

Role of Hepatocyte RIPK1 in Maintaining Liver Homeostasis during Metabolic Challenges

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Abstract

As a central hub for metabolism, the liver exhibits strong adaptability to maintain homeostasis in response to food fluctuations throughout evolution. However, the mechanisms governing this resilience remain incompletely understood. Receptor interacting protein kinase 1 (RIPK1) plays a key role in regulating cell survival, cell death, and inflammation. Despite extensive research on its involvement in various pathological conditions, the physiological role of RIPK1 remains relatively unexplored. In this study, we identified RIPK1 in hepatocytes as a critical regulator in preserving hepatic homeostasis during metabolic challenges, such as short-term fasting or high-fat dieting. Our results demonstrated that hepatocyte-specific deficiency of RIPK1 sensitized the liver to short-term fasting-induced liver injury and hepatocyte apoptosis in both male and female mice. Despite being a common physiological stressor that typically does not induce liver inflammation, short-term fasting triggered hepatic inflammation and compensatory proliferation in hepatocyte-specific RIPK1-deficient (*Ripk1^{Δhep}*) mice. Transcriptomic analysis revealed that short-term fasting oriented the hepatic microenvironment into an inflammatory state in *Ripk1^{Δhep}* mice, with upregulated expression of inflammation and immune cell recruitment-associated genes. Single-cell RNA sequencing further confirmed the altered cellular composition in the liver of *Ripk1^{Δhep}* mice during fasting, highlighting the increased recruitment of macrophages to the liver. Mechanically, our results indicated that ER stress was involved in fasting-induced liver injury in *Ripk1^{Δhep}* mice. Overall, our findings revealed the role of RIPK1 in maintaining liver homeostasis during metabolic fluctuations and shed light on the intricate interplay between cell death, inflammation, and metabolism.

eLife assessment

The study presents **valuable** findings on the role of RIPK1 in maintaining liver homeostasis under metabolic stress. Strengths include the intriguing findings that RIPK1 deficiency sensitizes the liver to acute liver injury and apoptosis, but because the conclusions require additional experimental support, the evidence is **incomplete**.

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Introduction

During the long evolutionary history, mammals have frequently encountered various metabolic stresses arising from fluctuations in food availability. The liver, functioning as the central hub for metabolism, exhibits remarkable adaptability in maintaining both hepatic and systemic homeostasis when faced with these metabolic challenges [1]. Acute fasting, as one of the main causes of metabolic stress, is known to trigger hepatic glycogen depletion, increased production of hepatic glucose and ketone bodies, adipose tissue lipolysis, and the influx and accumulation of lipids in the liver [2–4]. Under normal circumstances, the liver possesses the capability to shield itself from hepatic lipotoxicity caused by the elevated lipid influx and accumulation during fasting [5, 6]. However, in specific situations, such as observed in patients with anorexia nervosa, fasting can result in severe liver damage and dysfunction [7]. Nevertheless, the underlying mechanisms that dictate the liver's adaptive ability to mitigate or succumb to lipotoxicity induced by metabolic stress are not fully understood.

The serine/threonine kinase RIPK1 is a crucial mediator of cell death and inflammation [8]. It manifests two distinct, even opposing functions: its scaffold function regulates cell survival and activation of NF- κ B pathway, while its kinase activity promotes cell death including apoptosis and necroptosis [9, 10]. Both functions play crucial regulatory roles across a spectrum of physiological and pathological scenarios. Notably, the kinase activity of RIPK1 has been extensively studied due to its association with the deleterious effects in pathological situations [11–14]. In contrast, the scaffold function of RIPK1 is less studied and current evidences suggest that it plays an essential role in maintaining tissue homeostasis within physiological context [15, 16]. The scaffold function of RIPK1 has been studied using *Ripk1*^{-/-} cells or RIPK1 deficient mice [15–19]. The postnatality death of *Ripk1*^{-/-} mice suggested that the scaffold function of RIPK1 plays an essential role in normal development, acting as a brake to prevent cell death and inflammation in different tissues [17–19]. Deletion of RIPK1 results in the loss of its scaffold function and unleashes the release of subsequent deleterious part with cell death and inflammation. Generation of conditional knockout mice with RIPK1 deletion in different tissues or cell types results in different phenotypes, suggesting that the role of RIPK1 scaffold in different tissues is dependent on the context in tissue or cells [20–23]. In contrast to the severe phenotype observed in mice with RIPK1 deletion in epithelial cells, mice lacking RIPK1 in hepatocytes (*Ripk1* ^{Δ hep}) exhibit a surprising state of health and normal under steady conditions [20, 21]. Nonetheless, when confronted with pathogen-associated molecular patterns (PAMPs) or viral challenges, *Ripk1* ^{Δ hep} mice display heightened inflammation and cell death [20, 24, 25]. However, the precise role of hepatocellular RIPK1 in liver physiology remains unknown.

This study aimed to investigate the physiological functions of RIPK1 in the liver. Hepatocyte-specific RIPK1-deficient mice (*Ripk1* ^{Δ hep}) were generated and subjected to a 12-hour fasting period. Surprisingly, we observed that RIPK1 deficiency in hepatocytes sensitized the liver to acute liver injury and hepatocyte apoptosis. Remarkably, both male and female *Ripk1* ^{Δ hep} mice exhibited

fasting-induced liver injury and apoptosis, emphasizing the robustness of this phenotype across genders. Furthermore, short-term fasting triggered hepatic inflammation in *Ripk1^{Δhep}* mice, as indicated by elevated expression of inflammatory markers and increased compensatory proliferation, potentially linked to hepatocellular carcinoma markers.

Transcriptomic profiling of liver tissues revealed that RIPK1 deficiency amplified the proinflammatory response during fasting, with upregulated expression of inflammation-associated genes and enhanced recruitment of immune cells to the liver. Single-cell RNA sequencing further dissected the altered cellular composition in *Ripk1^{Δhep}* mice, highlighting an expansion of recruited macrophages and NK&T cells. Sub-clustering of macrophages unveiled an increased presence of recruited macrophages in *Ripk1^{Δhep}* mice during fasting, suggesting their role in driving the fasting-induced inflammatory state. In summary, our study sheds light on the critical role of RIPK1 in maintaining liver homeostasis during short-term fasting and provides insights into the intricate interplay between cell death, inflammation, and liver adaptation to metabolic challenges.

Material & Methods

2.1 Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Serum alanine transaminase (ALT)	Nanjing Jiancheng Bioengineering Institute	C009-2-1
Serum aspartate transaminase (AST)	Nanjing Jiancheng Bioengineering Institute	C010-2-1
Serum triglyceride (TG)	Nanjing Jiancheng Bioengineering Institute	A110-1-1
Serum cholesterol (TC)	Nanjing Jiancheng Bioengineering Institute	A111-1-1
Serum free fatty acid (FFA)	Solarbio	BC0595
Ketone Body Assay Kit	Abcam	ab272541
Hepatic triglyceride (TG)	Solarbio	BC0625
TUNEL Assay Kits	Vazyme	A113-02
Antibodies		

anti-cleaved-Caspase-3	Cell Signaling Technology	9661S
anti-phospho-MLKL (S345)	Cell Signaling Technology	37333S
anti-F4/80	Servicebio	GB113373
anti-CD11b	Servicebio	GB11058
anti-CCR2	Servicebio	GB11326
anti-CX3CR1	Servicebio	GB11861
anti-Ki67	Bioss	bs-2130R
anti-Alb	Servicebio	GB122080
anti-TNF α	proteintech	60291-1-Ig
anti-RIPK1	Cell Signaling Technology	3493S
anti-phospho-IRE1 α	Abcam	ab48187
anti-IRE1 α	Cell Signaling Technology	3294S
anti-GRP78	Cell Signaling Technology	3177S
anti-CHOP	Cell Signaling Technology	2895S
anti-GAPDH	Servicebio	GB12002

2.2 Mouse models

Ripk1^{fl/fl} and *Albumin-cre* transgenic mice on C57BL/6J background as previously described were kindly provided by Prof. Haibing Zhang (Chinese Academy of Sciences, Shanghai, China), both male and female littermates were used in the experiments [26]. Liver-specific RIPK1 knockout mice (*Ripk1^{Δhep}* mice) were generated by crossing *Ripk1^{fl/fl}* and *Albumin-cre* mice. Then *Ripk1^{fl/fl}* mice were bred with *Ripk1^{fl/fl} Cre⁺* mice to generate *Ripk1^{fl/fl}* and *Ripk1^{fl/fl} Cre⁺* mice as littermates. All mice were maintained in the standard laboratory conditions with temperature 22±2°C, strict 12 hours dark-light cycles (7:00 a.m. to 7:00 p.m.). All mice had ad libitum access to food and water, and before the start of experiments all mice were maintained on a regular chow diet (LAD 0011, Trophic Animal Feed, China). All experiments were conducted following the Animal Care and Use Committee at Nanjing University of Science & Technology.

For the fasting studies involving *Ripk1^{fl/fl}* and *Ripk1^{Δhep}* mice, 6-week-old littermates of *Ripk1^{fl/fl}* or *Ripk1^{Δhep}* were randomly assigned to groups. The fed group was allowed ad libitum access to both food and water. The fasted group, on the other hand, was deprived of food but not water from 8:00 p.m. since then, and this deprivation continued for a period of 12 hours.

For the endoplasmic reticulum stress inhibitor experiment, 6-week-old *Ripk1^{fl/fl}* and *Ripk1^{Δhep}* mice were divided into separate groups and administered either 80 mg/kg of 4-PBA (SML0309, Sigma, Germany) or saline vehicle via intraperitoneal injection at 8:00 a.m. Fasting was initiated for all groups by removing food at 8:00 p.m., while water was still available, for a duration of 12 hours. At 10:00 p.m., the mice were intraperitoneally injected with 80 mg/kg of 4-PBA.

For the high-fat diet (HFD) experiment, separate groups of *Ripk1^{fl/fl}* and *Ripk1^{Δhep}* mice were fed either a HFD (TP201482; Trophic Animal Feed, China) or a normal diet (LAD 0011, Trophic Animal Feed, China) for a period of 6 days. On the last day, a 3-hour fast was initiated at 7 a.m. and the mice were sacrificed thereafter.

For the adenovirus-associated virus (AAV) experiment, 6-week-old littermates of *Ripk1^{fl/fl}* were randomly divided into two groups. One group was administered serotype 8 AAV-thyroxine-binding globulin (TBG)-Cre (AAV-TBG-Cre, 1.5×10¹¹ gene copies per mouse, intravenously) (H5721, Obio

Technology, China), while the other group was injected with AAV-TBG-null (H20594, Obio Technology, China). After 4 weeks, the mice were subjected to the same fasting rationale as described earlier.

At the end of the experiment of each different model, mice were sacrificed following blood collection. Subsequently, the mice were dissected and the liver tissues were harvested. Some portions of the tissues were fixed in 4% paraformaldehyde tissue fixation solution for further histological examination, while others were stored at -80°C for additional detection. All mice used for experiments displayed general health. The total number of mice analyzed for each experiment is detailed in the figure legends.

2.3 Serum and liver assays

Plasma was maintained at room temperature for 1 h, and then centrifuged at 4,000 rpm for 10 min to take the supernatant as serum. Serum alanine transaminase (ALT), aspartate transaminase (AST), triglyceride (TG), cholesterol (TC), free fatty acid (FFA) and β -hydroxybutyrate (BOH) were measured according to the manufacturer's instructions. For measurements of liver triglyceride (TG) levels, 50 mg liver was homogenized in lysis buffer (isopropanol: chloroform = 1:1) using a high speed low temperature tissue grinding machine (Servicebio, China) and supernatant was centrifuged 8,000 g for 10 min at 4°C for measurement according to the manufacturer's instructions.

2.4 TUNEL assay

TUNEL Assay Kits were used to measure apoptotic cells in livers. Briefly, paraffin-embedded liver tissue sections were dried for 30 min at 60°C , and soaked in xylene to deparaffinize completely. Deparaffinized sections were pretreated by proteinase K for 20 min at 25°C . Then PBS was used to wash tissue sections and the mixture of reaction buffer with TdT enzyme was used to incubate in dark for 60 min at 25°C . Meanwhile the cell nucleuses were stained with DAPI. The tissue sections were observed and photographed using the fluorescence microscope (NIKON ECLIPSE 80i). The number of apoptotic cells was quantified in at least three random fields of the same size.

2.5 RNA isolation and RT-qPCR

Liver tissue (10–20 mg) was homogenized directly in TRIzol (Invitrogen) using a high speed low temperature tissue grinding machine (Servicebio, China), then total mRNA was extracted according to the manufacturer's instructions. Next cDNA was synthesized using one-step reverse transcription kit (5x All-In-One-RT Naster Mix, Abm). RT-qPCR reactions were performed on the iQ5 real-time PCR system (Bio-Rad, USA) using Hieff[™] qPCR SYBR[®] Green Master Mix (Yeasen Biotech, China). GAPDH mRNA was used as an internal control to normalize mRNA expression. The sequences of primers for qPCR were as follows:

Name	Forward (5'-3')	Reverse (5'-3')
<i>Mcp-1</i>	CACTCACCTGCTGCTACTCA	AGACCTTAGGGCAGATGCAG
<i>Tnfa</i>	GTAGCCACGTCGTAGCAAA	TAGCAAATCGGCTGACGGTG
<i>Il-1β</i>	GCCACCTTTTGACAGTGATGAG	ACGGGAAAGACACAGGTAGC
<i>Ifnγ</i>	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
<i>Il-6</i>	TGATGGATGCTACCAAAGTGA	GGAAATTGGGGTAGGAAGGACT
<i>Afp</i>	ACCTCCAGGCAACAACCATT	GTTTGACGCCATTCTCTGCG
<i>Ki67</i>	AACCATCATTGACCGCTCCT	AGGCCCTTGGCATAACAAAA
<i>Grp78</i>	CGAGGAGGAGGACAAGAAGG	TCAAGAACGGGCAAGTTCCAC
<i>Xbp1s</i>	GAGTCCGCAGCAGGTG	AGGGTACCTGAGACTGTG
<i>Chop</i>	CTGCCTTTACCTTGAGAC	ATAGAGTAGGGGTCCTTTGC
<i>Gapdh</i>	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

2.6 Histological analysis

Liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry/immunofluorescence. For histopathology, frozen sections of liver tissues were stained by Oil Red O working solution to determine the steatosis in liver tissues.

2.7 Immunohistochemistry and immunofluorescence analysis

For immunohistochemistry in liver tissues, paraffin embedded mouse liver sections (3.5 - 4 μ m) were dried for 30 min at 60 °C, and soaked in xylene to deparaffinized completely. Then boiled the liver sections in citrate buffer (pH = 6.0) for antigen retrieval, followed by hydrogen peroxide blocking of endogenous peroxidase. Then sections were incubated with primary antibody (anti-Ki67) at 4 °C overnight. The slides were then washed with PBS (PH=7.4) and incubated with secondary antibody for 1 h at 25°C, and then stained with DAB substrate after 5 min with streptavidin-HRP. The cell nuclei were stained with hematoxylin for 3 min at 25°C. For immunofluorescence, after being incubated with primary antibodies (anti-cleaved-Caspase-3, anti-phospho-MLKL (S345), anti-F4/80, anti-CD11b, anti-CCR2 and anti-CX3CR1) at 4 °C overnight, slides were washed with PBS (PH = 7.4) for three times and then incubated with fluorescent secondary antibodies in dark for 60 min at 25°C. The cell nuclei were stained in dark by DAPI for 5 min at 25°C. Finally, the slides were observed and photographed using the fluorescence microscope (NIKON ECLIPSE 80i). The number of positive cells was quantified in at least three random fields of the same size.

2.8 Western blotting

Total protein was extracted from liver tissues with RIPA lysis buffer (Beyotime, China) with protease inhibitor (P1005; Beyotime, China) and phosphatase inhibitor (P1081; Beyotime, China). BCA Protein Assays kits(Yeasen, China) were used to measure the protein concentrations. Equal amounts of proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 4–12% acrylamide gel (Yeasten, China) and transferred onto 0.22 μ m

PVDF membranes (Millipore Corporation, USA). Membranes were incubated with primary antibodies (anti-RIP1, anti-TNF α , anti-phospho-IRE1 α , anti-IRE1 α , anti-GRP78 and anti-CHOP) overnight at 4°C, washed with TBS-T (0.1% TWEEN-20) and incubated with suitable secondary antibodies (Goat anti-Mouse IgG HRP or Goat anti-Rabbit IgG HRP) at room temperature for 1 h. A chemiluminescent reagent (Beyotime, China) and a ChemiDoc MP Imaging System (CLiNX, China) were used for detection.

2.9 Liver tissue transcriptome sequencing

2.9.1 Isolation of RNA for sequencing

6-week-old *Ripk1^{fl/fl}* and *Ripk1^{Δhep}* male mice were fasted for 6 weeks. At end of the fast, mice were sacrificed and dissected. Approximately 200 mg of liver tissue at the left lateral lobe was cut, weighed and harvested for each mouse. Total RNA was extracted from mouse liver using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The isolated RNA was further quantified using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). Then the total RNA was subjected to sequencing for transcriptome analysis.

2.9.2 cDNA Library construction and sequencing

The RNA-seq library was generated using the TruSeq Stranded Total RNA Sample Preparation kit from Illumina (NEB, USA) following the manufacturer's recommendations for 150-bp paired-end sequencing. Poly-A-containing mRNA were purified and fragmented from DNase-treated total RNA using oligo(dT) magnetic beads. Following the purification step, mRNAs were fragmented to 300 bp nucleotides and used for synthesis of single-stranded cDNAs by means of random hexamer priming. With the constructed single-stranded cDNAs as templates, second strand cDNA synthesis was performed to prepare double-stranded cDNAs. These cDNAs were then amplified with end-pair repair, addition of A-tail, and adapter ligation using polymerase chain reaction (PCR). The quality control of cDNA library was evaluated by Agilent 2100 BioAnalyzer (Agilent Technologies). After quality control, sequencing was performed with Illumina HiSeq3000.

2.9.3 Transcript data analysis

Illumina sequencing analysis viewer was used for data quality check and Illumina Bcl2fastq2 program was used for demultiplexing. Then the high-quality filtered sequencing data was aligned with mouse reference genome (GRCm38) by TopHat (version 2.1.1). To profile differential gene expression (DEG), reads per kilo-base per million mapped reads (RKM) were calculated for each transcript. Paired sample t-tests followed by Benjamini-Hochberg correction were performed to identify DEGs. Genes with >2-fold changes ($|\log_2 \text{fold change}| > 1$) and false discovery rate <0.05 were defined as DEGs by DESeq2 (version 1.18.1). Enrichment analysis of DEGs was performed by Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.kegg.jp/>) pathway enrichment analysis to identify the affected metabolic pathways and signal transduction pathways.

2.10 Single-cell RNA sequencing

Male mice of 6 weeks old with *Ripk1^{fl/fl}* and *Ripk1^{Δhep}* genotypes were subjected to a 12-hour fast, after which they were sacrificed and dissected. Liver tissue samples were collected from the left lateral lobe, right lateral lobe, and right medial lobe of each mouse and weighed, with approximately 600 mg of tissue per sample. Liver tissue samples were mechanically dissociated and digested in lysis buffer containing collagenase/dispase and 0.001% deoxyribonuclease I, after which the samples were processed. Isolated cells passed through 70 μm cell strainer were treated with Red Blood Cell Lysis Solution (10 $\times$) (Miltenyi Biotec) for 5 min to lyse blood cells. To acquire cells with >90% viability, dead cells were removed with Dead Cell Removal Kit (Miltenyi Biotec) according to the manufacturer's instructions and cell pellet was resuspended in 2% FBS/phosphate-buffered saline. Then live cells were counted with Countstar and adjusted the cell

concentration to 1000 cells/ μ l. For scRNA-seq, chromium microfluidic chips were loaded with cell suspension with 3' chemistry and barcoded with a 10 $\times$ Chromium Controller (10 $\times$ Genomics). RNA was reverse-transcribed from the barcoded cells. Qubit dsDNA HS Assay Kit (Invitrogen) was used to quantify cDNA concentration and single-cell transcriptome libraries were constructed using the 10 $\times$ Chromium Single Cell 3' Library (10 $\times$ Genomics, v3 barcoding chemistry). Quality control was performed with Agilent 2100(Agilent Technologies). Libraries were then purified, pooled, and analyzed on Illumina NovaSeq 6000 S2 Sequencing System with 150 bp paired-end reads according to the manufacturer's instructions.

For analysis of scRNA-seq data, briefly, cellRanger single-cell software suite (v3.1.0) were used to process and analysis scRNA-seq reads with the default parameters. Base calling files generated by Illumina sequencer were demultiplexed according to the sample index. Sequences were then aligned to mouse reference genome mm10 reference for whole transcriptome analysis. CellRanger 3.1.0 were used to conducted Filtering, barcode counting, and UMI counting. For the following analysis multiple samples were aggregated. Visualization, quality control, normalization, scaling, PCA dimension reduction, clustering, and differential expression analysis were used by Loupe browser and Seurat (v4.0.0) to perform. Cells with a detected gene number of <200 or >10000, mitochondrion gene percentage of <10, hemoglobin gene percentage of <10, and doublet nucleus were removed by package Seurat (v4.0.0). Data were subsequently log-normalized (divided by the total expression and amplified scaling factor 10,000) before further analyses. The remaining 22,274 cells were unsupervised clustered after aligning the top 30 dimensions and setting resolution to 0.9. Loupe browser software (10X Genomics) were used to demonstrate UMAP and T-SNE. The identity for each cluster was assigned according to marker genes for known cell types in the mouse liver. Differentially expressed genes with absolute log-fold change greater than 1.5 and p value less than 0.05 were used for pathway and network enrichment analysis on the Kyoto Encyclopedia of Genes and Genomes website (KEGG) (<http://www.kegg.jp/> .

2.11 Statistical analysis

All the results in this study are expressed as mean $\pm$ standard error of the mean (SEM). Unpaired Student's t test or one/two-way ANOVA (for more than 2 groups) analysis were used to calculate the differences in mean values, using GraphPad Prism $\text{\textcircled{R}}$ 8.2.1 software (San Diego, USA). $p < 0.05$ was considered as statistically significant.

Results

3.1. RIPK1 deficiency in hepatocytes sensitizes the liver to short-term fasting-induced liver injury and hepatocyte apoptosis

To investigate the physiological functions of RIPK1 in liver homeostasis, hepatocyte-specific RIPK1 deficient mice (*Ripk1* ^{Δ hep} mice) were generated by crossing *Ripk1*^{fl/fl} mice with Albumin-Cre transgenic mice. Deletion of RIPK1 in hepatocytes was confirmed by the analysis of RIPK1 expression in the liver tissue (**Fig. S1 A** ). Consistent with previous reports ^{[20 }, *Ripk1* ^{Δ hep} mice were normal and healthy. Then both *Ripk1* ^{Δ hep} mice and their control littermates were subjected to short-term 12-hour fasting from 8:00 pm-8:00 am. To our surprise, short-term fasting induced a significant increase in serum transaminase (alanine amino-transferase (ALT) and aspartate amino-transferase (AST)) levels in *Ripk1* ^{Δ hep} mice but not in their wild type controls, suggesting that RIPK1 deficiency sensitizes the liver to short-term fasting, inducing acute liver injury (**Fig. 1A**  and **1B** ). Since RIPK1 is a key player regulating cell death including caspase-8-mediated apoptosis and RIPK3-MLKL-mediated necroptosis, we next examined whether the liver injury was due to cell apoptosis or necroptosis. As **Fig. 1C&D**  shown, the number of TUNEL-positive cells in the liver of *Ripk1* ^{Δ hep} mice was significantly higher compared to the control mice. Cleaved caspase-3 and phospho-MLKL are widely recognized as key proteins involved in the execution of apoptosis

and necroptosis, respectively, and are commonly used as markers to detect these types of cell death. As indicated in **Fig. 1E&F**, the number of cleaved caspase-3-positive cells was significantly higher in *Ripk1^{Δhep}* mice compared to controls. However, we did not observe any change in the number of phospho-MLKL-positive cells in *Ripk1^{Δhep}* mice (**Fig. 1G**). These results suggest that fasting-induced acute liver injury in *Ripk1^{Δhep}* mice might be due to apoptosis rather than necroptosis. To further investigate which cell types were undergoing apoptosis, we conducted the co-staining with TUNEL and Alb immunofluorescence, and the results demonstrated that most of TUNEL-positive cells co-localized with Alb-positive staining (**Fig. 1H**), suggesting that short-term fasting induced apoptosis mainly in hepatic parenchymal cells in *Ripk1^{Δhep}* mice.

It is known that fasting induces hypoglycemia and adipose tissue lipolysis, leading to the release of free fatty acids, which are transported to liver to facilitate the production of ketone bodies through fatty acid beta oxidation. We next investigated whether RIPK1 deficiency in hepatocytes affected the metabolic parameters during fasting. As **Fig. 1I-O** shown, short-term fasting significantly decreased the blood glucose level, elevated the plasma levels of triglycerides (TG), total cholesterol (TC), free fatty acids (FFA) and β -hydroxybutyrate (BHB), and there was no obvious difference between wild type control and *Ripk1^{Δhep}* mice, suggesting that RIPK1 deficiency did not affect the lipid metabolism in liver during fasting.

We next investigated whether sex difference affects fasting-induced acute liver injury in *Ripk1^{Δhep}* mice. Female *Ripk1^{fl/fl}* mice and their *Ripk1^{Δhep}* littermates were subjected to short-term fasting. Consistent with male mice, we observed a significant increase in serum ALT/AST levels and hepatic TUNEL-positive cells in *Ripk1^{Δhep}* female mice (**Fig. S1B-D**), suggesting that RIPK1 deficiency sensitizes the liver to short-term fasting-induced liver injury and hepatocyte apoptosis in both male and female mice.

3.2. Short-term fasting induced hepatic inflammation and compensatory proliferation in *Ripk1^{Δhep}* mice

As a central hub for metabolism and many other physiological processes, liver coordinates a series of adaptations to maintain tissue homeostasis during food and energy fluctuations. Normally, short-term or temporary fasting will not disrupt liver homeostasis and cause tissue cell death and inflammation. However, consistent with liver injury and hepatocyte death, short-term fasting induced hepatic inflammation in *Ripk1^{Δhep}* mice. As demonstrated in **Fig. 2A**, the transcriptional expression of inflammatory markers, including MCP-1, TNF- α , IFN- γ , and IL-6, was all significantly induced in *Ripk1^{Δhep}* mouse liver tissue, in contrast to wild type littermates. We also examined the protein level of TNF α by histological staining (**Fig. 2B&C**). As liver damage is known to activate compensatory proliferation, which is thought to promote hepatocarcinogenesis [27, 28], we also evaluated the expression of AFP, a marker gene of hepatocellular carcinoma, and Ki67, a proliferation-related antigen, and found a significant increase in both markers in *Ripk1^{Δhep}* mice after fasting (**Fig. 2D**). To validate these findings at the protein level, we used immunohistochemistry of Ki67 to measure the rate of compensatory proliferation in livers and found a significant increase in the number of Ki67-positive cells in *Ripk1^{Δhep}* mice after 12 hours of fasting when liver injury was detected (**Fig. 2E**). Taken together, these results suggested that short-term fasting ignited inflammation and caused compensatory proliferation in *Ripk1^{Δhep}* mice.

3.3 Transcriptomic profile of the mouse liver tissue upon short-term fasting

We next analyzed the liver transcriptome profiles to further characterize how short-term fasting oriented the hepatic microenvironment into an inflammatory state in *Ripk1^{Δhep}* mice. Consistent with previous reports, fasting significantly remodeled the transcriptional expression of various genes associated with metabolism in wild type mice, among which 454 genes were up-regulated

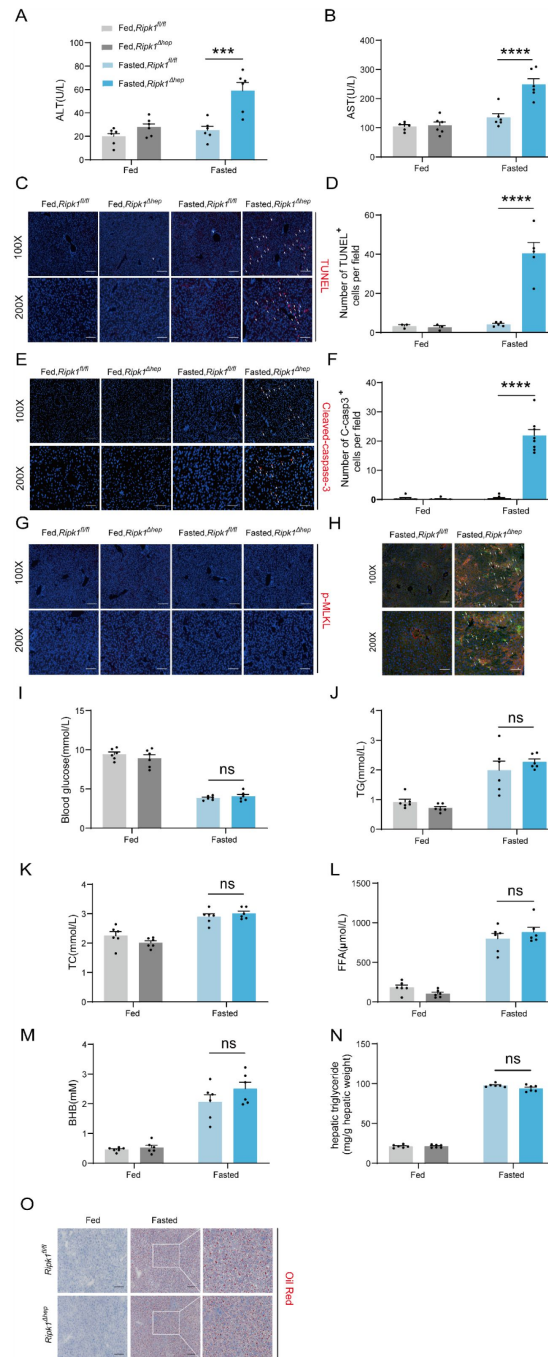


Figure 1.

RIPK1 deficiency in hepatocytes sensitizes the liver to short-term fasting-induced liver injury and hepatocyte apoptosis. (A) Serum alanine amino-transferase (ALT) levels. (B) Serum aspartate amino-transferase (AST) levels. (C&D) Representative images and quantification of TUNEL staining. Scale bar, 50 or 100 μ m. (E&F) Representative images and quantification of liver sections stained with anti-cleaved caspase 3 antibody (red) and DAPI (blue). Scale bar, 50 or 100 μ m. (G) Representative images of liver sections stained with anti-phospho-MLKL antibody (red) and DAPI (blue). Scale bar, 50 or 100 μ m. (H) Fluorescence microscopy images of the liver stained with anti-Alb antibody (red), TUNEL (green), and DAPI (blue). Scale bar, 50 or 100 μ m. (I) Blood glucose levels. (J) Serum triglycerides (TG) levels. (K) Serum total cholesterol (TC) levels. (L) Serum free fatty acids (FFA) levels. (M) Serum β -hydroxybutyrate (BHB) levels. (N) Hepatic triglyceride (TG) levels (mg/g tissue). (O) Liver tissue was stained by Oil Red O. Scale bar, 100 μ m. The data was analyzed via two-way ANOVA or one-way ANOVA. Data are expressed as mean $\pm$ SEM (n=6 per group). Asterisks denote statistical significance. ns, no significant, * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

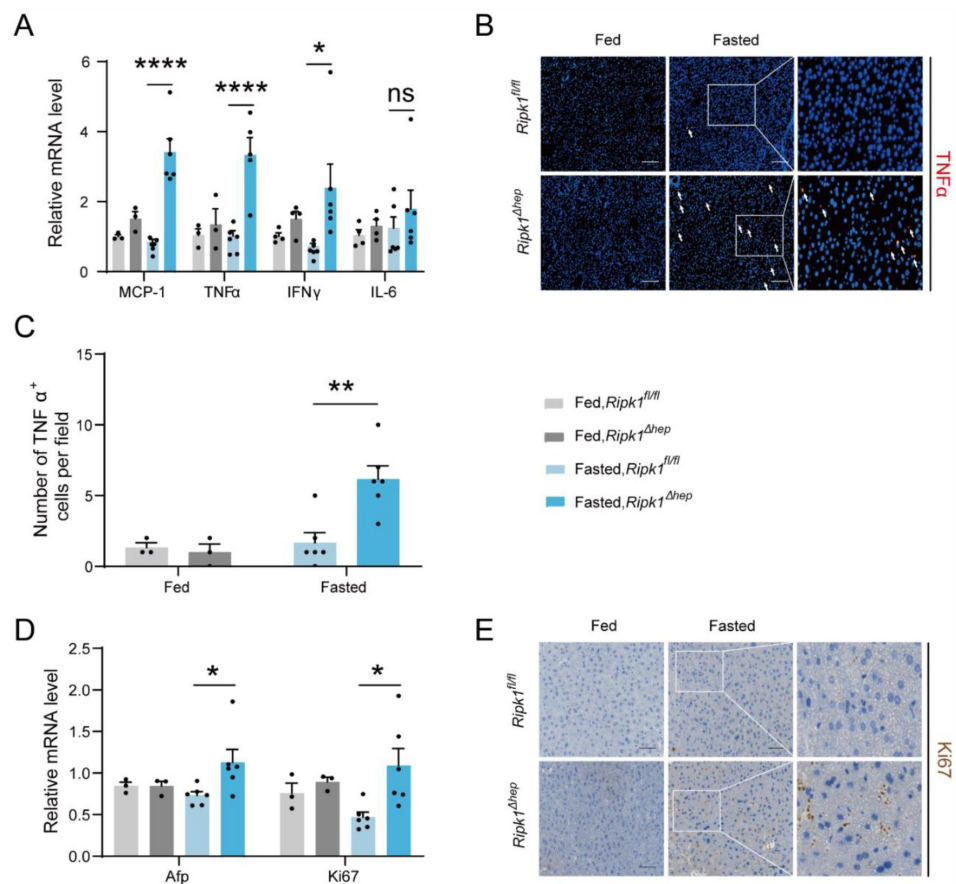


Figure 2.

Short-term fasting induced hepatic inflammation and compensatory proliferation in *Ripk1^{Δhep}* mice. (A) Hepatic mRNA expression of the inflammatory molecules. (B&C) Representative images and quantification of the liver stained with anti-TNFα antibody (red) and DAPI (blue). Scale bar, 100 μm. (D) Transcriptional expression of Afp and Ki67 in liver tissue. (E) Representative images of the liver stained with anti-Ki67 antibody (brown) and hematoxylin (blue). Scale bar, 100 μm. The data was analyzed via two-way ANOVA or one-way ANOVA. Data are expressed as mean ± SEM (n=6 per group). ns, no significant, * P < 0.05, *** P < 0.001, **** P < 0.0001.

and 545 genes were down-regulated (**Fig. S2A**). As expected, pathway enrichment (KEGG) analysis revealed that these differentially expressed genes were primarily enriched in metabolism-associated pathways, including fatty acid degradation, PPAR signaling pathway and MAPK signaling pathway (**Fig. S2B**). Cluster analysis showed that fasting significantly altered metabolism-related genes in *Ripk1^{fl/fl}* mice (**Fig. S2C**). To be noteworthy, in wild type mice, fasting normally suppressed the expression of immune- or inflammation-related genes, in agreement with the shifted energy to guarantee the need for basic survival other than immune protection during food deprivation. RIPK1 deficiency in hepatocytes did not greatly affect the gene expression profiles when food was available and there were 25 up-regulated and 54 down-regulated genes which were mostly associated with alcoholic liver disease, vascular smooth muscle contraction and cardiomyopathy in *Ripk1^{Δhep}* mice compared to wild type control at fed state (**Fig. S2D&E**). However, when subjected to metabolic fluctuation with short-term fasting, loss of RIPK1 in hepatocytes amplified the affect, and there were additional 85 up-regulated and 29 down-regulated genes identified in *Ripk1^{Δhep}* mice compared to the *Ripk1^{fl/fl}* group ($p < 0.05$, $|\log_2(\text{fold change})| \geq 1$) (**Fig. 3A**). Pathway enrichment (KEGG) analysis revealed that these differentially expressed genes (DEGs) were primarily enriched in inflammation-associated pathways, including TNF signaling pathway, IL-17 signaling pathway, toll-like receptor signaling pathway and NF- κ B signaling pathway, etc. (**Fig. 3B**). Cluster analysis of the differentially expressed genes revealed that hepatocyte-specific deletion of RIPK1 significantly induced the expression of many genes involved in the inflammatory process after fasting, including *Ccl2*, *Clec4e*, *Ikbke*, and *Tnfaip3* etc (**Fig. 3C**). Through further analysis of the transcriptome data, we observed a significant increase in the expression chemokine-related genes involved in the recruitment of immune cells, such as *ITGAM*, *CCR2*, and *CX3CR1* (**Fig. 3C**). To validate these findings at the protein level, we conducted immunofluorescence staining for F4/80, a marker for liver-resident immune cells, and *CD11b*, *CCR2*, and *CX3CR1*, markers for liver-recruited immune cells. Our results demonstrated a significant increase in the number of *CD11b*, *CCR2*, and *CX3CR1* positive cells in *Ripk1^{Δhep}* mice after fasting, while F4/80 expression remained unchanged (**Fig. 3D** and **3E**). These results suggested that in contrast to the immunosuppression state of liver during fasting, RIPK1 deficiency sensitize the liver into a proinflammatory state, with induced expression of inflammation and immune cell recruitment-associated genes. Hepatic gene expression profile reflected the dynamics of recruited macrophages into liver in *Ripk1^{Δhep}* mice. RIPK1 might be act as an important protein to maintain the immune tolerant homeostasis in liver during metabolism changes.

3.4. Single-cell RNA sequencing analysis of the mouse liver tissue

Transcriptomic profiling of whole liver tissue revealed that hepatic inflammation induced by fasting in *Ripk1^{Δhep}* mice was likely arising from enhanced recruitment of immune cells to the liver. To confirm this hypothesis, we performed single-cell RNA sequencing of liver non-parenchymal cells isolated from *Ripk1^{Δhep}* or *Ripk1^{fl/fl}* mice upon fasting. A total of 22274 single-cell transcriptomes (10374 *Ripk1^{fl/fl}*, 11900 *Ripk1^{Δhep}*) were obtained, and thirty major clusters were identified by T-distributed stochastic neighbor embedding (t-SNE) visualization, representing different cell types based on marker gene expression (**Fig. 4A&B**). As the result shown, short-term fasting led to significant changes in the cellular composition of the liver in *Ripk1^{Δhep}* mice, characterized by an increase in the number of cells in clusters 12, 14, 19, 22, and 28 and a decrease in the number of cells in clusters 9, 11, 22, 26, and 29, compared to control mice (**Fig. 4C** & **4D**). In order to further explore the significance of these changes, we identified 8 major cellular clusters in 12-hour fasted control liver, including B cells (*Cd19*, *Cd79a*, *Fcmmr*, *Cd79b*, *Ebf1*), neutrophils (*S100a8*, *Csf3r*, *S100a9*, *Mmp9*), ECs (*Igfbp7*, *Ptprb*, *Clec4g*, *Kdr*), PCs (*SDC1*, *CD138*), DCs (*Ly6d*, *Siglech*, *Rnase6*, *H2-Ab1*), NK&T cells (*Nkg7*, *Xcl1*, *Cd3d*, *Gzma*), macrophages (*Adgre1*, *Csf1r*, *Sdc3*, *Ifitm2*) and hepatocytes (*Alb*, *Saa1*, *Apoc1*, *Mup20*) (**Fig. 4E**) [29, 30]. As the result shown, the numbers of macrophages and NK&T cells were increased in the liver of *Ripk1^{Δhep}* mice

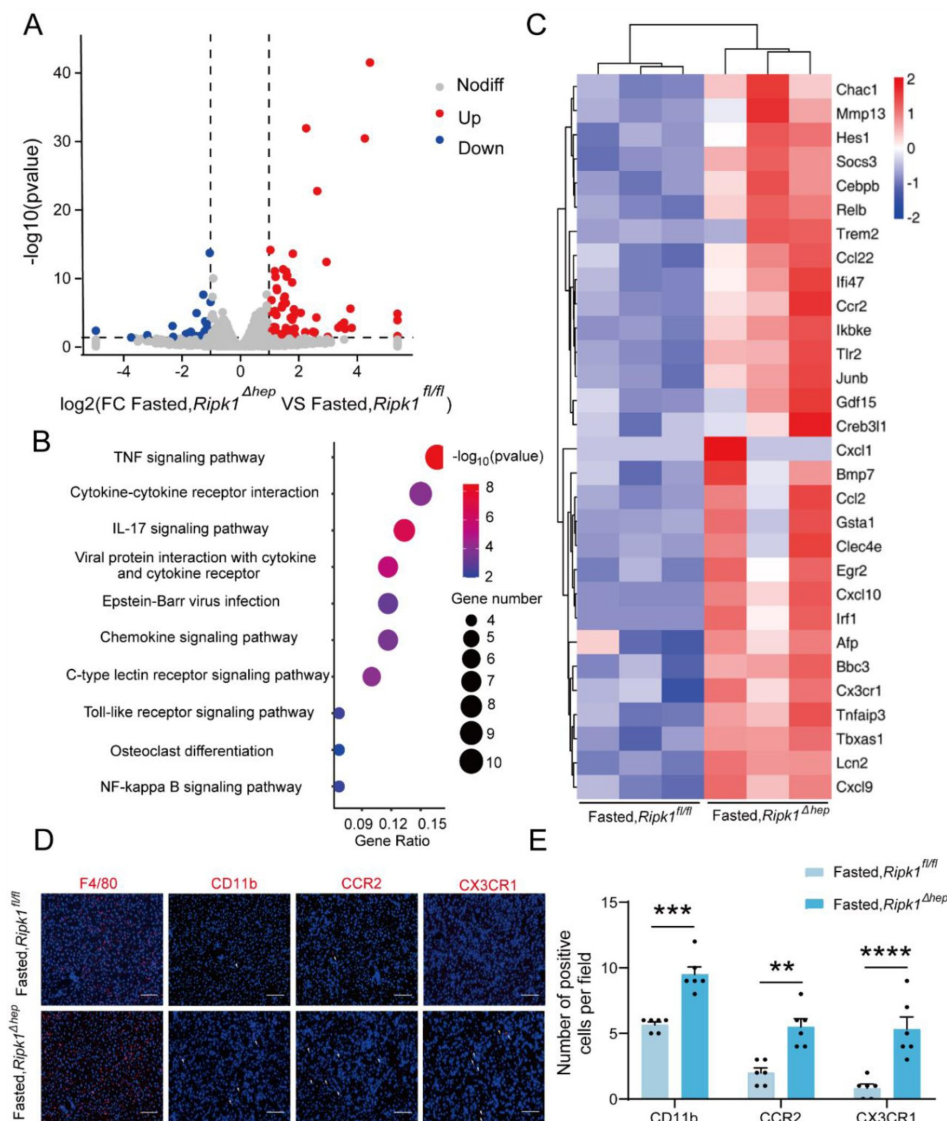


Figure 3.

Transcriptome sequencing of the liver tissue from $Ripk1^{fl/fl}$ and $Ripk1^{\Delta hep}$ mice. (A) The volcano plot of differentially expressed genes was illustrated. The blue spots represent the down-regulated genes in $Ripk1^{\Delta hep}$ group compared with control ($Ripk1^{fl/fl}$) group, and the red spots represent the up-regulated genes in $Ripk1^{\Delta hep}$ group. (B) The altered signaling pathways were enriched by KEGG analysis. (C) The genes which expression were significantly altered in $Ripk1^{\Delta hep}$ group were depicted in the heat map. (D&E) Representative fluorescence microscopy images and quantification of the liver stained with anti-F4/80 antibody (red), anti-CD11b antibody (red), anti-CCR2 antibody (red), anti-CX3CR1 antibody (red) and DAPI (blue), respectively. Scale bar, 100 μm . The data was analyzed via two-way ANOVA or one-way ANOVA. Data are expressed as mean $\pm$ SEM (n=6 per group). ns, no significant, * P < 0.05, *** P < 0.001, **** P < 0.0001.

compared to that of *Ripk1^{fl/fl}* mice upon short-term fasting, suggesting the dynamics of recruited immune cells into liver in *Ripk1^{Δhep}* mice (**Fig. 4F**). We next focused on liver macrophages, which might contribute to the fasting-induced inflammatory state in *Ripk1^{Δhep}* mice.

As expected, pathway enrichment (KEGG) analysis based on macrophage differential genes revealed that these differentially expressed genes were primarily enriched in inflammation-associated pathways, including IL-17 signaling pathway, Antigen processing and presentation and TNF signaling pathway, etc. (**Fig. 4G**). We performed sub-clustering of macrophages to further dissect changes in the landscape of macrophages upon RIP1 deficiency in hepatocytes. This cluster can be further divided into two groups of cells representing liver-resident macrophages (LrMs: KCs) and recruited macrophages (RMs) based on their marker gene expression profile (**Fig. 4H**). LrMs were characterized by high expression of *Adgre1* (encoding F4/80) and *Clec4f*, whereas RMs exhibited high expression of *Itgam* (encoding Cd11b, an important marker gene of bone marrow derived macrophages), *Ccr2* (a chemokine receptor important for infiltration of circulating monocytes) and *CX3CR1*^[31]. We observed that while the number of LrMs remained almost unchanged in *Ripk1^{Δhep}* mice after short-term fasting compared to *Ripk1^{fl/fl}* mice, the number of RMs significantly increased (**Fig. 4I**). These results suggested that in *Ripk1^{Δhep}* mice compared to wild type mice, fasting induced hepatic inflammation was characterized by altered hepatic gene expression profiles and changed liver cell compartment, with increased immune cells in liver.

3.5. Endoplasmic reticulum stress is required for fasting-induced liver injury and inflammation in *Ripk1^{Δhep}* mice

We next aimed to investigate the underlying mechanism of short-term fasting-induced liver injury and inflammation in *Ripk1^{Δhep}* mice. It is known that fasting-induced lipolysis in peripheral adipose tissue will lead to hepatic lipid burden, and excessive free fatty acids deposition has been shown to induce endoplasmic reticulum stress (ER stress) in liver. In addition, previous studies have shown that RIPK1 promotes apoptosis in response to unresolved ER stress^[32]. As **Fig. 1N&O** indicated, the fasting strategy we employed indeed increased the lipid content in liver tissue. We also detected the expression of ER stress markers in the liver tissues of fed- or fasted-mice, respectively. As **Fig. 5A** indicated, short-term fasting indeed increased the expression of CHOP, GRP78, IRE1a and phosphor-IRE1a at protein levels, suggesting that short-term fasting caused ER stress in liver. To investigate whether ER stress is involved in the fasting-induced liver injury in *Ripk1^{Δhep}* mice, *Ripk1^{fl/fl}* and *Ripk1^{Δhep}* mice were pretreated with the ER stress inhibitor 4-PBA and then the mice were subjected to short-term fasting for 12 hours (**Fig. 5B**). As **Fig. 5C** shown, 4-PBA treatment successfully reduced the expression of ER stress markers, confirming the efficiency of 4-PBA to inhibit ER stress. Interestingly, 4-PBA pretreatment effectively reduced serum ALT/AST levels and the number of TUNEL-positive cells in the liver of fasted *Ripk1^{Δhep}* mice, suggesting that ER stress was involved in fasting-induced liver injury and hepatocyte apoptosis in *Ripk1^{Δhep}* mice (**Fig. 5D**–**5F**). Furthermore, 4-PBA pretreatment also effectively inhibited the increased expression of inflammatory markers (MCP-1, TNF-α, IFN-γ and IL-6) and proliferation markers AFP in the liver tissue of fasted *Ripk1^{Δhep}* mice (**Fig. 5G&H**). These results indicated that 4-PBA not only prevented hepatocyte apoptosis and liver injury induced by fasting in *Ripk1^{Δhep}* mice, but also mitigated the hepatic inflammation and compensatory proliferation, suggesting that ER stress was involved in the process of fasting-induced liver injury and inflammation in *Ripk1^{Δhep}* mice.

3.6. AAV-TBG-Cre-mediated liver-specific RIPK1 knockout confirms fasting-induced acute liver injury in mice

We employed another different approach to conditionally delete RIPK1 from hepatocytes by injecting AAV8-TBG-Cre virus into *Ripk1^{fl/fl}* mice. Four weeks after virus injection, the mice were subjected for short-term fasting as above studies, and then serum and liver tissue were harvested

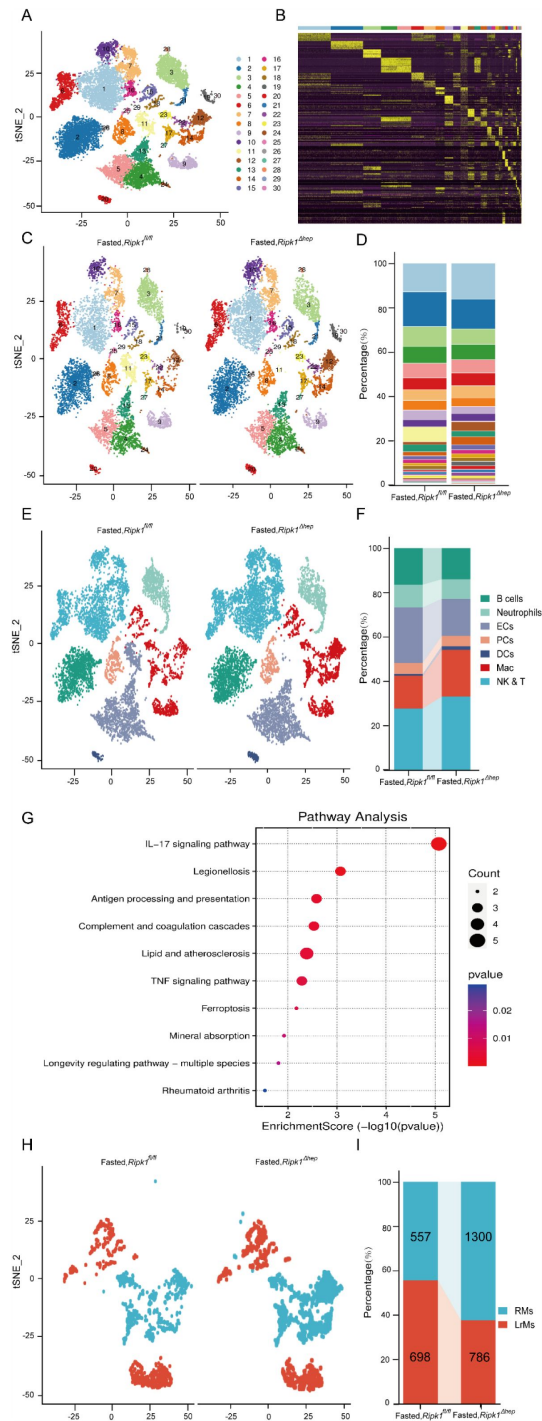


Figure 4.

Single-cell RNA sequencing of the liver tissue from *Ripk1^{fl/fl}* and *Ripk1^{Δhep}* mice. (A) t-SNE visualization of liver cell clusters based on 22,274 single-cell transcriptomes. (B) Heatmap of cluster marker genes. (C) t-SNE plots display color-coded cell clusters of cells in the *Ripk1^{fl/fl}* (left) and *Ripk1^{Δhep}* (right) mice liver tissues. (D) Bar charts show the proportion of major cell clusters among all cells at different genotypes after fasting by scRNA-seq. (E) t-SNE plots display color-coded cell subtypes of cells in the *Ripk1^{fl/fl}* (left) and *Ripk1^{Δhep}* (right) mice liver tissues. (F) Bar charts display the proportion of major cell subtypes within all different genotypes after fasting. (G) The altered signaling pathways associated with differential gene changes in macrophages were enriched by KEGG analysis. (H) t-SNE plots display color-coded cell subtypes of macrophages in the *Ripk1^{fl/fl}* (left) and *Ripk1^{Δhep}* (right) mice liver tissues. (I) Bar charts display the proportion of major cell subtypes within macrophages after fasting.

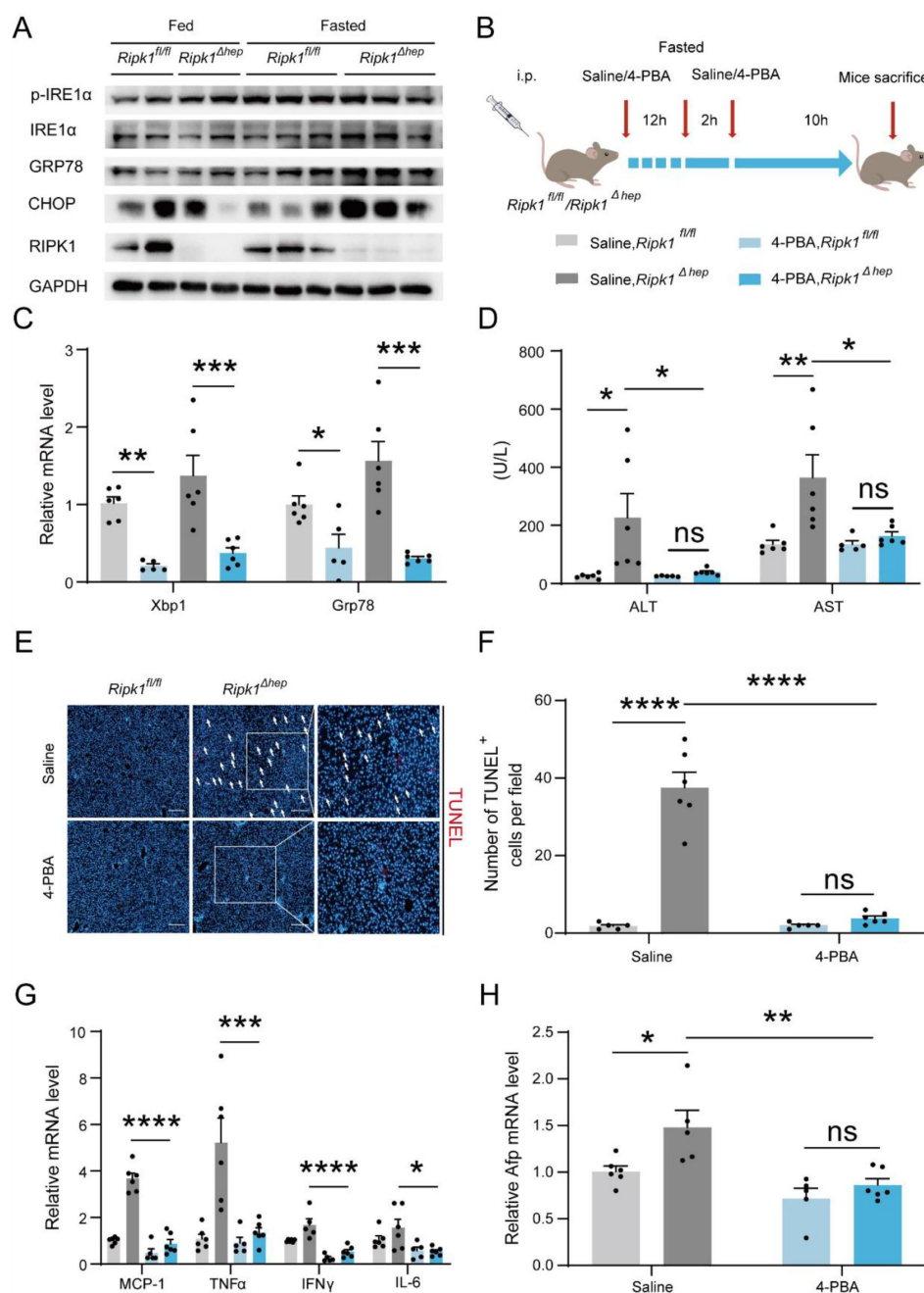


Figure 5.

Inhibitor of ER stress 4-PBA effectively rescued the fasting-induced liver injury and inflammation in *Ripk1^{Δhep}* mice. (A) Western blot analysis of p-IRE1α, IRE1α, GRP78, CHOP, RIPK1 and GAPDH in liver tissue. (B) Experiment schema. (C) Transcriptional expression of ER stress genes in mouse liver. (D) Serum alanine amino-transferase (ALT) and aspartate amino-transferase (AST) levels. (E&F) Fluorescence microscopy image and quantification of TUNEL staining. Scale bar, 100 μm. (G) Expression (qPCR) of inflammatory genes in the livers. (H) Expression (qPCR) of Afp in the livers. The data was analyzed via two-way ANOVA or one-way ANOVA. Data are expressed as mean ± SEM (n=4-6 per group). ns, no significant, * P < 0.05, *** P < 0.001, **** P < 0.0001.

(Fig. 6A). Immunoblot analysis confirmed that RIPK1 was specifically deleted in hepatocytes by AAV8-TBG-Cre virus treatment, in contrast to the control virus AAV8-TBG-null (Fig. 6B). Our results indicated that AAV8-TBG-Cre virus-induced RIPK1 deficient mice phenocopied *Ripk1^{Δhep}* mice, that hepatocyte-specific loss of RIPK1 sensitized the mice to short-term fasting-induced acute liver injury and hepatocyte apoptosis (Fig. 6C-F). Consistently, there was no significant alteration in serum TG, TC and liver TG levels of *Ripk1^{fl/fl}* mice injected with AAV8-TBG-Cre or control AAV8-TBG-null virus (Fig. 6G-I). We also detected the expression of ER stress markers in the liver tissue of AAV8-TBG-null or AAV8-TBG-Cre mice, respectively. As Fig. 6B&J indicated, short-term fasting indeed increased the expression of CHOP, GRP78, IRE1α and phosphor-IRE1α at both mRNA and protein levels, indicating that fasting induced the occurrence of ER stress in RIPK1-deleted livers. As demonstrated in Fig. 6B&K, transcriptional expression of inflammatory markers, including MCP-1, TNF-α and IL-1β, were also significantly induced in AAV8-TBG-Cre-mouse liver tissue, in contrast to AAV8-TBG-null-mouse. Consistent with previous results, the number of cleaved caspase-3-positive cells was significantly higher in AAV8-TBG-Cre-mouse compared to controls (Fig. 6L&M). Taken together, these results suggested that hepatocyte-specific loss of RIPK1, achieved by different strategies, made the mice fragile to metabolic disturbance, and even short-term fasting would result in liver injury, hepatocyte cell death, hepatic inflammation and ER stress.

3.7. Short-term high fat diet feeding induced liver injury, hepatic apoptosis, inflammation and endoplasmic reticulum stress in *Ripk1^{Δhep}* mice

We next aimed to explore whether other metabolic disturbance exhibited similar effect as short-term fasting in *Ripk1^{Δhep}* mice. It's known that short-term fasting leads to lipolysis in adipose tissue and temporary lipid burden to hepatocytes. We therefore created a short-term high fat diet (HFD) feeding model in both wild-type control and *Ripk1^{Δhep}* mice, to mimic the dietary-induced lipid metabolism disturbance. *Ripk1^{fl/fl}* and *Ripk1^{Δhep}* mice were fed with HFD or normal diet (ND) for 6 days respectively (Fig. 7A). We observed a significant increase in liver lipid deposition, liver triglyceride (TG), and serum total cholesterol (TC) in mice fed with HFD compared to those fed with ND (Fig. 7B-D). Previous studies reported that short-term HFD feeding was not enough to induce hepatic disorder, without obvious effects on the serum ALT/AST, liver histology, and our results with *Ripk1^{fl/fl}* mice were in agreement with these findings (Fig. 7E&F). In contrast, upon 6-day HFD feeding, the serum ALT/AST levels, hepatocyte apoptosis as measured by TUNEL and hepatic inflammation were significantly elevated in *Ripk1^{Δhep}* mice (Fig. 7E-I). Additionally, HFD feeding significantly induced the expression of AFP in *Ripk1^{Δhep}* mice, suggesting that HFD feeding promoted tumorigenesis in *Ripk1^{Δhep}* mice (Fig. 7J). We also found that the expression of ER stress-related genes was obviously increased in *Ripk1^{Δhep}* mice after 6 days of HFD feeding (Fig. 7K).

Taken together, these results suggest that hepatocyte RIPK1 is essential for the maintenance of hepatic homeostasis against the disturbance induced by different metabolism behaviours, including fasting and temporary high-fat diet feeding.

Discussion

In this study, we demonstrated that the specific deletion of RIPK1 in hepatocytes exacerbated the liver's vulnerability to metabolic disturbances, such as short-term fasting and high-fat diet feeding, resulting in increased liver damage, apoptosis, inflammation, and compensatory proliferation. We utilized single-cell RNA sequencing and bulk RNA sequencing to characterize the hepatic cellular profiles and transcriptional profiles in response to the physiological inflammation induced by the

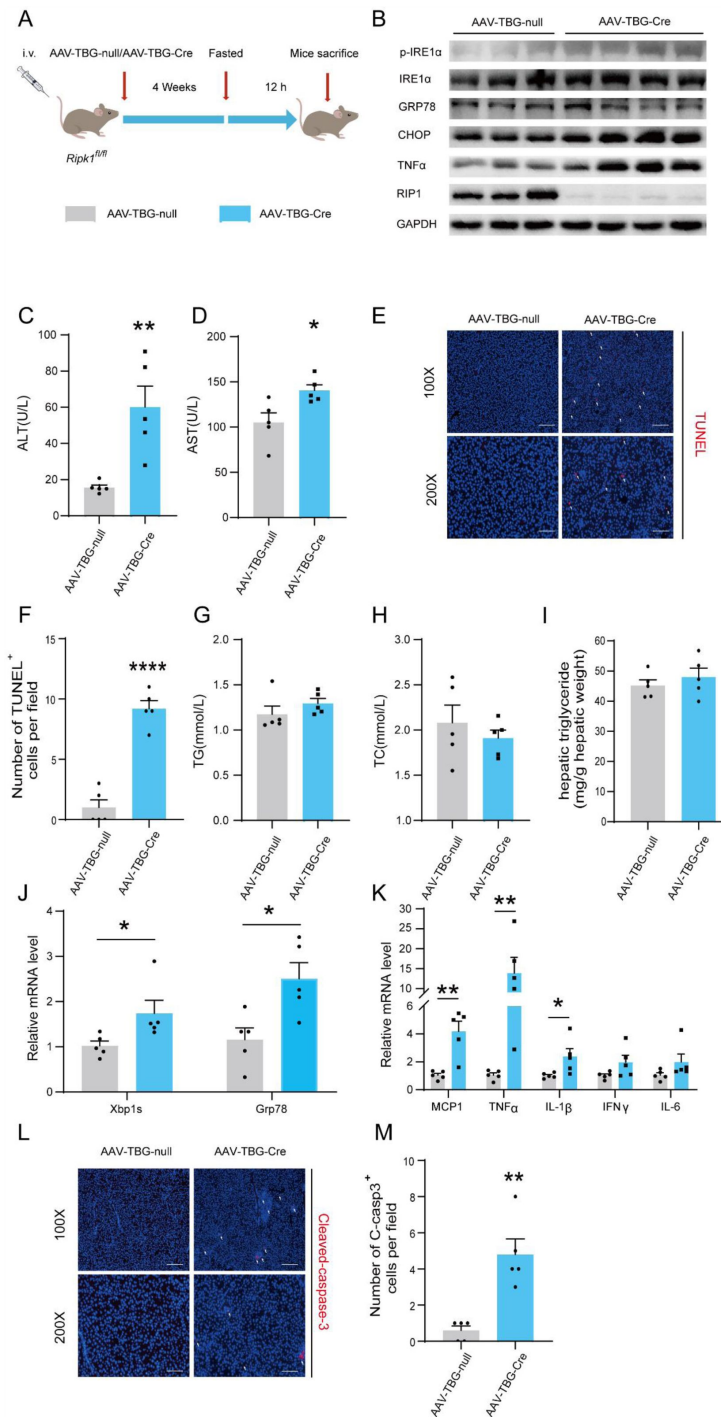


Figure 6.

AAV-TBG-Cre-mediated liver-specific RIPK1 knockout confirms fasting-induced acute liver injury in mice (A) Schema of AAV8-TBG-Cre administration. (B) Western blot analysis of p-IRE1α, IRE1α, GRP78, CHOP, TNFα, RIPK1 and GAPDH in liver tissue. (C) Serum alanine amino-transferase (ALT) levels. (D) Serum aspartate amino-transferase (AST) levels. (E&F) Fluorescence microscopy image and quantification of TUNEL staining. Scale bar, 50 or 100 μm. (G) Serum triglycerides (TG) levels. (H) Serum total cholesterol (TC) levels. (I) Hepatic triglyceride (TG) levels (mg/g tissue). (J) Expression (qPCR) of ER stress genes in the livers. (K) Expression (qPCR) of inflammatory genes in the livers. (L&M) Fluorescence microscopy images and quantification of liver of *Ripk1*^{Δhep} and control mice stained with anti-cleaved caspase 3 antibody (red) and DAPI (blue). Scale bar, 50 or 100 μm. The data was analyzed via two-way ANOVA or one-way ANOVA. Data are expressed as mean ± SEM (n=4-6 per group). ns, no significant, * P < 0.05, *** P < 0.001, **** P < 0.0001.

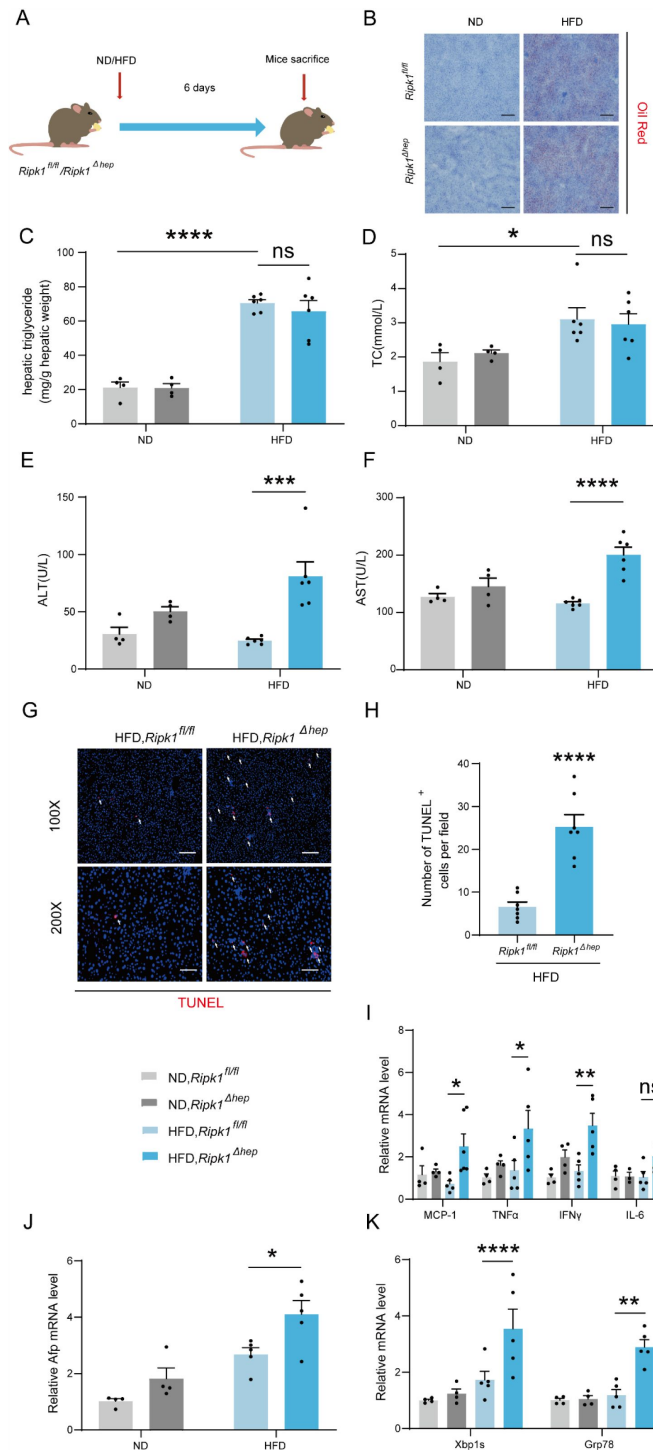


Figure 7.

Short-term high fat diet feeding induced liver injury, hepatic apoptosis, inflammation and endoplasmic reticulum stress in *Ripk1^{Δhep}* mice. (A) Schema of HFD administration. (B) Liver tissue was stained by Oil Red O. Scale bar, 100 μ m. (C) Hepatic triglyceride (TG) levels (mg/g tissue). (D) Serum total cholesterol (TC) levels. (E) Serum alanine amino-transferase (ALT) levels. (F) Serum aspartate amino-transferase (AST) levels. (G&H) Fluorescence microscopy image and quantification of TUNEL staining. Scale bar, 50 or 100 μ m. (I) Expression (qPCR) of inflammatory genes in the livers. (J) Expression (qPCR) of Afp in the livers. (K) Expression (qPCR) of ER stress markers in the livers. The data was analyzed via two-way ANOVA or one-way ANOVA. Data are expressed as mean $\pm$ SEM (n=4-6 per group). ns, no significant, * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

disruption of homeostasis. Furthermore, we presented evidence indicating the involvement of ER stress in sensitizing RIPK1-deleted liver tissue. In summary, we revealed a novel physiological role of RIPK1 as a scaffold in maintaining liver homeostasis during fasting and other nutritional disturbance. These findings could prove valuable in tailoring intermittent fasting or calorie restriction regimens for specific populations based on their *Ripk1* gene polymorphism or expression profiles.

Our work shed light on the intricate interplay between cell death, inflammation, and metabolism. While the pathophysiological roles of RIPK1 have primarily been studied in inflammatory diseases and pathogen infections, emerging evidence suggests its involvement in metabolism-related pathways [15, 16, 33]. Previous studies, including our own, have linked RIPK1's kinase activity to the pathogenesis of metabolic diseases like NASH, indicating a metabolic regulatory role of RIPK1 kinase [13, 14, 34]. Subsequently, UDP-glucose 6-dehydrogenase (UGDH) and UDP-glucuronate metabolism are found to suppress NASH pathogenesis and control hepatocyte apoptosis through inhibiting RIPK1 kinase activity, further solidifying the connection between RIPK1 kinase activity and metabolism during the pathogenesis of NASH [35]. In addition to its pathological roles, RIPK1's involvement in metabolism physiology has become increasingly apparent. Mei et al. found that the postnatal lethality of *Ripk1*^{-/-} mice was attributed to dysregulated aspartate metabolism, leading to impaired starvation-induced autophagy [15]. Zhang et al. recently reported that the classical energy sensor AMPK is able to phosphorylate RIPK1 at S416 in response to metabolic stress like glucose deprivation in vitro and fasting in vivo, and this phosphorylation of RIPK1 by AMPK represents a survival mechanism to keep the kinase activity of RIPK1 in check to prevent RIPK1 kinase-mediated cell death. AMPK deficiency sensitized cells to glucose deprivation-induced cell death [33]. Their results directly linked RIPK1 to key metabolism regulator AMPK. Together with our results, it suggested that hepatic AMPK-RIPK1 axis function as a mechanism to maintain liver homeostasis during suffering metabolic stress. Moreover, *Tak1*^{-/-} mice has been shown to exhibit similar phenotypes, suggesting that the key components in the complex I faction in the scaffold complex, all contribute to maintain tissue homeostasis [2]. These findings, together with our study, emphasize the critical role of RIPK1's scaffold function in sensing nutrient stress and maintaining metabolic homeostasis during various starvation conditions, both in neonatal stages and adulthood. Zhang et al. also noted that RIPK1 knockout MEFs were more susceptible to cell death induced by glucose starvation compared to WT MEFs, further underscoring the importance of RIPK1 in nutrient stress responses.

Our study also posited that hepatocyte RIPK1 plays a crucial role in preventing liver damage and inflammation triggered by metabolic stress. Prior investigations have indicated that deficiencies in TAK1 or NEMO in hepatocytes result in spontaneous liver injury and carcinogenesis in mice, with a more severe phenotype than that observed in RIPK1-deficient mice [27, 36, 37]. This is attributed to the deletion of TAK1 or NEMO unleashing the kinase activity of RIPK1, leading to the activation of RIPK1 kinase and the associated cell death pathways. Our findings, combined with these earlier studies, suggested that the RIPK1-TAK1-NF-κB axis constitutes an essential scaffold platform necessary for the liver's adaptation to metabolic fluctuations. Any improper inactivation or deletion of any component within this scaffold axis disrupts the delicate balance between cell death, inflammation, and normal function, rendering the liver vulnerable to metabolic changes and resulting in liver damage, hepatic inflammation, and compensatory proliferation.

Moreover, in contrast to organs such as the small intestine and lungs, the liver typically maintains immune tolerance and does not incite inflammation in response to various endogenous and exogenous pathogen-associated molecular patterns (PAMPs) or antigens present in the bloodstream. Recent years have witnessed the increasing attention towards physiological inflammation, which implications have been greatly broadened [38, 39]. Our research revealed that hepatocyte RIPK1 serves as a critical mechanism for preserving immune tolerance within the liver microenvironment. Furthermore, we identify an instance of physiological inflammation induced by loss of regulation in normal tissue. The physiological inflammation observed in RIPK1-

deficient livers during short-term fasting is milder but akin to hepatic inflammation induced by pathogenic infections and other pathological conditions. It is characterized by the upregulation of typical molecules, including MCP-1, TNF- α , IFN- γ , and IL-6 etc, and the recruitment of macrophages into liver tissue, indicating systemic adaptive responses.

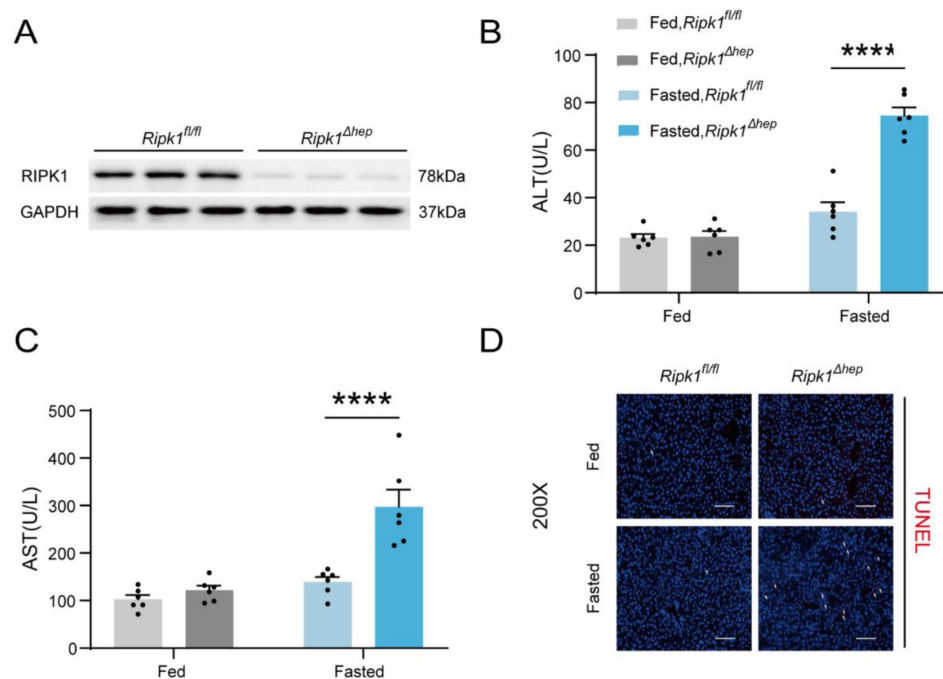
In summary, our study revealed the multifaceted role of RIPK1 in maintaining liver homeostasis in the face of metabolic challenges, shedding light on the intricate interplay between cell death, inflammation, and metabolism. Our findings provide new insights into the role of RIPK1 in various physiological contexts.

Acknowledgements

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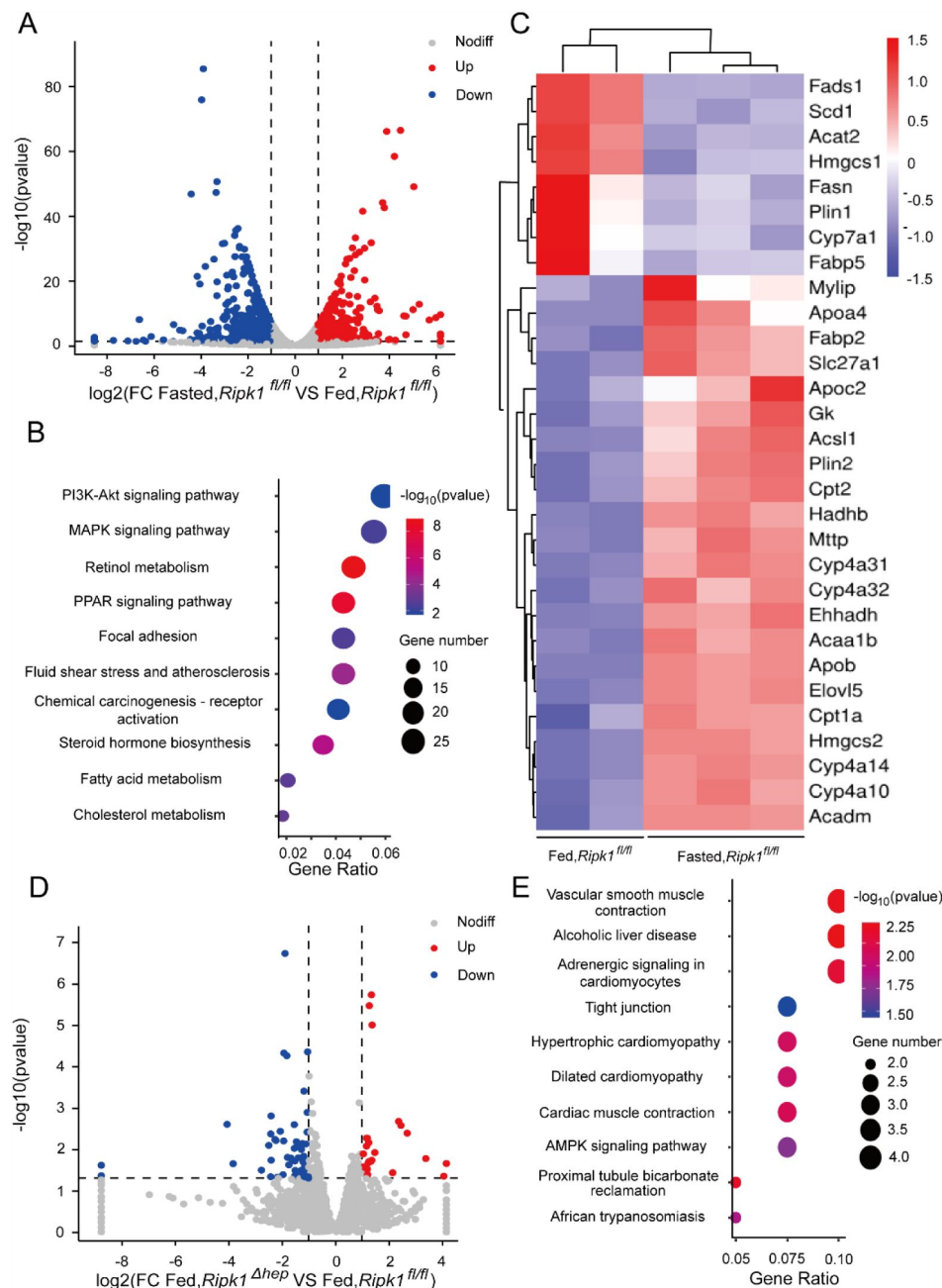
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Supplementary Figure 1.

RIPK1 deficiency in hepatocytes sensitizes the liver to short-term fasting-induced liver injury and hepatocyte apoptosis in female mice. (A) Western blot analysis of RIPK1 and GAPDH in liver tissue. (B) Serum alanine amino-transferase (ALT) levels. (C) Serum aspartate amino-transferase (AST) levels. (D) Representative fluorescence microscopy images of TUNEL staining. Scale bar, 50 μm. The data was analyzed via two-way ANOVA or one-way ANOVA. Data are expressed as mean ± SEM (n=4-6 per group). Asterisks denote statistical significance. ns, no significant, * P < 0.05, *** P < 0.001, **** P < 0.0001.



Supplementary Figure 2.

Transcriptome sequencing of the liver tissue from *Ripk1^{fl/fl}* and *Ripk1^{Δhep}* mice. (A) The volcano plot of differentially expressed genes was illustrated. The blue spots represent the down-regulated genes in fasted group compared with control (fed) group and the red spots represent the up-regulated genes in *Ripk1^{fl/fl}* mice. (B) The altered signaling pathways were enriched by KEGG analysis. (C) The genes which expression were significantly altered in fasted group were depicted in the heat map. (D) The volcano plot of differentially expressed genes was illustrated. The blue spots represent the down-regulated genes in *Ripk1^{Δhep}* group compared with control (*Ripk1^{fl/fl}*) group and the red spots represent the up-regulated genes in *Ripk1^{Δhep}* group. (E) The altered signaling pathways were enriched by KEGG analysis.

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Reviewer #1 (Public Review):

This study presents an investigation into the physiological functions of RIPK1 within the context of liver physiology, particularly during short-term fasting. Through the use of hepatocyte-specific Ripk1-deficient mice (Ripk1 Δ hep), the authors embarked on an examination of the consequences of Ripk1 deficiency in hepatocytes under fasting conditions. They discovered that the absence of RIPK1 sensitized the liver to acute injury and hepatocyte apoptosis during fasting, a finding of significant interest given the crucial role of the liver in metabolic adaptation. Employing a combination of transcriptomic profiling and single-cell RNA sequencing techniques, the authors uncovered intricate molecular mechanisms underlying the exacerbated proinflammatory response observed in Ripk1 Δ hep mice during fasting. While the investigation offers valuable insights into the consequences of Ripk1 deficiency in hepatocytes during fasting conditions, there appears to be a primarily descriptive nature to the study with a lack of clear connection between the experiments. Thus, a stronger focus is warranted, particularly on understanding the dialogue between

hepatocytes and macrophages. Moreover, the data would benefit from reinforcement through additional experiments such as Western blotting, flow cytometry, and rescue experiments, which would offer a more quantitative aspect to the findings. By incorporating these enhancements, the study could achieve a more comprehensive understanding of the underlying mechanisms and ultimately strengthen the overall impact of the research.

Detailed major concerns:

Related to Figure 1.

It is imperative to ensure consistency in the number of animals analyzed across the different graphs. The current resolution of the images appears to be low, resulting in unsharp visuals that hinder the interpretation of data beyond the presence of "white dots". To address this issue, it is recommended to enhance the resolution of the images and consider incorporating zoom-in features to facilitate a clearer visualization of the observed differences. Moreover, it would be beneficial to include a complete WB analysis for the cell death pathways analyzed. These adjustments will significantly improve the clarity and interpretability of Figure 1.

Related to Figure 2.

It is essential to ensure consistency in the number of animals analyzed across the different graphs, as indicated by $n=6$ in the figure legend (similar to Figure 1). Additionally, it is crucial to distinguish between male and female subjects in the dot plots to assess any potential gender-based differences, which should be consistent throughout the paper. To achieve this, the dots plot should be harmonized to clearly differentiate between males and females and investigate if there are any disparities between the genders. Moreover, it is imperative to correlate hepatic inflammation with the activation of Kupffer cells, infiltrating monocytes, and/or hepatic stellate cells (HSCs). Therefore, conducting flow cytometry would be instrumental in achieving this correlation. Additionally, the staining for Ki67 appears to be non-specific, showing a granular pattern reminiscent of bile crystals rather than the expected nuclear staining of hepatocytes or immune cells. It is crucial to ensure specific staining for Ki67, and conducting *in vitro* experiments on primary hepatocytes could further elucidate the proliferation process. These experiments are relatively straightforward to implement and would provide valuable insights into the mechanisms underlying hepatic inflammation and proliferation.

Related to Figure 3 & related to Figure 4.

The immunofluorescence data presented are not entirely convincing and are insufficient to conclusively demonstrate the recruitment of monocytes. Previous suggestions for flow cytometry studies remain pertinent and are indeed necessary to bolster the robustness of the data and conclusions. Conducting flow cytometry analyses would provide more accurate and quantitative assessments of monocyte recruitment, ensuring the reliability of the findings and strengthening the overall conclusions of the study. Regarding the single-cell RNA sequencing analysis presented in the manuscript, it's worth questioning its relevance and depth of information provided. While it successfully identifies a quantitative difference in the cellular composition of the liver between control and knockout mice, it may fall short in elucidating the intricate interactions between different cell populations, which are crucial for understanding the underlying mechanisms of hepatic inflammation. Therefore, I propose considering alternative bioinformatic analyses, such as CellPhone-CellChat, which could potentially provide a more comprehensive understanding of the cellular dynamics and interactions within the liver microenvironment. By examining the dialogue between different cell clusters, these analyses could offer deeper insights into the functional consequences of Ripk1 deficiency in hepatocytes and its impact on hepatic inflammation during fasting.

Related to Figure 5.

What additional insights do the data from Figure 5 provide compared to the study published in *Nat Comms*, which demonstrated that RIPK1 regulates starvation resistance by modulating aspartate catabolism (PMID: 34686667)?

Related to Figure 6.

The data presented in Figure 7 are complementary and do not introduce new mechanistic insights.

Related to Figure 7.

The data from Figure 7 suggest that RIPK1 in hepatocytes is responsible for the observed damage. However, it has been previously demonstrated that inhibition of RIPK1 activity in macrophages protects against the development of MASLD (PMID: 33208891). One possible explanation for these findings could be that the overreaction of macrophages to fasting, coupled with the absence of RIPK1 in hepatocytes (an indirect effect), contributes to the observed damage. Considering this, complementing hepatocytes with a kinase-dead version of RIPK1 could be a valuable approach to further refine the molecular aspect of the study. This would allow for a more precise investigation into the specific role of RIPK1's scaffolding or kinase function in response to starvation in hepatocytes. Such experiments could provide additional insights into the mechanisms underlying the observed effects and help delineate the contributions of RIPK1 in different cell types to metabolic stress responses.

<https://doi.org/10.7554/eLife.96798.1.sa1>

Reviewer #2 (Public Review):

Summary:

Zhang et al. analyzed the functional role of hepatocyte RIPK1 during metabolic stress, particularly its scaffold function rather than kinase function. They show that Ripk1 knockout sensitizes the liver to cell death and inflammation in response to short-term fasting, a condition that would not induce obvious abnormality in wild-type mice.

Strengths:

The findings are based on a knockout mouse model and supported by bulk RNA-seq and scRNA-seq. The work consolidates the complex role of RIPK1 in metabolic stress.

Weaknesses:

However, the findings are not novel enough because the pro-survival role of RIPK1 scaffold is well-established and several similar pieces of research already exist. Moreover, the mechanism is not very clear and needs additional experiments.

<https://doi.org/10.7554/eLife.96798.1.sa0>

Author response:

We wish to express our sincere acknowledgement to the reviewers and the editors for the time and the effort spent in reviewing our manuscript. We highly appreciate the positive feedback and the thorough and constructive comments.

We plan to conduct additional experiments to address the reviewers' concerns.

(1) We plan to utilize the RIPK1 kinase dead mice to investigate the role of RIPK1 kinase activity in these metabolic stress responses.

(2) We plan to conduct flow cytometry analysis to detect the percentage or number of different cell types in fasted liver tissue, to provide more accurate and quantitative assessments of monocyte recruitment.

- (3) We plan to conduct more western blotting to detect the expression of related molecules in the signal transduction pathway, to further clarify the underlying mechanisms.
- (4) Regarding the single-cell RNA sequencing analysis, we plan to conduct CellChat analysis to provide information about the interactions between different cell populations.
- (5) We will fix the issues regarding the data graphs and image resolutions.

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