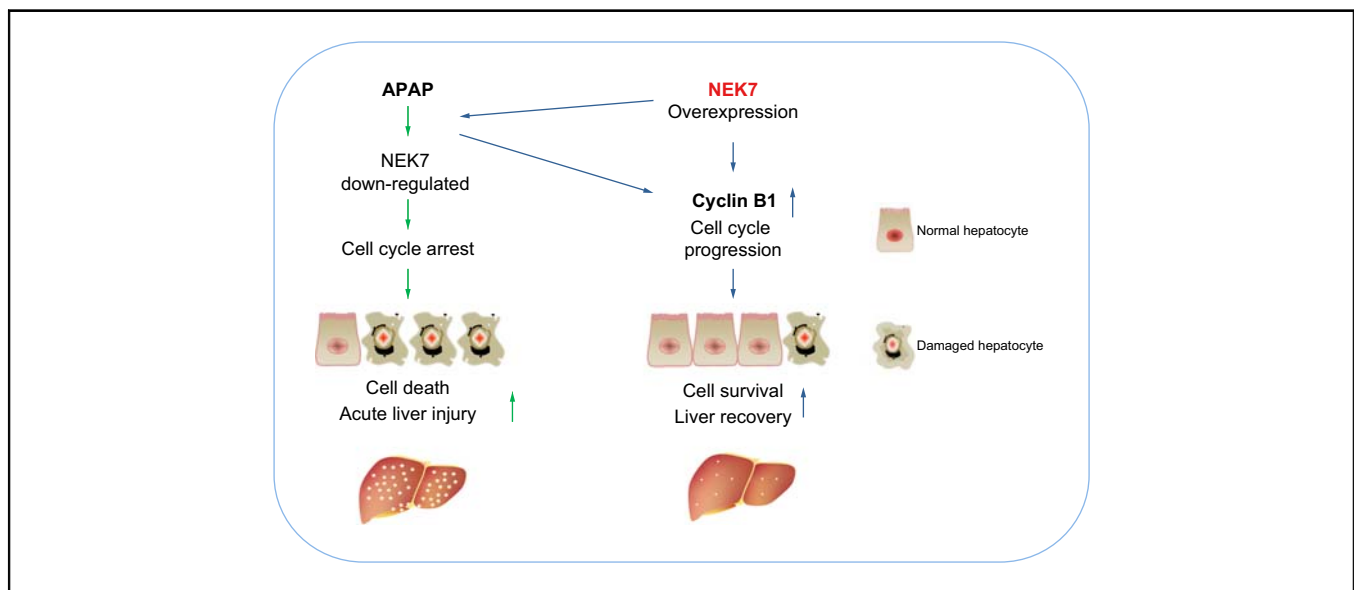
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Graphical abstract



Highlights

- NEK7 was downregulated in livers following APAP overdose challenge.
- Reduced NEK7 worsened APAP-induced acute liver injury.
- Reduced NEK7 dysregulated cyclins and cell cycle progression.
- Cyclin B1 overexpression attenuated NEK7 reduction-related worsening of APAP-induced acute liver injury.

Lay summary

Acetaminophen-induced acute liver injury is one of the major global health issues, owing to its high incidence, potential severity, and limited therapeutic options. Our current understanding of its pathogenesis is incomplete. Herein, we have shown that reduced NEK7 (a protein with a key role in the cell cycle) exacerbates acetaminophen-induced acute liver injury. Hence, NEK7 could be a possible therapeutic target for the prevention or treatment of this condition.



Acetaminophen-induced reduction of NIMA-related kinase 7 expression exacerbates acute liver injury

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Background & Aims: Acetaminophen (APAP)-induced acute liver injury (ALI) is a global health issue characterised by an incomplete understanding of its pathogenesis and unsatisfactory therapies. NEK7 plays critical roles in both cell cycle regulation and inflammation. In the present study, we investigated the role and mechanism of NEK7 in APAP-induced ALI. **Methods:** In mice with NEK7 overexpression (hydrodynamic tail vein injection of NEK7 plasmids), hepatocyte-specific NEK7 knockout (cKO), and inducible NEK7 knockout (iKO), an overdose of APAP was administered to induce ALI. Liver injury was determined by an analysis of serum liver enzymes, pathological changes, inflammatory cytokines, and metabolomic profiles. *In vitro*, hepatocyte damage was evaluated by an analysis of cell viability, the reactive oxygen species levels, and mitochondrial function in different cell lines. Hepatocyte proliferation and the cell cycle status were determined by Ki-67 staining, EdU staining, and the cyclin levels.

Results: NEK7 was markedly downregulated in APAP-induced injured liver and damaged hepatocytes. NEK7 overexpression in the liver significantly alleviated APAP-induced liver injury, as shown by the restored liver function, reduced pathological injury, and decreased inflammation and oxidative stress, which was confirmed in a hepatocyte cell line. Moreover, both NEK7 cKO and iKO mice exhibited exacerbation of APAP-induced ALI. Finally, we determined that cyclin B1-mediated cell cycle progression could mediate the protective effect of NEK7 against APAP-induced ALI.

Conclusions: Reduced NEK7 contributes to APAP-induced ALI, possibly by dysregulating cyclins and disturbing cell cycle progression.

Lay summary: Acetaminophen-induced acute liver injury is one of the major global health issues, owing to its high incidence, potential severity, and limited therapeutic options. Our current understanding of its pathogenesis is incomplete. Herein, we have shown that reduced NEK7 (a protein with a key role in the cell cycle) exacerbates acetaminophen-induced acute liver injury. Hence, NEK7 could be a possible therapeutic target for the prevention or treatment of this condition.

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Introduction

Acute liver injury (ALI) is a common and serious clinical problem caused by various insults, including drug hepatotoxicity, ischaemia hypoxia, metabolic disorders, and infectious and immune diseases.^{1,2} Once ALI progresses to life-threatening acute liver failure (ALF), a therapeutic choice of high specificity is

unavailable.^{3–5} Acetaminophen (APAP), which is an antipyretic and analgesic medication, has been the leading cause (over 50%) of ALI/ALF worldwide regardless of the recommended dose or overdose during treatment.^{4,6–8} Therefore, APAP-induced ALI/ALF is a growing concern and challenge for exploring effective therapeutic targets.

Generally, APAP hepatotoxicity is mainly attributable to the excessive accumulation of its reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is metabolised by cytochrome P450 enzymes (mainly CYP2E1).^{9,10} NAPQI overload damages the plasma membrane system (especially mitochondria) by forming protein adducts,^{11,12} which results in a series of pathological manifestations, such as glutathione (GSH) depletion, mitochondrial oxidative stress, sterile inflammation, metabolic dysfunction, hepatocellular necrosis surrounding the centrilobular region and even the loss of liver function.^{10,13–16} Numerous studies have also identified that ALI induced by

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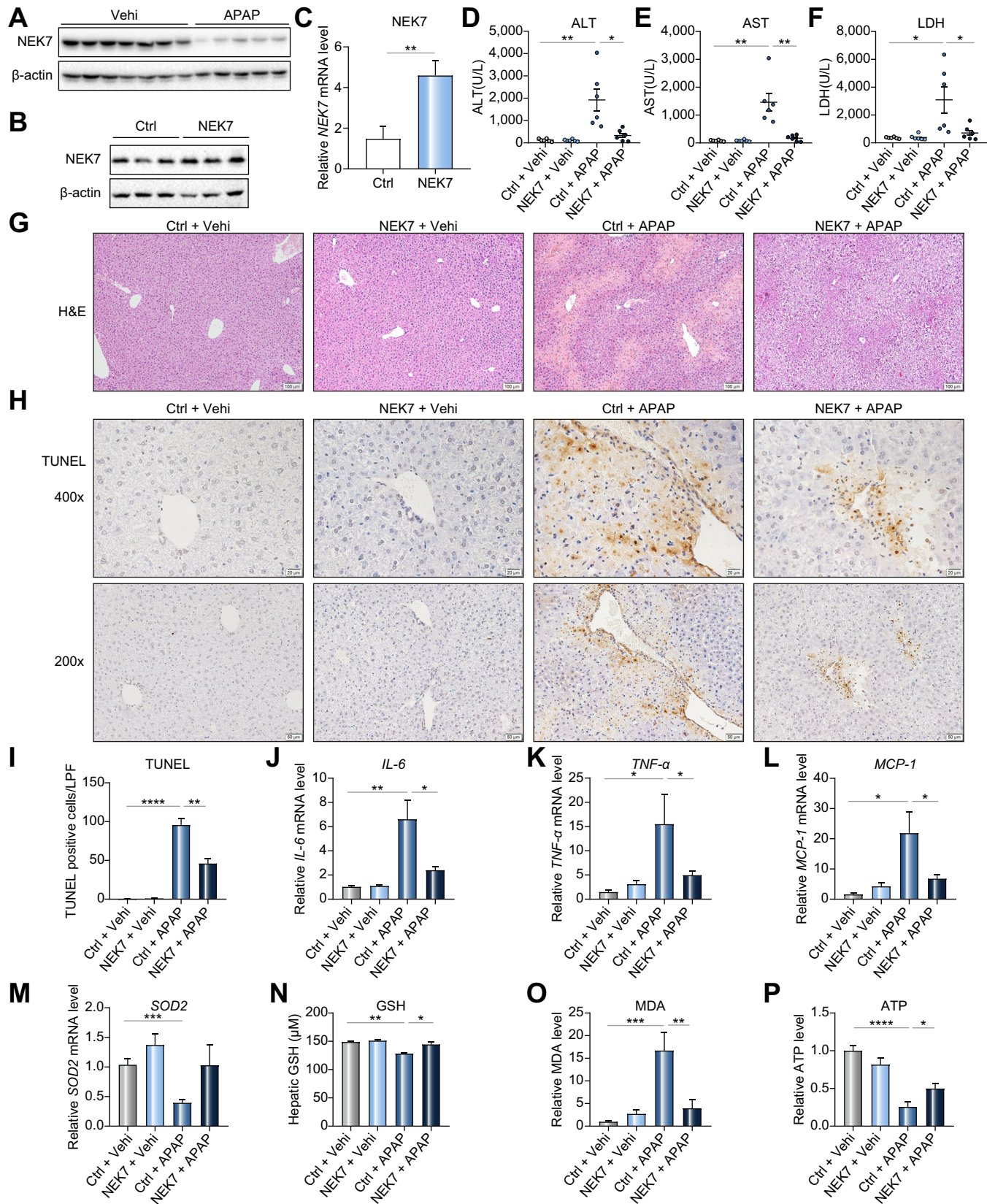


Fig. 1. NEK7 overexpression alleviates APAP-induced ALL. (A) Expression of NEK7 protein in the liver from control mice and APAP mice by a Western blot analysis, $n = 5-7$ per group. (B, C) Levels of NEK7 protein and mRNA in liver tissue determined by a Western blot analysis and qRT-PCR after hydrodynamic tail vein injection with NEK7 and empty plasmids, $n = 6$ per group. (D-F) Levels of ALT, AST, and LDH in the serum from 4 groups of mice, $n = 6$ per group. (G) Representative images of H&E-stained liver sections from each group, scale bars, 100 μ m. (H) TUNEL staining of liver sections, scale bars, 20 μ m for 400x, 50 μ m

APAP is subsequently followed by the initiation of liver compensatory regeneration,^{17–19} which might involve several proliferation-promoting signalling mediators, such as certain cytokines (IL-6 and tumour necrosis factor- α [TNF- α]), mediators of cell cycle arrest (transforming growth factor- β and p53/p21), growth factors (HGF, EGF, and VEGF) and other paracrine mediators that induce the upregulation of cyclins and trigger the entry of quiescent hepatocytes into mitosis.^{17–20} Given the limited understanding of the pathogenesis and effective clinical treatment of APAP-induced ALI, exploring novel molecular mechanisms and therapeutic targets in this disease is of critical importance.

NIMA-related kinase 7 (NEK7) is the smallest member of the NEK family (including NEK1–NEK11) and is widely expressed in the heart, liver, kidney, lung, brain, and muscle.²¹ Initially, NEK7 was identified as an important mediator of various aspects of mitotic progression.²² Established studies have demonstrated that the aberrant expression and modification of NEK7 finally leads to cell cycle disorder, which is reflected in more multipolar or monopolar spindle phenotypes, cell arrest, and even uncontrolled cell division.^{21,23} The mechanism by which NEK7 regulates mitosis has been attributed to centriole duplication, separation, and proper spindle assembly, which are related to microtubule modulation.^{24–27} Meanwhile, NEK7 may also participate in the regulation of cyclins, such as cyclin B1, cyclin D1, and cyclin E, and probably contribute to the genesis and development of tumours.^{28,29} Additionally, several studies discovered the indispensable role of NEK7 in mediating the activation of the NLRP3 inflammasome and confirmed the physical binding site and the molecular conformation between NEK7 and NLRP3, even though there are currently several controversies.^{30–32}

With emerging knowledge regarding the function of NEK7 in the cell cycle and inflammation, NEK7 has been identified as being involved in many diseases in recent years.^{33–35} As mentioned above, inflammation (including NLRP3 inflammasome activation) and cell cycle progression are important for the progression and prognosis of APAP-induced ALI.^{15,17,19} However, to date, few studies have reported the function of NEK7 in the field of liver disease. Thus, the above findings prompted us to investigate whether NEK7 is involved in the pathophysiology of APAP-induced ALI.

In the present study, to uncover the role of hepatocyte NEK7 in APAP-induced ALI, we constructed genetic NEK7 knockout mice in both the whole body and hepatocytes and overexpressed NEK7 in the liver by tail vein injection. Liver injury characterised by liver dysfunction, cell death, inflammation, and oxidative stress was evaluated comprehensively. Finally, the potential molecular mechanism was also explored. These results strongly suggest that NEK7 in hepatocytes plays a protective role against APAP-induced ALI.

Materials and methods

More detailed materials and methods can be found in the [Supplementary material](#).

Cell lines and gene-edited mice

HepaRG cells were used for the *in vitro* studies and cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) medium, with 10% foetal bovine serum (Gibco). All cells were maintained in a 37 °C humidified incubator filled with 5% CO₂.

The NEK7 hepatocyte conditional knockout mice (cKO) were obtained by crossbreeding NEK7^{fl/fl} mice (GemPharmatech, Nanjing, China) with Alb-cre mice (Shanghai Model Organisms Center, Shanghai, China). The NEK7 whole body knockout mice with tamoxifen inducibility (iKO) were generated by crossbreeding NEK7^{fl/fl} mice with CAG-cre (GemPharmatech).

All mice used in this study, including male wild-type (WT) C57BL/6J and male gene-edited mice, were maintained under a 12-h light/12-h dark cycle in a controlled specific pathogen-free environment with a standard *ad libitum* diet. All animal experiments complied with ethical regulations and were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University.

APAP-induced ALI and hepatocyte damage

APAP-induced ALI

APAP was purchased from MCE (Monmouth Junction, NJ, USA), administered to the mice by i.p. injection (250 mg/kg), and prepared according to a previous report.³⁶ Before the APAP treatment, all mice were fasted for 16 h. Twenty-four hours after the APAP administration, all mice were sacrificed under anaesthesia with isoflurane inhalation. Serum and liver tissue were collected and immediately preprocessed for the subsequent experiments.

APAP-induced hepatocyte damage

For the *in vitro* study, APAP was prepared in the corresponding medium and filtered for sterilisation. Different doses (1 mM, 2.5 mM, 5 mM, and 10 mM) of APAP were administered to immortalised hepatocytes for toxicity screening. In addition, the dose of 10 mM APAP was chosen as the final dose for the subsequent functional studies *in vitro* because of obvious cell injury induced by 10 mM APAP for 24 h. For the modulation of NEK7 or cyclin B1 *in vitro*, we pretransfected cells with 1 μ g individual plasmids or their control plasmids and 5 μ l (20 μ M) siRNA or negative control for 24 h. Then, the cells were treated with 10 mM APAP for another 24 h. After the treatments, the cells were collected, and a series of tests, including cell counting kit 8 (CCK-8), TMRM (tetramethylrhodamine methyl ester), EdU, etc., were conducted. The detailed methods of these tests are provided in the [Supplementary material](#).

for 200 \times . (I) Quantification of positive cells in liver sections stained by TUNEL, n = 3. (J–L) mRNA levels of inflammatory genes (TNF- α , IL-6, and MCP-1), n = 5–6 per group. (M) mRNA level of SOD2 in the livers of APAP-injured mice pretreated with NEK7 plasmids or control plasmids, n = 6 per group. (N–P) Total hepatic levels of GSH, ATP, and MDA as determined by the corresponding kits, n = 6 per group. Experimental design: Mice were injected with NEK7 or control plasmids for 24 h, followed by APAP or saline treatment for 24 h. All values are represented as the means \pm SEM. Significant differences were analysed using a 1-way ANOVA or Student *t* test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. ALI, acute liver injury; ALT, alanine aminotransferase; APAP, acetaminophen; AST, aspartate aminotransferase; Ctrl, control; GSH, glutathione; LDH, lactate dehydrogenase; MCP-1, monocyte chemoattractant protein 1; MDA, malondialdehyde; NEK7, NIMA-related kinase 7; SOD2, superoxide dismutase 2; TNF- α , tumour necrosis factor- α ; Vehi, vehicle.

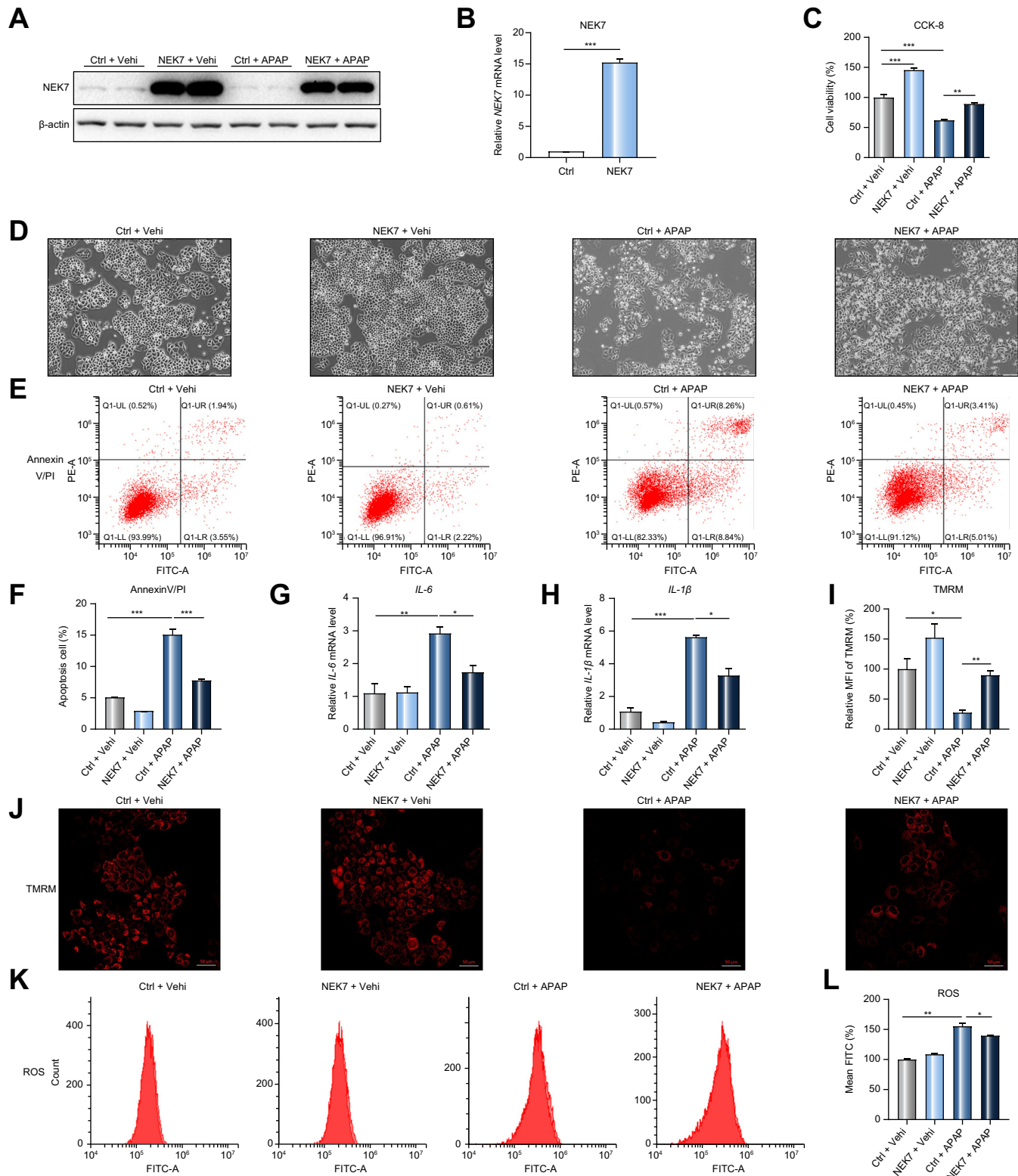


Fig. 2. NEK7 overexpression significantly mitigates APAP-induced hepatocyte damage *in vitro*. (A, B) Overexpression efficiency of NEK7 in HepaRG cells as shown by protein and mRNA levels, $n = 3-4$. (C) Cell viability of HepaRG cells in different groups measured by a CCK-8 assay, $n = 6$. (D) Cell images acquired by microscopy; scale bars, 100 μ m, $n = 3$. (E, F) Flow cytometry analysis and quantification of HepaRG cells after FITC Annexin V staining, $n = 3$. (G, H) mRNA levels of inflammatory genes by qRT-PCR, $n = 3$. (I, J) Mitochondrial membrane potential of HepaRG cells with different treatments as indicated by TMRM staining, $n = 3$. (K, L) Flow cytometry analysis and quantification of the levels of ROS in HepaRG cells, $n = 3$. Experimental design: cells were transfected with NEK7 or control plasmids for 24 h, followed by APAP or vehicle for 24 h. All values are represented as the means \pm SEM. Significant differences were analysed using a 1-way ANOVA or Student t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. APAP, acetaminophen; CCK-8, cell counting kit 8; Ctrl, control; NEK7, NIMA-related kinase 7; PI, propidium iodide; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester; Vehi, vehicle.

Results

NEK7 overexpression alleviates APAP-induced ALI

First, we investigated the expression of NEK7 in adult mouse livers from an open single-cell database.³⁷ The preliminary screening showed that NEK7 was highly expressed in both hepatocytes and other non-parenchymal cells (Fig. S1A). APAP overdose notably induced acute liver injury and hepatocyte damage *in vivo* and *in vitro* (Fig. S1B, D, and E), as reported previously.^{14,38} We observed the expression of NEK7 in APAP-injured livers and hepatocytes and found that the protein and mRNA levels of NEK7 were remarkably downregulated in the livers injured by APAP (Fig. 1A and Fig. S1C). The reduction in NEK7 after the treatment with APAP was further confirmed in HepaRG cells (Fig. S1F).

Subsequently, to explore the function of NEK7 in ALI, we overexpressed NEK7 in the livers of C57BL/6J mice by hydrodynamic tail vein injection of its plasmids (Fig. 1B and C). Then, we induced ALI in these mice by i.p. injection of APAP (250 mg/kg) for 24 h. We first examined the levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) and found that NEK7 overexpression in the liver almost normalised the serum levels of these liver enzymes following an overdose of APAP (Fig. 1D–F). Along with the striking protection of liver function, NEK7 overexpression also significantly alleviated the pathological damage (Fig. 1G–I), inflammation (Fig. 1J–L) and oxidative damage (Fig. 1M–P) induced by APAP. Additionally, we examined the effect of NEK7 overexpression on APAP-induced ALI at earlier and later time points. We found that liver injury in both groups exhibited no difference at earlier time points (1.5 h and 6 h) after the APAP treatment as evidenced by the serum biochemical analysis (Fig. S2A–C). At 16 h, the NEK7-overexpressing mice exhibited attenuated liver injury compared with the control (Ctrl) mice. Thus, these data suggest that NEK7 plays a protective role in the liver against APAP-induced ALI.

NEK7 overexpression significantly mitigates APAP-induced hepatocyte damage *in vitro*

To further investigate the role of NEK7 in hepatocytes following APAP administration, we overexpressed NEK7 in HepaRG cells followed by APAP treatment. Obviously, compared with the control, NEK7 overexpression alone increased the hepatocyte viability (Fig. 2A–D). Meanwhile, NEK7 significantly weakened the hepatocyte toxicity of APAP, as evidenced by decreased cell death (Fig. 2C–F), inflammation (Fig. 2G and H), mitochondrial dysfunction (Fig. 2I and J) and reactive oxygen species (ROS) levels (Fig. 2K and L). Altogether, these data provide further evidence that NEK7 protects against APAP-induced hepatocyte damage.

cKO of hepatocyte NEK7 exacerbates APAP-induced ALI

To better determine the hepatocyte-specific role of NEK7 in APAP-induced ALI, we established conditional hepatocyte NEK7 knockout mice (NEK7 cKO) by crossing NEK7^{fl/fl} mice with Alb-cre mice. However, after a period of crossbreeding, we obtained only 4 surviving adult homozygous mice, whereas the other mice died either in the embryonic period or shortly after birth (Table S1). Therefore, we performed the subsequent studies using heterozygous mice (Het).

The Het mice exhibited a profound depletion of NEK7 expression in the liver, as evidenced by measuring the protein and mRNA levels of liver tissue (Fig. 3A and B). Notably, compared with the control mice, the Het mice showed modest liver injury without exposure to APAP as determined by the increased serum levels of liver enzymes (ALT, AST, and LDH) (Fig. 3C–E), pathological changes (Fig. 3F), more dead cells (Fig. 3G and H), upregulated inflammation (Fig. 3I), and disordered function of mitochondria (Fig. 3J–L). The above data suggest that reduced NEK7 in APAP-treated mice could be pathogenic. Furthermore, the mice in the Het + APAP group exhibited greater liver injury than the Ctrl + APAP mice (Fig. 3C–I). *In vitro*, consistent with the *in vivo* results, inhibiting the expression of NEK7 with si-NEK7 in HepaRG cells (Fig. S3A) caused obvious cell damage as reflected by increased dead cells, the generation of ROS and a decreased mitochondrial membrane potential (Fig. S3C–G). After exposure to APAP, the NEK7-depleted cells exhibited more serious cell damage than the control cells (Fig. 3M and N, Fig. S3B–G).

As the liver is a critical organ for metabolism, we performed an untargeted metabolome analysis in the liver. By a cluster analysis of differential metabolites in the liver tissue from Het and control mice, we observed 35 upregulated and 8 downregulated metabolites as shown in the heatmap and volcano graph (Fig. S4A and B). These affected metabolites are involved in various metabolic pathways, including the biosynthesis and metabolism of amino acids, carbon metabolism, glutathione metabolism, lipolysis in adipocytes, hormone and energy metabolism response to stress and other pathways, as shown in the KEGG pathway impact graph along with some representative differential metabolites (Fig. S4C–H). These results suggest that NEK7 depletion in hepatocytes leads to disturbed metabolism of the liver, which may contribute to the susceptibility of the liver to APAP hepatotoxicity.

Tamoxifen-induced KO of whole-body NEK7 in adult mice also exacerbates APAP-induced ALI

Because of the low birth and yield rates of NEK7 cKO homozygous mice, obvious liver defects in heterozygous mice as shown above, and a previous report showing growth retardation or embryonic lethality occurring in NEK7 KO mice,³⁹ we speculate that the depletion of NEK7 in hepatocytes may affect the development and maturation of the liver. Therefore, to exclude the influence of NEK7 deficiency on the basal function of the liver, we established tamoxifen-inducible whole body NEK7 KO mice (iKO) by crossing NEK7^{fl/fl} mice with CAG-cre (tamoxifen-inducible) mice. NEK7 in the liver of adult mice was almost completely knocked out by the administration of tamoxifen for 5 consecutive days (Fig. 4A). Then, both the control mice and iKO mice were treated with APAP to induce liver injury. We did not observe obvious liver injury in the iKO mice compared with the control mice (Fig. 4B–G). However, the iKO mice exhibited more severe liver injury than the control mice after the APAP administration, as evidenced by further increased levels of serum liver enzymes (ALT, AST, and LDH) (Fig. 4B–D), enlarged necrotic areas of the liver (Fig. 4E), increased TUNEL-stained positive cells (Fig. 4F and G) and enhanced inflammation (Fig. 4H–J) in the livers of the iKO + APAP mice. These data better illustrated the pathogenic role of NEK7 reduction under APAP treatment.

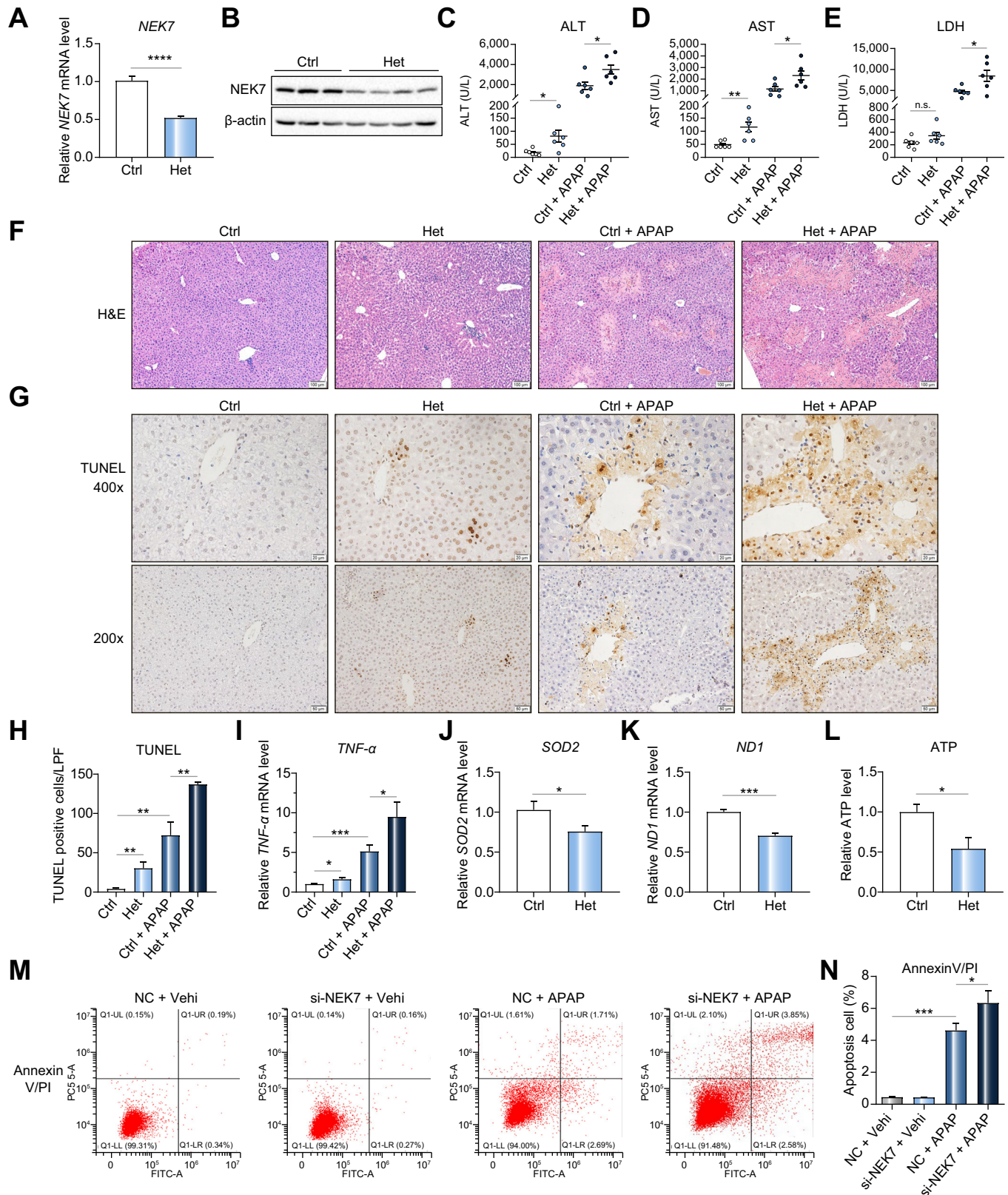


Fig. 3. Conditional knockdown of hepatocyte NEK7 exacerbates APAP-induced ALL. (A, B) Levels of NEK7 protein and mRNA in livers from control mice and Het mice. (C–E) Levels of ALT, AST, and LDH in the serum from each group of mice, $n = 6$. (F) Representative images of H&E-stained liver sections from each group; scale bars, 100 μ m. (G) TUNEL staining of liver sections; scale bars, 20 μ m for 400 \times , 50 μ m for 200 \times . (H) Quantification of positive cells in liver sections stained by TUNEL, $n = 3$. (I–K) mRNA levels of inflammatory gene (*TNF- α*), antioxidant gene (*SOD2*) and mitochondrial gene (*ND1*), $n = 5$ –6 per group. (L) Hepatic levels of total ATP in control and Het mice, $n = 6$ per group. (M, N) Flow cytometry analysis and quantification of HepaRG cells after FITC Annexin V staining, $n = 3$. All

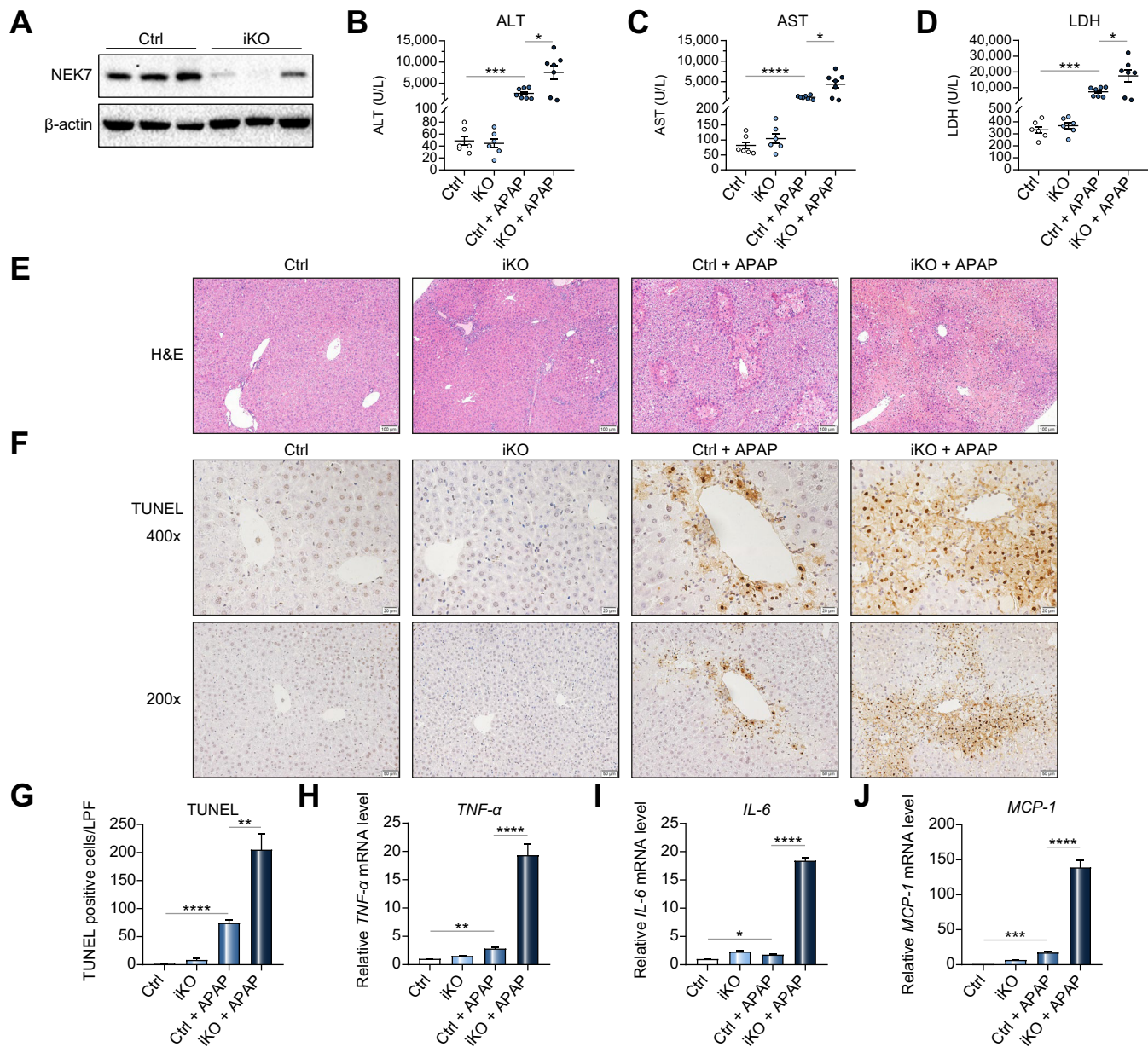


Fig. 4. Tamoxifen-induced knockout of whole-body NEK7 in adult mice also exacerbates APAP-induced ALI. (A) Protein levels of NEK7 in livers from control and iKO mice. (B–D) Serum levels of ALT, AST, and LDH in mice from 4 groups, Ctrl, Ctrl + APAP, iKO, and iKO + APAP, $n = 6-7$. (E) Representative images of H&E-stained liver sections; scale bars, 100 μ m. (F) TUNEL staining showing damaged cells in liver sections, scale bars, 20 μ m for 400 \times , 50 μ m for 200 \times , $n = 3$. (G) Quantification of the positive cells in low-power fields by TUNEL staining. (H–J) mRNA levels of inflammatory genes by qRT-PCR, $n = 6-7$. Experimental design: mice were treated with APAP or saline for 24 h. All values are represented as the means \pm SEM. Significant differences were analysed using a 1-way ANOVA or Student t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ALI, acute liver injury; ALT, alanine aminotransferase; APAP, acetaminophen; AST, aspartate aminotransferase; Ctrl, control; iKO, inducible knockout; LDH, lactate dehydrogenase; MCP-1, monocyte chemoattractant protein 1; NEK7, NIMA-related kinase 7; $TNF-\alpha$, tumour necrosis factor- α .

Furthermore, we performed an untargeted metabolome analysis of liver tissue from iKO, Ctrl (C), iKO + APAP (iKO + AP), and Ctrl + APAP (AP) mice. Thirty-seven markedly differential

metabolites were shown in the heatmap by standard cluster analysis (Fig. S5A). The analysis of the KEGG pathways of the differential metabolites between the iKO and Ctrl mice suggested

values are represented as the means \pm SEM. Experimental design: mice were treated with APAP or saline for 24 h. Cells were transfected with si-NEK7 or negative control for 24 h, followed by APAP or vehicle for 24 h. Significant differences were analysed using a 1-way ANOVA or Student t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ALI, acute liver injury; ALT, alanine aminotransferase; APAP, acetaminophen; AST, aspartate aminotransferase; Ctrl, control; Het, heterozygous; LDH, lactate dehydrogenase; NC, negative control; ND1, NADH dehydrogenase 1; NEK7, NIMA-related kinase 7; n.s., no significance; PI, propidium iodide; si, small-interfering; SOD2, superoxide dismutase 2; $TNF-\alpha$, tumour necrosis factor- α ; Vehi, vehicle.

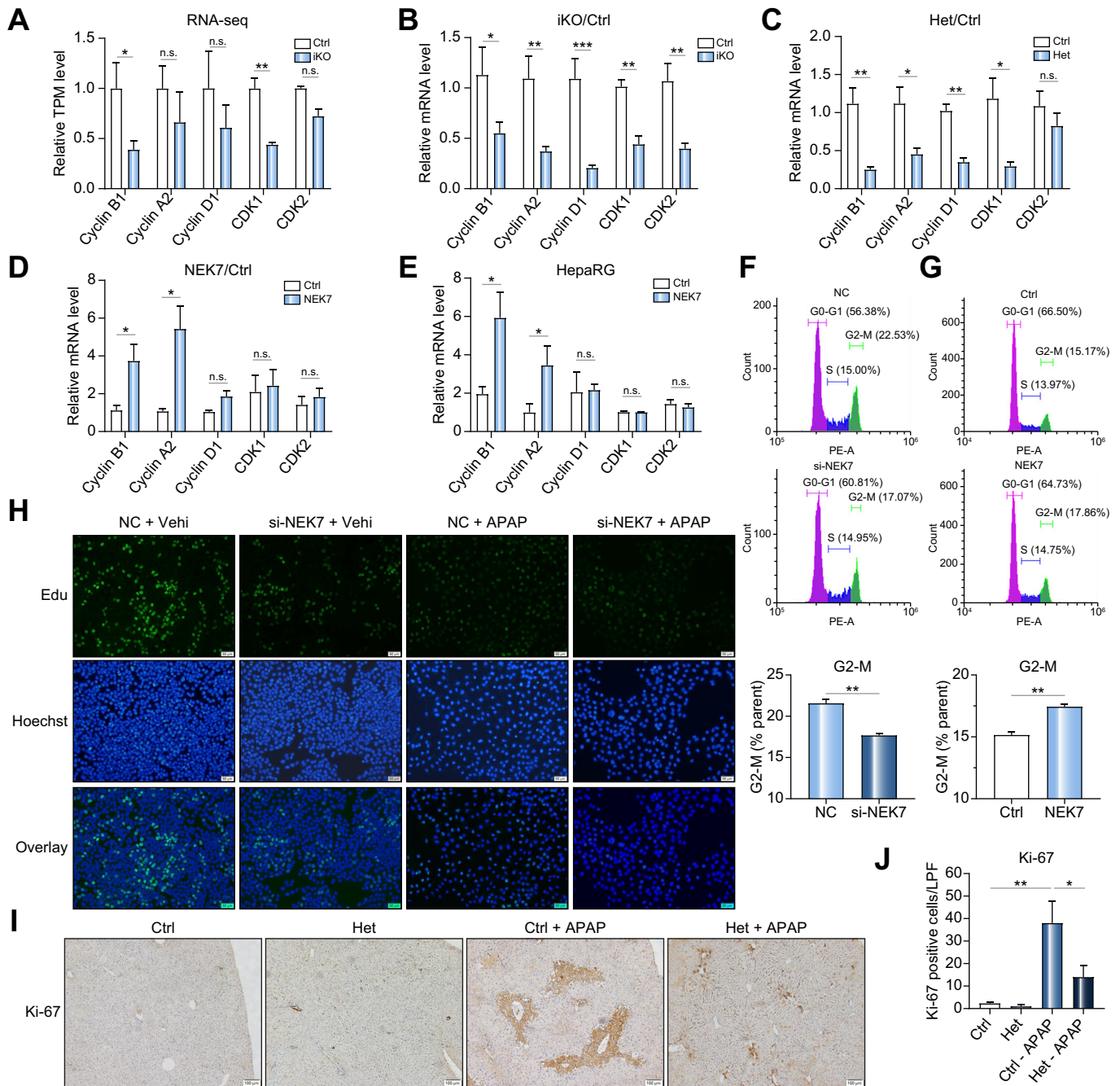


Fig. 5. Reduced NEK7 impairs cell cycle progression. (A) Relative transcript levels of cyclin B1, cyclin D1, cyclin A2, CDK1, and CDK2 profiled from the transcriptome of liver tissue (iKO mice and Ctrl mice, $n = 3$). Relative mRNA levels of cyclin B1, cyclin A2, cyclin D1, CDK1, and CDK2, (B) in the livers of iKO mice with respect to control mice (Ctrl), $n = 6$, (C) in the livers of Het mice and control mice, $n = 6$, (D) in NEK7 overexpressing livers and control livers, $n = 6$, (E) in HepaRG cells transfected with NEK7 plasmids (NEK7) and control plasmids (Ctrl), $n = 3$. (F, G) Flow cytometry analysis of the cell number in each phase of the cell cycle and quantification of the cell number in G2-M, $n = 3$. (H) EdU staining showing the proliferation activity of HepaRG cells in each group, $n = 3$. (I) Immunohistochemical staining of Ki-67 in liver sections; scale bars, 100 μ m; quantification of the positive cells, $n = 3$ (J). Experimental design: mice were treated with APAP or saline for 24 h. Cells were transfected with si-NEK7 or negative control for 24 h, followed by APAP or vehicle for 24 h. All values are represented as the means \pm SEM. Significant differences were analysed using a 1-way ANOVA or Student t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. APAP, acetaminophen; Ctrl, control; Het, heterozygous; iKO, inducible knockout; NC, negative control; NEK7, NIMA-related kinase 7; n.s., no significance; TPM, transcripts per kilobase of exon model per million mapped reads; Vehi, vehicle.

that NEK7 depletion in the adult mouse liver also impaired metabolic homeostasis in the liver (Fig. S5B). Moreover, the iKO + APAP mice exhibited worse metabolic conditions than the Ctrl + APAP mice (Fig. S5C). Then, we displayed several differential

metabolites among the 4 groups (Fig. S5D–G), which were mainly involved in nucleotide metabolism (GMP and uridine), carbohydrate metabolism (galactitol), and biosynthesis of amino acids (L-asparagine), respectively. We found that the iKO + APAP

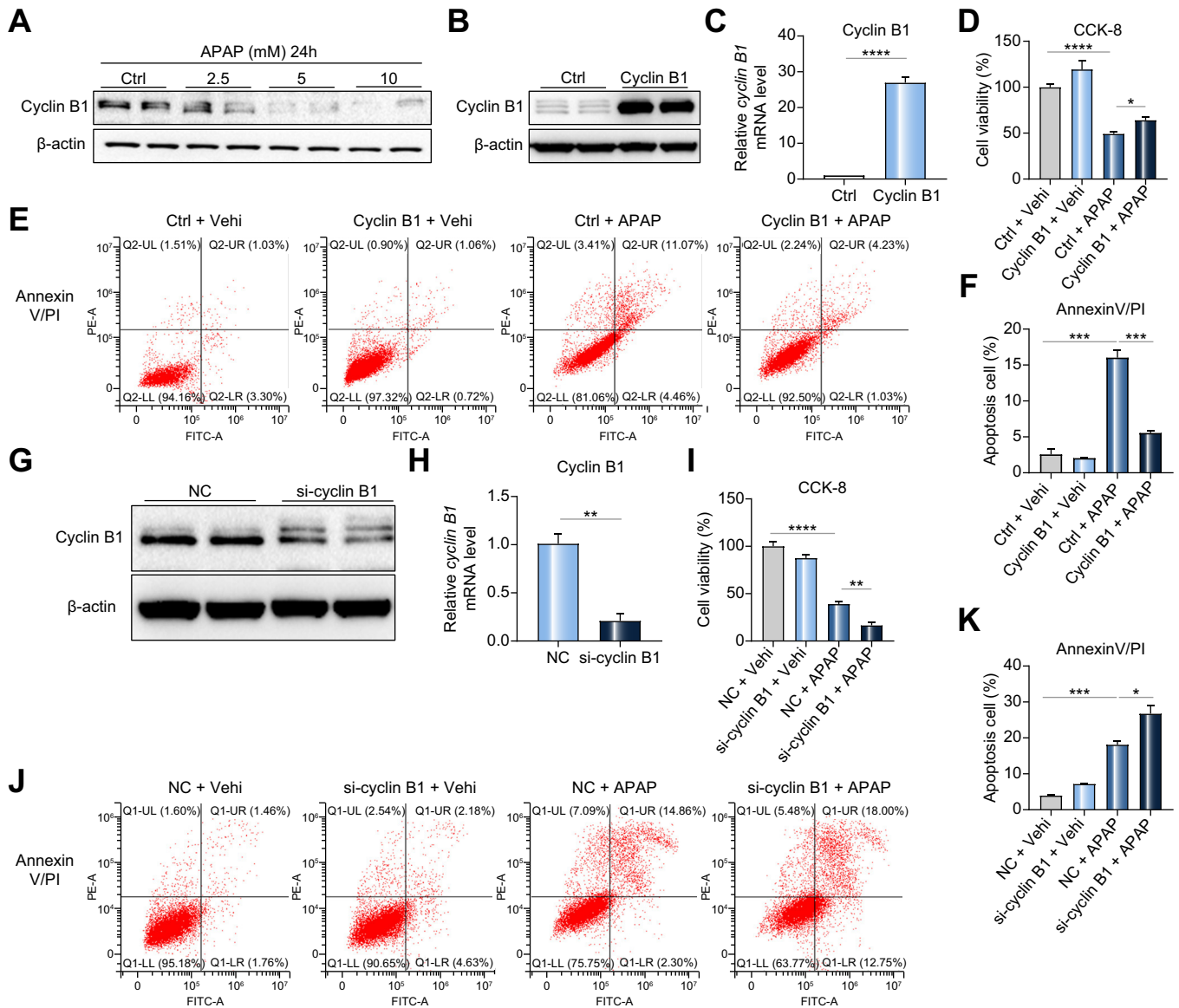


Fig. 6. Effects of cyclin B1 modulation on hepatocyte damage caused by APAP *in vitro*. (A) Cyclin B1 protein decreased dose-dependently in HepaRG cells treated with APAP for 24 h, $n = 4$. (B, C) Western blot analysis and RT-PCR displaying the overexpression efficiency of cyclin B1 in HepaRG cells, $n = 3-4$. (D) Cell viability of HepaRG cells in different groups measured by a CCK-8 assay, $n = 6$. (E, F) Flow cytometry analysis and quantification of HepaRG cells after FITC Annexin V staining, $n = 3$. (G, H) The efficiency of si-cyclin B1 (cyclin B1 siRNA) in HepaRG cells was examined by a Western blot analysis and RT-PCR, $n = 3-4$. (I) Cell viability of HepaRG cells in each group by a CCK-8 assay, $n = 6$. (J, K) Degree of cell damage as shown by FITC Annexin V staining and the corresponding quantification, $n = 3$. Experimental design: cells were transfected with si-cyclin B1 or cyclin B1 plasmids for 24 h, followed by APAP or vehicle treatment for 24 h. All values are represented as the means \pm SEM. Significant differences were analysed using a 1-way ANOVA or Student t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. APAP, acetaminophen; Ctrl, control; CCK-8, cell counting kit 8; NC, negative control; n.s., no significance; PI, propidium iodide; si, small-interfering; Vehi, vehicle.

mice exhibited a further loss of liver function associated with mitotic progression (DNA biosynthesis dysfunction), protein generation (RNA transcription and amino acid biosynthesis) and carbohydrate metabolism compared with the Ctrl + APAP mice. Altogether, these data show that NEK7 is an important protein for maintaining normal liver function and protecting against liver injury induced by APAP.

Reduced NEK7 impairs cell cycle progression

NEK7 is a known regulator of mitosis.²¹ By analysing an RNA-seq profile of the liver regarding regeneration and development

included in PubMed,⁴⁰ the results show a significant increase in NEK7 in the liver even 1 h after two-thirds hepatectomy, reaching a peak at 12–18 h compared with the preoperative stage, suggesting a probable role of NEK7 in promoting liver regeneration (Fig. S6). Additionally, we profiled the transcriptome of liver tissue from iKO mice and Ctrl mice and found that the relative transcript levels of cyclin B1 and CDK1 in the iKO livers were decreased significantly compared with those in the control livers, with a decreasing trend in other cell cycle-related genes (Fig. 5A), and these results were validated by qRT-PCR (Fig. 5B). Moreover, we observed similar results in the

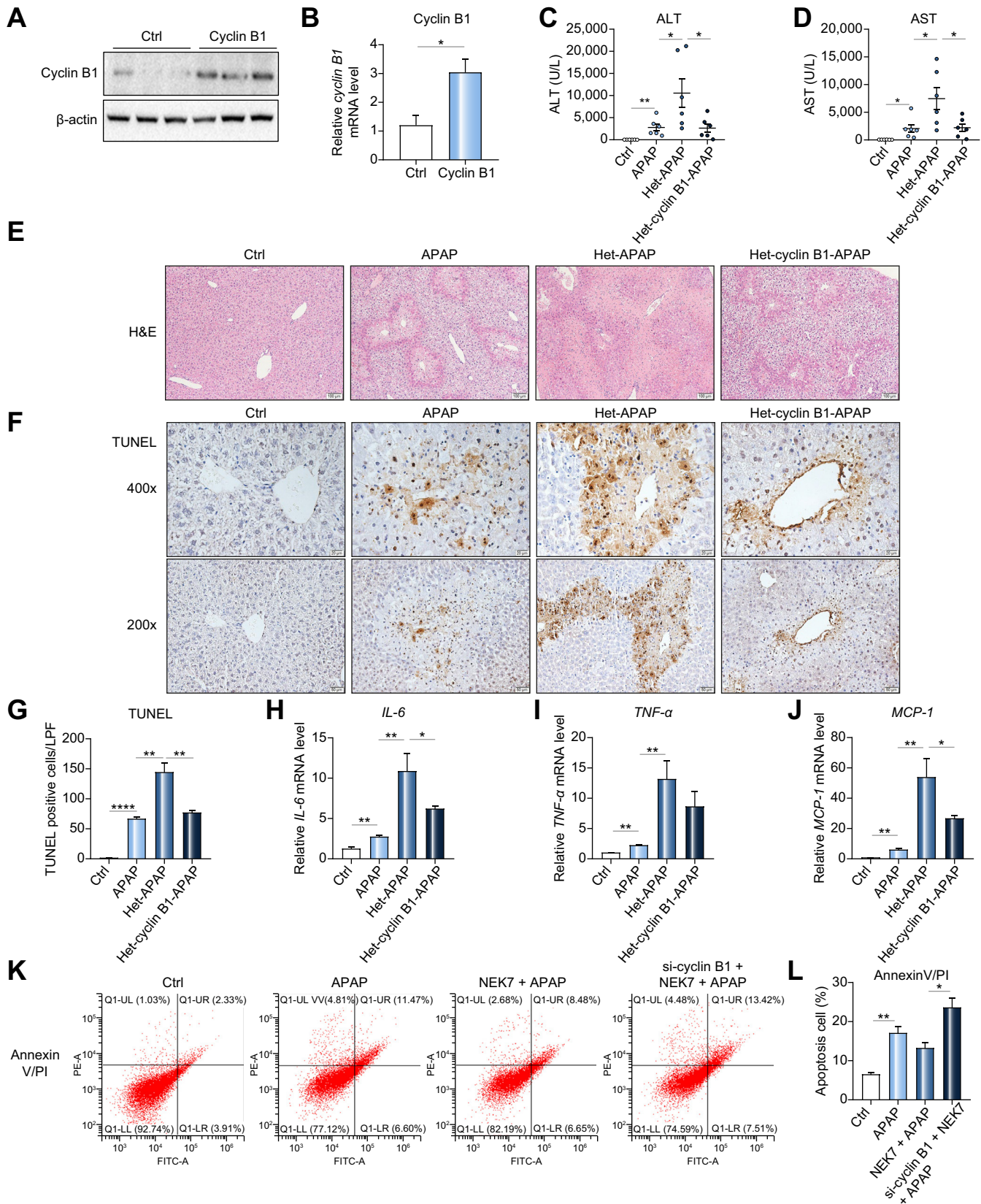


Fig. 7. Cyclin B1 reverses the aggravation of APAP-induced liver injury in mice with NEK7-deficient hepatocytes. (A, B) Cyclin B1 mRNA and protein expression levels in the livers of mice after injection with Ctrl (control) or cyclin B1 plasmids, $n = 3-6$ per group. (C, D) Serum levels of ALT and AST; $n = 6$ per group. (E) Representative images of H&E-stained liver sections; scale bars, 100 μ m. (F) TUNEL-stained liver sections, scale bars, 20 μ m for 400 \times , 50 μ m for 200 \times , $n = 3$. (G) Quantification of positive cells by TUNEL staining. (H-J) mRNA levels of inflammatory genes in the liver, $n = 6$ per group. (K, L) Flow cytometry and

NEK7 cKO Het mice (Fig. 5C). Nevertheless, the overexpression of NEK7 in both the liver and hepatocytes (HepaRG) only induced levels of cyclin B1 and cyclin A2 (Fig. 5D and E). Consistently, we observed that NEK7 deficiency suppressed the proliferative activity of hepatocytes (Fig. 5H, Fig. S7C) and decreased the number of cells in the G2-M phase (Fig. 5F). Furthermore, the depletion of NEK7 in hepatocytes also inhibited cell proliferation after the APAP treatment as evidenced by Ki-67 staining (Fig. 5I–J). In contrast, NEK7 overexpression increased the cell number in the G2-M phase (Fig. 5G) and markedly promoted the proliferative activity of hepatocytes (Fig. S7A and B). Altogether, these data show that reduced NEK7 impairs the cell cycle progression of hepatocytes after APAP challenge.

Cyclin B1 reverses the aggravation of APAP-induced liver injury in mice with NEK7-deficient hepatocytes

The above evidence strongly suggests that NEK7 is involved in the regulation of the cyclin-mediated cell cycle under this disease condition. However, the role of cyclins, especially cyclin B1, in APAP-induced ALI is still unclear. We first found a gradual decrease in cyclin B1 expression, which is consistent with the variation trend of NEK7 in HepaRG cells treated with increasing concentrations of APAP (Fig. 6A). The overexpression of cyclin B1 significantly alleviated the degree of hepatocyte damage triggered by APAP as determined by CCK-8 and flow cytometry assays (Fig. 6B–F). However, the depletion of cyclin B1 exacerbated APAP-triggered hepatocyte damage (Fig. 6G–K). To evaluate whether exogenous cyclin B1 could rescue the liver injury triggered by APAP in NEK7 cKO Het mice, we overexpressed cyclin B1 in the liver by the injection of its plasmids into Het mice, followed by APAP (Fig. 7A and B). After the analysis of liver function markers (Fig. 7C and D), pathological changes (Fig. 7E–G) and inflammation (Fig. 7H–J), we found that cyclin B1 reversed NEK7 deficiency-related ALI under APAP treatment. Moreover, in HepaRG cells, the depletion of cyclin B1 significantly abolished the protective effect of NEK7 against APAP-induced ALI as evidenced by the levels of damaged cells and ROS (Fig. 7K and L, Fig. S8A–C).

Neither NLRP3 inflammasome activation nor CYP2E1 expression is affected by NEK7 under APAP-induced liver injury

In addition to its role in regulating the cell cycle, NEK7 was demonstrated to be involved in the assembly of the NLRP3 inflammasome complex, which has been considered crucial for the activation of the NLRP3 inflammasome.²¹ However, in this study, we found evidence of the time-dependent upregulation of NLRP3 and Caspase 1 and enhanced activity of Caspase 1, indicating the activation of the NLRP3 inflammasome in contrast to the decreased expression of NEK7 *in vitro* (Fig. S9A and B). *In vivo*, the level of NEK7 in the liver decreased after APAP overdose for 16 h, which was largely consistent with the *in vitro* results (Fig. S9C and D). However, overexpression of NEK7 did not increase the levels of Caspase 1 p10 (*i.e.* the activated form of Caspase 1) or IL-1 β mRNA in the liver (Fig. S9E and F). These

results suggest that NLRP3 inflammasome activation in APAP-induced ALI can be independent of NEK7.

CYP2E1 is a crucial enzyme for APAP metabolism to NAPQI, which accounts for hepatic GSH depletion and subsequent liver injury.⁹ To explore the potential effect of NEK7 on APAP metabolism in the liver, we examined the levels of CYP2E1 and GSH *in vivo* and *in vitro* after NEK7 modulation. As shown by the data, CYP2E1 was not affected by NEK7 *in vivo* (Fig. S10A and B). Meanwhile, the GSH contents in the liver after the APAP treatment for 1.5 h or 1 h were similar between the NEK7 overexpressing/knockdown mice and their control mice (Fig. S11A and B). *In vitro*, NEK7 overexpression in HepaRG cells also did not affect the GSH levels at early time points (Fig. S11C). Thus, NEK7 prevented liver injury induced by APAP probably independently of regulation of CYP2E1 and APAP metabolism.

Discussion

APAP-induced ALI is of growing public concern because of its high incidence and limited therapeutic options, and further exploration is urgently needed.^{5,10,13} The liver is one of few organs that has the potential to regenerate upon injury.⁴¹ Therefore, exploring new molecules responsible for liver regeneration and restoration is a promising endeavour.^{19,42,43} In the present study, we are the first to determine the role of NEK7 in APAP-triggered ALI. Here, we found that NEK7 is highly expressed in hepatocytes. Moreover, the level of NEK7 was markedly decreased in livers and hepatocytes injured by APAP. The NEK7 reduction might result from the hepatotoxicity of APAP metabolites or the subsequent insults of APAP hepatotoxicity.

Subsequently, we overexpressed NEK7 in the livers of WT mice by hydrodynamic tail vein injection, which was verified as an effective method for delivering constructs to modulate the expression of genes.^{44,45} After the APAP treatment, the mice with NEK7 overexpression displayed mild injury compared with the control mice. *In vitro*, the overexpression of NEK7 in hepatocyte cell lines also alleviated the cell damage induced by APAP. Thus, we speculated that NEK7 might play a crucial role in hepatocytes preventing APAP-induced ALI, and the protective effect of NEK7 on APAP-triggered liver injury is reflected in various aspects, including the striking improvement in liver function, milder pathological changes, and relief of inflammation and oxidative damage. Because of the limitations of hydrodynamic injection and the current lack of a specific activator of NEK7, we could not overexpress or activate NEK7 during the acute phase after APAP treatment to investigate the therapeutic effect of NEK7 overexpression. This issue is also a weakness of this study, which needs further exploration in the future.

We also used 2 gene-edited mice to deplete the expression of NEK7 in hepatocytes and the whole body. Both mouse strains exhibited exacerbated APAP-induced liver injury compared with their control mice. As mentioned above, NEK7 cKO homozygous mice did not survive well, whereas heterozygous mice displayed moderate liver dysfunction. These results further support that a decrease in NEK7 could contribute to the development of APAP-induced liver injury. Combined with a previous report showing

quantitative analysis of cell damage in HepaRG cells with different treatments, n = 3. Experimental design: mice were injected with cyclin B1 or control plasmids for 24 h, followed by APAP or saline for 24 h. All values are represented as the means \pm SEM. Significant differences were analysed using a 1-way ANOVA or Student *t* test, **p* < 0.05, ***p* < 0.01, *****p* < 0.0001. ALT, alanine aminotransferase; APAP, acetaminophen; AST, aspartate aminotransferase; Ctrl, control; Het, heterozygous; MCP-1, monocyte chemoattractant protein 1; NEK7, NIMA-related kinase 7; TNF- α , tumour necrosis factor-alpha.

that NEK7 KO mice exhibited a phenotype of embryonic death,³⁹ we concluded that NEK7 plays a protective role against APAP hepatotoxicity.

Numerous studies have identified various molecules that might play an important role in liver proliferation under injury by either direct targets on hepatocytes or the crosstalk between liver cells.^{18,19} NEK7 belongs to the NEK family and is involved in the regulation of the cell cycle.²³ Here, we also found that the overexpression of NEK7 upregulated cyclins, accompanied by an increase in hepatocyte proliferative activity, which has been suggested in previous studies.^{28,29} Additionally, the depletion of NEK7 inhibited the proliferative activity of the liver after the APAP challenge, which was also confirmed by the *in vitro* results. Among the cyclins, cyclin B1 seems to be the most sensitive to NEK7. However, there is still no direct evidence showing that cyclin B1 protects against APAP-induced ALI despite its role in proliferation and regeneration. In this study, we demonstrated that cyclin B1 displayed a protective effect similar to that of NEK7 and rescued the exacerbation of APAP-induced liver injury resulting from NEK7 depletion in Het mice. Moreover, cyclin B1 depletion largely diminished the protective effect of NEK7 overexpression on APAP-induced hepatocyte damage. One limitation is how NEK7 regulates cyclin B1 or other cyclins in hepatocytes remains unknown and requires further exploration. Thus, altogether, we elucidated the important role of the cyclin-mediated cell cycle downstream of NEK7 in APAP-induced ALI.

In addition to its role in mitosis regulation, NEK7 has also been demonstrated to be involved in the assembly of the NLRP3 inflammasome complex in certain diseases.^{21,33} In acute liver injury and hepatocyte damage induced by APAP, the NLRP3 inflammasome was significantly activated, which is consistent with previous studies.^{46,47} However, the decreased expression of NEK7 in APAP-induced ALI, inhibited cleavage of pro-caspase1 and decreased levels of IL-1 β upon NEK7 overexpression suggest that the activation of the NLRP3 inflammasome might not depend on NEK7 here, which is consistent with a recently reported study about ALI induced by cisplatin.⁴⁸

CYP2E1 is a crucial enzyme responsible for the metabolism of APAP to NAPQI.⁹ Interestingly, the expression of CYP2E1 in APAP-induced ALI has not been consistent in different reports, variously showing an increase, decrease, or no change.^{36,49} In the present study, we found that the protein level of CYP2E1 was significantly decreased both *in vivo* and *in vitro*, which is consistent with a previous study aiming to determining the changes in various CYP450s in the liver in an APAP-induced liver injury model.⁴⁹ Meanwhile, we found that the expression of CYP2E1 was not affected by NEK7. Thus, CYP2E1 might also not act as the downstream molecular mechanism of NEK7 against APAP-induced ALI.

In conclusion, we explored and demonstrated a new function of NEK7 in the liver against APAP-induced ALI, which not only sheds light on the novel role of NEK7 in the field of liver disease, but also suggests that NEK7 is a potential target in the prevention and treatment of APAP-related ALI.

Abbreviations

ALF, acute liver failure; ALI, acute liver injury; ALT, alanine aminotransferase; APAP, acetaminophen; AST, aspartate aminotransferase; CCK-8, cell counting kit 8; cKO mice, conditional knockout mice; Ctrl, control; GSH, glutathione; Het, heterozygous; iKO mice, inducible knockout mice; LDH, lactate dehydrogenase; MCP-1, monocyte chemoattractant protein-1; MDA, malondialdehyde; NAPQI, *N*-acetyl-*p*-benzoquinone imine; ND1, NADH dehydrogenase 1; NEK7, NIMA-related kinase 7; PI, propidium iodide; ROS, reactive oxygen species; si, small interfering; TNF- α , tumour necrosis factor- α ; TPM, transcripts per kilobase of exon model per million mapped reads; WT, wild-type.

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Conflicts of interest

The authors declare no conflicts of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Designed the experiments and wrote the manuscript: ZS, ZJ. Performed experiments, acquired and analysed data: ZS, QW, LS, MW, TN, CZ. Assisted in conducting the experiment: SL, HH. Contributed helpful discussions on the project and manuscript: YS. Conceived the concept, supervised the experiment design and execution, and revised the manuscript: ZJ, YZ, AZ, SH.

Data availability statement

Data that support this study are available from the corresponding author on reasonable request.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhepre.2022.100545>.

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