Rev-erbα: The circadian guardian against NLRP3-driven liver fibrosis

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Abstract. Liver fibrosis is a pivotal pathological process in the progression of various chronic liver diseases toward cirrhosis, primarily driven by the activation of hepatic stellate cells. Recent studies have implicated dysregulation of circadian clock genes in the pathogenesis of hepatic disorders. The present investigation focused on the role of the circadian regulator nuclear receptor subfamily 1 group D member 1 (Rev-erbα) in liver fibrosis and its mechanistic interplay with the NLR family domain containing protein 3 (NLRP3) inflammasome. A mouse model of liver fibrosis was established via carbon tetrachloride (CCl_4) administration. The expression of Rev-erbα was modulated pharmacologically using the agonist GSK4112 and the antagonist SR8278 to assess its impact on fibrogenesis. In parallel, lentiviral vectors were employed in *in vitro* studies to generate LX-2 cell lines with Rev-erbα overexpression or knockout. Transforming growth factor-β1 (TGF-β1) was applied to induce cellular activation, and subsequent effects on the NLRP3 inflammasome and its downstream mediators were analyzed. The extent of fibrosis and molecular alterations were evaluated using Masson's trichrome staining, Sirius Red staining, immunohistochemistry, western blotting and reverse transcription-quantitative PCR. Rev-erbα expression was significantly downregulated in both CCl_4-induced murine models and TGF-β1-activated LX-2 cells. Pharmacological activation of Rev-erbα attenuated hepatic fibrosis, evidenced by reduced collagen accumulation and suppression of fibrogenic markers (α-smooth muscle actin, collagen 1 and TGF-β1). By contrast, inhibition of Rev-erbα exacerbated fibrotic responses. Mechanistically, Rev-erbα activation inhibited NLRP3 inflammasome signaling and downstream pro-inflammatory cytokines [interleukin (IL)-18 and IL-1β], underscoring its anti-fibrotic function via NLRP3 pathway modulation. Rev-erbα functions as a key negative regulator of hepatic fibrosis by suppressing NLRP3 inflammasome activation, representing a promising therapeutic target for the management of liver fibrosis.

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Abbreviations: ASC, apoptosis associated speck; α-SMA, α-smooth muscle actin; Bmal1, brain and muscle arnt-like protein 1; CCl4, carbon tetrachloride; Cry, cryptochrome; cGAS, cGMP-AMP synthase; COL-1, collagen 1; HSC, hepatic stellate cell; IL-1β, interleukin-1β; IL-18, interleukin-18; NLRP3, NLR family domain containing protein 3; Per, period circadian regulator; Rev-erbα, nuclear receptor subfamily 1 group D member 1; TGF-β1, transforming growth factor-β1

Key words: Rev-erbα, NLRP3, inflammation, liver fibrosis, clock genes

Introduction

Liver fibrosis represents a reparative response to sustained hepatic injury caused by autoimmune hepatitis, primary cholangitis, alcoholic liver disease and hepatocellular carcinoma, with advanced fibrotic stages often progressing to cirrhosis (1,2). The activation of hepatic stellate cells (HSCs) is a central event in the onset and progression of fibrosis, and its inhibition has been shown to attenuate disease severity (3,4). Disruption of hepatic circadian regulation has also been implicated in the pathogenesis of both acute and chronic liver disorders (5,6). The circadian clock, a ubiquitous endogenous regulatory mechanism, is conserved across nearly all living organisms and enables physiological adaptation to temporal environmental fluctuations (7). Core components of the circadian machinery include Clock, brain and muscle arnt-like protein 1 (Bmal1), period circadian regulator (Per)1/2/3, cryptochrome (Cry)1/2 and nuclear receptor subfamily 1 group D member 1 (Rev-erbα), among others (6,8). Genetic ablation of Per1/2 or Bmal1 in mouse models resulted in impaired hepatic function, persistent inflammation and fibrotic remodeling (9-11). In a cholestasis-induced liver injury model, Per2 deficiency led to upregulation of liver fibrosis-related genes

and excessive extracellular matrix deposition (11,12), indicating that circadian disruption contributes to chronic hepatic inflammation and fibrogenesis.

Rev-erbα regulates transcriptional networks involved in metabolism, circadian rhythm and inflammation, positioning it as a therapeutic target for metabolic disorders, malignancies, epilepsy, inflammatory conditions and neurodegenerative diseases (13-19). Notably, a marked reduction in both the baseline expression and oscillatory amplitude of Rev-erbα has been observed in carbon tetrachloride (CCl_4)-induced mouse liver fibrosis models and *in vitro* activated HSCs (10,20). Previous functional studies indicated that Rev-erbα knockdown activated the cyclic GMP-AMP synthase (cGAS) pathway, promoting a pro-inflammatory microenvironment and accelerating fibrogenic progression (21), whereas its overexpression suppressed cGAS signaling and mitigated fibrosis (21). Additionally, Rev-erbα has been identified as a key negative regulator of NLR family domain containing protein 3 (NLRP3) inflammasome expression, with evidence showing its protective role against ulcerative colitis in mice (22). Given that NLRP3 inflammasomes can directly activate HSCs and exacerbate liver fibrosis (23,24), the mechanistic association between Rev-erbα and NLRP3 signaling in hepatic fibrosis warrants further elucidation.

To investigate the regulatory role of Rev-erbα in liver fibrosis and its interaction with the NLRP3 inflammasome, in the present study, *in vivo* experiments employed the Rev-erbα agonist GSK4112 and antagonist SR8278 to pharmacologically enhance or suppress Rev-erbα expression in a CCl_4-induced mouse model of hepatic fibrosis. In parallel, stable LX-2 cell lines with Rev-erbα knockdown or overexpression were generated via lentiviral transduction. Following 24-h transforming growth factor-β1 (TGF-β1) stimulation, cellular activation and downstream signaling events were evaluated. Based on a comprehensive analysis of the molecular mechanisms underlying hepatic fibrosis pathogenesis, the present study aims to elucidate the role of key regulatory factors in disease progression, thus laying the theoretical groundwork for developing more effective therapeutic strategies.

Materials and methods

Reagents. CCl_4 (cat. no. 5623-5) for liver fibrosis induction was obtained from National Pharmaceutical Chemical Reagent (https://www.reagent.com.cn/). TGF-β1 (cat. no. PRT221-0020), used to stimulate LX-2 cell activation, was sourced from Beyotime Institute of Biotechnology. The Rev-erbα agonist GSK4112 (cat. no. 1216744-19-2) and antagonist SR8278 (cat. no. 1254944-66-5) were purchased from MedChemExpress. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were quantified using ALT (cat. no. C009-2-1) and AST (cat. no. C010-2-1) assay kits from Nanjing Jiancheng Bioengineering Institute. For liver tissue staining, Masson's trichrome kit (cat. no. G1006), hematoxylin solution (cat. no. G1077) and diaminobenzidine (DAB) chromogenic kit (cat. no. G1212) were obtained from Wuhan Servicebio Technology Co., Ltd., while the Sirius Red staining kit (cat. no. 2610-10-8) was sourced from ChemicalBook Inc. Xylene (cat. no. 1330-20-7), glycerol (cat. no. 56-81-5), citrate buffer (cat. no. 6132-04-3) and glacial acetic acid (cat. no. 64-19-7), used for immunohistochemistry, were supplied by Sinopharm Chemical Reagent Co., Ltd. Cell culture reagents included DMEM (cat. no. 8119054) and FBS (cat. no. A5670701) from Gibco; Thermo Fisher Scientific, Inc. The CCK-8 cell viability assay kit (cat. no. 40203ES80) was provided by Shanghai Yeasen Biotechnology Co., Ltd. Custom lentiviral transfection reagents for Rev-erbα overexpression and knockdown were obtained from Jikai Biotechnology, and puromycin (cat. no. ST551-50mg) used for selection was purchased from Beyotime Institute of Biotechnology. Total RNA was extracted using a commercial RNA extraction kit (cat. no. 19211ES60) from Shanghai Yeasen Biotechnology Co., Ltd., followed by reverse transcription with a kit from Tiangen Biotech Co., Ltd. (cat. no. KR118). SYBR Green qPCR mix (cat. no. 11201E03) was purchased from Shanghai Yeasen Biotechnology Co., Ltd. Primers targeting α-smooth muscle actin (α-SMA), TGF-β1, Rev-erbα, NLRP3, Caspase-1, apoptosis associated speck (ASC), interleukin (IL)-18, IL-1β and β-actin were designed based on GenBank cDNA sequences and synthesized by Shanghai Shanjing Molecular Biotechnology Co., Ltd. Protein extraction was performed using RIPA lysis buffer (cat. no. WB0102) with protease inhibitors (cat. no. WB0122), both from Shanghai Weiao Biotechnology Co., Ltd. Protein concentration was determined using a BCA assay kit (cat. no. MA-0082-2) from Dalian Meilun Biology Technology Co., Ltd., and chemiluminescent detection was performed with an ECL kit (cat. no. SW-WB012) from Shanghai Weiao Biotechnology Co., Ltd. Primary antibodies against Rev-erbα (cat. no. ab305753), Caspase-1 (cat. no. ab207802), IL-18 (cat. no. ab243091), IL-1β (cat. no. ab254360), α-SMA (cat. no. ab124964), collagen 1 (COL-1) (cat. no. ab34710) and TGF-β1 (cat. no. ab27937) were obtained from Abcam. Antibodies against NLRP3 (cat. no. 15101) and ASC (cat. no. 67824S) were sourced from Cell Signaling Technology, Inc. β-actin (cat. no. GB15003) and HRP-conjugated Affinipure Goat Anti-rabbit IgG (cat. no. GB22303) were provided by Wuhan Servicebio Technology Co., Ltd.

Animal experiments. A total of 42 male C57BL/6 mice, SPF grade, 6 weeks (22±2 g) were obtained from Shanghai Jiesijie Experimental Animal Co., Ltd., and housed under standard conditions at the Animal Facility of Shanghai Municipal Hospital of Traditional Chinese Medicine. Mice were kept at a standard temperature of 25-27˚C and a humidity of 55-65%, in a light/dark cycle of 12/12 h, and with *ad libitum* access to sterilized food and water. After a 1-week acclimatization period, animals were randomly assigned to experimental groups as follows: i) A total of 12 mice were divided into a control group (Con) and a CCl_4-induced model group (CCl_4) (n=6 per group). Liver fibrosis was induced by intraperitoneal injection of a 20% CCl_4 solution in olive oil (5 ml/kg), administered twice weekly for 6 weeks (25). Control mice received an equal volume of physiological saline. ii) The remaining 30 mice were randomly allocated into three groups: Control (n=12), GSK4112 (n=12) and SR8278 (n=12). For pharmacological modulation of Rev-erbα, lyophilized GSK4112 and SR8278 were reconstituted in DMSO. Mice in the SR8278 group received daily intraperitoneal injections of SR8278 (25 mg/kg) (26), while those in the GSK4112 group were

administered GSK4112 (20 μg/mouse) (27,28). Control mice were treated with equivalent volumes of DMSO. Injections were performed once daily for 14 consecutive days. After treatment, six mice from each group were randomly selected and reassigned to receive CCl_4 treatment as described above, forming the CCl_4 group (n=6), SR8278 + CCl_4 group (n=6) and GSK4112 + CCl_4 group (n=6).

Upon completion of modeling, mice were anesthetized with 1% pentobarbital sodium (50 mg/kg). Livers were harvested, with a 1x1-cm section from the central lobe fixed in 4% paraformaldehyde for histological analysis at ~25˚C. The remaining tissue was snap-frozen in liquid nitrogen and stored at -80˚C for subsequent experiments. Euthanasia was performed via cervical dislocation (29). All animal procedures were approved by the Ethics Committee of Shanghai Municipal Hospital of Traditional Chinese Medicine (Shanghai, China; approval no. 2022033).

Cell culturing and treatment. The human HSC LX-2 cell line was obtained from Shanghai Kanglang Biological Technology Co., Ltd., and cultured in DMEM supplemented with 10% FBS under standard conditions (37˚C and 5% CO_2).

For fibrogenic induction, LX-2 cells were divided into a control group and a TGF-β1-treated group, with the latter exposed to 5 ng/ml TGF-β1 for 24 h (25).

The Rev-erbα overexpression plasmid and knockdown shRNA plasmid utilized in this study were constructed by Shanghai Jikai Biotechnology, employing a triple-plasmid co-transfection system in 293T cells for lentiviral production; the lentiviral supernatant was concentrated and purified into high-titer stocks within 48-72 h post-transfection. For Rev-erbα overexpression lentivirus, the plasmid combination consisted of the transfer plasmid GV208 carrying the target gene Rev-erbα (4 μg), the packaging plasmid Helper 1.0 (3 μg), and the envelope plasmid Helper 2.0 (1 μg). For Rev-erbα knockdown lentivirus, the plasmids included the transfer plasmid pLKO.1-shRNA (4 μg), the packaging plasmid psPAX2 (3 μg) and the envelope plasmid pMD2.G (1 μg). LX-2 cells were seeded in 24-well plates at $5x10^4$ cells/ml (1 ml/well) and were assigned to the following groups: Con522 (negative control for Rev-erbα overexpression), con313 (negative control for Rev-erbα knockdown), OvRev-erbα (Rev-erbα overexpression; MOI=10) and Rev-erbα sh1/sh2/sh3 (shRev-erbα knockdown groups; MOI=30/20/10, respectively). After adherence to 24-well plates and reaching 20-30% confluency, transduction was performed with Rev-erbα lentiviruses at different multiplicities of infection. The viral volume was calculated using the formula: Virus volume (μl)=(MOI x cell number)/viral titer (TU/ml). After 12 h of incubation at 37˚C, the DMEM was replaced with culture medium containing 10% FBS, and cells were cultured for an additional 72 h. Transduction efficiency was assessed via fluorescence inverted microscopy (Olympus IX73; Olympus Coporation) to determine optimal conditions. Puromycin selection (2 μg/ml) was initiated at 72 h post-transduction and maintained for 7-14 days. Successful Rev-erbα modulation was validated by RT-qPCR for mRNA expression and western blotting for protein expression, as performed below, with successfully transfected cells subsequently used for further experiments.

To assess the effect of Rev-erbα on TGF-β1-induced activation, LX-2 cells were categorized into TGF-β1, OvRev-erbα + TGF-β1 and shRev-erbα + TGF-β1 groups. Based on preliminary findings, OvRev-erbα + TGF-β1 was transduced with Rev-erbα at MOI=10 and shRev-erbα + TGF-β1 received interference at MOI=20. The TGF-β1 group was treated with DMEM without viral particles. All groups were subsequently treated with 5 ng/ml TGF-β1 for 24 h at 37˚C and in 5% CO_2.

Serum liver function analysis. For serum collection, blood samples from each mouse group were left at room temperature (25˚C) for 4 h, centrifuged at 3,000 rpm and 4˚C for 15 min, and serum was harvested. AST and ALT levels were measured using the aforementioned commercially available detection kits, following the manufacturer's protocols.

Masson and Sirius red staining. Fresh liver specimens (~1x1 cm) were fixed in 4% paraformaldehyde at room temperature for 24 h. After paraffin embedding, tissue sections were stained using Masson's trichrome and Sirius red protocols. For liver tissues, sections of 5-7 μm in thickness were used. Collagen fiber staining was performed at room temperature, with Masson's trichrome staining for 10 min and Sirius red staining for 30 min. Histopathological alterations were assessed using a light microscope at x200 magnification (BX41, Beijing Ruike Zhongyi Technology Co., Ltd.).

Cellular immunofluorescence detection. For immunofluorescence, cells from the TGF-β1, OvRev-erbα + TGF-β1 and shRev-erbα + TGF-β1 groups were fixed with 4% paraformaldehyde at room temperature for 15 min and subjected to 10% FBS blocking at 37˚C for 30 min. The TGF-β1, OvRev-erbα + TGF-β1 and shRev-erbα + TGF-β1 groups were incubated with an α-SMA antibody (1:200) at 4˚C for 16 h, followed by secondary antibody incubation at room temperature for 50 min. After PBS washing (pH 7.4), nuclei were counterstained with DAPI at 25˚C for 10 min in the dark. Slides were rinsed, spin-dried and mounted using an anti-fade glycerol medium. Fluorescence imaging was performed with a fluorescence inverted microscope (Olympus IX73 microscope). Quantification of positive staining was carried out using ImageJ 1.8.0 software (National Institutes of Health), and statistical analyses of protein expression levels were performed using GraphPad Prism 8.3.0 (Dotmatics).

Immunohistochemical detection of liver tissue. Following fixation (4% paraformaldehyde at room temperature for 24 h), liver tissues were dehydrated at room temperature for 30 min and embedded in paraffin (melting point ~60˚C). Paraffin blocks were sectioned into 5-μm slices, rinsed and air-dried prior to staining. The procedure included the following steps: i) At room temperature, dewaxing with xylene for 30 min and graded ethanol hydration (100, 95, 80 and 75%) for 20 min; ii) rehydration through a descending ethanol series followed by distilled water rinses; iii) nuclear staining with hematoxylin at room temperature for 10 min; iv) rinsing in distilled water; v) antigen retrieval by heating sections in 200 ml 0.01M citrate buffer (pH6.0) at 70˚C for 10 min; vi) washing in PBS three times (5 min each), then incubating in 3% hydrogen peroxide at room temperature for 10 min to

quench endogenous peroxidase activity; vii) blocking with 2% FBS in PBS at 37˚C for 2 h; viii) incubation with primary antibodies, α-SMA (1:500), COL-1 (1:600) and TGF-β1 (1:800), at 4˚C for 16 h; ix) after PBS washes, incubation with HRP-conjugated goat anti-mouse secondary antibody (1:200) at 37˚C for 2 h; x) DAB chromogenic development for 10 min; xi) rinsing with distilled water; and xii) clearing with xylene for 10 min, followed by mounting with neutral resin and drying at 70˚C. Microscopic images were captured using a Horiba BX41 light microscope (Beijing Ruike Zhongyi Technology Co., Ltd.) from five randomly selected fields per section at magnifications of x100 and x400. Quantitative analysis of antibody-positive staining areas was performed using ImageJ 1.8.0 software (National Institutes of Health). Statistical analysis of target protein expression levels was conducted using GraphPad Prism 8.3.0 (Dotmatics).

Cell viability detection. Cell viability was assessed using the CCK-8 assay. A suspension of $2x10^3$ cells in 100 μl complete culture medium containing 10% FBS was seeded into each well of a 96-well plate and incubated under standard conditions (37˚C and 5% CO_2) for 6 h. Subsequently, 10 μl CCK-8 solution were added to each well. After 2 h of incubation, optical density (OD) was measured at 450 nm using a microplate reader (BioTek Epoch2; Agilent Technologies, Inc.). OD values were used to calculate cell viability, and results were presented in histogram form for comparative analysis.

Reverse transcription-quantitative PCR (RT-qPCR). For RT-qPCR analysis, 40-50 mg of liver tissue was collected from each group, and total RNA was extracted using TRIzol® (Thermo Fisher Scientific, Inc.) and precipitated in ethanol. Reverse transcription was performed using the FastKing gDNA Dispelling RT SuperMixJun (cat. no. KR118; Tiangen Biotech Co., Ltd.), and the resulting cDNA was diluted 10-fold with ddH_2O for amplification. qPCR was performed using SYBR Green PCR Mastermix (cat. no. 11201E03). The results were analyzed on an ABI StepOnePlus real-time PCR system (Applied Biosystems) using the $2^{-\Delta\Delta Cq}$ method (30). Values were normalized to β-actin. The reaction conditions were as follows: 95˚C predenaturation once for 5 min, followed by denatured at 95˚C for 10 sec, annealing at 60˚C for 20 sec and extension at 72˚C for 20 sec, for 40 cycles. Primer sequences are provided in Table SI.

Western blotting analysis. The quantity of whole-protein extracts from cells and livers of mice, extracted using RIPA lysis buffer (cat. no. WB0102) with protease inhibitors (cat. no. WB0122), were determined using the BCA assay. Equal amounts of total protein (20-30 μg) were resolved by 7.5% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% skimmed milk at room temperature for 2 h, the membranes were incubated with specific primary antibodies for 16 h at 4˚C [Rev-erbα (1:1,000), Caspase-1 (1:1,000), IL-18 (1:1,000), IL-1β (1:1,000), α-SMA (1:1,000), collagen 1 (1:1,000) , TGF-β1 (1:1,000) , NLRP3 (1:1,000), ASC (1:1,000) and β-actin (1:3,000)], followed by incubation with HRP-conjugated Affinipure Goat Anti-rabbit IgG (1:5,000) at room temperature for 2 h. Signal detection was performed using a chemiluminescence imaging system (Amersham Imager 600; Cytiva), and band intensities were semi-quantified using ImageJ 1.8.0 software.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 8.3.0 (Dotmatics). Comparisons between two groups were performed using unpaired, two-tailed t-tests, while multiple-group comparisons were evaluated by one-way ANOVA. When comparing ≥3 groups (e.g., shRev-erbα 1, shRev-erbα 2 and shRev-erbα3) requiring assessment of all possible pairwise comparisons (shRev-erbα 1 vs. shRev-erbα 2, shRev-erbα 1 vs. shRev-erbα 3 and shRev-erbα 2 vs. shRev-erbα 3), Tukey's test was employed for post-hoc evaluation. When analyzing ≥3 groups (e.g., CCl_4, GSK4112 + CCl_4 and SR8287 + CCl_4) focusing on specific pairwise comparisons between predetermined groups (CCl_4 vs. GSK4112 + CCl_4 and CCl_4, vs. SR8287 + CCl_4), Bonferroni's correction was applied for post-hoc statistical assessment. Data are expressed as the mean ± SEM. $P<0.05$ was used to indicate a statistically significant difference.

Results

Reduced expression of circadian clock gene Rev-erbα in liver fibrosis. To investigate the expression dynamics of the circadian clock gene Rev-erbα in liver fibrosis, a mouse model was established using CCl_4 administration. Compared with those in the Con group, serum ALT and AST levels were significantly elevated in the CCl_4-treated mice, indicating substantial hepatic injury (Fig. 1A and B). Histological analysis via Masson's trichrome and Sirius red staining revealed well-preserved hepatic architecture in the control group, whereas CCl_4-treated mice exhibited marked collagen deposition and expanded fibrotic areas, consistent with progressive fibrosis (Fig. 1C). In parallel, mRNA and protein expression levels of TGF-β1 and α-SMA were significantly upregulated in the livers of CCl_4-treated mice compared with levels in the controls (Fig. 1D-F), confirming successful model induction. Notably, Rev-erbα mRNA and protein levels were significantly reduced in the liver tissue of CCl_4 group mice relative to the controls (Fig. 1G and H). This downregulation was further corroborated *in vitro*, where TGF-β1-stimulated LX-2 cells showed significant upregulation of fibrotic markers (α-SMA and TGF-β1) (Fig. 1I-K) and a concomitant decrease in Rev-erbα expression at both the transcript and protein levels (Fig. 1L and M). These results suggest that Rev-erbα may act as a negative regulator in the progression of liver fibrosis, with its reduced expression potentially facilitating fibrogenic and pro-inflammatory responses in hepatic tissue.

Rev-erbα has a significant inhibitory impact on liver fibrosis. Rev-erbα serves as a critical transcriptional regulator of metabolic, circadian and inflammatory pathways, rendering it a promising therapeutic target for metabolic disorders, malignancies, epilepsy, inflammatory conditions and neurodegenerative diseases (22,31). To evaluate its role in liver fibrosis progression, C57BL/6 mice received intraperitoneal injections of the Rev-erbα agonist GSK4112 (25 mg/kg) or the antagonist SR8278 (20 μg/mouse). Western blot analysis confirmed

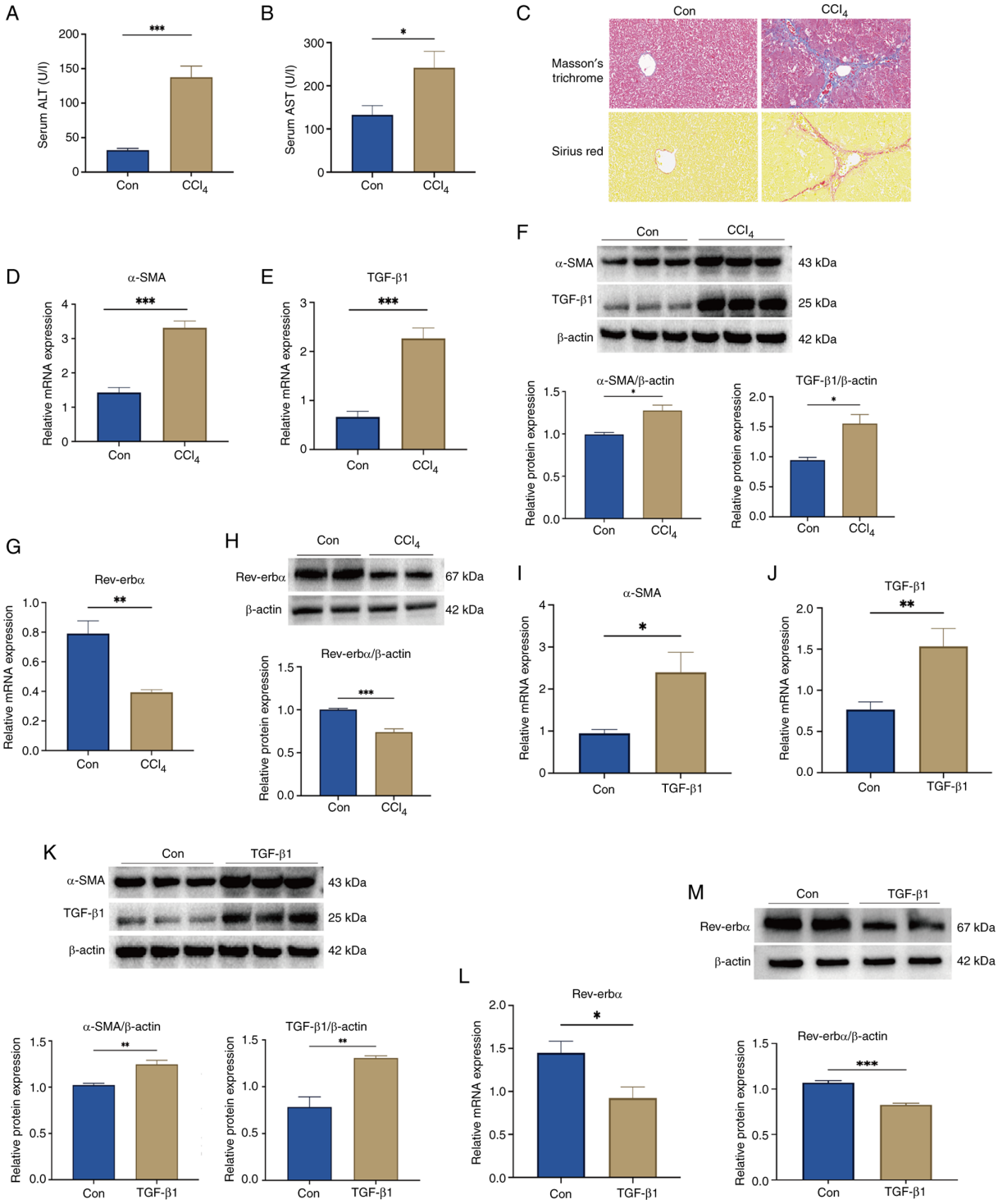

Figure 1. Decreased expression of circadian clock gene Rev-erbα in liver fibrosis. (A) Serum ALT levels in Con and CCl_4-treated mice (n=6). (B) Serum AST levels in Con and CCl_4-treated mice (n=6). (C) Masson's trichrome and Sirius red staining of liver tissue in Con and CCl_4-treated mice (x200 magnification). (D) The mRNA expression levels of α-SMA in liver tissue in Con and CCl_4-treated mice (n=6). (E) The mRNA expression levels of TGF-β1 in liver tissue in Con and CCl_4-treated mice (n=6). (F) Protein expression levels of α-SMA and TGF-β1 in liver tissue from Con and CCl_4-treated mice (n=3). (G) The mRNA expression levels of Rev-erbα in liver tissue from Con and CCl_4-treated mice (n=6). (H) Protein expression levels of Rev-erbα in liver tissue from Con and CCl_4-treated mice (n=4). (I) The mRNA expression levels of α-SMA in Con and TGF-β1-treated cell groups (n=6). (J) The mRNA expression levels of TGF-β1 in Con and TGF-β1-treated cell groups (n=6). (K) Protein expression levels of α-SMA and TGF-β1 in Con and TGF-β1-treated cell groups (n=3). (L) The mRNA expression levels of Rev-erbα in Con and TGF-β1-treated cell groups (n=6). (M) Protein expression levels of Rev-erbα in Con and TGF-β1-treated cell groups (n=4). Data are expressed as the mean ± SEM; *P<0.05, **P<0.01 and ***P<0.001. Con, control; Rev-erbα, nuclear receptor subfamily 1 group D member 1; TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin; CCl_4, carbon tetrachloride.

successful pharmacological modulation, with Rev-erbα expression significantly reduced in the SR8278 group and significantly elevated in the GSK4112 group (Fig. 2A).

Following model establishment, liver fibrosis was induced using CCl_4. Histological staining with Masson's trichrome and Sirius red revealed pronounced pseudolobule formation,

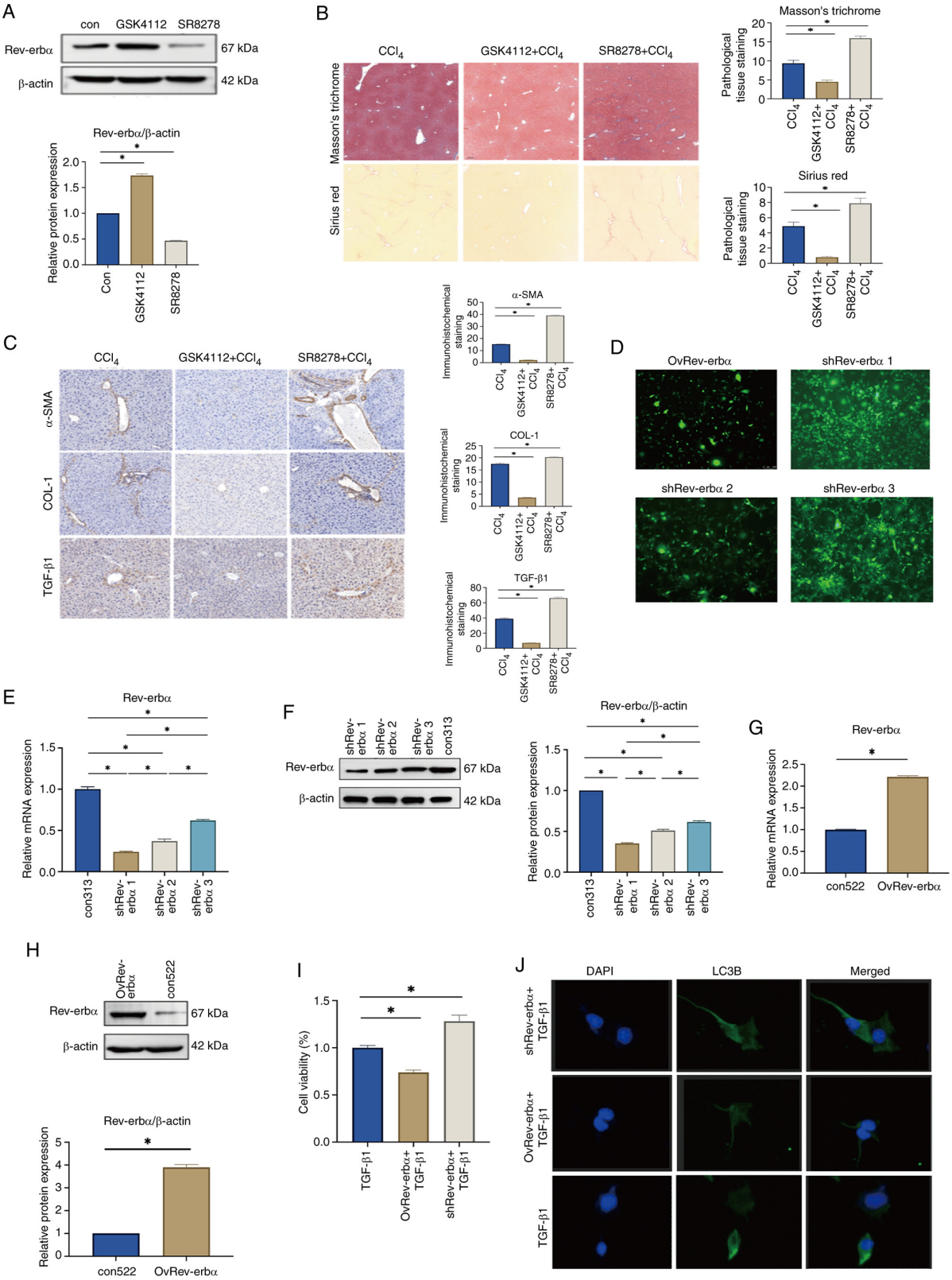

Figure 2. Rev-erbα significantly inhibits liver fibrosis. (A) Rev-erbα protein expression in mouse liver tissue following administration of Rev-erbα agonist GSK4112 or inhibitor SR8278 (n=3). (B) Liver histopathology in the CCl_4, GSK4112 + CCl_4 and SR8278 + CCl_4 groups (Masson's trichrome and Sirius red staining; x100 magnification) (n=3). (C) Expression of α-SMA, COL-1 and TGF-β1 in liver tissue of CCl_4-induced liver fibrosis mice from in the CCl_4, GSK4112 + CCl_4 and SR8278 + CCl_4 groups (x400 magnification) (n=3). (D) Lentiviral fluorescence expression in stable Rev-erbα gene knockdown or overexpression cell lines (x200 magnification). The greater the extent of green fluorescent areas, the better the lentiviral transduction efficacy. (E) The mRNA expression of Rev-erbα in shRev-erbα cell groups (n=6). (F) Protein expression of Rev-erbα in the shRev-erbα cell group (n=6). (G) The mRNA expression of Rev-erbα in the OvRev-erbα cell group (n=6). (H) Protein expression of Rev-erbα in the OvRev-erbα cell group (n=6). (I) LX-2 cell viability in the TGF-β1, OvRev-erbα + TGF-β1 and shRev-erbα + TGF-β1 groups (n=3). (J) Expression of α-SMA in the TGF-β1, OvRev-erbα + TGF-β1 and shRev-erbα + TGF-β1 groups (immunofluorescence; x200 magnification). Data are expressed as the mean ± SEM; $^*P<0.05$. Con, control; Rev-erbα, nuclear receptor subfamily 1 group D member 1; TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin; CCl_4, carbon tetrachloride; COL-1, collagen 1; DAPI, 4',6-diamidino-2-phenylindole; LC3B, microtubule-associated protein 1A/1B light chain 3β.

extensive collagen accumulation and expanded fibrotic areas in the CCl_4 + SR8278 group. By contrast, the CCl_4 + GSK4112 group exhibited more preserved hepatic architecture, reduced fibrous proliferation and an absence of steatosis compared with the CCl_4 group (Fig. 2B). Immunohistochemical analysis further demonstrated that expression levels of α-SMA, COL-1 and TGF-β1 were significantly upregulated in the CCl_4 + SR8278 group, whereas these markers were significantly downregulated in the CCl_4 + GSK4112 group (Fig. 2C). These results indicate that Rev-erbα suppression exacerbates fibrogenesis, while its activation attenuates hepatic inflammation, collagen deposition and fibrotic remodeling. Lentiviral transduction was employed to establish LX-2 cell lines with stable Rev-erbα knockdown or overexpression for subsequent *in vitro* investigations. Fluorescence microscopy confirmed that Rev-erbα sh1 (MOI=20) achieved the most effective knockdown (Fig. 2D), with significantly reduced mRNA and protein expression levels of Rev-erbα in LX-2 cells (Fig. 2E and F). This condition was subsequently selected for downstream experiments. Fluorescence microscopy confirmed efficient overexpression of Rev-erbα in the MOI=10 group (Fig. 2D), accompanied by a significant increase in both mRNA and protein levels of Rev-erbα in LX-2 cells (Fig. 2G and H). LX-2 cells were then allocated into three groups: TGF-β1, OvRev-erbα + TGF-β1 and shRev-erbα + TGF-β1. Following lentiviral transduction at the defined MOIs, cells were stimulated with 5 ng/ml TGF-β1 for 24 h to induce activation. CCK-8 assays revealed a significant reduction in cell viability in the OvRev-erbα + TGF-β1 group, while the shRev-erbα + TGF-β1 group exhibited a significant increase in viability, compared with the TGF-β1 group (Fig. 2I). Immunofluorescence analysis further demonstrated that α-SMA expression was substantially downregulated in the OvRev-erbα + TGF-β1 group, whereas notable upregulation was observed in the shRev-erbα + TGF-β1 group (Fig. 2J). These results suggest that decreased Rev-erbα expression promotes HSC activation and fibrogenic responses, whereas its overexpression exerts a suppressive, anti-fibrotic effect.

Rev-erbα inhibits NLRP3 and alleviates liver fibrosis. The NLRP3 inflammasome consists of ASC, caspase-1 and NLRP3, with ASC serving as a key adaptor that promotes the recruitment and activation of caspase-1 and NLRP3. Caspase-1 processes the inactive precursors of IL-1β and IL-18 into their mature, bioactive forms, thereby initiating pyroptosis and inflammatory responses that play central roles in the progression of alcoholic liver disease, viral hepatitis and liver fibrosis (32,33).

In the present study, following CCl_4 induction in three groups of mice, Rev-erbα protein expression was assessed. Compared with those in the CCl_4 group, the protein levels of Rev-erbα in the GSK4112 + CCl_4 group were significantly elevated and those in the SR8278 + CCl_4 group were significantly decreased (Fig. 3A). To examine whether the anti-fibrotic effect of Rev-erbα is mediated through modulation of the NLRP3 inflammasome, liver fibrosis models were generated in C57BL/6 mice subjected to CCl_4 combined with either GSK4112 or SR8278 treatment. Expression levels of NLRP3, caspase-1, ASC and downstream proinflammatory cytokines IL-18 and IL-1β were analyzed in liver tissue. Relative to the CCl_4 group, mRNA expression of NLRP3, caspase-1, ASC, IL-18 and IL-1β was significantly reduced in the GSK4112 + CCl_4 group, whereas significant increases were observed in the SR8278 + CCl_4 group (Fig. 3B), with protein expression showing consistent trends (Fig. 3C). To further substantiate these findings, validation was performed in three groups of LX2 cells. Following TGF-β1 stimulation, Rev-erbα protein expression was assessed. Compared with that in the TGF-β1 group, Rev-erbα expression was significantly upregulated in the OvRev-erbα + TGF-β1 group and markedly downregulated in the shRev-erbα + TGF-β1 group (Fig. 3D). Subsequent analysis focused on the regulatory effect of Rev-erbα on the NLRP3 inflammasome in LX2 cells. RT-qPCR and western blotting results demonstrated that, relative to the TGF-β1 group, mRNA and protein levels of NLRP3, caspase-1, ASC and the downstream cytokines, IL-18 and IL-1β, were significantly suppressed in the OvRev-erbα + TGF-β1 group, whereas marked increases were observed in the ShRev-erbα + TGF-β1 group (Fig. 3E and F). These results indicate that Rev-erbα knockdown promotes inflammatory responses during liver fibrosis by enhancing NLRP3 inflammasome activation in HSCs, thereby elevating the expression of IL-18 and IL-1β. By contrast, overexpression of Rev-erbα reverses these effects.

Collectively, the data support a role for Rev-erbα as an upstream negative regulator of the NLRP3 inflammasome, contributing to its anti-fibrotic function by attenuating inflammasome-mediated inflammation in liver fibrosis and activated HSCs.

Discussion

Liver fibrosis represents a key pathological process in the development of cirrhosis. Hepatic injury and inflammation, triggered by factors such as drug toxicity, excessive alcohol intake, viral infections and autoimmune responses, drive this progression (34). Upon stimulation, HSCs undergo transdifferentiation into myofibroblast-like cells, which secrete extracellular matrix components that accumulate as scar tissue, thereby advancing fibrogenesis (1,3). Accumulating evidence indicates that disruptions in circadian rhythms significantly impact the pathogenesis of various liver disorders, including fibrosis (10,35,36). While the master circadian clock resides in the suprachiasmatic nucleus of the hypothalamus, peripheral tissues and organs, including the liver, kidney, myocardium, pancreas and skeletal muscle, possess autonomous clocks that regulate local rhythmic gene expression to maintain physiological homeostasis (37).

Alterations in hepatic circadian regulation profoundly affect the initiation and progression of liver disease. Bmal1 is a core component of the molecular clock and plays a pivotal role in regulating lipid, bile acid and glucose metabolism; its suppression leads to metabolic dysregulation and hepatic dysfunction (38). Bmal1-deficient mice, especially under chronic alcohol exposure, exhibit exacerbated hepatic steatosis and injury (39). Clock gene dysfunction promotes lipid accumulation in the liver, contributing to the development of non-alcoholic steatohepatitis (NASH) (40). In addition, NAD-dependent protein deacetylase sirtuin-1 modulates hepatic insulin sensitivity through the Clock/Bmal1 axis (41). Genetic ablation of Clock and Bmal1 results in hypoglycemia,

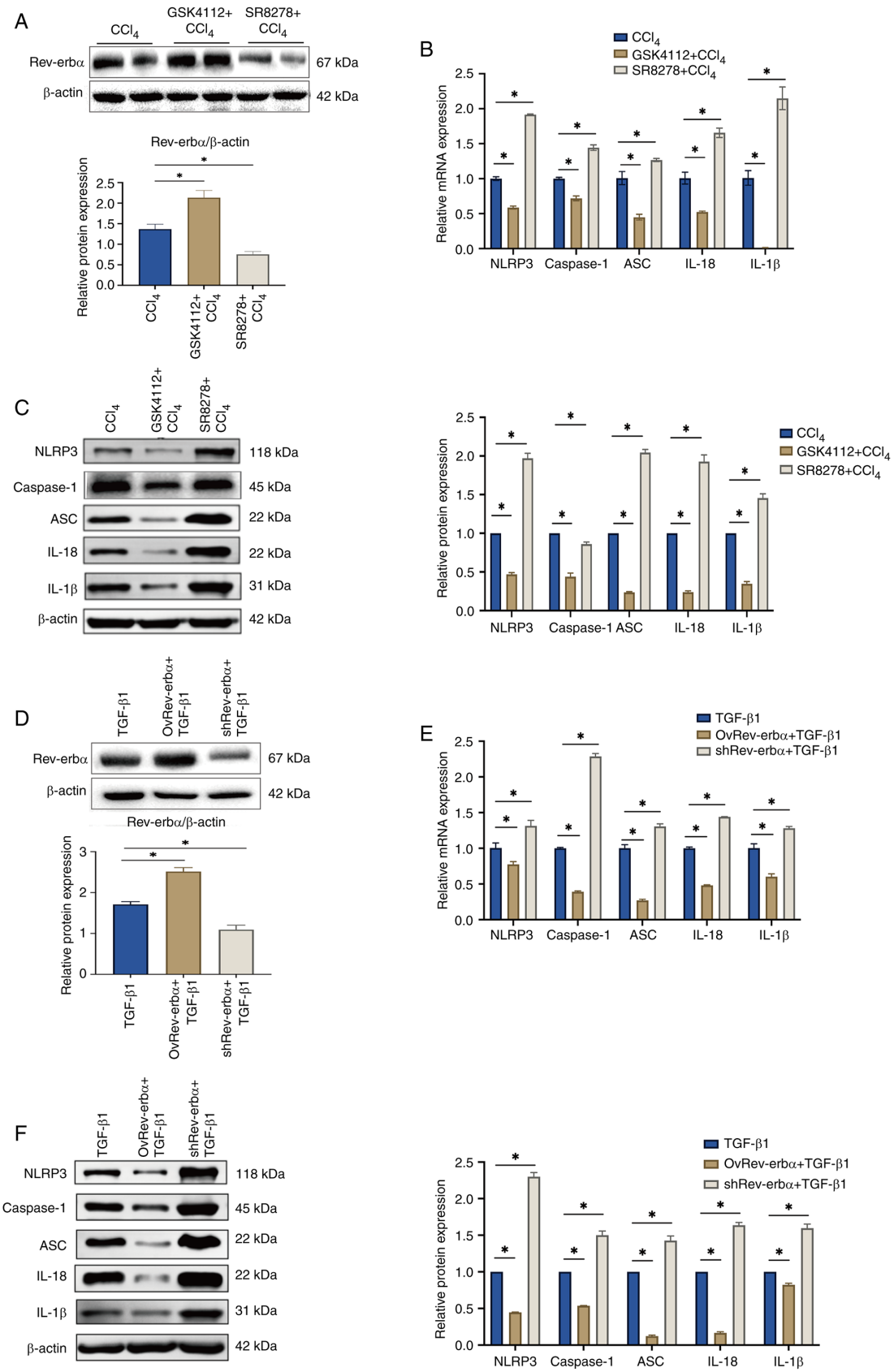

Figure 3. Rev-erbα inhibits NLRP3 and reduces liver fibrosis. (A) Protein expression of Rev-erbα in the CCl_4, GSK4112 + CCl_4 and SR8278 + CCl_4 groups (n=3). (B) The mRNA expression of NLRP3, caspase-1, ASC, IL-18 and IL-1β in the CCl_4, GSK4112 + CCl_4 and SR8278 + CCl_4 groups (n=3). (C) Protein expression of NLRP3, caspase-1, ASC, IL-18 and IL-1β in the CCl_4, GSK4112 + CCl_4 and SR8278 + CCl_4 groups (n=3). (D) Protein expression of Rev-erbα in the TGF-β1, OvRev-erbα + TGF-β1 and shRev-erbα + TGF-β1 groups (n=3). (E) The mRNA expression of NLRP3, caspase-1, ASC, IL-18 and IL-1β in the TGF-β1, OvRev-erbα + TGF-β1 and shRev-erbα + TGF-β1 groups (n=3). (F) Protein expression of NLRP3, caspase-1, ASC, IL-18 and IL-1β in the TGF-β1, OvRev-erbα + TGF-β1 and shRev-erbα + TGF-β1 groups (n=3). Data are expressed as the mean ± SEM; ns, not significant; *P<0.05. Rev-erbα, nuclear receptor subfamily 1 group D member 1; TGF-β1, transforming growth factor-β1; CCl_4, carbon tetrachloride; NLRP3, NLR family domain containing protein 3; ASC, apoptosis associated speck; IL, interleukin.

whereas loss of Per and Cry leads to hyperinsulinemia (42). Notably, Rev-erbα knockdown activates the cGAS pathway, induces a localized proinflammatory microenvironment and accelerates liver fibrosis progression (21).

The liver clock genes Rev-erbα and Rev-erbβ, both classified as orphan nuclear receptors, cooperatively regulate circadian rhythms and metabolic homeostasis (14,43). Rev-erbα has been shown to modulate bile acid and cholesterol biosynthesis by regulating SREBP regulating gene protein expression in mice, and its genetic deletion leads to impaired bile acid metabolism (44). Simultaneous ablation of both Rev-erbα and Rev-erbβ in double-knockout models severely disrupts the rhythmic expression of lipid metabolism-related genes (14,45). Beyond hepatic functions, Rev-erbα also plays a critical role in maintaining intestinal barrier permeability and treating NASH (46). In NASH mouse models induced by a high-cholesterol, high-fat diet, intestinal expression of Rev-erbα and tight junction-associated genes is downregulated, resulting in increased intestinal permeability. Pharmacological activation of Rev-erbα with SR9009 ameliorates hepatic lipid accumulation, insulin resistance, inflammation and fibrosis, in part by enhancing intestinal barrier function (46). Additionally, Rev-erbα has been implicated in the regulation of the long non-coding RNA Platr4, which attenuates NASH progression by suppressing NLRP3 inflammasome activation (47). Another study demonstrated that intestinal Rev-erbα deficiency exacerbates high-fat diet (HFD)-induced obesity. Pharmacological modulation of the Rev-erbα/Bmal1 axis using small-molecule compounds alleviated HFD-induced metabolic dysfunction (48). Collectively, these findings highlight the critical role of the gut circadian clock, particularly Rev-erbα, in regulating lipid absorption and energy balance, offering potential therapeutic targets for obesity and related metabolic disorders.

Rev-erbα also plays a pivotal role in liver ischemia-reperfusion (I/R) injury (49). Additionally, Rev-erbα deficiency exacerbates hepatic I/R injury in mice, as evidenced by elevated plasma levels of ALT and AST, higher histopathological injury scores and increased hepatic myeloperoxidase activity. Moreover, the absence of Rev-erbα leads to heightened expression of pro-inflammatory cytokines, enhanced NLRP3 inflammasome activation and greater infiltration of inflammatory cells. Pharmacological activation of Rev-erbα significantly mitigates hepatic injury and the associated inflammatory response, indicating its protective role through suppression of inflammation (49). Rev-erbα is also critically involved in the pathogenesis of alcoholic fatty liver (AFL) (50). In ethanol-fed mice and ethanol-treated L02 hepatocytes, Rev-erbα expression is markedly upregulated, and its activation promotes hepatic steatosis. Inhibition or downregulation of Rev-erbα alleviates lipid accumulation, primarily by restoring autophagic activity. Mechanistically, Rev-erbα impairs autophagy via Bmal1-dependent pathways (50). Treatment with the Rev-erbα antagonist SR8278 reduces hepatic lipid deposition and enhances autophagic flux in ethanol-fed mice (50), suggesting that Rev-erbα acts as a key modulator in AFL progression and represents a potential therapeutic target. Furthermore, Rev-erbα deficiency in ALD mice increases CYP4A expression, lipid accumulation and oxidative stress. Intervention with either the Rev-erbα agonist SR9009 or the CYP4A inhibitor HET0016 attenuates alcohol-induced hepatic steatosis and injury, highlighting both Rev-erbα and CYP4A as viable therapeutic targets in ALD management (51). In addition, the Rev-erbα agonist GSK4112 has demonstrated protective effects in Fas-induced acute liver injury (26). In a mouse model established by administration of the anti-Fas antibody Jo2, GSK4112 treatment (25 mg/kg, intraperitoneally) significantly reduced plasma ALT and AST levels, ameliorated liver histological damage and improved survival. Mechanistically, GSK4112 suppressed caspase-3 and caspase-8 activity, decreased hepatocyte apoptosis, downregulated Fas expression and enhanced Akt phosphorylation (26).

In the context of liver fibrosis research, Wang *et al* (52) reported that Rev-erbα binds directly to the promoter region of the pro-fibrotic coagulation regulator PAI-1, thereby suppressing its expression and exerting anti-fibrotic effects. A recent study further revealed rhythmic oscillations in the expression of TGF-β signaling components and fibrosis-associated genes in liver tissue from hepatocyte-specific Rev-erbα/β knockout mice (53). In LX2 cells subjected to circadian rhythm synchronization, silencing of Rev-erbα led to upregulation of pro-fibrotic gene expression. Activation of Rev-erbα by SR9009 was shown to reduce SMAD2/3 phosphorylation in TGF-β-stimulated human lung myofibroblasts, indicating that Rev-erbα activation impairs TGF-β signaling in both HSCs and myofibroblasts (53). To evaluate the therapeutic potential of targeting Rev-erbα in MASH-induced fibrosis, the pharmacological agonist SR9009 was administered to mice fed a choline-deficient, amino acid-defined, HFD. SR9009 treatment significantly attenuated hepatic fibrosis, as evidenced by reduced collagen deposition (Sirius red staining), diminished HSC activation (α-SMA expression) and downregulation of fibrosis-related genes (53). These findings support the therapeutic efficacy of Rev-erbα agonists in mitigating fibrosis progression. Consistent with these observations, the present study identified a significant decrease in Rev-erbα expression in both CCl_4-induced liver fibrosis mouse models and TGF-β-stimulated LX2 cells. These results suggest that reduced Rev-erbα expression may contribute to the progression of liver fibrosis.

Studies have identified porphyrin heme as the endogenous ligand of Rev-erbα. Binding of heme is essential for Rev-erbα to recruit corepressor complexes, such as nuclear receptor co-repressor (NCoR), enabling it to actively repress transcription of its target genes, including Bmal1, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (26,54). GSK4112 (also known as SR6452), a synthetic non-porphyrin small molecule, functions as a Rev-erbα agonist by mimicking the action of heme (54). Conversely, SR8278 acts as an antagonist, inhibiting the transcriptional repression activity of Rev-erbα. Structural analyses show that SR8278 closely resembles agonist molecules, and treatment of HepG2 cells with SR8278 results in increased expression of Rev-erbα target genes such as Bmal1, G6Pase and PEPCK, consistent with its ability to block the effects of endogenous heme (28,55). Both GSK4112 and SR8278 serve as valuable chemical tools for probing Rev-erbα functions in transcriptional repression, circadian regulation and metabolic pathways (26,27). The repressive function of Rev-erbα is mediated through recruitment of the NCoR/HDAC3 corepressor

complex (56,57). In addition to transcriptional regulation, protein stability is a critical mechanism controlling Rev-erbα activity. Rev-erbα is intrinsically unstable, with a half-life of less than 1 h, indicating continuous proteolytic turnover (58). Phosphorylation by glycogen synthase kinase 3β (GSK3β) at serine residues 55 and 59 has been implicated in modulating Rev-erbα stability (59). Furthermore, a ubiquitin E3 ligase complex composed of ADP-ribosylation factor binding protein 1 and MYC binding protein 2 has been shown to mediate lithium-induced degradation of Rev-erbα (58). SR8278 (administered via micro slow injection at 20 μg/mouse) has also been reported to restore the DNA-binding activity of Rev-erbα and nuclear receptor subfamily 4 group A member 2 at the tyrosine hydroxylase promoter, promoting enrichment at R/N motifs [The 'R/N motif' specifically refers to a DNA sequence pattern recognized by transcription factors, wherein R denotes a purine base (adenine (A) or guanine (G)), and N represents any nucleotide (A, T, C or G)] recognized by both transcription factors (28). Additionally, SR8278 exerts anti-depressant and anxiolytic effects in 6-OHDA-induced mouse models in a circadian rhythm-dependent manner, restoring rhythmic patterns of emotion-related behaviors (27).

To investigate the role of Rev-erbα in liver fibrosis, *in vivo* experiments were conducted using the Rev-erbα agonist GSK4112 (25 mg/kg) and the antagonist SR8278 (20 μg/mouse) to pharmacologically activate or inhibit Rev-erbα signaling in mice. Liver fibrosis was induced through intraperitoneal administration of a CCl_4 mixture. In parallel, *in vitro* experiments involved stable lentiviral transfection to generate LX-2 cell lines with either Rev-erbα overexpression or knockdown, followed by TGF-β1 stimulation for 24 h to induce cellular activation. Inhibition of Rev-erbα expression in mice resulted in increased collagen deposition and elevated hepatic levels of α-SMA, COL-1 and TGF-β1, while activation of Rev-erbα reversed these pathological features. Comparable effects were observed in LX-2 cells, consistent with the established role of activated HSCs in upregulating α-SMA and COL-1 expression (60), with α-SMA serving as a well-recognized marker of HSC activation and fibrosis severity (61,62). TGF-β1, a key pro-fibrotic cytokine, is known to drive HSC activation and sustain fibrogenesis (63,64). Taken together, these findings indicate that downregulation of Rev-erbα promotes liver fibrosis, whereas its upregulation exerts a protective, anti-fibrotic effect.

The NLRP3 inflammasome has been implicated in various inflammatory conditions, including colitis, type 2 diabetes and atherosclerosis. Loss of Rev-erbα has been shown to exacerbate atherosclerotic plaque vulnerability and rupture by activating the NF-κB/NLRP3 axis, thereby increasing macrophage infiltration, oxidative stress and inflammatory cytokine production (65). In breast cancer cells, Rev-erbα inhibition activates the cGAS-stimulator of interferon genes pathway, leading to elevated levels of type I interferons and downstream chemokines, chemokine (C-C motif) ligand 5 and CXC motif chemokine ligand 10, enhancing antitumor immune responses (66). In HSCs, Rev-erbα degradation impairs mitochondrial fission and promotes mitochondrial DNA (mtDNA) release, triggering cGAS activation and contributing to a pro-inflammatory microenvironment that accelerates liver fibrosis (21). Additionally, Rev-erbα regulates the circadian rhythm of the NLRP3 inflammasome activity, and its activation has been shown to alleviate hepatitis in mouse models (31). The Rev-erbα agonist SR9009 exerts potent anti-inflammatory effects by suppressing pro-inflammatory cytokine expression and preventing monocyte infiltration during inflammation. Notably, SR9009 alleviated colitis in wild-type mice but failed to confer protection in NLRP3- or Rev-erbα-deficient models (67). In models of neuroinflammation, SR9009 inhibited NLRP3 activation, and reduced IL-6, IL-1β and IL-18 production, attenuating microglial and astrocyte activation, and ameliorating status epilepticus (31). Similarly, in degenerative disc disease, Rev-erbα activation suppressed NLRP3 inflammasome assembly and IL-1β production, reducing local inflammation (68). These findings underscore the central role of the Rev-erbα/NLRP3 axis in inflammatory disease progression. Consistent with this mechanistic framework, the present study demonstrated that Rev-erbα inhibition in both *in vivo* and *in vitro* liver fibrosis models led to upregulation of NLRP3, caspase-1, ASC, IL-18 and IL-1β. Conversely, pharmacological or genetic activation of Rev-erbα suppressed the expression of these inflammasome components. These results support the hypothesis that Rev-erbα functions as a negative upstream regulator of the NLRP3 inflammasome in activated HSCs and fibrotic liver tissue, thereby mediating its anti-fibrotic effects through suppression of inflammasome-driven inflammation (Fig. 4).

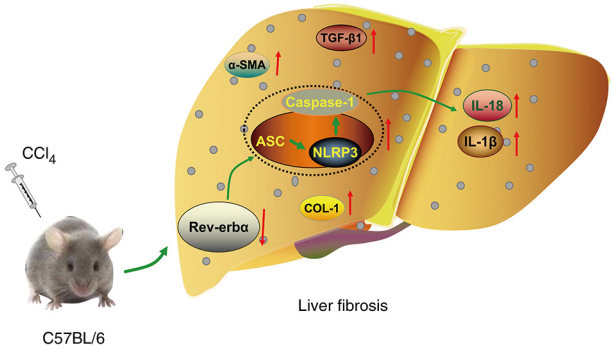

Figure 4. Mechanism of Rev-erbα promoting the progression of liver fibrosis. The decrease in the expression of the biological clock gene Rev-erbα promotes the activation of NLRP3 inflammasomes (ASC, caspase-1 and NLRP3) and downstream inflammatory pathways (IL-18 and IL-1β), and promotes the progression of liver fibrosis. ASC, apoptosis-associated speck; α-SMA, α-smooth muscle actin; CCl_4, carbon tetrachloride; COL-1, collagen 1; IL, interleukin; NLRP3, NLR family domain containing protein 3; Rev-erbα, nuclear receptor subfamily 1 group D member 1; TGF-β1, transforming growth factor-β1.

Although significant progress has been made in elucidating the role of Rev-erbα in liver fibrosis and its association with the NLRP3 inflammasome, several limitations remain that require further investigation. First, the molecular mechanisms underlying these observations have not been fully explored. While the present study demonstrates that Rev-erbα negatively regulates the NLRP3 inflammasome, the specific molecular interactions remain unclear. Future research should focus on understanding how Rev-erbα interacts with the various components of the NLRP3 inflammasome and how this interaction

influences the onset and progression of liver fibrosis. Second, the animal model used in this study, CCl_4-induced liver fibrosis, only partially mimics the pathological processes of human liver fibrosis and does not fully reflect clinical conditions. Third, the study's focus on HSCs limits its scope. Liver fibrosis is a complex, multifactorial condition involving interactions between various cell types, including hepatocytes, fibroblasts and immune cells. A broader examination of the role of these other cell types would provide a more comprehensive understanding of the disease process. Finally, while Rev-erbα shows promise as a therapeutic target in liver fibrosis, translating this potential into clinical applications faces significant challenges. These include the optimization of drug delivery systems, addressing inter-individual variability, and ensuring safety and efficacy in clinical settings.

In light of these limitations, several future research directions are proposed. Regarding the molecular mechanisms, building upon existing findings and recent international advancements, a preliminary hypothesis can be formed concerning how Rev-erbα regulates the NLRP3 inflammasome in liver fibrosis. The first potential mechanism involves direct transcriptional regulation. As a nuclear receptor, Rev-erbα may bind directly to the promoter regions of NLRP3 inflammasome-related genes and suppress their transcription. For instance, Rev-erbα could bind to the promoters of NLRP3, caspase-1 or ASC, inhibiting their expression and thus reducing NLRP3 inflammasome activity. The second mechanism involves indirect signaling pathways. Rev-erbα may modulate NLRP3 inflammasome activity by influencing other cellular signaling pathways, such as inhibiting the NF-κB pathway, thereby reducing NLRP3 activation. Additionally, Rev-erbα could regulate oxidative stress and mitochondrial function, indirectly affecting inflammasome activity. The third mechanism entails protein-protein interactions, where Rev-erbα may physically interact with one or more components of the NLRP3 inflammasome, interfering with its assembly and function. For example, Rev-erbα might bind to NLRP3, caspase-1 or ASC, preventing the formation of active inflammasome complexes. To validate these hypotheses and elucidate the precise molecular mechanisms by which Rev-erbα regulates the NLRP3 inflammasome in liver fibrosis, advanced molecular biology techniques such as chromatin immunoprecipitation and RNA sequencing should be employed. These tools will enable the exploration of how Rev-erbα interacts with each component of the inflammasome and how these interactions influence the progression of liver fibrosis. Furthermore, the development of more clinically relevant animal models is needed. Models that better replicate the etiology of human liver fibrosis, such as cholestatic liver disease or viral hepatitis models, would provide more accurate insights into the role of Rev-erbα in different types of liver fibrosis. The interaction between various cell types should also be explored. Specifically, studying Rev-erbα expression and function in hepatocytes, immune cells and other cell types will help clarify how these interactions contribute to the progression of liver fibrosis. Finally, efforts to promote clinical translation are essential. Optimizing drug delivery systems and improving the stability and bioavailability of Rev-erbα modulators will be key to advancing their therapeutic potential *in vivo*. Clinical trials should also be conducted to assess the efficacy and safety of Rev-erbα regulators in patients with liver fibrosis.

In summary, by deepening our understanding of the function and regulatory network of Rev-erbα, novel drug targets can be identified, leading to the development of more effective anti-fibrotic therapies. This progress not only promises to advance medical research but also offers new hope for the treatment of fibrotic diseases, ultimately contributing to a transformative shift in clinical practice.

In conclusion, expression of the circadian clock gene Rev-erbα is significantly suppressed in liver fibrosis, which contributes to fibrogenesis by driving the upregulation of the downstream NLRP3 inflammasome.

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Availability of data and materials

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

Authors' contributions

JW was responsible for conceptualization, validation, writing the original draft of the manuscript, using software, visualization, and reviewing and editing the manuscript. YW and LL contributed to investigation and the methodology. WP and YL were responsible for the acquisition of funding, project administration and formal analysis. All authors have read and approved the final version of the manuscript. WP and YL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All animal experiment protocols were approved by the Ethics Committee of Shanghai Municipal Hospital of Traditional Chinese Medicine (Shanghai, China; approval no. 2022033).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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