Intracellular CIRP promotes liver regeneration while extracellular CIRP induces ER stress after partial hepatectomy in mice

Tao Wang
The First Affiliated Hospital of Xi'an Jiaotong University
Mengzhou Wang
The First Affiliated Hospital of Xi'an Jiaotong University
Wuming Liu
The First Affiliated Hospital of Xi'an Jiaotong University
Lin Zhang
The First Affiliated Hospital of Xi'an Jiaotong University
Jia Zhang
Xi'an Jiaotong University Second Affiliated Hospital
Jianbin Bi
Xi'an Jiaotong University Second Affiliated Hospital
Junzhou Zhao
The First Affiliated Hospital of Xi'an Jiaotong University
Zheng Wu
The First Affiliated Hospital of Xi'an Jiaotong University
Yi Lv
The First Affiliated Hospital of Xi'an Jiaotong University
Rongqian Wu (rwu001@mail.xjtu.edu.cn)
Xi'an Jiaotong University https://orcid.org/0000-0003-2316-0106

Research Article

Keywords: Extracellular CIRP, Intracellular CIRP, Hepatectomy, Liver regeneration, ER stress

Posted Date: January 12th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3840462/v1

Abstract

Background

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, its role in liver regeneration and injury after hepatectomy has not been investigated. This study was therefore designed to explore whether CIRP was involved in liver regeneration after hepatectomy and its specific role and underlying molecular mechanism.

Methods

Male adult mice were subjected to two-thirds partial hepatectomy. Hepatic and serum levels of CIRP were measured after hepatectomy. 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 shRNA plasmids were transfected to HepG2 cells to study the effect of intracellular CIRP on cell proliferation. The effect of extracellular CIRP on cell proliferation and injury was determined using recombinant CIRP protein to stimulate HepG2 cells *in vitro*.

Results

Both hepatic and serum CIRP levels were significantly increased after partial hepatectomy. CIRP deficiency impaired liver regeneration, while alleviated liver injury after partial hepatectomy in mice. C23 administration attenuated liver injury, suppressed ER stress and oxidative stress. However, it had no effects on liver regeneration after partial hepatectomy. Loss- and gain-of-function analyses in hepG2 cells indicated that upregulation of intracellular CIRP promoted cell proliferation via activation of the STAT3 signaling pathway. On the other hand, recombinant CIRP had no effects on cell proliferation and STAT3 phosphorylation, but induced ER stress via a TLR4-dependent pathway in hepG2 cells.

Conclusion

Taken together, our results demonstrated that intracellular CIRP promotes liver regeneration by activating the STAT3 pathway, while extracellular CIRP induces ER stress via interacting with TLR4 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(Fausto, Campbell, & Riehle, 2006). The process of liver regeneration is regulated by a set of complicated signaling pathways, which are still largely unknown. Hence, an in-depth understanding of this process will help to rationalize the use of specific therapies to promote recovery of liver function and liver regeneration.

Cold-inducible RNA-binding protein (CIRP) is a 172-amino acid cold shock protein discovered in 1997(Sheikh, et al., 1997). It is constitutively expressed at low levels in various tissues, but can be upregulated by hypoxia(Wellmann, et al., 2004), UV radiation(C. Yang & Carrier, 2001), glucose deprivation(Zhong & Huang, 2017) and heat stress(Z. P. Xia, et al., 2012), suggesting that CIRP is a general stress-response protein. In response to stress, CIRP can migrate from the nucleus to the cytoplasm and regulate mRNA stability through its binding site on the 3'-UTR of its targeted mRNAs(Liao, Tong, Tang, & Wu, 2017). CIRP is now known to regulate diverse biological processes depending on its cellular localization. Intracellular CIRP (iCIRP) is implicated in mRNA stability(Z. Xia, et al., 2012), cell proliferation(Masuda, et al., 2012), cell survival(Roilo, Kullmann, & Hengst, 2018), circadian modulation(Morf, et al., 2012), telomere maintenance(Y. Zhang, et al., 2016), and tumor formation and progression(Lujan, Ochoa, & Hartley, 2018). During hypoxia and inflammation, CIRP in the cytoplasm can be released to the extracellular space. Extracellular CIRP (eCIRP) as a danger-associated molecular patter (DAMP) induces inflammatory responses and tissue injury(Z. Li, et al., 2017; Liu, et al., 2023; Qiang, et al., 2013). The effects of eCIRP and its mechanism involved have been studied in a number of immune cells, including macrophages(Z. Li, et al., 2017), lymphocyte(Bolognese, et al., 2018), neutrophils(Godwin, et al., 2015; W. L. Yang, et al., 2016), and dendritic cells(Villanueva, et al., 2018).

Previous studies indicated that CIRP is associated with several types of human cancers and plays important roles in cell proliferation and survival under stress condition(Guo, Wu, & Hartley, 2010; Sakurai, et al., 2014; Sakurai, et al., 2015). Signal transducer and activator of transcription 3 (STAT3) is a key transcriptional mediator for cytokines and growth hormone and plays an important role in liver regeneration(Gao, Wang, Lafdil, & Feng, 2012; Welte, et al., 2003). CIRP has been shown to activate the STAT3 signaling pathway(Sharma, Brenner, Jacob, Marambaud, & Wang, 2021; Zhou, et al., 2020). However, the role of CIRP on liver regeneration and injury after hepatectomy remains largely unknown. In this study, we found that iCIRP promoted liver regeneration by positively regulating hepatocyte proliferation via STAT3 signaling, while eCIRP induced ER stress via interacting with TLR4. The role of CIRP in liver regeneration provided a potential strategy for the management of liver restoration after hepatectomy.

Materials and methods

Experimental animals

In this study, 8- to 10-week-old male C57BL/6 mice weighing 20 to 25 g obtained from the experimental animal center of Xi'an Jiaotong University were used. CIRP knockout (KO) mice were obtained from Shanghai Model Organisms Center, Inc. All the 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.

Partial hepatectomy

The liver regeneration research was conducted based on the model of two-thirds partial hepatectomy in mice. Briefly, age and weight matched mice were anesthetized with 2% isoflurane and 2 L/min oxygen flow and maintained anesthetic by isoflurane during the whole surgical procedure. 1-2cm 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. Then the abdominal cavity was closed, the mice were placed on a warming pad for palinesthesia. In the sham group, the mice just were performed with laparotomy but without liver resection. 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 remaining liver samples were harvested at 1, 3, 7 days, then fixed with 4% paraformaldehyde for at least for 24h for further histological examination or stored at -80 ℃ for molecular and biochemical analyses. Blood samples were obtained by eyeball extirpating, and centrifuged at a low speed (3000rpm/min) for serum.

Cell culture and transfection

HepG2 cell line was purchased from iCell Bioscience Inc. (Shanghai, China). The cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin mixture and incubated at 37 ℃ with 5% CO2. Human CIRP overexpression and shRNA plasmids were purchased

from the Shanghai Genechem Co.Ltd. (Shanghai, China). The plasmid was constructed as previous described(Y. Zhang, et al., 2016). Plasmid DNA or shRNA was transfected using Lipofectamine 3000 reagent (Thermofisher) following the manufacturer's instructions. CIRP cDNA clone (NM_001280) in GV712 vector was designed by the Shanghai Genechem Co.Ltd. (Shanghai, China). The targeting sequences for various CIRP shRNAs were as follows:

CIRP-RNAi1: 5'-GCCATGAATGGGAAGTCTGTA-3'

CIRP-RNAi2: 5'-CTTCTCAAAGTACGGACAGAT-3'

CIRP-RNAi3: 5'CGGGTCCTACAGAGACAGTTA-3'

Overexpression and knockdown of CIRP in these cells was verified by performing Western blotting using anti-CIRP antibody as described in latter section.

Measurement of liver function

The serum alanine aminotransferase (ALT) assay Kit (C009-2), aspartate aminotransferase (AST) assay Kit (C010-2), and lactic dehydrogenase (LDH) assay Kit (A020-2) were purchased from NanJing

JianCheng Bioengineering Institute, Nanjing, China. The levels of serum ALT, AST, LDH were measured according to the manufacturer's instructions.

Measurement of oxidative stress

To quantify oxidative stress, malonaldehyde (MDA)assay Kit (A003-1), superoxide dismutase (SOD) assay Kit (A001-3) and glutathione peroxidase activity (GSH-PX) assay Kit (A005) were purchased from NanJing JianCheng Bioengineering Institute, Nanjing, China. The levels of liver MDA, SOD, GSH-PX were measured according to the instructions of the manufacturer.

Western blotting

Liver lysates were prepared with RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Beyotime). The proteins were extracted for Western blotting. Briefly, the proteins were separated by sodium dodecyl sulphatepolyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA). The proteins were then transferred to PVDF membranes (Bio-Rad, Hercules, CA) and blocked with 5% skimmed milk or BSA at room temperature for 1 h. According to our research, the membranes were incubated with the indicated primary antibodies at 4 ℃ overnight and with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Anti-STAT3, anti-phospho-STAT3 were purchased from Abcam. Anti-CIRP, Anti-GAPDH were purchased from Proteintech Group Inc. Anti-cyclin D1, anti-PCNA, anti-BIP, anti-β-actin were purchased from Affinity Biosciences. Anti-phospho-IRE1α, anti-XBP1s, anti-PDI and anti-BAX were purchased from Cell Signaling Technology, Inc. HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Proteintech Group Inc. The protein expression was detected using a chemiluminescence system (Bio-Rad, Hercules, CA) and quantified using ImageJ2x software.

Histological analysis

The liver tissues fixed in paraformaldehyde (4%) for at least 24h were embedded in paraffin. Liver samples were cut into 5μm thick sections for Hematoxylin and Eosin (H&E) staining. For immunohistochemistry, the sections were first preprocessed for antigen exposure and then incubated with primary antibodies at 4 ℃ overnight followed by incubation with secondary antibodies at room temperature for 1 h. These sections were counterstained and dehydrated for further observation. All the images were then obtained under an optical microscope. The primary antibody of F4/80, CD68, CO11b, CD20, MPO and CIRP were purchased from Abcam.

Cell viability assay

Cell viability assay was determined using the CCK8 Assay Kit. HepG2 cells with overexpressed and knockdown CIRP were seeded at a density of $5X10^3$ cells/well in 100μl MEM medium in the 96-well plate. After 24 and 48h, 10μl CCK-8 was added to each well and the cells were subsequently incubated at 37℃ for 2 h. For the administration to HepG2 cells, the antagonist (Stattic, Selleck) and activator (Colvelin, Selleck) of p-STAT3 were added to the well for 24h. To examine the effect of eCIRP on HepG2 cells, different concentrations of recombinant CIRP (0, 100, 500 and 1000ng/ml) were added to 96-well plate

for 24h. Then 10μl CCK-8 was added for further incubation at 37℃ for 2 h. Absorbance was measured at 450 nm using a microplate reader.

Enzyme-linked immunosorbent assays (ELISA)

The cold-inducible RNA binding protein (CIRP) ELISA kit (CSB-EL005440MO, Cusabio, Wuhan, China) was used for measuring the levels of serum CIRP according to the instructions of the manufacturer.

Statistical analysis

All the experiments data were expressed as mean ± SEM. Statistical significance was evaluated by unpaired two-tailed student *t*-test and by oneway ANOVA analysis of variance in comparison among more than two groups. Differences were considered significant at a P value of less than 0.05.

Results

CIRP expression was up-regulated after partial hepatectomy in mice

To determine the characteristics of CIRP expression after hepatectomy in mice, we first examined its expression in mice liver after two-thirds partial hepatectomy, an in vivo system that has been used widely in the study of liver regeneration(Pibiri, 2018). As shown in Fig. 1A, the amount of CIRP in serum was increased after hepatectomy and then reduced afterwards (Fig. 1A). Simultaneously, the relative expression of CIRP in the liver was up-regulated dramatically in the first day after hepatectomy, while then gradually decreased in the following days (Fig. 1B-1D). These results indicate that CIRP might be involved in liver regeneration after partial hepatectomy.

CIRP deficiency restrained liver regeneration after partial hepatectomy in mice

To confirm the role of CIRP on liver regeneration, CIRP KO mice and WT mice were subjected to two-thirds partial hepatectomy. As shown in Ki67 staining, CIRP deficiency exhibited decreased hepatocyte proliferation after hepatectomy compared with WT mice (Fig. 2A, B). Meanwhile, compared with WT mice, the ratio of liver to body weight of CIRP KO mice was lower after hepatectomy (Fig. 2C). Then we examined proteins related to liver regeneration. As is shown in Fig. 2D-2H, the CyclinD1 and PCNA were dramatically up-regulated after hepatectomy, while their expression in CIRP KO mice were lower than the WT mice. Next, the underlying mechanism of CIRP in liver regeneration was explored. Previous research found that the STAT3 signaling is responsible for hepatocyte proliferation after partial hepatectomy in mice(da Silva, et al., 2013). Once the transcription factor STAT3 is activated and phosphorylated, it will translocate into the nucleus and regulate gene related to liver regeneration(He & Karin, 2011; W. Li, Liang, Kellendonk, Poli, & Taub, 2002). The phosphorylation of STAT3 was dramatically increased in 1,3 and 7 days after hepatectomy, while CIRP KO significantly restricted its phosphorylation (Fig. 2D and Fig.S1).

These data demonstrate that CIRP deficiency impaired liver regeneration and CIRP may regulate liver regeneration through STAT3 phosphorylation.

Liver function was alleviated after partial hepatectomy in CIRP-KO mice

Several studies have suggested that CIRP deficiency contributes to the recovery of liver function after liver injury, such as liver I/R and hepatic sepsis(Aziz, Brenner, & Wang, 2019; F. Zhang, Brenner, Yang, & Wang, 2018). As shown in Fig. 3A-C, ALT, AST and LDH serum levels were significantly increased after hepatectomy, whereas they were higher in WT mice than that in CIRP KO mice. Reduced liver injury in CIRP KO mice was also associated with less inflammatory cell infiltration in the liver tissue than WT mice (Fig. S2 and S3). Meanwhile, Western blotting results demonstrated that BIP, p-IRE1α, XBP1s, PDI and BAX were markedly increased after hepatectomy. However, a profound decrease in the CIRP KO mice compared with WT group was observed (Fig. 3D-3I), suggesting that CIRP deficiency suppressed ER stress during liver injury after hepatectomy. Then, we examined MDA, SOD and GPx-sH. As is shown in Fig. 3J-3L, the MDA was increased, but SOD and GPX-sH were decreased in WT mice after hepatectomy. Notably, these changes were reversed by CIRP Knockout, indicating that CIRP deficiency significantly relieved oxidative stress after hepatectomy. These finding demonstrated that CIRP deficiency can somehow protects mice from liver injury after partial hepatectomy.

C23 protected liver injury after partial hepatectomy while had 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 preventing eCIRP from binding to TLR4. As is shown in Fig. 4A and 4B, AST and ALT increased significantly after hepatectomy in WT mice, while C23 reduced their level. Meanwhile, the protein expression of BIP, p-IRE1α, XBP1s, PDI and BAX were lower in WT mice treated with C23 than control group after hepatectomy (Fig. 4C-4H). Similarly, C23 significantly mitigated against the change of MDA, SOD and GPXsH (Fig. 4I-4K). However, C23 had no impact on the ratio of liver to body weight after hepatectomy (Fig. 5A). Likewise, C23 administration did not influence hepatocyte proliferation, as shown by Ki67 results (Fig. 5F, G). And, there was no difference in protein expression of cyclinD1, PCNA and p-STAT3 no matter C23 was given or not (Fig. 5B-5E). These results indicated that C23 protected liver injury after hepatectomy but had no impact on liver regeneration.

Intracellular CIRP promoted hepatocyte proliferation via regulating STAT3 phosphorylation

To examine whether intracellular CIRP promoted liver regeneration through regulating STAT3 phosphorylation, we over-expressed and low-expressed CIRP in HepG2 cells (Fig. 6A, B). As shown by CCK8 results, the proliferation of HepG2 cells was significantly increased by overexpressed CIRP but was

rescued by 24-h treatment with Stattic, a STAT3 antagonist (Fig. 6C, D). Western blotting was performed to further confirm the activation of STAT3 in hepG2 cells ectopically expressing CIRP. As expected, pSTAT3, cyclin D1 were significantly up-regulated in CIRP TG cells, while additional administration of Stattic abolished the effect of CIRP overexpression (Fig. 6E). In contrast, knockdown of CIRP significantly decreased the cell viability of HepG2, but this was rescued by administration of Colivelin, a STAT3 activator (Fig. 6F, G). Meanwhile, CIRP knockdown down-regulated the expression of p-STAT3, cyclin D1, which was counteracted by Colivelin (Fig. 6H). These data demonstrated that intracellular CIRP played an important role in hepatocyte proliferation by partially regulating the phosphorylation and activation of STAT3.

Extracellular CIRP had no impact on hepatocyte proliferation while promoted ER stress.

To further investigate the direct effects of extracellular CIRP on hepatocytes, HepG2 cells were treated with different concentrations of recombinant CIRP (rCIRP). Interestingly, as shown by CCK8 results (Fig. 7A), rCIRP had no promotive or suppressive effect on hepatocyte proliferation. Meanwhile, protein expression of p-STAT3, CyclinD1 and PCNA showed no differences between rCIRP-treated HepG2 and control cells (Fig. 7B, C). However, the protein expression of BIP, p-IRE1α, XBP1s, PDI and BAX was significantly up-regulated in HepG2 cells treated with rCIRP (Fig. 7D, E), which was reversed by TAK242, a small molecule TLR4-inhibitor (Fig. 7F, G). Taken together, these results proved that extracellular CIRP had no proliferative effects on hepatocytes but promoted ER stress in hepatocytes partly through binding to TLR4, which was in accordance with the in vivo results.

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, even used in living donor liver transplantation(Anugwom, Leventhal, & Debes, 2022; X. Li, et al., 2018). Clinical outcomes of patients received hepatectomy are strongly dependent on the proliferative ability of remaining hepatocytes(Michalopoulos & Bhushan, 2021). Deficiency of liver regeneration will lead to serious complications, for example, hepatectomy liver failure, which further causes severe clinical problem. Improving the liver regeneration comes to be a promising and available therapy to prevent hepatectomy liver failure and could reduce the degree of morbidity and mortality after hepatectomy(Golse, et al., 2013; Ray, Mehta, Golhar, & Nundy, 2018). Although a growing number of researches have been conducted to reveal this complex regulatory process, the mechanism of liver regeneration still keeps obscure and no clinically available therapeutic agents exist, which all contribute to a high degree of morbidity and mortality resulted from impaired/dysfunctional liver regeneration.

Although CIRP plays a positive role through up-regulation gene related to hepatocyte proliferation during certain carcinoma development, no researches have been performed to explore whether CIRP can regulate liver regeneration after hepatectomy(Pibiri, 2018). In this study, we have shown that CIRP protein level in the liver of WT mice was increased during regeneration, with a peak at 24 ~ 48 h after hepatectomy,

which suggested an important role of CIRP during the progression into G1/S phase of the cell cycle. Further analysis is necessary to determine how and by which mechanisms CIRP is regulated during regeneration. We also conducted two-thirds partial hepatectomy on WT and CIRP KO mice to investigate the relation of CIRP and liver regeneration. The ratio of liver to body weight and Ki6 staining level are commonly used to evaluate the ability of liver regeneration(B. Zhang, et al., 2022). Our research results demonstrated that the ratio of liver to body weight and Ki67-positive staining level peaked at the 3rd day, while those levels were lower in CIRP KO group compared to control group, indicating that CIRP deficiency inhibited liver regeneration. A wide variety of cytokines, growth factors, and hormones and their downstream signaling pathways are involved in liver regeneration(Ozaki, 2020). Among these regulatory factors, the STAT3 pathway, as a principle signaling pathway, has been extensively studied(Fazel Modares, et al., 2019; Hu, et al., 2020). Meanwhile, previous studies demonstrated that CIRP expression is positively correlated with the activation of STAT3, further promoting cell proliferation and survival in tumor(Sakurai, et al., 2015). Therefore, we focused on investigating whether CIRP promoted hepatocyte proliferation through regulating STAT3 after hepatectomy. Results from loss- and gain-of-function experiments confirmed the effect of CIRP on STAT3 signaling, in which CIRP overexpression significantly up-regulated p-STAT3 level and promoted cells proliferation. Moreover, the effect of CIRP overexpression on hepatocyte proliferation was restored by the p-STAT3 antagonist Stattic. Conversely, CIRP knockdown dramatically reduced p-STAT3 level and inhibited cells proliferation, but the inhibition of cell proliferation was reversed with the STAT3 activator Colivelin. These results indicated that CIRP positively regulated hepatocyte proliferation via STAT3 signaling.

Paradoxically, CIRP deficiency restrained hepatocyte proliferation, but alleviated liver injury and oxidation stress. As shown in previous researches, CIRP can perform different functions depending on its location inside or outside the cells(Liao, et al., 2017). 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(Corre & Lebreton, 2023). After released, eCIRP induces inflammatory responses, causing tissue injury(Godwin, et al., 2015). The mechanism underlying the pro-inflammatory effects of eCIRP has been previously revealed as a DAMP to activate TLR4/MD to trigger inflammation, which can be blocked by C23(Denning, Yang, Hansen, Prince, & Wang, 2019; F. Zhang, Yang, Brenner, & Wang, 2017). Therefore, we hypothesized that CIRP played an intracellular role in promoting hepatocyte proliferation. However, once secreted, CIRP acts as a pro-inflammatory factor to promote inflammation, leading to liver injury after hepatectomy. To better understand the underlying mechanisms, the mice were treated with C23 after hepatectomy. The results showed that C23 administration did not affect the ability of liver regeneration, but it could alleviate liver damage. Meanwhile, *in vitro* experiments, CIRP deficiency suppressed hepatocyte proliferation, and CIRP overexpression accelerated hepatocyte proliferation. This observation indicated that CIRP may be a potential therapy target to promote liver regeneration after hepatectomy, and that simultaneous administration of C23 counteracted the effects of CIRP released as DAMPs.

Previous study showed that eCIRP has an ability to bind with IL-6 receptor, activating p-STAT3 to promote macrophage endotoxin tolerance(Zhou, et al., 2020). Therefore, we speculated whether eCIRP can

promote hepatocyte proliferation by binding to the IL-6R on the hepatocyte. However, administration of different concentrations of rCIRP to HepG2 cell did not increase the cell vitality. Meanwhile, protein expression of p-STAT3, cyclinD1 and PCNA remained on the baseline, indicating that eCIRP had no direct effect on hepatocyte proliferation. Our previous study has shown that eCIRP treatment can lead to persistent ER stress in pancreatic acinar cell(Liu, et al., 2023). In this study, up-regulated BIP, p-IRE1α, XBP1s and PDI were observed after rCIRP stimulation in HepG2 cells, which were reversed by TAK242. Therefore, eCIRP may cause ER stress in hepatocyte partially through TLR4 receptor.

There were some limitations in our research. Firstly, we just adopted the model of partial hepatectomy to prove the role of CIRP, but whether CIRP can promote liver regeneration in other models such as APAP or CCL4-induced acute liver injury remained unknown. Secondly, though CIRP has been identified as a positive regulator in liver regeneration, partial hepatectomy should be conducted in mice with over-expressed CIRP to verify the effect of CIRP overexpression on liver regeneration *in vivo*. Thirdly, the mechanism and signaling pathway via which CIRP exerted its effects on STAT3 phosphorylation were still vague. Meanwhile, whether other signal pathways of liver regeneration were involved in the regulation of CIRP keep unsolved. All of these mentioned above require further investigation.

Conclusion

In conclusion, we demonstrated that intracellular CIRP promotes liver regeneration by activating the STAT3 pathway, while extracellular CIRP induces ER stress via interacting with TLR4 after hepatectomy. Pharmacological and genetic approaches for the modulation of intracellular CIRP activity may be beneficial for enhancing liver regeneration and management of extracellular CIRP can be a protective method for liver injury after hepatectomy.

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 patter

TNFα Tumor necrosis factor-α

HMGB1 High-mobility group protein B1

MD2 Myeloid differentiation 2

IL-6 Interleukin-6 receptor

IL-6R Interleukin-6 receptor

JAK Janus kinase

NF-κB Nuclear factor kB

Cdk5 Cyclin-dependent kinases 5

DMEM Dulbecco's Modified Eagle's Medium

PCNA Proliferating cell nuclear antigen

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agreed to publish this manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declared that they have no competing interests

Funding

This work was supported by grants from Natural Science Foundation of China (82370659, 82172167)

Authors' contributions

Design: TW and RW. Animal operation: TW, MW and WL. Cell culture: TW, MW, and WL. Western blotting: TW and LZ. Histology: TW, JZ and JB. Data analysis and writing: TW, JZ, YR and RW. ZW, YL and RW supervised the study and edited the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References

1. Anugwom, C. M., Leventhal, T. M., & Debes, J. D. (2022). Understanding immune perspectives and options for the use of checkpoint immunotherapy in HCC post liver transplant. *Hepatoma Res, 8*.
2. Aziz, M., Brenner, M., & Wang, P. (2019). Extracellular CIRP (eCIRP) and inflammation. *J Leukoc Biol, 106*, 133-46.
3. Bolognese, A. C., Sharma, A., Yang, W. L., Nicastro, J., Coppa, G. F., & Wang, P. (2018). Cold-inducible RNA-binding protein activates splenic T cells during sepsis in a TLR4-dependent manner. *Cell Mol Immunol, 15*, 38-47.
4. Corre, M., & Lebreton, A. (2023). Regulation of cold-inducible RNA-binding protein (CIRBP) in response to cellular stresses. *Biochimie*.
5. da Silva, C. G., Studer, P., Skroch, M., Mahiou, J., Minussi, D. C., Peterson, C. R., Wilson, S. W., Patel, V. I., Ma, A., Csizmadia, E., & Ferran, C. (2013). A20 promotes liver regeneration by decreasing SOCS3 expression to enhance IL-6/STAT3 proliferative signals. *Hepatology, 57*, 2014-25.
6. Denning, N. L., Yang, W. L., Hansen, L., Prince, J., & Wang, P. (2019). C23, an oligopeptide derived from cold-inducible RNA-binding protein, suppresses inflammation and reduces lung injury in neonatal sepsis. *J Pediatr Surg, 54*, 2053-60.
7. Fausto, N., Campbell, J. S., & Riehle, K. J. (2006). Liver regeneration. *Hepatology, 43*, S45-53.
8. Fazel Modares, N., Polz, R., Haghighi, F., Lamertz, L., Behnke, K., Zhuang, Y., Kordes, C., Häussinger, D., Sorg, U. R., Pfeffer, K., Floss, D. M., Moll, J. M., Piekorz, R. P., Ahmadian, M. R., Lang, P. A., & Scheller, J. (2019). IL-6 Trans-signaling Controls Liver Regeneration After Partial Hepatectomy. *Hepatology, 70*, 2075-91.
9. Gao, B., Wang, H., Lafdil, F., & Feng, D. (2012). STAT proteins - key regulators of anti-viral responses, inflammation, and tumorigenesis in the liver. *J Hepatol, 57*, 430-41.
10. Godwin, A., Yang, W. L., Sharma, A., Khader, A., Wang, Z., Zhang, F., Nicastro, J., Coppa, G. F., & Wang, P. (2015). Blocking cold-inducible RNA-binding protein protects liver from ischemia-reperfusion injury. *Shock, 43*, 24-30.
11. Golse, N., Bucur, P. O., Adam, R., Castaing, D., Sa Cunha, A., & Vibert, E. (2013). New paradigms in post-hepatectomy liver failure. *J Gastrointest Surg, 17*, 593-605.
12. Guo, X., Wu, Y., & Hartley, R. S. (2010). Cold-inducible RNA-binding protein contributes to human antigen R and cyclin E1 deregulation in breast cancer. *Mol Carcinog, 49*, 130-40.
13. He, G., & Karin, M. (2011). NF-κB and STAT3 - key players in liver inflammation and cancer. *Cell Res, 21*, 159-68.
14. Hu, Z., Han, Y., Liu, Y., Zhao, Z., Ma, F., Cui, A., Zhang, F., Liu, Z., Xue, Y., Bai, J., Wu, H., Bian, H., Chin, Y. E., Yu, Y., Meng, Z., Wang, H., Liu, Y., Fan, J., Gao, X., Chen, Y., & Li, Y. (2020). CREBZF as a Key Regulator of STAT3 Pathway in the Control of Liver Regeneration in Mice. *Hepatology, 71*, 1421-36.
15. Li, W., Liang, X., Kellendonk, C., Poli, V., & Taub, R. (2002). STAT3 contributes to the mitogenic response of hepatocytes during liver regeneration. *J Biol Chem, 277*, 28411-7.

16. Li, X., Sun, J., Fan, X., Guan, L., Li, D., Zhou, Y., Zeng, X., Chen, Y., Zhang, H., Xu, L., Jiang, F., Huang, M., & Bi, H. (2018). Schisandrol B promotes liver regeneration after partial hepatectomy in mice. *Eur J Pharmacol, 818*, 96-102.
17. Li, Z., Fan, E. K., Liu, J., Scott, M. J., Li, Y., Li, S., Xie, W., Billiar, T. R., Wilson, M. A., Jiang, Y., Wang, P., & Fan, J. (2017). Cold-inducible RNA-binding protein through TLR4 signaling induces mitochondrial DNA fragmentation and regulates macrophage cell death after trauma. *Cell Death Dis, 8*, e2775.
18. Liao, Y., Tong, L., Tang, L., & Wu, S. (2017). The role of cold-inducible RNA binding protein in cell stress response. *Int J Cancer, 141*, 2164-73.
19. Liu, W., Bi, J., Ren, Y., Chen, H., Zhang, J., Wang, T., Wang, M., Zhang, L., Zhao, J., Wu, Z., Lv, Y., Liu, B., & Wu, R. (2023). Targeting extracellular CIRP with an X-aptamer shows therapeutic potential in acute pancreatitis. *iScience, 26*, 107043.
20. Lujan, D. A., Ochoa, J. L., & Hartley, R. S. (2018). Cold-inducible RNA binding protein in cancer and inflammation. *Wiley Interdiscip Rev RNA, 9*.
21. Masuda, T., Itoh, K., Higashitsuji, H., Higashitsuji, H., Nakazawa, N., Sakurai, T., Liu, Y., Tokuchi, H., Fujita, T., Zhao, Y., Nishiyama, H., Tanaka, T., Fukumoto, M., Ikawa, M., Okabe, M., & Fujita, J. (2012). Cold-inducible RNA-binding protein (Cirp) interacts with Dyrk1b/Mirk and promotes proliferation of immature male germ cells in mice. *Proc Natl Acad Sci U S A, 109*, 10885-90.
22. Michalopoulos, G. K., & Bhushan, B. (2021). Liver regeneration: biological and pathological mechanisms and implications. *Nat Rev Gastroenterol Hepatol, 18*, 40-55.
23. Morf, J., Rey, G., Schneider, K., Stratmann, M., Fujita, J., Naef, F., & Schibler, U. (2012). Cold-inducible RNA-binding protein modulates circadian gene expression posttranscriptionally. *Science, 338*, 379-83.
24. Ozaki, M. (2020). Cellular and molecular mechanisms of liver regeneration: Proliferation, growth, death and protection of hepatocytes. *Semin Cell Dev Biol, 100*, 62-73.
25. Pibiri, M. (2018). Liver regeneration in aged mice: new insights. *Aging (Albany NY), 10*, 1801-24.
26. Qiang, X., Yang, W. L., Wu, R., Zhou, M., Jacob, A., Dong, W., Kuncewitch, M., Ji, Y., Yang, H., Wang, H., Fujita, J., Nicastro, J., Coppa, G. F., Tracey, K. J., & Wang, P. (2013). Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis. *Nat Med, 19*, 1489-95.
27. Ray, S., Mehta, N. N., Golhar, A., & Nundy, S. (2018). Post hepatectomy liver failure - A comprehensive review of current concepts and controversies. *Ann Med Surg (Lond), 34*, 4-10.
28. Roilo, M., Kullmann, M. K., & Hengst, L. (2018). Cold-inducible RNA-binding protein (CIRP) induces translation of the cell-cycle inhibitor p27Kip1. *Nucleic Acids Res, 46*, 3198-210.
29. Sakurai, T., Kashida, H., Watanabe, T., Hagiwara, S., Mizushima, T., Iijima, H., Nishida, N., Higashitsuji, H., Fujita, J., & Kudo, M. (2014). Stress response protein cirp links inflammation and tumorigenesis in colitis-associated cancer. *Cancer Res, 74*, 6119-28.
30. Sakurai, T., Yada, N., Watanabe, T., Arizumi, T., Hagiwara, S., Ueshima, K., Nishida, N., Fujita, J., & Kudo, M. (2015). Cold-inducible RNA-binding protein promotes the development of liver cancer.

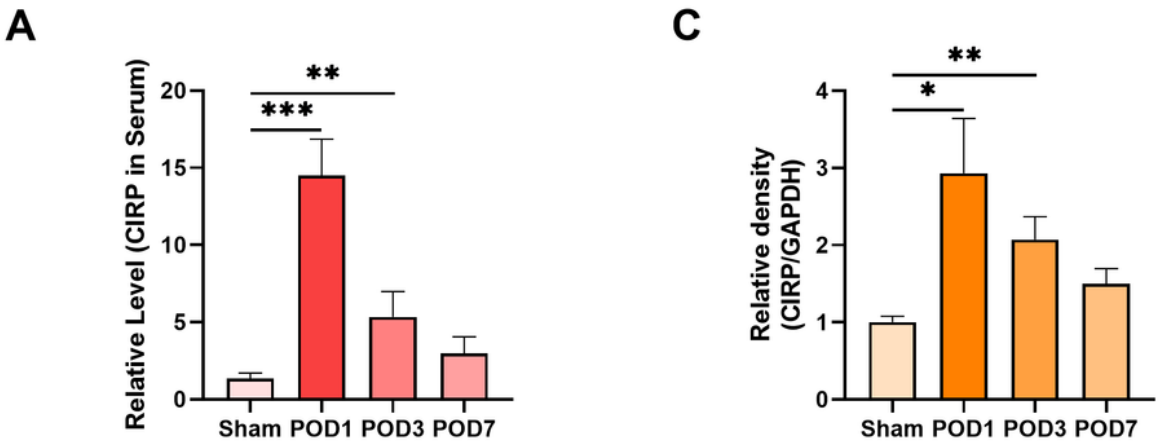
Cancer Sci, 106, 352-8.

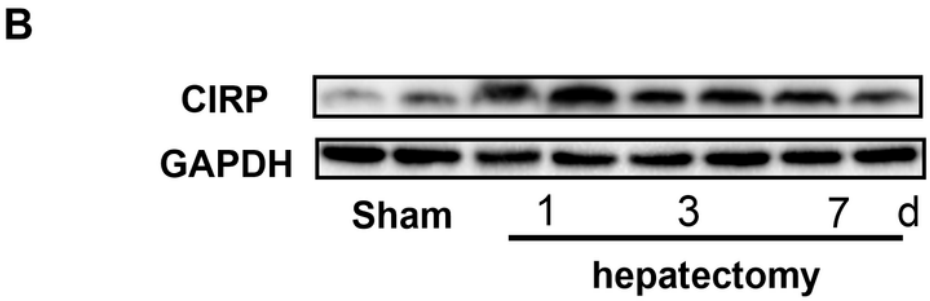
31. Sharma, A., Brenner, M., Jacob, A., Marambaud, P., & Wang, P. (2021). Extracellular CIRP Activates the IL-6Rα/STAT3/Cdk5 Pathway in Neurons. *Mol Neurobiol, 58*, 3628-40.
32. Sheikh, M. S., Carrier, F., Papathanasiou, M. A., Hollander, M. C., Zhan, Q., Yu, K., & Fornace, A. J., Jr. (1997). Identification of several human homologs of hamster DNA damage-inducible transcripts. Cloning and characterization of a novel UV-inducible cDNA that codes for a putative RNA-binding protein. *J Biol Chem, 272*, 26720-6.
33. Villanueva, L., Silva, L., Llopiz, D., Ruiz, M., Iglesias, T., Lozano, T., Casares, N., Hervas-Stubbs, S., Rodríguez, M. J., Carrascosa, J. L., Lasarte, J. J., & Sarobe, P. (2018). The Toll like receptor 4 ligand cold-inducible RNA-binding protein as vaccination platform against cancer. *Oncoimmunology, 7*, e1409321.
34. Wellmann, S., Bührer, C., Moderegger, E., Zelmer, A., Kirschner, R., Koehne, P., Fujita, J., & Seeger, K. (2004). Oxygen-regulated expression of the RNA-binding proteins RBM3 and CIRP by a HIF-1-independent mechanism. *J Cell Sci, 117*, 1785-94.
35. Welte, T., Zhang, S. S., Wang, T., Zhang, Z., Hesslein, D. G., Yin, Z., Kano, A., Iwamoto, Y., Li, E., Craft, J. E., Bothwell, A. L., Fikrig, E., Koni, P. A., Flavell, R. A., & Fu, X. Y. (2003). STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: a critical role of STAT3 in innate immunity. *Proc Natl Acad Sci U S A, 100*, 1879-84.
36. Xia, Z., Zheng, X., Zheng, H., Liu, X., Yang, Z., & Wang, X. (2012). Cold-inducible RNA-binding protein (CIRP) regulates target mRNA stabilization in the mouse testis. *FEBS Lett, 586*, 3299-308.
37. Xia, Z. P., Zheng, X. M., Zheng, H., Liu, X. J., Liu, G. Y., & Wang, X. H. (2012). Downregulation of cold-inducible RNA-binding protein activates mitogen-activated protein kinases and impairs spermatogenic function in mouse testes. *Asian J Androl, 14*, 884-9.
38. Yang, C., & Carrier, F. (2001). The UV-inducible RNA-binding protein A18 (A18 hnRNP) plays a protective role in the genotoxic stress response. *J Biol Chem, 276*, 47277-84.
39. Yang, W. L., Sharma, A., Wang, Z., Li, Z., Fan, J., & Wang, P. (2016). Cold-inducible RNA-binding protein causes endothelial dysfunction via activation of Nlrp3 inflammasome. *Sci Rep, 6*, 26571.
40. Zhang, B., Lv, D., Chen, Y., Nie, W., Jiao, Y., Zhang, J., Zhou, X., Wu, X., Chen, S., & Ma, T. (2022). Aquaporin-9 facilitates liver regeneration following hepatectomy. *Redox Biol, 50*, 102246.
41. Zhang, F., Brenner, M., Yang, W. L., & Wang, P. (2018). A cold-inducible RNA-binding protein (CIRP)-derived peptide attenuates inflammation and organ injury in septic mice. *Sci Rep, 8*, 3052.
42. Zhang, F., Yang, W. L., Brenner, M., & Wang, P. (2017). Attenuation of hemorrhage-associated lung injury by adjuvant treatment with C23, an oligopeptide derived from cold-inducible RNA-binding protein. *J Trauma Acute Care Surg, 83*, 690-97.
43. Zhang, Y., Wu, Y., Mao, P., Li, F., Han, X., Zhang, Y., Jiang, S., Chen, Y., Huang, J., Liu, D., Zhao, Y., Ma, W., & Songyang, Z. (2016). Cold-inducible RNA-binding protein CIRP/hnRNP A18 regulates telomerase activity in a temperature-dependent manner. *Nucleic Acids Res, 44*, 761-75.

44. Zhong, P., & Huang, H. (2017). Recent progress in the research of cold-inducible RNA-binding protein. *Future Sci OA, 3*, Fso246.

45. Zhou, M., Aziz, M., Denning, N. L., Yen, H. T., Ma, G., & Wang, P. (2020). Extracellular CIRP induces macrophage endotoxin tolerance through IL-6R-mediated STAT3 activation. *JCI Insight, 5*.

Figures





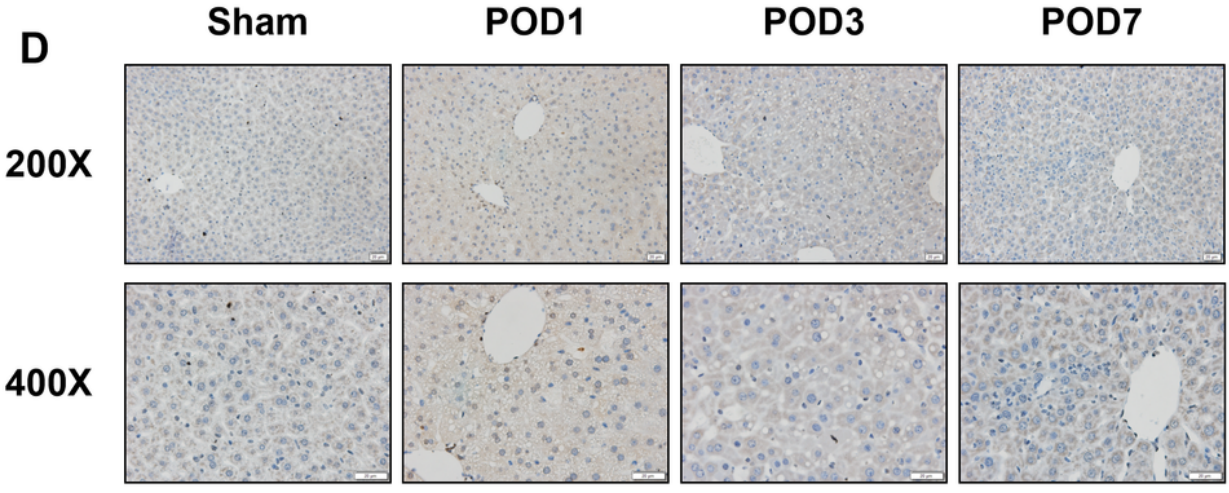


Figure 1

CIRP expression was up-regulated after partial hepatectomy in mice.

(A) CIRP level in WT mice serum at 1, 3 and 7 days after *partial* hepatectomy. **(B, C)** CIRP expression in WT mice liver at 1, 3 and 7 days after *partial* hepatectomy. **(D)** Immunohistochemical staining of CIRP in liver sections from WT mice livers at 1, 3 and 7 days after hepatectomy (400X magnification). The sham operated mice were used as the baseline control. All data are shown as the means ± SEM; n = 6; *p < 0.05; **p < 0.01; ***p < 0.001 compared with sham mice.

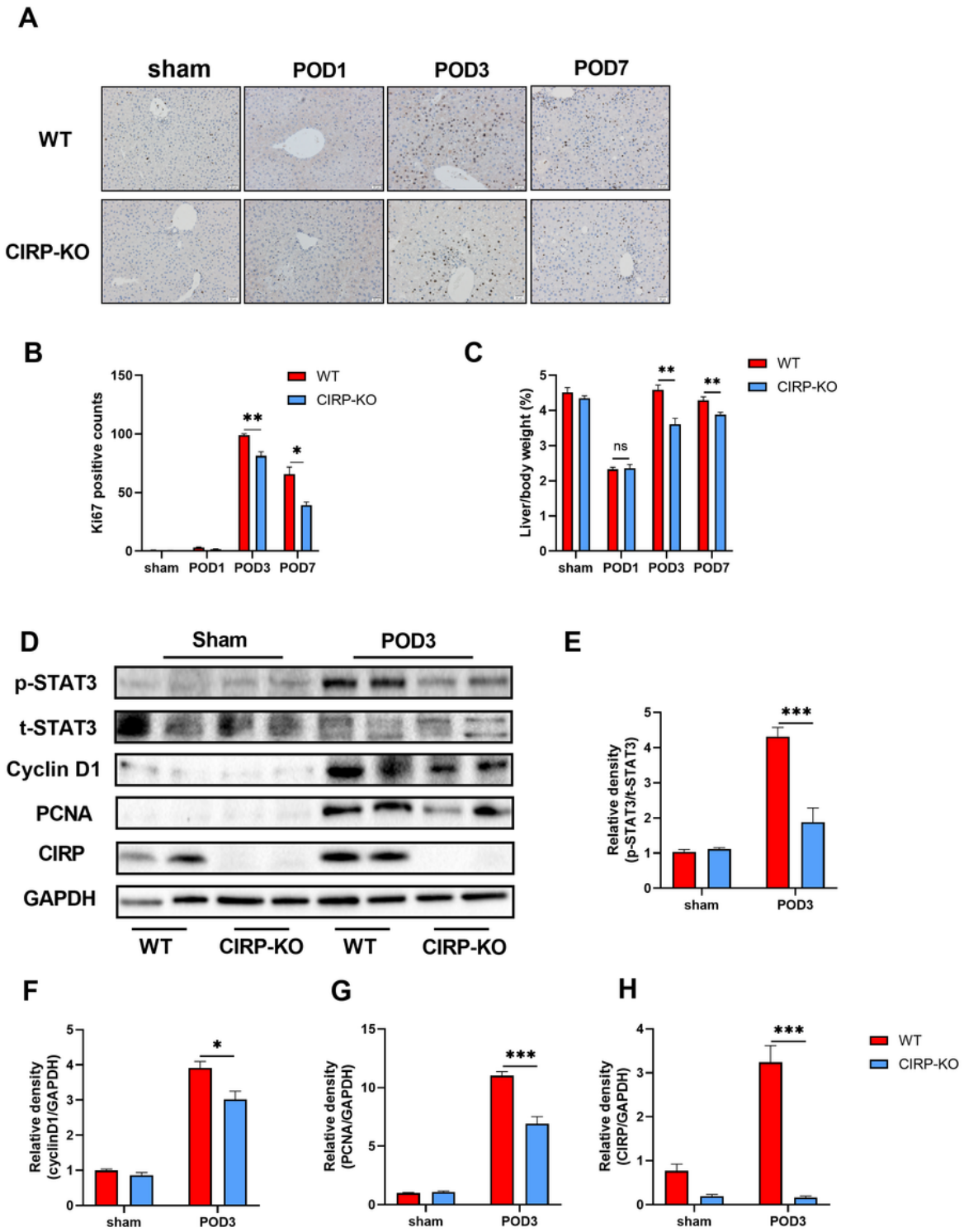


Figure 2

CIRP deficiency restrained liver regeneration after partial hepatectomy in mice.

(A, B) Immunohistochemical staining of Ki67 in liver sections from WT and CIRP KO mice livers at 1, 3 and 7 days after *partial* hepatectomy. **(C)**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. **(D-H)**Western

blotting analysis of p-STAT3, Cyclin D1, PCNA and CIRP in WT and CIRP KO mice livers at 3 day after partial hepatectomy. GAPDH was used as loading controls. All data are shown as the means ± SEM; n = 6; *p < 0.05; **p < 0.01; ***p <0 .001 compared with WT mice.

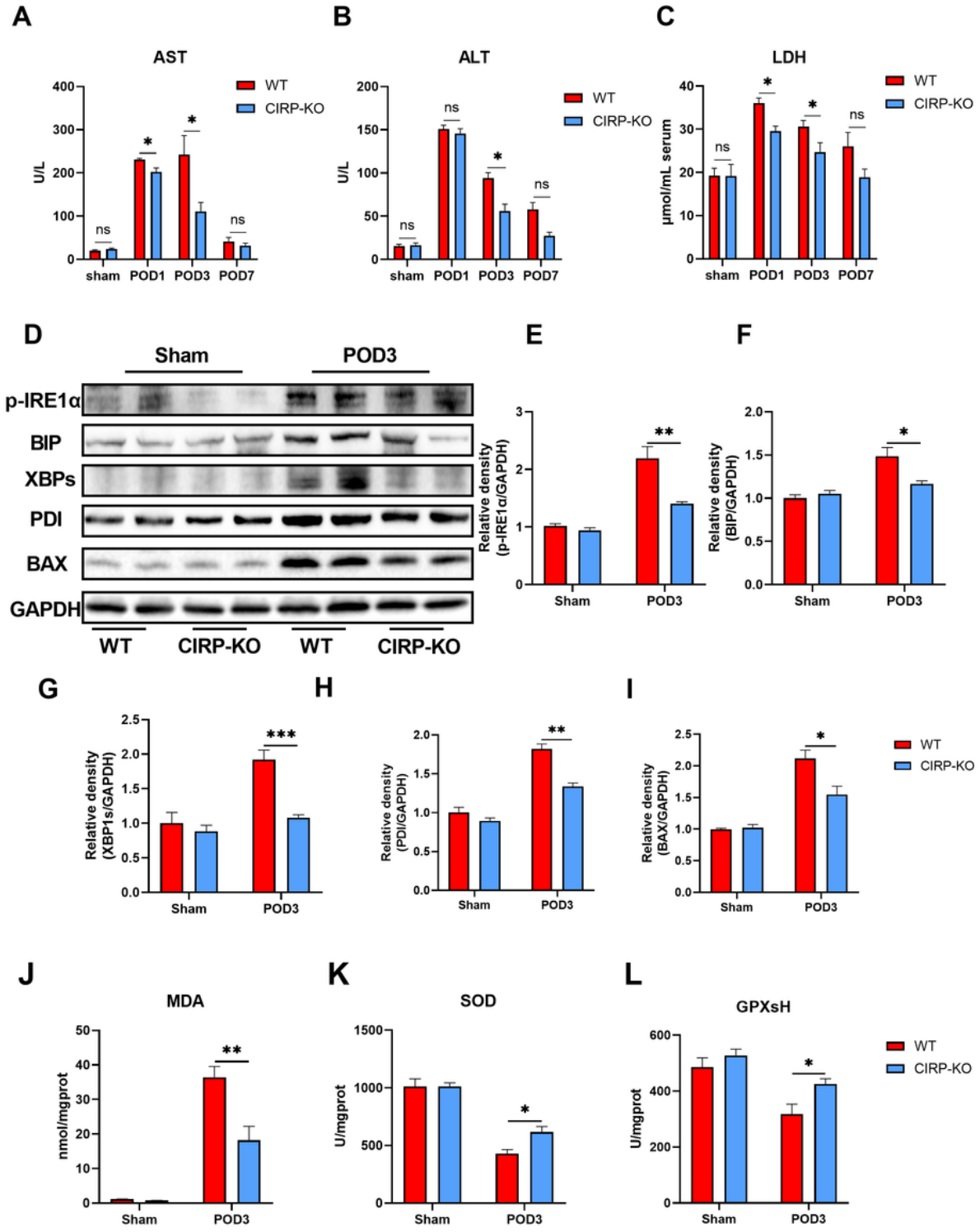


Figure 3

CIRP deficiency alleviated liver injury after partial hepatectomy.

(A, B) Serum AST and ALT at 1, 3 and 7 days after *partial* hepatectomy. **(C)**Serum LDH at 1, 3 and 7 day after *partial* hepatectomy. **(D-I)**Western blotting analysis of BIP, p-IRE1α, XBP1s, PDI and BAX in WT and CIRP KO mice livers at 3 day after *partial* hepatectomy. GAPDH was used as loading controls. **(J-L)** Examination of MDA, SOD and GPXsH in WT and CIRP KO mice livers at 3 day after *partial* hepatectomy. All data are shown as the means ± SEM; n = 6; *p < 0.05; **p < 0.01; ***p <0 .001 compared with WT mice.

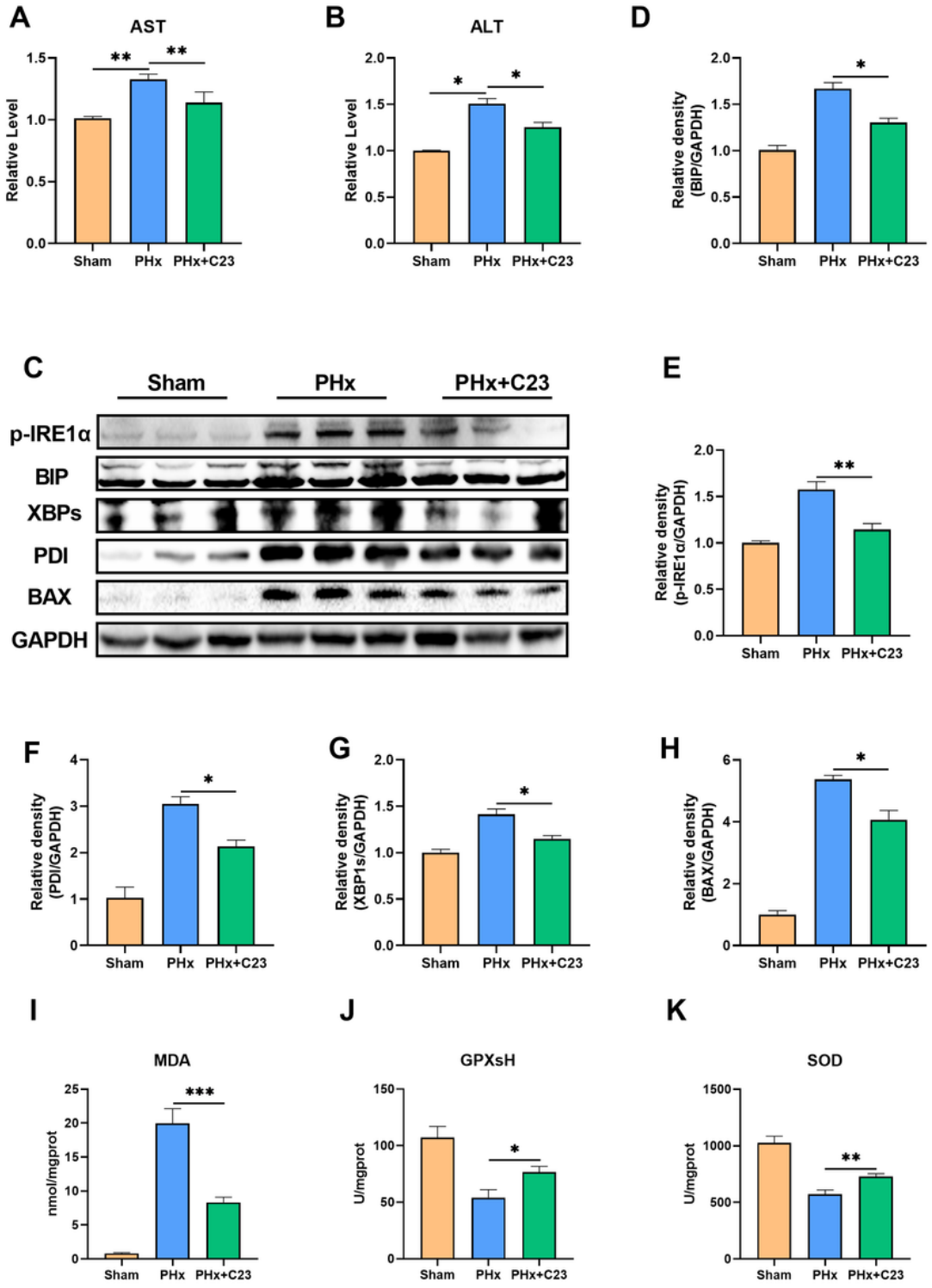


Figure 4

C23 protected liver injury after partial hepatectomy.

C23 was administrated intraperitoneally in WT mice (8mg/kg mice weight) after *partial* hepatectomy. **(A, B)** Serum AST and ALT at 3 day after *partial* hepatectomy. **(C-H)** Western blotting analysis of p-IRE1α, XBP1s, PDI and BAX in WT and WT+ C23 mice livers at 3 day after partial hepatectomy. GAPDH was used as loading controls. **(I-K)** Examination of MDA, SOD and GPXsH in WT and WT+ C23 mice livers at 3 day after partial hepatectomy. All data are shown as the means ± SEM; n = 6; *p < 0.05; **p < 0.01; ***p <0 .001 compared with sham mice.

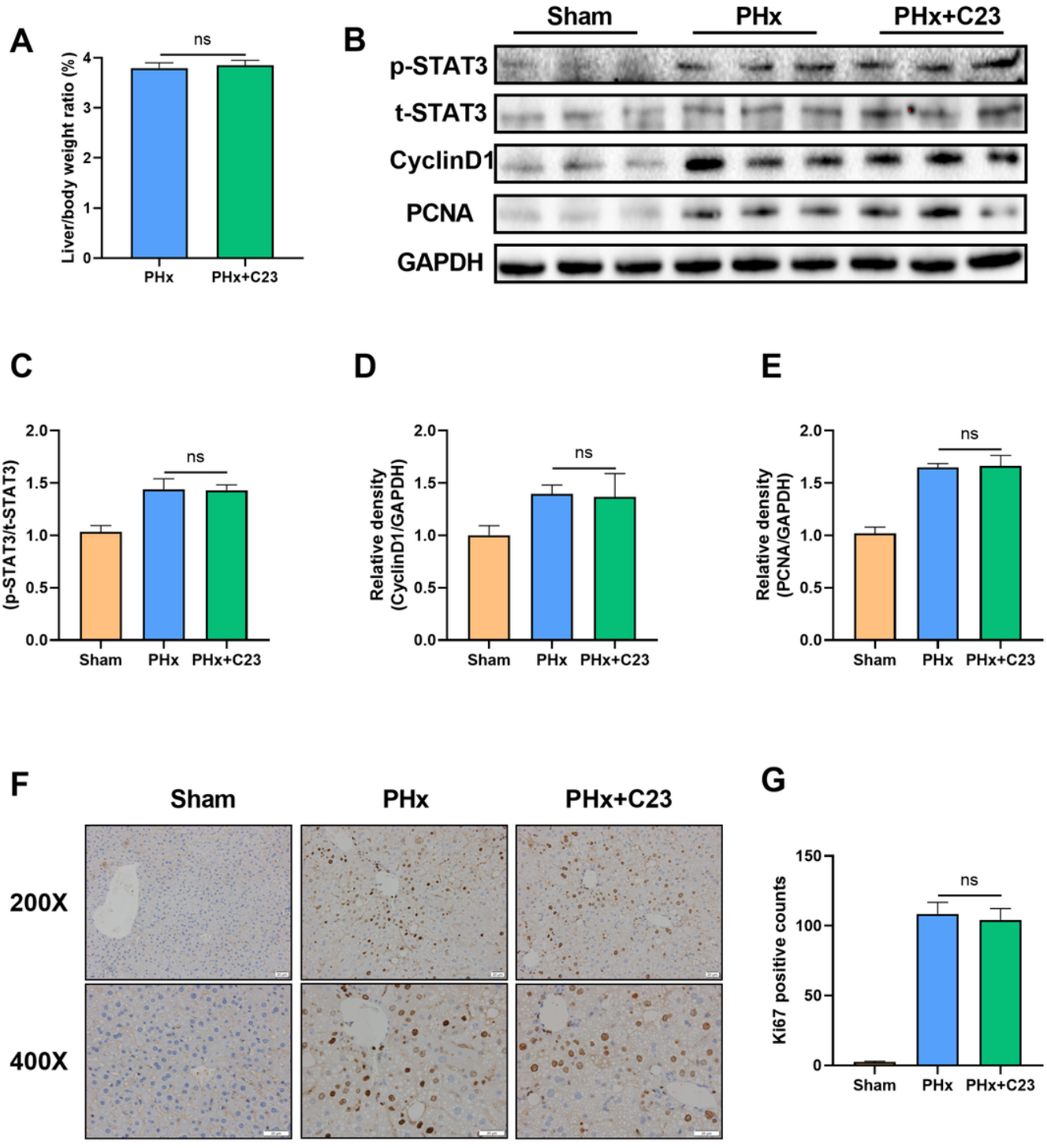


Figure 5

C23 adiministration had no impact on liver regeneration after partial hepatectomy.

(A) Liver-to-body-weight ratio in WT and WT+C23 mice at 3 day after *partial* hepatectomy. **(B-E)**Western blotting analysis of p-STAT3, CyclinD1and PCNA in WT and WT+ C23 mice livers at 3 day after partial hepatectomy. GAPDH was used as loading controls. **(F-G)**Mice Immunohistochemical staining of Ki67 staining in liver sections from WT and WT+C23 mice livers at 3 day after hepatectomy. All data are shown as the means ± SEM; n = 6; *p < 0.05; **p < 0.01; ***p <0 .001 compared with sham mice.

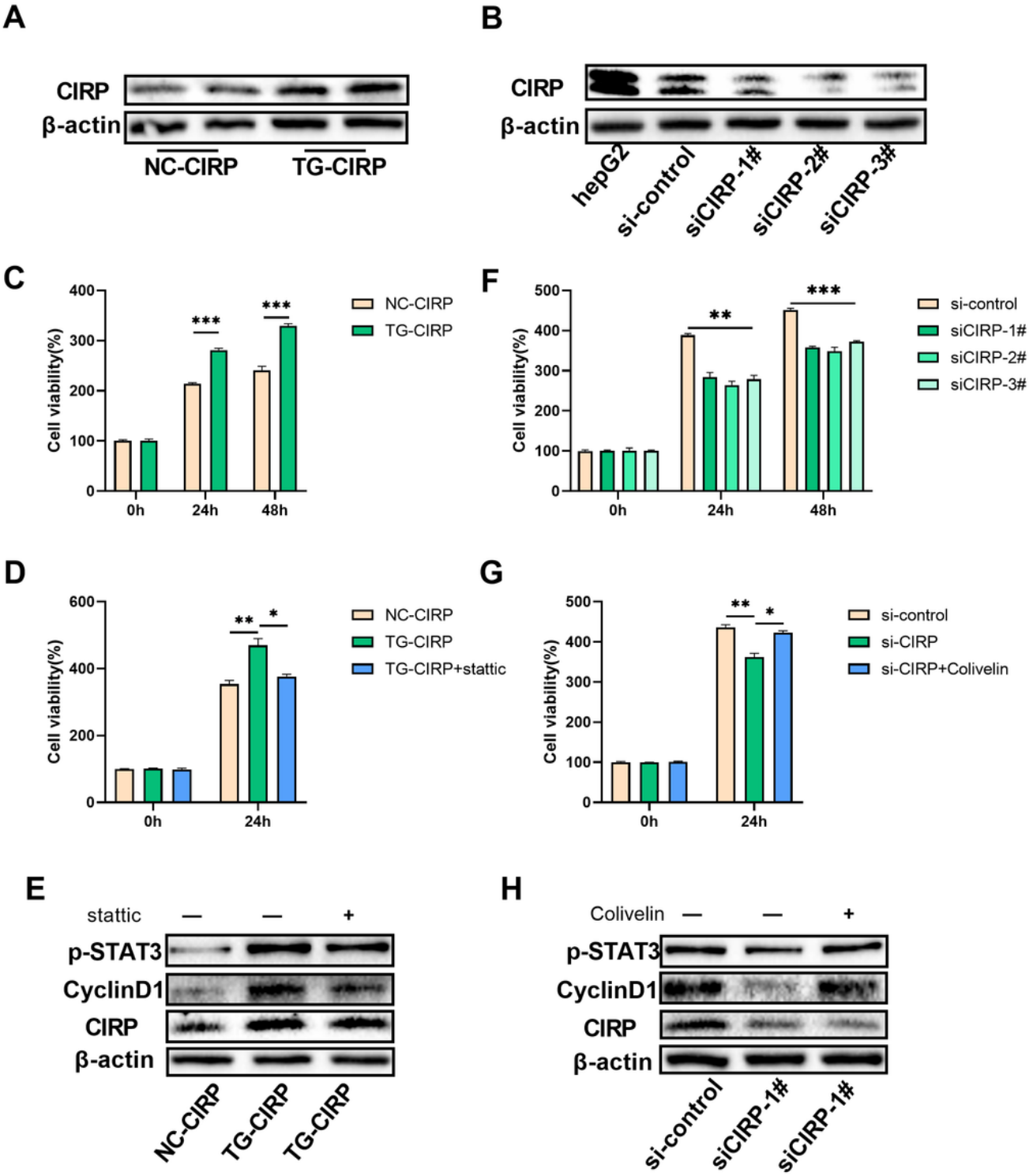


Figure 6

CIRP promoted liver regeneration via regulating STAT3 phosphorylation.

(A , B) Western blotting analysis of CIRP expression in CIRP overexpression and CIRP Knockdown hepG2 cells. β-actin was used as loading controls. **(C , F)** Cell proliferation ability of NC and CIRP TG and CIRP KD hepG2 cells were determined by the CCK8 assay at 24 and 48h (n=3). **(D)** An CCK8 assay

demonstrated that the STAT3 activator Colivelin reversed the suppressive effect of CIRP KD on the proliferation of hepG2 cells. **(G)** An CCK8 assay demonstrated that the STAT3 antagonist stattic reversed the promotive effect of CIRP TG on the proliferation of hepG2 cells. **(E , H)** Western blotting analysis of CIRP and STAT3 signaling proteins in the above hepG2 cells. All data are shown as the means ± SEM; n = 3; *p < 0.05; **p < 0.01; ***p <0 .001 compared with NC group.

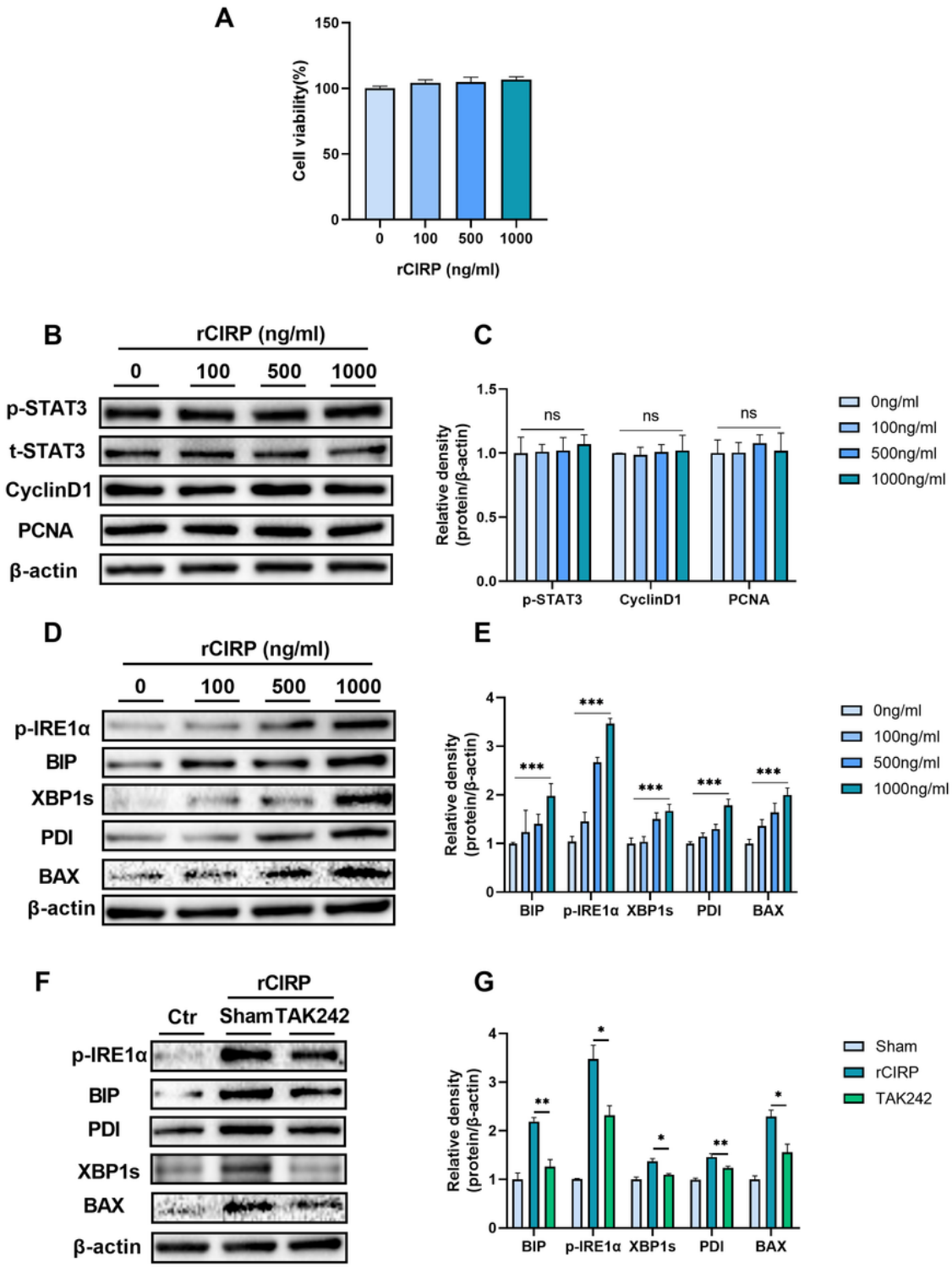


Figure 7

r*CIRP treatment led to ER stress via TLR4 in hepatocytes.*

(A) An CCK8 assay of hepG2 proliferation after treatment with different concentration rCIRP (0, 100, 500 and 1000ng/ml) in 24h. **(B , C)** Western blotting analysis of p-STAT3, CyclinD1and PCNA in hepG2 treated with different concentration of rCIRP. **(D , E)** Western blotting analysis of BIP, p-IRE1α, XBP1s, PDI and BAX in hepG2 treated with different concentration of rCIRP. β-actin was used as loading controls. HepG2 cells were treated with rCIRP (1000ng/ml) with or without TAK242 (5μM) for 24h. **(F , G)** Western blotting analysis of BIP, p-IRE1α, XBP1s, PDI and BAX in hepG2 cells. All data are shown as the means ± SEM; n = 3; *p < 0.05; **p < 0.01; ***p <0 .001 compared with control group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstract.png
- SupplemmtaryFigure13.docx