

Intracellular CIRP promotes liver regeneration via STAT3 signaling pathway activation after partial hepatectomy in mice

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Abstract. Cold-inducible RNA-binding protein (CIRP) is a cold shock protein implicated in the regulation of multiple biological processes depending on its cellular localization. However, to the best of our knowledge, the role of CIRP in liver regeneration and injury after hepatectomy has not been investigated. The present study was therefore designed to explore whether CIRP is involved in liver regeneration after hepatectomy and its specific role and underlying molecular mechanism. The overall involvement of CIRP in liver regeneration and injury after hepatectomy was evaluated in CIRP-deficient mice. C23, an antagonist of extracellular CIRP, was used to assess the effect of extracellular CIRP on liver regeneration and injury after hepatectomy. CIRP overexpression and short hairpin RNA plasmids were transfected into HepG2 cells to study the effect of intracellular CIRP on cell proliferation. The effects of extracellular CIRP on cell proliferation and injury were determined via the use of recombinant CIRP protein to stimulate HepG2 cells *in vitro*. The results indicated that both hepatic and serum CIRP levels significantly increased after partial hepatectomy. Additionally, CIRP deficiency impaired liver regeneration but alleviated liver

injury after partial hepatectomy in mice. C23 administration attenuated liver injury and suppressed endoplasmic reticulum (ER) stress and oxidative stress. Loss- and gain-of-function analyses in HepG2 cells indicated that an increase in intracellular CIRP promoted cell proliferation via signal transducers and activation of transcription 3 (STAT3) signaling pathway activation. Moreover, recombinant CIRP had no effect on cell proliferation or STAT3 phosphorylation but induced ER stress, which was blocked by TAK242, an inhibitor of Toll-like receptor 4 (TLR4), in HepG2 cells. Taken together, the results of the present study demonstrated that intracellular CIRP promotes liver regeneration by activating the STAT3 pathway, whereas extracellular CIRP induces ER stress possibly via the TLR4 signaling pathway after hepatectomy.

Introduction

The liver is the only organ in the human body that has the ability to regenerate rapidly and abundantly. Even if nearly two-thirds of the liver is surgically removed, the remaining liver can quickly return to its original size (1). The process of liver regeneration is regulated by a set of signaling pathways that are still largely unknown. Hence, an in-depth understanding of this process will help rationalize the use of specific therapies to promote liver function recovery and liver regeneration.

Cold-inducible RNA-binding protein (CIRP) is a 172-amino acid cold shock protein that was discovered in 1997 (2). CIRP is constitutively expressed at low levels in various tissues; however, CIRP expression can be upregulated by hypoxia (3), UV radiation (4), glucose deprivation (5) and heat stress (6), indicating that CIRP is a stress-response protein. In response to stress, CIRP migrates from the nucleus to the cytoplasm and regulates mRNA stability by binding to CIRP-specific binding sites on the 3'-untranslated region of target mRNAs (7). CIRP is also known to regulate diverse biological processes depending on its cellular localization. Intracellular CIRP (iCIRP) has been implicated in mRNA stability (8), cell proliferation (9), cell survival (10), circadian modulation (11), telomere

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Abbreviations: CIRP, cold-inducible RNA-binding protein; ER, endoplasmic reticulum; STAT3, signal transducers and activation of transcription 3; TLR4, Toll-like receptor 4; DAMP, danger-associated molecular pattern; PCNA, proliferating cell nuclear antigen

Key words: extracellular CIRP, intracellular CIRP, hepatectomy, liver regeneration, ER stress

maintenance (12) and tumor formation and progression (13). During hypoxia and inflammation, CIRP in the cytoplasm can be released into the extracellular space. Extracellular CIRP (eCIRP), a danger-associated molecular pattern (DAMP), induces inflammatory responses and tissue injury by binding with its receptor, Toll-like receptor 4 (TLR4) (14-16). C23, a CIRP-derived short peptide, blocks the interaction between CIRP and TLR4 and targets cells that express TLR4 (15). The effects and mechanism of eCIRP have been studied in numerous immune cells, including macrophages (16), lymphocytes (17), neutrophils (18,19) and dendritic cells (20).

Previous studies have indicated that CIRP is associated with several human cancer types and plays important roles in cell proliferation and survival under stress conditions (21-23). Signal transducer and activator of transcription 3 (STAT3) is a key transcriptional mediator of cytokines and growth hormone and serves a notable role in liver regeneration (24,25). CIRP has been shown to activate the STAT3 signaling pathway (26,27); however, the role of CIRP in liver regeneration and injury after hepatectomy remains largely unknown. Therefore, in the present study, *in vivo* and *in vitro* experiments were conducted to elucidate these effects of CIRP and explore the mechanism involved. In conclusion, promoting the role of CIRP in liver regeneration is a potential strategy for the management of liver restoration after hepatectomy.

Materials and methods

Experimental animals. A total of 23 wild-type (WT) C57BL/6 male mice (8-10 weeks old, weighing 20-25 g) were obtained from the experimental animal center of Xi'an Jiaotong University (Xi'an, China). Additionally, 23 CIRP knockout (KO) C57BL/6 male mice (8-10 weeks old, weighing 20-25 g) were obtained from Shanghai Model Organisms Center, Inc. All animals were housed in the same specified pathogen-free environment under a 12-h light/dark cycle and food and water were provided *ad libitum*. All the animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Ethics Committee of Xi'an Jiaotong University Health Science Center (approval no. XJTUAE2023-2145).

Partial hepatectomy. The mice were divided into the sham (WT mice, n=5; KO mice, n=5) and partial hepatectomy (WT mice, n=18; KO mice, n=18) groups. The liver regeneration experiment was conducted based on the model of two-thirds partial hepatectomy in mice according to a method previously described (28). Briefly, age and weight-matched mice were anesthetized by isoflurane inhalation (2% induction and 2% maintenance) during the whole surgical procedure. A 1-2 cm incision was performed in the midline abdominal skin to expose the liver. Then, the left lateral and median hepatic lobes were removed after ligating the stem of the hepatic lobes with sterile threads. After closing the abdominal cavity, the mice were placed on a warming pad for palinesthesia. In the sham group, laparotomy without liver resection was performed. For C23 treatment, the mice were intraperitoneally injected with C23 (8 mg/kg) after hepatectomy, and the vehicle group received an equivalent volume of normal saline. The animal health and behavior were monitored every day

after hepatectomy. The duration of the experiment was 7 days. The mice were euthanized (by cervical dislocation following isoflurane anesthesia) on days 1, 3 and 7 after hepatectomy. Mice death was verified by cardiac arrest and the cessation of breathing. A total of 23 WT and 23 CIRP KO mice were used and euthanized in the experiment, and no death occurred before the end of the experiment. All animal welfare considerations were fully considered, including efforts to minimize suffering and distress, use of or anesthetics, or special housing conditions.

Sample collection. At the end of the experiment, ~500 μ l blood samples were obtained by eyeball extirpation after the mice were anesthetized with isoflurane. The samples were centrifuged at a low speed (4°C, 845 x g, 15 min) to collect the serum. After blood collection, the mice were euthanized by cervical dislocation. Liver samples were harvested at the indicated timepoints, then fixed with 4% paraformaldehyde at room temperature for at least 24 h for further histological examination or stored at -80°C for molecular and biochemical analyses.

Cell culture and transfection. The HepG2 hepatoblastoma cell line was purchased from iCell Bioscience Inc., which was authenticated by STR profiling. Although the HepG2 cell line is a liver cancer cell line, it retains a number of functions and features of normal human hepatocyte cells and it has been extensively used to study liver regeneration (29-31). The cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (both from Cytiva) and 1% penicillin/streptomycin and incubated at 37°C with 5% CO₂. Human CIRP overexpression and short hairpin (sh)RNA plasmids were purchased from Shanghai GeneChem Co. Ltd. The plasmids were constructed as previously described (12). Plasmid DNA or shRNA and its corresponding negative control vectors were transfected using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Briefly, 3 μ g nucleic acid and Lipofectamine 3000 was added to cells in a 6-well plate that contained 2 ml DMEM for 24 h. CIRP cDNA (NM_001280) in the GV712 vector (CMV enhancer-MCS-SC40-puromycin) and the GV712 vector without CIRP cDNA (the negative control vector), CIRP shRNA in the GV493 vector (hU6-MCS-CBh-gcGFP-IRES-puromycin) and the GV493 vector without CIRP shRNA (the negative control vector) were designed by Shanghai GeneChem Co. Ltd. The targeting sequences for the various CIRP shRNAs were as follows: CIRP-RNA interference (RNAi)1, 5'-GCCATGAATGGG AAGTCTGTA-3'; CIRP-RNAi2: 5'-CTTCTCAAAGTA CGGACAGAT-3'; and CIRP-RNAi3: 5'CGGGTCCTACAG AGACAGTTA-3'. Overexpression or knockdown of CIRP in these cells was verified by western blotting using anti-CIRP antibody as described below.

Measurement of liver function. The serum alanine aminotransferase (ALT) assay (cat. no. C009-2), aspartate aminotransferase (AST) assay (cat. no. C010-2) and lactic dehydrogenase (LDH) assay (cat. no. A020-2) kits were purchased from Nanjing Jiancheng Bioengineering Institute. The levels

of serum ALT, AST and LDH were measured according to the manufacturer's instructions.

Measurement of oxidative stress. To quantify oxidative stress, malonaldehyde (MDA) assay (cat. no. A003-1), superoxide dismutase (SOD) assay (cat. no. A001-3) and glutathione peroxidase (GSH-PX) activity assay (cat. no. A005) kits were purchased from Nanjing Jiancheng Bioengineering Institute. The levels of liver MDA, SOD and GSH-PX were measured according to the manufacturer's instructions.

Western blotting. Liver lysates were prepared with RIPA buffer containing a protease inhibitor cocktail and phosphatase inhibitor cocktail (Beyotime Institute of Biotechnology). The proteins were extracted for western blotting, whose concentration was determined by a BCA protein concentration determination kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, the proteins (25 μ g per lane) were separated by sodium dodecyl sulphate-polyacrylamide gel (10%) electrophoresis (Bio-Rad Laboratories, Inc.). The proteins were then transferred to PVDF membranes (Bio-Rad Laboratories, Inc.) and blocked with 5% skimmed milk or BSA (Beyotime Institute of Biotechnology) at room temperature for 1 h. The membranes were then incubated with the indicated primary antibodies at 4°C overnight and with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Anti-STAT3 (1:1,000; cat. no. ab109085), anti-phosphorylated (p)-STAT3 (1:2,000; cat. no. ab76315) and anti-p-inositol-requiring enzyme 1 α (p-IRE1 α ; 1:1,000; cat. no. ab48187) were purchased from Abcam. Anti-CIRP (1:1,000; cat. no. 10209-2-AP) and anti-GAPDH (1:10,000; cat. no. 60004-1-Ig) were purchased from Proteintech Group Inc. Anti-cyclin D1 (1:1,000; cat. no. AF0931), anti-proliferating cell nuclear antigen (PCNA; 1:1,000; cat. no. AF0239), anti-immunoglobulin heavy-chain-binding protein (-BIP; 1:1,000; cat. no. AF5366) and anti- β -actin (1:1,000; cat. no. T0022) were purchased from Affinity Biosciences. Anti-IRE1 α (1:1,000; cat. no. 3294), anti-X-box binding protein (XBP1s; 1:1,000; cat. no. 40435), anti-protein disulfide isomerase (PDI; 1:1,000; cat. no. 2446) and anti-BAX (1:1,000; cat. no. 2772) were purchased from Cell Signaling Technology, Inc. HRP-conjugated goat anti-mouse (1:4,000; cat. no. SA00001-1) and goat anti-rabbit (1:4,000; cat. no. SA00001-2) secondary antibodies were purchased from Proteintech Group Inc. Protein expression was detected using a chemiluminescence system (Bio-Rad Laboratories, Inc) and quantified using ImageJ software (version 1.53q; National Institutes of Health).

Histological analysis. The liver tissues fixed in paraformaldehyde (4%) for at least 24 h (as aforementioned) were embedded in paraffin. Liver samples were cut into 5- μ m thick sections for hematoxylin (5 min) and eosin (15 sec) staining at room temperature. For immunohistochemistry, the sections were firstly dewaxed and rehydrated by dewaxing solution (Wuhan Servicebio Technology Co., Ltd.), ethanol at 100, 95, 85 and 75%, and distilled water for 5 min respectively at room temperature. Then the sections were preprocessed for antigen retrieval by microwave heating at 100°C for 10 min and cooled at room temperature for 2 h. After washing with PBS three times, the sections were blocked with 5% BSA (Wuhan Servicebio

Technology Co., Ltd.) at 37°C for 30 min and then incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies against HRPconjugated goat anti-rabbit IgG H&L (1:200; cat. no. ab205718; Abcam) at room temperature for 1 h. The primary antibodies for F4/80 (1:5,000; cat. no. ab300421), CD68 (1:100; cat. no. ab283654), CO11b (1:4,000; cat. no. ab133357), CD20 (1:100; cat. no. ab64088), myeloperoxidase (MPO; 1:1,000; cat. no. ab208670), Ki67 (1:1,000; cat. no. ab15580) and CIRP (1:500; cat. no. ab106230) were purchased from Abcam. These sections were stained with DAB for 5 min and counterstained with hematoxylin for 3 min at room temperature. Finally, the sections were dehydrated for further observation. All the images were then obtained under an optical microscope.

Cell viability assay. Cell viability was determined using the Cell Counting Kit-8 (CCK-8) Assay Kit (Dojindo Laboratories, Inc.). HepG2 cells with overexpressed or knockdown CIRP were seeded at a density of 5×10^3 cells/well in 100 μ l DMEM in a 96-well plate. After 24 and 48 h, 10 μ l CCK-8 was added to each well and the cells were subsequently incubated at 37°C for 2 h. For the treatment of HepG2 cells, the antagonist (static; 5 μ M; Selleck Chemicals) and activator (Colivelin; 5 μ M Selleck Chemicals) of p-STAT3 were added to the wells and incubated at 37°C for 24 h. To examine the effect of eCIRP on HepG2 cells, different concentrations of recombinant CIRP (rCIRP; 0, 100, 500 and 1,000 ng/ml; Wuhan USCN Business Co., Ltd.) or TAK242 (10 μ M; Selleck Chemicals) were added to the wells and incubated at 37°C for 24 h. Then 10 μ l CCK-8 was added for further incubation at 37°C for 2 h. The absorbance was measured at 450 nm using a microplate reader.

Cell cycle analysis. HepG2 cells were washed with PBS three times and fixed with 70% ethanol at -4°C overnight. After a single wash with PBS, the cells were stained with the cell cycle assay solution (Cell Cycle Assay Kit; Dojindo Laboratories, Inc.) according to the manufacturer's instruction. The cell cycle distribution was obtained using a flow cytometer (NovoCyt 2020R; NovoCyt Flow Cytometer; ACEA Biosciences, Inc.; Agilent) with NovoExpress software (version 1.3.1; ACEA Biosciences, Inc.; Agilent).

Enzyme-linked immunosorbent assays (ELISA). The CIRP ELISA kit (cat. no. CSB-EL005440MO; Cusabio Technology, LLC) was used to measure the levels of serum CIRP according to the instructions of the manufacturer.

Statistical analysis. All the experimental data are presented as the mean $\pm$ SEM. Statistical analysis between two groups was performed by unpaired two-tailed Student's t-test. Statistical analysis among more than two groups was performed by one-way ANOVA followed by SNK or Tukey (>3 groups) post hoc tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CIRP expression is upregulated after partial hepatectomy in mice. To determine the characteristics of CIRP expression after hepatectomy in mice, CIRP expression levels in

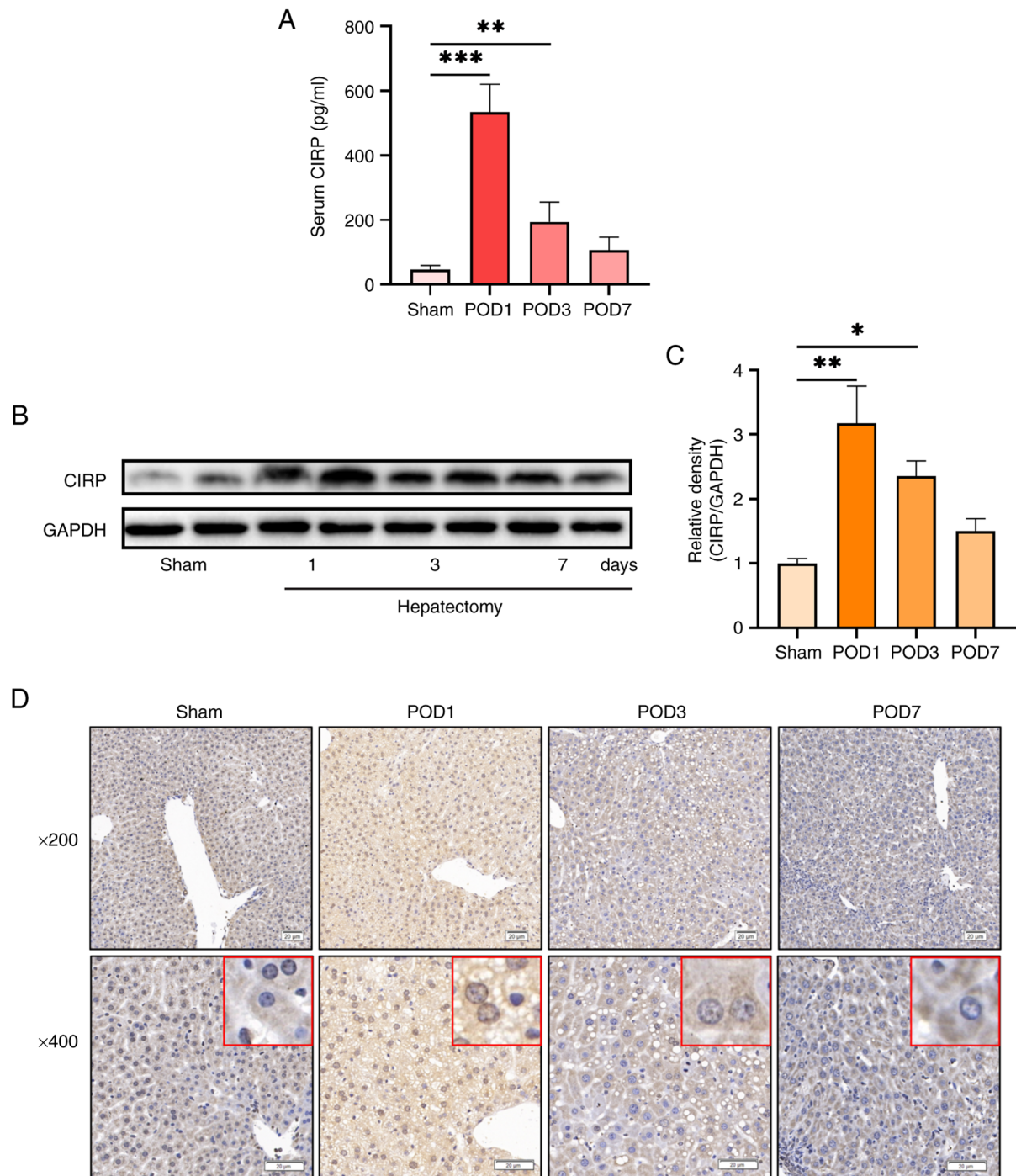


Figure 1. CIRP expression is upregulated after partial hepatectomy in mice. (A) CIRP level in WT mice serum at 1, 3 and 7 days after partial hepatectomy. (B) CIRP expression in WT mice livers at 1, 3 and 7 days after hepatectomy, (C) which was semi-quantified. (D) Immunohistochemical staining of CIRP in liver sections from WT mice livers at 1, 3 and 7 days after hepatectomy (magnification, x200, x400 and x1,600). The sham operated mice were used as the baseline control. All data are presented as the mean $\pm$ SEM; n=6. *P<0.05, **P<0.01, ***P<0.001 compared with sham mice. POD, postoperative day; CIRP, cold-inducible RNA-binding protein; WT, wild-type.

mouse livers after two-thirds partial hepatectomy were first examined, an *in vivo* system that has been widely used in the study of liver regeneration (32). As shown in Fig. 1A, the level of CIRP in the serum increased after hepatectomy but then decreased (Fig. 1A). Moreover, the relative expression of CIRP in the liver increased significantly on the first day after hepatectomy but then gradually decreased over the following days (Fig. 1B-D). These results indicated that CIRP may be involved in liver regeneration after partial hepatectomy.

CIRP deficiency restrains liver regeneration after partial hepatectomy in mice. To confirm the role of CIRP in liver regeneration, CIRP-KO mice and WT mice were subjected to two-thirds partial hepatectomy. As shown by Ki67 staining, CIRP deficient mice exhibited decreased hepatocyte proliferation after hepatectomy than WT mice (Fig. 2A and B). Moreover, compared with that of WT mice, the liver to body weight ratio of CIRP-KO mice was lower 3 days after hepatectomy (Fig. 2C and D). Proteins related to liver regeneration

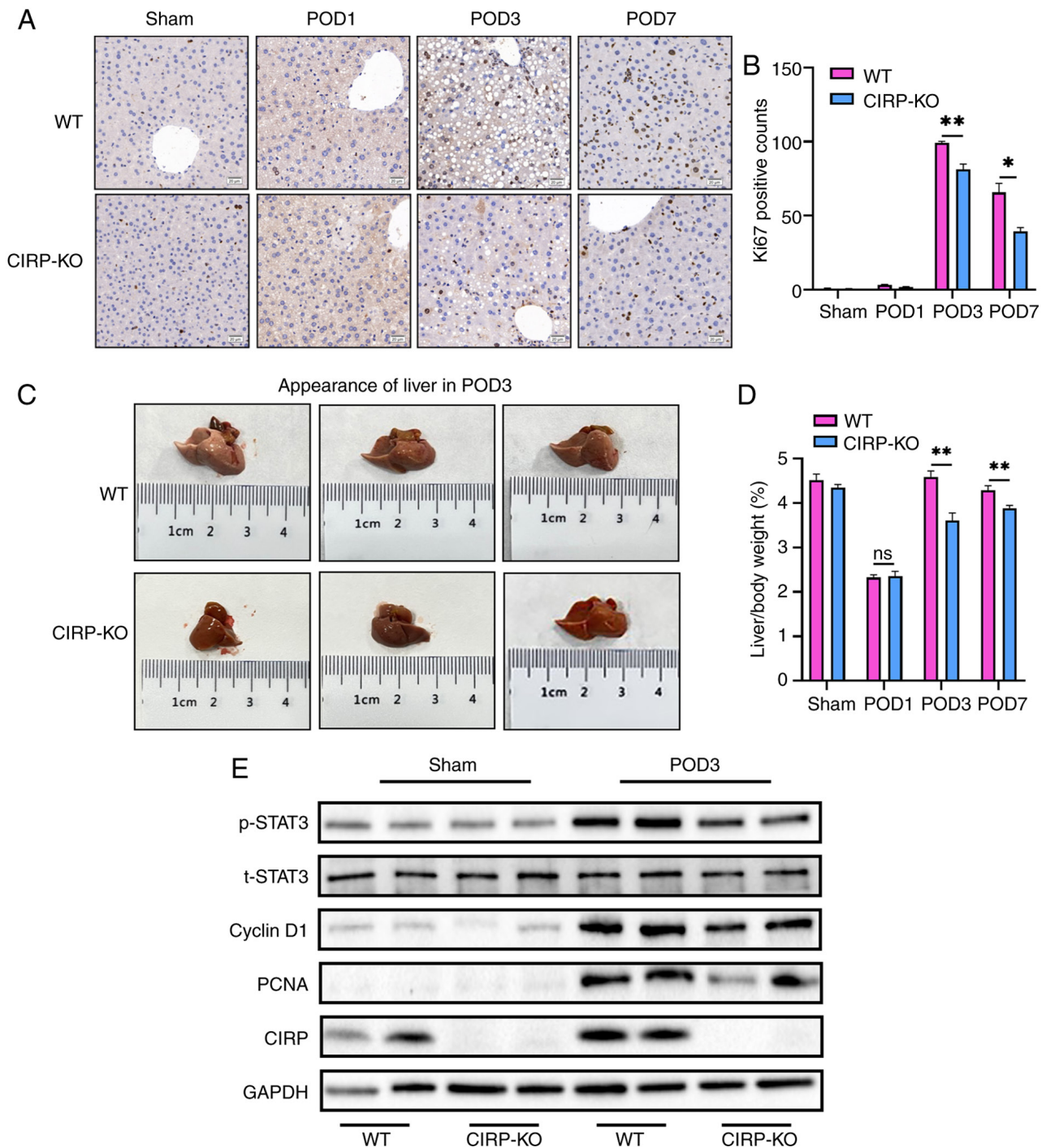


Figure 2. CIRP deficiency restrains liver regeneration after partial hepatectomy in mice. (A) Immunohistochemical staining of Ki67 in liver sections from WT and CIRP-KO mice livers at 1, 3 and 7 days after partial hepatectomy, (B) which was semi-quantified. (C) The appearance of the livers from WT and CIRP-KO mice on day 3 after partial hepatectomy. (D) Liver-to-body-weight ratio in WT and CIRP-KO mice at 1, 3 and 7 days after partial hepatectomy. The sham operated mice were used as the baseline control. (E) Western blotting analysis of p-STAT3, Cyclin D1, PCNA and CIRP in WT and CIRP-KO mice livers on day 3 after partial hepatectomy. GAPDH was used as the loading control. All data are presented as the mean $\pm$ SEM; n=6. *P<0.05, **P<0.01 compared with WT mice. POD, postoperative day; WT, wild-type; KO, knockout; CIRP, cold-inducible RNA-binding protein; p-, phosphorylated; t-, total; STAT3, signal transducers and activation of transcription 3; PCNA, proliferating cell nuclear antigen.

were subsequently examined. As shown in Figs. 2E and S1, Cyclin D1 and PCNA were markedly upregulated after hepatectomy, while their expression in CIRP-KO mice was lower than that in WT mice. Next, the underlying mechanism of CIRP in liver regeneration was explored. Previous research revealed that STAT3 signaling is responsible for hepatocyte proliferation after partial hepatectomy in mice (33). Once

the STAT3 transcription factor is activated and phosphorylated, it will translocate into the nucleus and regulate the expression of genes related to liver regeneration (34,35). The phosphorylation of STAT3 notably increased at 1, 3 and 7 days after hepatectomy, whereas CIRP KO significantly restricted STAT3 phosphorylation (Figs. 2E and S1). These data demonstrated that CIRP deficiency impaired liver regeneration and

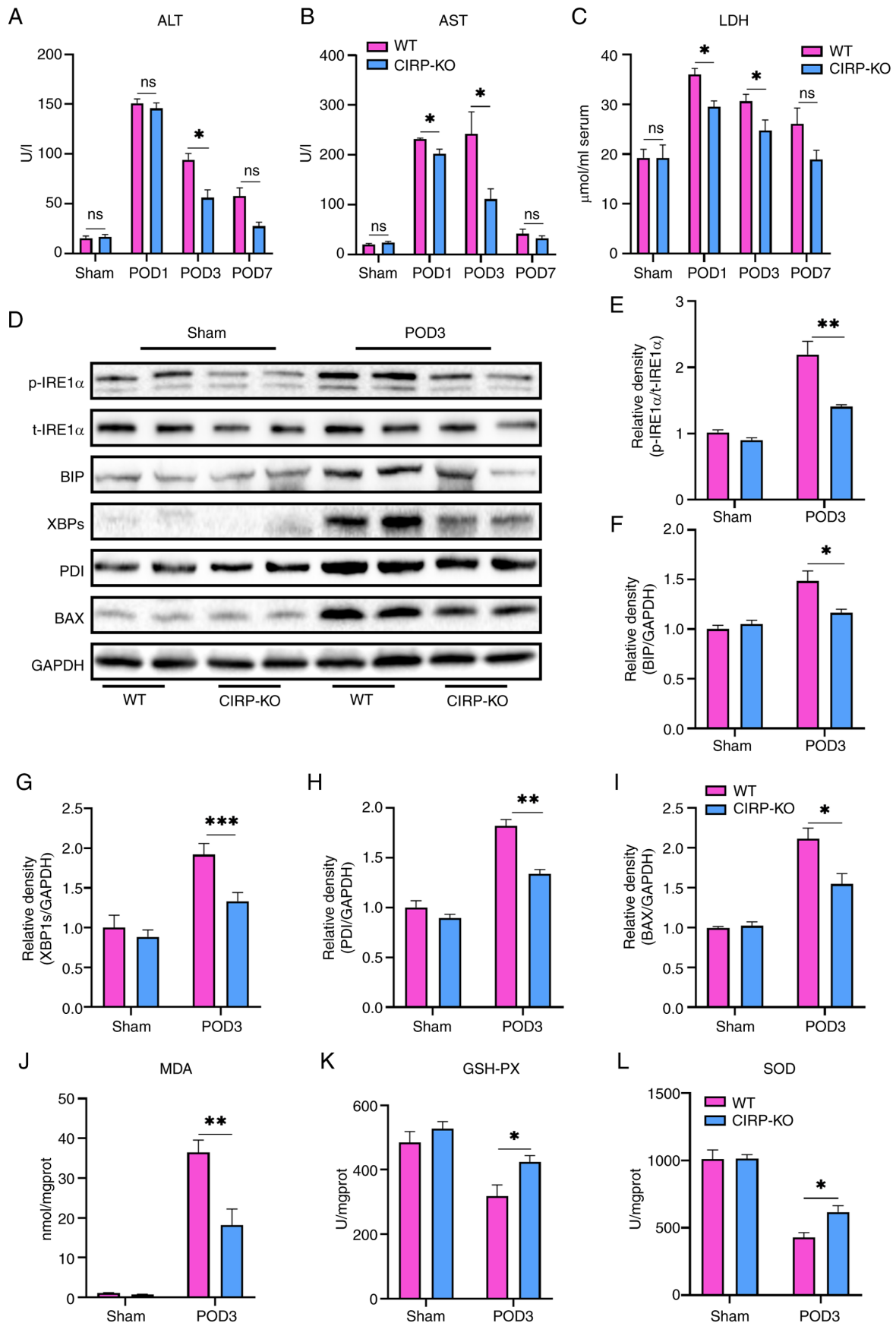


Figure 3. CIRP deficiency alleviates liver injury after partial hepatectomy. Serum (A) AST, (B) ALT and (C) LDH at 1, 3 and 7 days after partial hepatectomy. (D-I) Western blotting analysis of BIP, p-IRE1 α , XBPs, PDI and BAX in WT and CIRP-KO mice livers on day 3 after partial hepatectomy. GAPDH was used as the loading control. Examination of (J) MDA, (K) SOD and (L) GSH-PX levels in WT and CIRP-KO mice livers on the 3rd day after partial hepatectomy. All data are presented as the mean $\pm$ SEM; n=6. * P <0.05, ** P <0.01, *** P <0.001 compared with WT mice. POD, postoperative day; WT, wild-type; KO, knockout; CIRP, cold-inducible RNA-binding protein; p-, phosphorylated; t-, total; IRE1 α , inositol-requiring enzyme 1 α ; BIP, immunoglobulin heavy-chain-binding protein; XBPs, X-box binding proteins; PDI, protein disulfide isomerase; MDA, malonaldehyde; SOD, superoxide dismutase; GSH-PX, glutathione peroxidase.

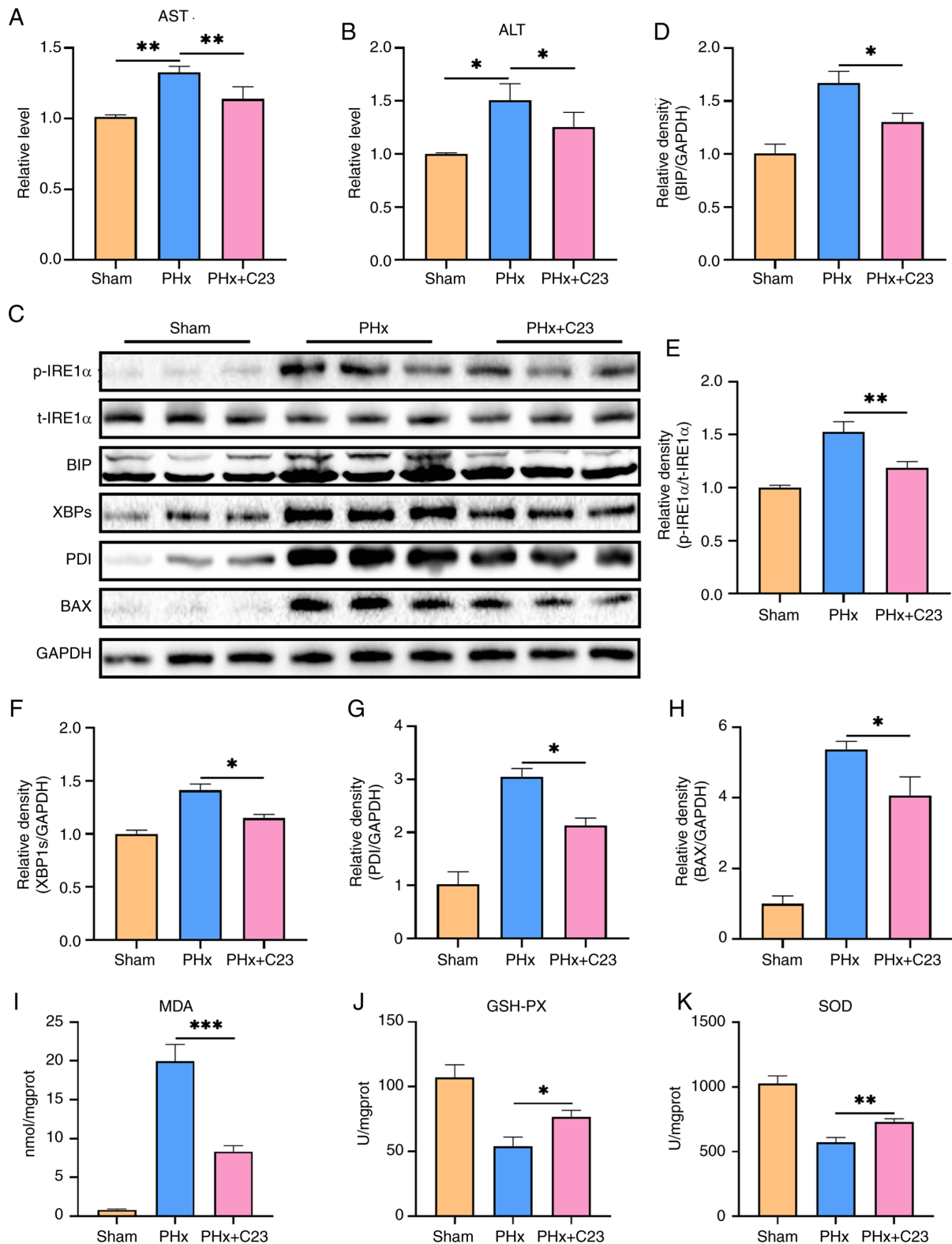


Figure 4. C23 protects against liver injury after partial hepatectomy. C23 was administered intraperitoneally in WT mice (8 mg/kg mice weight) after partial hepatectomy. Serum (A) AST and (B) ALT levels on day 3 after partial hepatectomy. (C-H) Western blotting analysis of p-IRE1 α , XBP1s, PDI and BAX in WT and WT + C23 mice livers on day 3 after partial hepatectomy. GAPDH was used as the loading control. (I-K) Examination of (I) MDA, (J) SOD and (K) GSH-PX in WT and WT + C23 mice livers on day 3 after partial hepatectomy. All data are presented as the mean $\pm$ SEM; n=6. *P<0.05, **P<0.01, ***P<0.001 compared with sham mice. PHx, partial hepatectomy. WT, wild-type; p-, phosphorylated; t-, total; IRE1 α , inositol-requiring enzyme 1 α ; BIP, immunoglobulin heavy-chain-binding protein; XBP1s, X-box binding proteins; PDI, protein disulfide isomerase; MDA, malonaldehyde; SOD, superoxide dismutase; GSH-PX, glutathione peroxidase.

that CIRP may regulate liver regeneration through STAT3 phosphorylation.

Liver function recovers after partial hepatectomy in CIRP-KO mice. Certain studies have suggested that CIRP

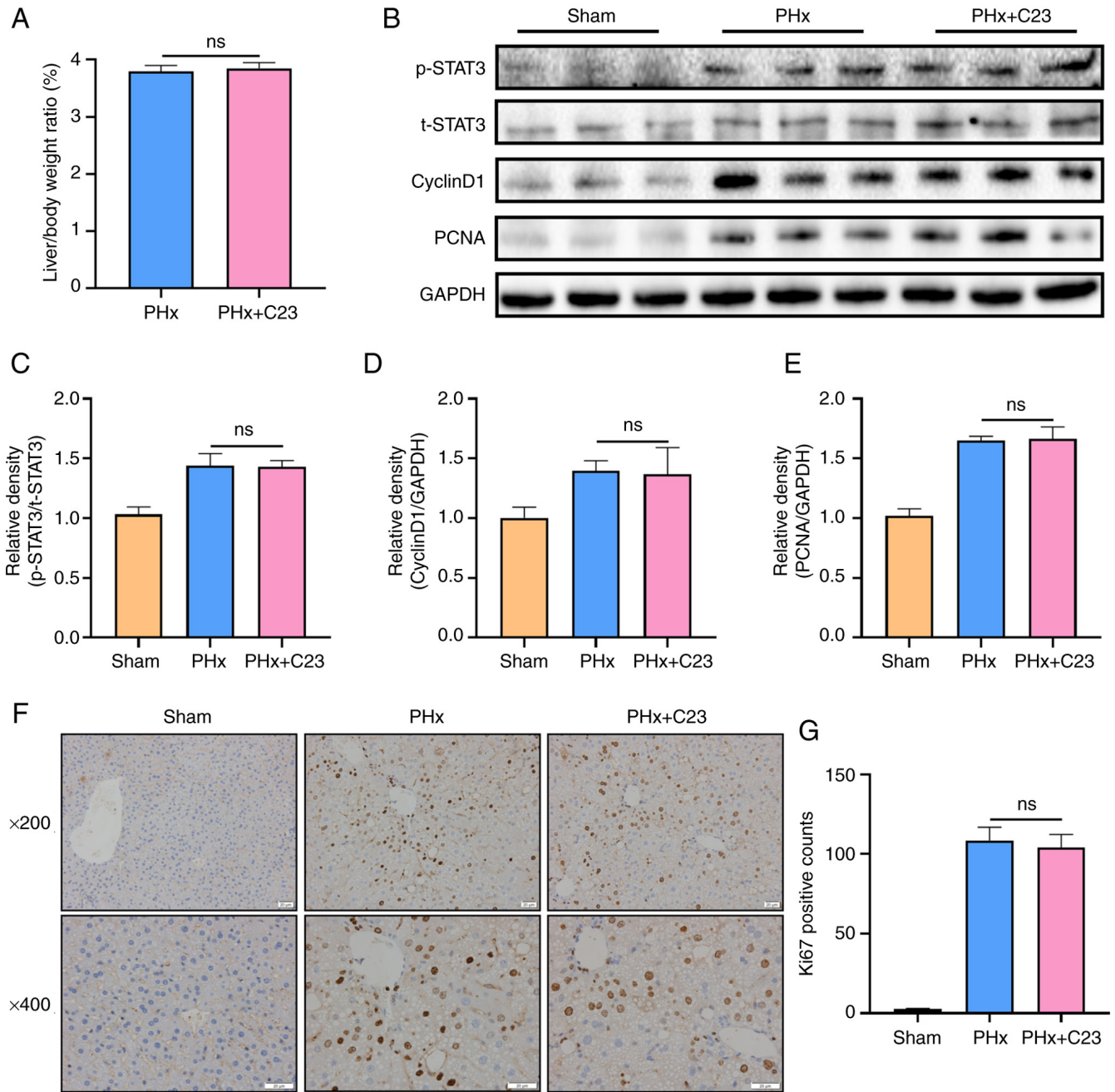


Figure 5. C23 administration has no effect on liver regeneration after partial hepatectomy. (A) Liver-to-body-weight ratio in WT and WT + C23 mice on day 3 after partial hepatectomy. (B-E) Western blotting analysis of p-STAT3, Cyclin D1 and PCNA in WT and WT + C23 mice livers on day 3 after partial hepatectomy. GAPDH was used as the loading control. (F) Ki67 immunohistochemical staining of sections from WT and WT + C23 mice livers on day 3 after hepatectomy, (G) which were semi-quantified. All data are presented as the mean $\pm$ SEM; n=6. PHx, partial hepatectomy; ns, no significance; p-, phosphorylated; t-, total; STAT3, signal transducers and activation of transcription 3; PCNA, proliferating cell nuclear antigen.

deficiency contributes to the recovery of liver function after liver injury, such as liver ischemia/reperfusion and hepatic sepsis (36,37). As shown in Fig. 3A-C, the ALT, AST and LDH serum levels markedly increased after hepatectomy, and were greater in WT mice than in CIRP-KO mice. Compared with that in WT mice, reduced liver injury in CIRP-KO mice was also associated with reduced inflammatory cell infiltration in liver tissue (Figs. S2 and S3). Moreover, the western blot results demonstrated that BIP, p-IRE1 α , XBP1s, PDI and BAX expression was markedly increased after hepatectomy; however, significant decreases in expression in the

CIRP-KO group compared with the WT group were observed (Fig. 3D-I), suggesting that CIRP deficiency suppressed endoplasmic reticulum (ER) stress during liver injury after hepatectomy. The level of MDA, SOD and GSH-PX was subsequently examined. As shown in Fig. 3J-L, the MDA content increased but the SOD and GSH-PX contents decreased in the WT mice after hepatectomy. Notably, these change in trends of the CIRP-KO mice appeared to mirror that of the WT mice, but to a lesser extent. These findings demonstrated that CIRP deficiency can protect mice from liver injury after partial hepatectomy.

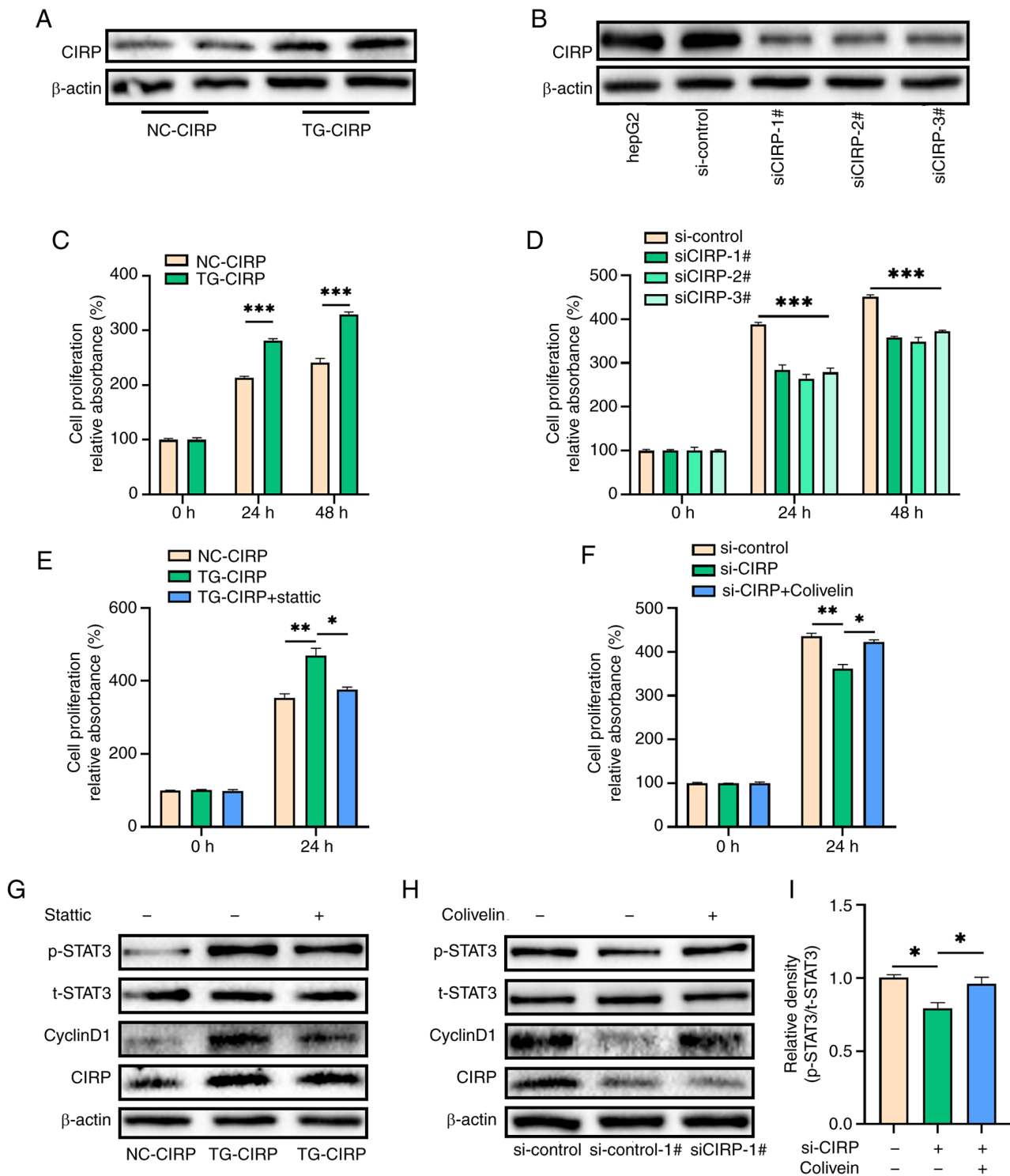


Figure 6. CIRP promotes liver regeneration by regulating STAT3 phosphorylation. Western blotting analysis of CIRP expression in (A) CIRP overexpression and (B) CIRP knockdown HepG2 cells. β-actin was used as the loading control. The cell proliferation ability of CIRP (C) overexpression and (D) KD HepG2 cells were determined by the CCK-8 assay at 24 and 48 h (n=3). (E) A CCK-8 assay demonstrated that the STAT3 antagonist, static, reversed the promotive effect of CIRP TG on the proliferation of HepG2 cells. (F) A CCK-8 assay demonstrated that the STAT3 activator, Colivelin, reversed the suppressive effect of CIRP KD on the proliferation of HepG2 cells. (G-I) Western blotting analysis of CIRP and STAT3 signaling proteins in the aforementioned HepG2 cells. All data are presented as the mean ± SEM; n=3. *P<0.05, **P<0.01, ***P<0.001 compared with NC group. CIRP, cold-inducible RNA-binding protein; NC, negative control; TG, transgenic; KO, knockout; p-, phosphorylated; t-, total; STAT3, signal transducers and activation of transcription 3; CCK-8, Cell Counting Kit-8.

C23 protects against liver injury after partial hepatectomy but has no impact on liver regeneration. To explore the role of eCIRP in liver regeneration and injury after hepatectomy, WT mice were treated with C23, an antagonist that prevents eCIRP from binding to TLR4. As shown in Fig. 4A and B, AST and

ALT increased significantly after hepatectomy in WT mice, whereas C23 reduced AST and ALT levels. Meanwhile, the protein expression levels of BIP, p-IRE1α, XBP1s, PDI and BAX were lower in the WT mice treated with C23 than that in mice in the control group after hepatectomy (Fig. 4C-H).

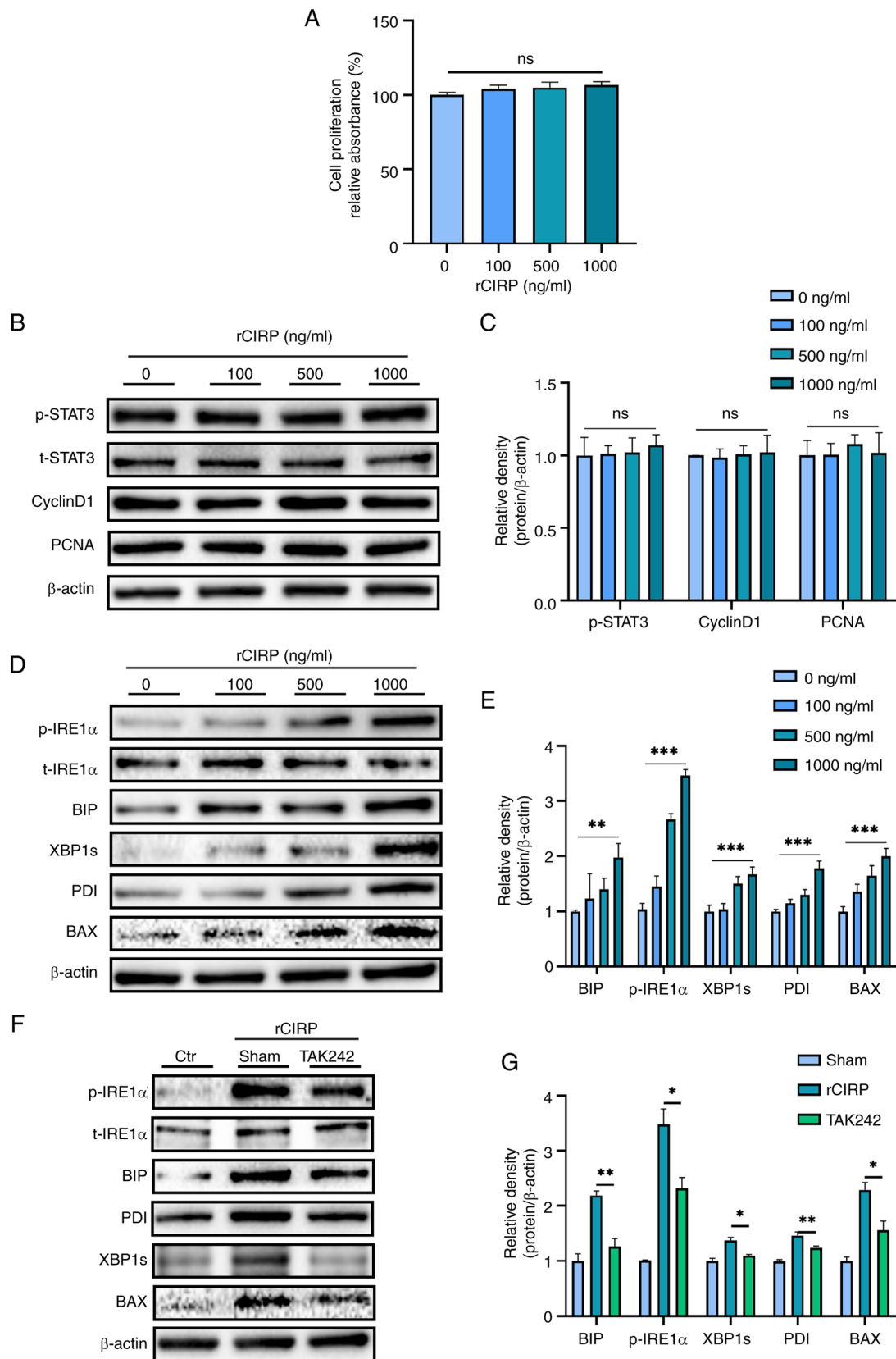


Figure 7. rCIRP treatment leads to ER stress via TLR4 in hepatocytes. (A) A Cell Counting Kit-8 assay of HepG2 proliferation after treatment with different concentrations rCIRP (0, 100, 500 and 1,000 ng/ml) for 24h. (B) Western blotting analysis of p-STAT3, Cyclin D1 and PCNA in HepG2 cells treated with different concentration of rCIRP, (C) which was semi-quantified. (D) Western blotting analysis of BIP, p-IRE1 α , XBP1s, PDI and BAX in HepG2 cells treated with different concentrations of rCIRP, (E) which was semi-quantified. (F) HepG2 cells were treated with rCIRP (1,000 ng/ml) with or without TAK242 (5 μ M; a TLR4 inhibitor) for 24 h, then (F) western blotting analysis of BIP, p-IRE1 α , XBP1s, PDI and BAX in HepG2 cells was performed, (G) which was semi-quantified. β -actin was used as the loading control. All data are presented as the mean $\pm$ SEM; n=3. * P <0.05, ** P <0.01, *** P <0.001 compared with Ctr group. Ctr, control; ns, no significance; rCIRP, recombinant CIRP; CIRP, cold-inducible RNA-binding protein; p-, phosphorylated; t-, total; STAT3, signal transducers and activation of transcription 3; PCNA, proliferating cell nuclear antigen; IRE1 α , inositol-requiring enzyme 1 α ; BIP, immunoglobulin heavy-chain-binding protein; XBP1s, X-box binding proteins; PDI, protein disulfide isomerase; TLR4, Toll-like receptor 4.

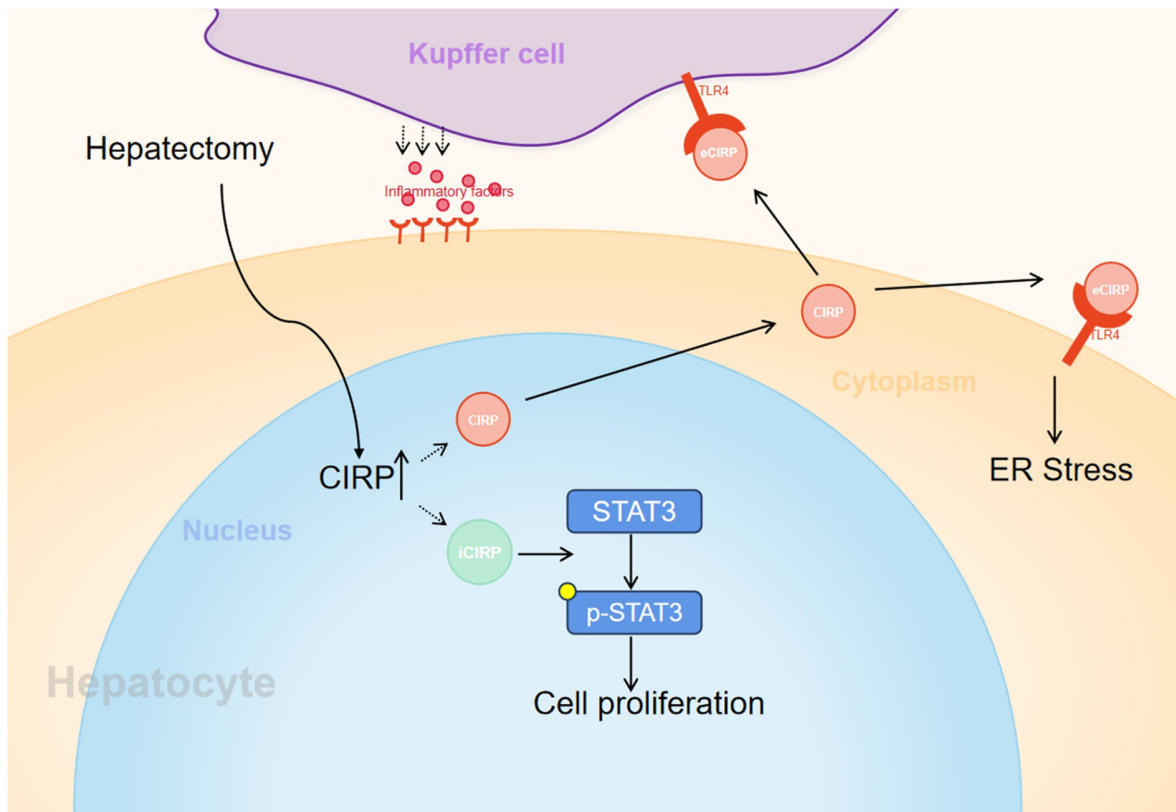


Figure 8. Graphical mechanism of how iCIRP regulates cell proliferation and eCIRP induces ER stress. The expression of CIRP is upregulated after hepatectomy, which can perform different functions depending on its location inside or outside the cells. iCIRP can promote cell proliferation by activating the STAT3 pathway, while eCIRP induces ER stress via interaction with TLR4 after hepatectomy. CIRP, cold-inducible RNA-binding protein; i, intracellular; e, extracellular; p-, phosphorylated; STAT3, signal transducers and activation of transcription 3; TLR4, Toll-like receptor 4.

Similarly, C23 significantly mitigated the changes in MDA, SOD and GSH-PX levels (Fig. 4I-K). However, C23 had no effect on the liver to body weight ratio after hepatectomy (Fig. 5A). Similarly, C23 administration did not influence hepatocyte proliferation, as shown by the Ki67 staining results (Fig. 5F and G). Moreover, there was no difference in the protein expression levels of cyclin D1, PCNA or p-STAT3 following C23 administration (Fig. 5B-E). These results indicated that C23 protected against liver injury after hepatectomy but had no impact on liver regeneration.

iCIRP promotes hepatocyte proliferation by regulating STAT3 phosphorylation. To examine whether iCIRP promotes liver regeneration by regulating STAT3 phosphorylation, CIRP was overexpressed or knocked down in HepG2 cells (Fig. 6A and B). As shown by the CCK-8 results, the proliferation of HepG2 cells significantly increased after CIRP overexpression, an effect that was reversed by a 24-h treatment with Stattic, a STAT3 antagonist (Fig. 6C and E). A cell cycle distribution assay was performed via flow cytometry and revealed that the percentage of cells in the G0/G1 phase decreased in cells with high CIRP expression (Fig. S4A and B). Meanwhile, western blotting confirmed STAT3 activation in HepG2 cells ectopically expressing CIRP. As expected, p-STAT3 and cyclin D1 expression was significantly upregulated in CIRP transgenic cells, while the administration of Stattic diminished the effect of CIRP overexpression (Fig. 6G). By contrast, CIRP knock-down significantly decreased the viability of HepG2 cells, an

effect that was reversed by the administration of Colivelin, a STAT3 activator (Fig. 6D and F). Moreover, CIRP knockdown downregulated the expression of p-STAT3 and cyclin D1, which was counteracted by Colivelin (Fig. 6H and I). These data demonstrated that iCIRP may play an important role in hepatocyte proliferation by partially regulating the phosphorylation and activation of STAT3.

eCIRP has no effect on hepatocyte proliferation but promotes ER stress. Previous studies have shown that CIRP can induce ER stress in acute lung injury (38) and acute pancreatitis (14). To further investigate the direct effects of eCIRP on hepatocytes, HepG2 cells were treated with different concentrations of rCIRP. Notably, as shown by the CCK-8 results (Fig. 7A), rCIRP had no promoting or suppressive effect on hepatocyte proliferation. Meanwhile, the protein expression of p-STAT3, Cyclin D1 and PCNA did not differ between the rCIRP-treated and control cells (Fig. 7B and C). However, the protein expression levels of BIP, p-IRE1 α , XBP1s, PDI and BAX were significantly upregulated in HepG2 cells treated with rCIRP (Fig. 7D and E), which was reversed by TAK242 treatment, a small molecule TLR4-inhibitor (Fig. 7F and G). Notably, the change in iCIRP levels did not affect the protein expression of p-IRE1 α , BIP, XBP1s, PDI or BAX (Fig. S5). Taken together, these results demonstrated that eCIRP had no effects on the proliferation of hepatocytes but did promote ER stress possibly via the TLR4 signaling pathway, a mechanism that needs further investigation.

Discussion

Hepatectomy is a feasible and relatively safe procedure for managing different liver diseases, such as liver cancer, liver abscess, hepatic cyst and liver trauma, and is even used in living donor liver transplantation (39,40). The clinical outcomes of patients who undergo hepatectomy are strongly dependent on the proliferative ability of the remaining hepatocytes (41). Deficiencies in liver regeneration will lead to serious complications, such as hepatectomy-related liver failure, which further causes severe clinical problems. Improving liver regeneration will be a promising and effective therapy for preventing hepatectomy-related liver failure and could reduce the degree of morbidity and mortality after hepatectomy (42,43). Although a growing number of studies have been conducted to reveal this complex regulatory process (30,44,45), the mechanism of liver regeneration remains obscure, and no clinically available therapeutic agents exist, which contributes to a high degree of morbidity and mortality resulting from impaired/dysfunctional liver regeneration.

Although CIRP plays a positive role through the upregulated expression of genes related to hepatocyte proliferation during the development of certain carcinomas, no studies have explored whether CIRP can regulate liver regeneration after hepatectomy (32). In the present study, it was shown that the CIRP protein level in the livers of WT mice increased on the first day after hepatectomy but then gradually decreased over the following days, which suggested an important role of CIRP in liver regeneration. Further analysis is necessary to determine how and by which mechanisms CIRP is regulated during regeneration. Additionally, two-thirds partial hepatectomy was conducted on WT and CIRP-KO mice to investigate the relationship between CIRP and liver regeneration. The liver to body weight ratio and Ki67 staining are commonly used to evaluate liver regeneration ability (46). The results of the present study demonstrated that the liver to body weight ratio and Ki67-positive staining peaked on the 3rd day, whereas these indices were lower in the CIRP-KO group than that in the control group, indicating that CIRP deficiency inhibited liver regeneration. A number of cytokines, growth factors and hormones, as well as their downstream signaling pathways, are involved in liver regeneration (47). Among these regulatory factors, the STAT3 pathway, as a principle signaling pathway, has been extensively studied (30,48). A previous study has demonstrated that CIRP expression is positively correlated with STAT3 activation, further promoting cell proliferation and survival in tumors (21). Therefore, the present study focused on investigating whether CIRP promoted hepatocyte proliferation through regulating STAT3 after hepatectomy. The results of loss- and gain-of-function experiments confirmed the effect of CIRP on STAT3 signaling, in which CIRP overexpression notably upregulated p-STAT3 levels and promoted cell proliferation. Moreover, the effect of CIRP overexpression on hepatocyte proliferation was restored by the p-STAT3 antagonist, Stattic. Conversely, CIRP knockdown notably reduced the p-STAT3 levels and inhibited cell proliferation, but the inhibition of cell proliferation was reversed by the STAT3 activator, Colivelin. These results indicated that CIRP positively regulated hepatocyte proliferation via STAT3 signaling.

Paradoxically, it was demonstrated that CIRP deficiency restrained hepatocyte proliferation, but alleviated liver injury

and oxidative stress. As shown in previous studies, CIRP can perform different functions depending on its location inside or outside the cells (5,7,13). In response to stress, through regulating its targets, iCIRP is implicated in multiple cellular processes, such as cell proliferation, cell survival, circadian modulation, telomere maintenance and tumor formation and progression (49). After release, eCIRP induces inflammatory responses, causing tissue injury (18). The mechanism underlying the proinflammatory effects of eCIRP has been previously revealed: eCIRP acts as a DAMP and activates TLR4/myeloid differentiation protein to trigger inflammation, which can be blocked by C23 (50,51). Therefore, we hypothesized that CIRP plays an intracellular role in promoting hepatocyte proliferation; however, once secreted, CIRP acts as a proinflammatory factor to promote inflammation, leading to liver injury after hepatectomy. In the present study, CIRP-KO mice exhibited mitigated liver injury after hepatectomy, accompanied by decreased inflammatory cell infiltration. Reactive oxygen species (ROS) is the main product of oxidative stress, which plays an important role in disease-induced liver injury (52). When activated by ROS, MDA, a product of lipid peroxidation, can lead to the swelling and necrosis of cells. However, antioxidant enzymes, such as SOD and GSH-PX can counteract ROS, and then protect cells from damage. In the present study, MDA increased, but SOD and GSH-PX decreased in WT mice after hepatectomy, whereas the changes were reversed by CIRP KO, indicating that CIRP deficiency significantly relieved oxidative stress after hepatectomy. To improve the understanding of the underlying mechanisms, the mice were treated with C23 after hepatectomy. The results showed that C23 administration did not affect the liver regeneration ability but could alleviate liver injury and oxidative stress. Moreover, *in vitro* experiments revealed that CIRP deficiency suppressed hepatocyte proliferation and that CIRP overexpression accelerated hepatocyte proliferation. These findings indicate that CIRP may be a potential therapeutic target to promote liver regeneration after hepatectomy and that the simultaneous administration of C23 counteracts the effects of CIRP release as a DAMP.

A previous study showed that eCIRP has the ability to bind with the IL-6 receptor, activating p-STAT3 to promote macrophage endotoxin tolerance (27). Therefore, we considered that eCIRP could promote hepatocyte proliferation by binding to IL-6R on hepatocytes. However, the administration of different concentrations of rCIRP to HepG2 cells did not increase the cell viability. Meanwhile, the protein expression of p-STAT3, cyclin D1 and PCNA remained at the baseline levels, indicating that eCIRP had no direct effect on hepatocyte proliferation. Our previous study revealed that eCIRP treatment can lead to persistent ER stress in pancreatic acinar cells (14). In the present study, upregulated BIP, p-IRE1 α , XBP1s and PDI expression was observed after rCIRP stimulation in HepG2 cells, which were reversed by TAK242. However, neither the overexpression nor the knockdown of CIRP in HepG2 cells affect ER stress. Therefore, eCIRP may cause ER stress in hepatocytes possibly through the TLR4 signaling pathway (Fig. 8).

There were several limitations to the present study. First, a partial hepatectomy model was adopted to elucidate the role of CIRP, but whether CIRP can promote liver regeneration in other models, such as acetaminophen or carbon tetrachloride

induced acute liver injury, remains unknown. Second, although CIRP has been identified as a positive regulator of liver regeneration, partial hepatectomy should be conducted in mice with CIRP overexpression to verify the effect of on liver regeneration *in vivo*. Third, the mechanism and signaling pathway through which CIRP exerts its effects on STAT3 phosphorylation are still vague. Moreover, whether other signaling pathways of liver regeneration are involved in the regulation of CIRP remains unknown. All of these issues require further investigation.

In conclusion, the present study demonstrated that iCIRP promotes liver regeneration by activating the STAT3 pathway, whereas eCIRP induces ER stress possibly via the TLR4 signaling pathway after hepatectomy. Pharmacological and genetic approaches for the modulation of iCIRP activity may be beneficial for enhancing liver regeneration, and management of eCIRP may be a protective method for preventing liver injury after hepatectomy in the future.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

TW contributed to writing the original draft, visualization, methodology, investigation (experimental operation), formal analysis and conceptualization. MW contributed to reviewing and editing the manuscript, visualization and investigation (data collection). WL contributed to methodology and investigation (data collection). LZ contributed to data curation. JiZ contributed to supervision, methodology and investigation (experimental operation). JuZ contributed to validation and software. ZW contributed to reviewing and editing the manuscript, supervision, resources and conceptualization. YL contributed to reviewing and editing the manuscript, supervision, resources, funding acquisition and conceptualization. RW contributed to reviewing and editing the manuscript, supervision, resources, funding acquisition and conceptualization. TW and MW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals of the National Research Council and were approved by the Institutional Animal Care and Use Committee of the Ethics Committee of Xi'an Jiaotong University Health Science Center (Xi'an, China; approval no. XJTUAE2023-2145)

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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