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ISG15 deficiency in hepatic stellate cells promotes TGF β 2-induced liver fibrosis by counteracting CREB1 ISGylation

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ABSTRACT

Background Interferon (IFN)-stimulated gene 15 (ISG15) is a downstream molecule of the IFN pathways central to many cellular processes. ISG15 mainly exerts its function through a post-translational modification process known as ISGylation.

Objective In this study, the role of ISG15 in the activation of hepatic stellate cells (HSCs) and liver fibrosis was examined.

Design Liver fibrosis was established by carbon tetrachloride (CCl₄), bile duct ligation (BDL) surgery and metabolic dysfunction-associated steatohepatitis (MASH) diet between HSC-specific deletion of ISG15 (ISG15cKO) and wild type mice. Using genetic strategies in vitro, the role of ISG15 in HSCs was established. Immunoprecipitation, luciferase reporter assays and chromatin-immunoprecipitation assays (ChIP) in combination with proteomics sequencing in HSCs were used to study the associated downstream mechanisms.

Results ISG15 was underexpressed in activated HSCs and fibrotic livers, showing an inverse correlation with α -smooth muscle actin in patients with liver fibrosis. ISG15cKO mice developed spontaneous hepatic fibrosis and showed exacerbated CCl₄/BDL-induced fibrogenesis. In vitro, ISG15 modulated HSC activation, proliferation and excessive extracellular matrix production. ISG15 deficiency in HSCs promoted transforming growth factor β 2 (TGF β 2) transcription by enhancing phosphorylated cAMP responsive element binding protein 1 (CREB1) activity, thereby inducing CREB1 binding on TGF β 2 promoter regions to activate TGF β 2/SMAD2 signalling. ISGylation directly binds CREB1 on Lys-304 and Lys-305 to inhibit p-CREB1 activity. Overexpression of ISG15 in HSCs or pharmacological inhibition of CREB1 by 666–15 could abolish ISG15 deficiency-induced liver fibrosis in CCl₄-treated mice.

Conclusions ISG15 regulated HSC activation and liver fibrosis in part via the CREB1/TGF β 2/SMAD2 regulatory pathway. Utilisation of ISG15-CREB1 signalling may be a potential therapeutic target for liver fibrosis.

INTRODUCTION

Liver fibrosis, the pivotal stage of the wound-healing response against various injuries that culminates in liver failure, is associated with high mortality rates.¹ Extensive research efforts have focused on deciphering its cellular mechanisms and developing targeted hepatic therapies.² Central to

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Interferon (IFN)-stimulated gene 15 (ISG15) is mainly implicated in a post-translational modification process, ISGylation, while its role in the pathogenesis of liver fibrosis remains unclear.
- ⇒ ISG15 is one of the key downstream molecules of the type I IFN pathways.

WHAT THIS STUDY ADDS

- ⇒ ISG15 is underexpressed in activated HSCs from fibrotic livers, and IFN-triggered ISG15 inhibits HSC activation, proliferation and extracellular matrix production.
- ⇒ ISG15 deficiency in HSCs not only exacerbates liver fibrosis but also induces spontaneous fibrosis.
- ⇒ ISG15 inhibits TGF β 2/SMAD2 signalling and HSC activation by directly binding CREB1 on Lys-304 and Lys-305 via ISGylation.
- ⇒ The CREB1 inhibitor 666–15 effectively alleviates experimental liver fibrosis in mice.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ This study provides a scientific basis for future investigations into the impact of HSC within liver fibrosis.
- ⇒ Administration of 666–15 to target ISG15-CREB1 signalling may be a potential therapeutic target for liver fibrosis.

this process is the activation of hepatic stellate cells (HSCs), which transdifferentiate from quiescent vitamin A-storing stages into α -smooth muscle actin (α SMA)-positive myofibroblasts, driving excessive extracellular matrix (ECM) deposition and regenerative nodule formation.^{3,4} Their activation represents a cornerstone of fibrogenesis pathogenesis, underscoring the critical need to identify molecular regulators governing HSC activation and ECM accumulation as potential therapeutic targets.

Interferon (IFN)-stimulated gene 15 (ISG15), a pivotal downstream effector of the type I IFN (IFN-I) pathways and the first identified ubiquitin-like protein,⁵ primarily functions through ISGylation—a post-translational modification process involving covalent conjugation to the substrate

lysine residues through the carboxy-terminal LRLRG motif.⁵ ISGylation mirrors ubiquitination and can participate in various biological processes by regulating protein stability or function, including antiviral immunity, genomic stability and cellular homeostasis.^{5–6} But unlike ubiquitination modification, the substrate of ISGylation three-step enzymatic cascade is ISG15, requiring ubiquitin-like modifier activating enzyme 7 as an ISG15-activating E1 enzyme, ubiquitin-conjugating enzyme E2 L6 (UBE2L6) as an ISG15-conjugating E2 enzyme, and the Homologous to the E6AP Carboxyl Terminus (HECT) domain and RCC1-like domains 6 (HERC6) as an ISG15 E3 ligase,⁷ with ubiquitin-specific protease 18 (USP18) serving as the deconjugating isopeptidase of the ISGylation process by cleaving off ISG15 that is conjugated to the substrate protein.⁸ Beyond its established roles in viral defence, ISG15 modulates diverse pathologies including tumour progression, adipose thermogenesis and female fertility.^{7–10} Emerging evidence implicates ISG15 in tissue fibrosis, with excessive ISGylation exacerbating angiotensin II-induced cardiac fibrosis and accelerating acute kidney injury.^{11–12} In hepatic contexts, ISGylation regulates liver injury responses through modification of hepatocyte or HSC mediators like p53 and β -catenin.^{13–14} Hepatic viral infections, mainly hepatitis B and C virus infections, correlate with ISG15 expression.^{15–16} Despite these advances, the involvement of ISG15/ISGylation in liver fibrogenesis remains unexplored.

Here, we demonstrated that ISG15 protein/transcript levels were downregulated in human and murine fibrotic livers, particularly within activated HSCs localised to fibrotic foci. In vitro, ISG15 and its associated conjugating (UBE2L6)/deconjugating (USP18) enzymes modulated HSC activation, proliferation and ECM production. Targeted deletion of ISG15 in HSCs induced spontaneous hepatic fibrosis and exacerbated carbon tetrachloride (CCl₄)/bile duct ligation (BDL)-induced fibrogenesis. Mechanistically, ISG15 suppressed HSC activation through CREB1/TGF β 2/SMAD2 signalling. ISGylation covalently binds CREB1 on lysine 304/305, inhibiting the phosphorylated CREB1 on serine 133 and impairing CREB1's ability to engage the TGF β 2 promoter, thereby reducing TGF β 2 transcription. Therapeutically, overexpression of ISG15 in HSCs or pharmacological inhibition of CREB1 by 666–15 attenuated ISG15 deficiency-driven fibrotic progression in vivo.

MATERIALS AND METHODS

Human liver samples

All samples were obtained from two cohorts of patients from Tongji Hospital of Huazhong University of Science and Technology (HUST, Wuhan, China). Liver specimens were collected from 42 patients who underwent surgery due to hepatocellular carcinoma (n=28), cholangiocarcinoma (n=8) and focal benign lesion (n=6). Cohort 1 includes 21 patients with clinically diagnosed liver cirrhosis. The liver samples were used for q-PCR. In cohort 2, liver tissues were isolated from 13 patients with cirrhosis (Scheurer Score 4, S4) and 8 patients without cirrhosis (Scheurer Score 0–1, S0-1). These samples were used for immunohistochemistry (IHC) and immunofluorescence (IF) staining. The characteristics of these patients are shown in online supplemental table 1.

Patient and public involvement

All patients gave their written informed consent. Sample tissues were used following the appropriate regulations. Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

Further experimental details are available in the online supplemental materials and methods section.

RESULTS

ISG15 is underexpressed in activated HSCs from fibrotic livers

To assess the role of ISG15 in liver fibrosis, we analysed single-cell RNA sequencing (scRNA-seq) data (GSE134037)¹⁷ comparing normal and fibrotic mouse livers. The livers showed a distinct fibroblast cluster marked by actin alpha 2, smooth muscle (α SMA), Collagen Type I Alpha 1 (Col1 α 1), Collagen Type III Alpha 1 (Col3 α 1) and Platelet-derived growth factor receptor beta (PDGFR β) (figure 1A and online supplemental figure S1A). ISG15 expression was predominantly enriched in normal liver fibroblast clusters, as evidenced by both the larger dot size and darker colour, but was significantly downregulated in fibrotic liver fibroblasts (figure 1B,C, online supplemental figure S1B–D). Therefore, we verified ISG15 expression in liver tissue samples from CCl₄/BDL-induced fibrotic mice. Results showed the reduced hepatic ISG15 expression and ISGylation levels in fibrotic mice compared with control mice (online supplemental figure S1E). Primary HSCs isolated from fibrotic livers also exhibited suppressed ISG15 mRNA and protein levels (figure 1D and online supplemental figure S1F). Since α -SMA expression in healthy liver tissues is low or absent, we choose Desmin as the HSC marker in the staining experiment. The IHC staining on the sections from fibrotic mouse livers showed significantly decreased ISG15 expression in the fibrous cords, coinciding with the distribution of Desmin-positive HSCs (figure 1E). Quiescent HSCs can be spontaneously activated by in vitro culture. Consistently, in vitro activation of quiescent HSCs showed declining ISG15 and ISGylation levels alongside rising α -SMA expression during 5–7 day cultures (figure 1F), consistent with the microarray data (GSE34949)¹⁸ showing ISG15 reduction in activated HSCs from a 7-day in vitro culture (figure 1G). Finally, we further examined the expression of ISG15 and ISGylation-related molecules in the liver tissues from patients in our cohorts. mRNA levels of ISG15 were negatively correlated with α -SMA expression ($R=-0.44$, $p<0.05$) (figure 1H and online supplemental figure S1G). Double IF staining revealed ISG15+Desmin+ cells decreased from $78.33\pm10\%$ in liver sections from patients without cirrhosis in cohort 2 to $7.96\pm2.26\%$ in those from cirrhosis (figure 1I), revealing reduced ISG15 expression in activated HSCs.

IFN-dependent transcription factor IRF9 inhibits ISG15 expression in HSCs

To identify upstream mechanisms responsible for the downregulation of ISG15 in liver fibrosis, we analysed IFN pathway molecules, including IFNAR, IFN- β , IFN- γ , interferon induced transmembrane protein 1 (IFITM1), interferon induced protein with tetratricopeptide repeats 2 (IFIT2), interferon gamma inducible protein 16 (IFI16) and interferon regulatory factor 3 (IRF3), which were downregulated in primary HSCs isolated from CCl₄-treated mice (online supplemental figure S2A). The IFN-I pathway activates signal transducer and activator of transcription 1 (STAT1)/STAT2 phosphorylation to form interferon-stimulated gene factor 3 with interferon regulatory factor 9 (IRF9). This complex could trigger ISG15 transcription via binding to an IFN-dependent promoter, IFN-stimulated response element (ISRE);^{19–20} we hypothesise that type I IFN stimulates IRF9 to promote the expression of ISG15 in HSCs. IFN- β treatment increased IRF9 and ISG15 expression (online supplemental figure S2B), while IRF9 knockdown reduced

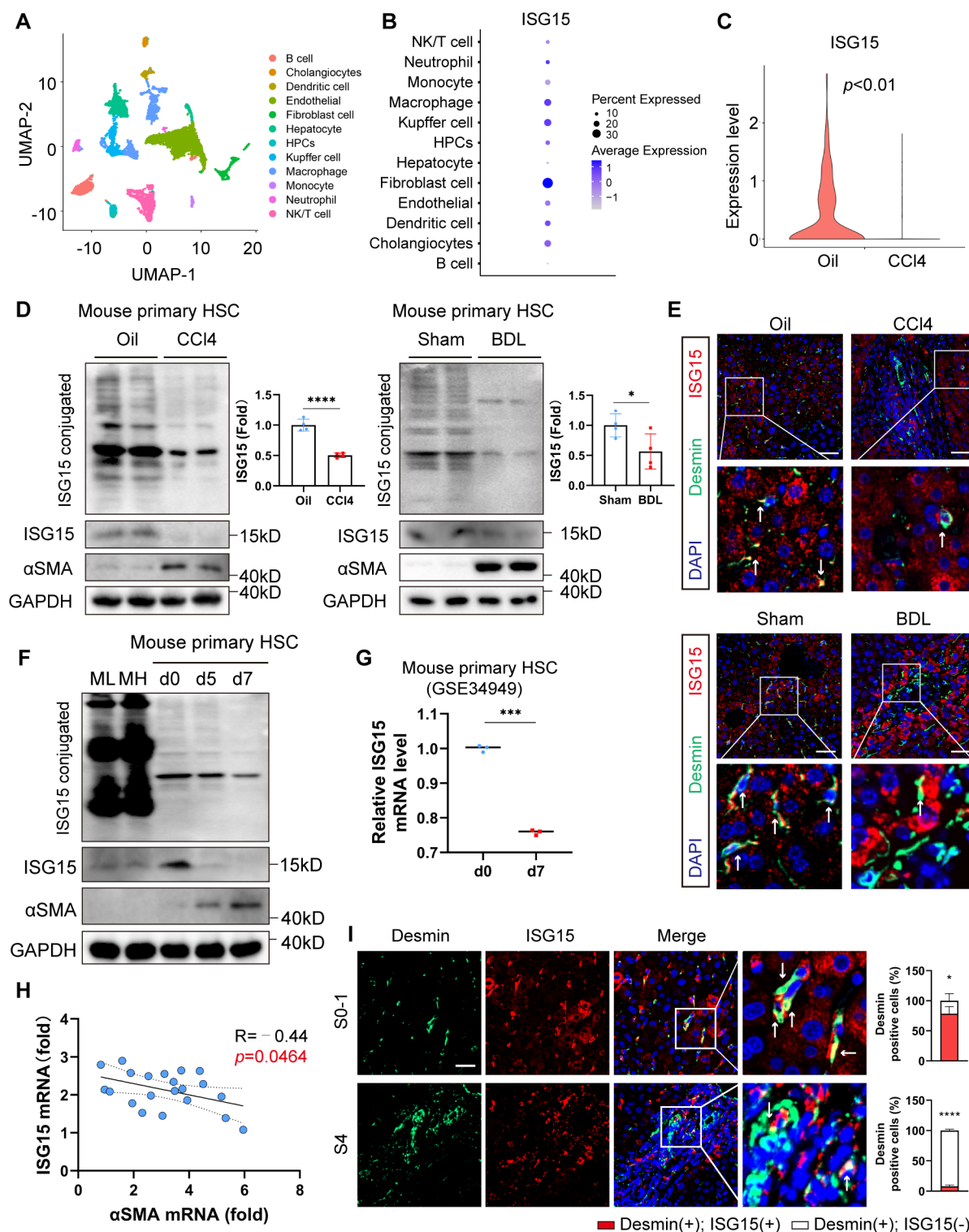


Figure 1 ISG15 is underexpressed in HSCs and negatively correlated with fibrosis. (A) Reclustering of scRNA-seq on the NPCs from control mice or CCl4-induced mice liver fibrosis samples (GSE134037). (B) The bubble map shows the distribution and expression of ISG15 in the liver subgroup of normal mice. (C) Violin plots of the ISG15 expression of HSCs from normal and CCl4-induced fibrotic livers. (D) The protein levels of ISG15 and αSMA in primary HSCs isolated from CCl4/BDL-treated mice (n=4). (E) Representative double immunofluorescence staining images of ISG15 and Desmin in liver tissues from the livers of CCl4/BDL-treated mice. (F) Immunoblot showing ISG15 and αSMA levels in mouse primary HSCs cultured for 5–7 days. (G) Microarray ISG15 expression in quiescent (d0) or 7 day culture-activated (d7) mouse primary HSCs (GSE34949). (H) Association of hepatic mRNA levels between αSMA and ISG15 in cohort 1 containing 21 patients with clinically diagnosed liver cirrhosis. (I) Representative double immunofluorescence staining images of ISG15 and Desmin in liver tissues from cohort 2 patients (cirrhosis: Scheurer Fibrosis Score 4, S4, n=13; non-cirrhosis: Scheurer Fibrosis Score 0–1, S0-1, n=8) (scale bars, 20 mm). The boxed regions are shown at higher magnification. For bar graphs, data are means±SEM. * $p < 0.05$, ** $p < 0.01$. αSMA, α-smooth muscle actin; BDL, bile duct ligation; CCl4, carbon tetrachloride; HSC, hepatic stellate cell; ISG15, interferon-stimulated gene 15; MH, mouse hepatocyte; ML, healthy mouse liver.

ISG15 in human immortalised HSC line LX-2 cells (online supplemental figure S2C). To determine their transcription activity, the ISG15 promoter regions and ISRE were separately cloned into the pGL3 basic vector to perform a luciferase reporter assay. Notably, IFN- β boosted the activity of both ISG15 promoter regions and ISRE in LX-2s, but this effect was reversed by IRF9 knockdown (online supplemental figure S2D), indicating that decreased expression of the IFN-dependent IRF9 impairs ISG15 transcription. Finally, we verified the expression of IRF9 in the fibrotic/normal mouse liver tissues from scRNA-seq data and found IRF9 expression was significantly downregulated in fibrotic liver fibroblasts (online supplemental figure S2E). And in liver tissues from patients with and without liver cirrhosis, double IF staining showed that IRF9+Desmin+ cells decreased from $59.62 \pm 9.76\%$ in liver sections from non-cirrhosis in cohort 2 to $9.25 \pm 3.77\%$ in those from cirrhosis (online supplemental figure S2F), revealing reduced IRF9 expression in activated HSCs. Overall, these results indicate that IFN-dependent transcription factor IRF9 inhibits ISG15 expression in activated HSCs.

ISG15 deficiency in HSCs induces spontaneous hepatic fibrosis in 18-month-old mice

We generated an HSC-specific ISG15 deletion mouse line (ISG15(flox/flox); Lrat-Cre, abbreviated as ISG15cKO) by crossing with Lrat-Cre mice (a highly efficient HSC deleter strain) and ISG15(flox/flox) mice (wild type (WT) mice) (figure 2A) to investigate the loss-of-function effect of ISG15 in vivo. The genotypes of the mice were determined through PCR analysis of tail DNA (online supplemental figure S3A). And the successful knockout of ISG15 was confirmed by western blotting (WB) analysis of isolated primary HSCs (online supplemental figure S3B). Although the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels had no difference in ISG15cKO mice at 9 months compared with WT mice, the liver hydroxyproline levels slightly increased, indicating that ISG15cKO mice developed fibrogenesis at 9 months of age (online supplemental figure S3C,D).

We then maintained WT and ISG15cKO mice for 18 months and observed a slightly irregular texture on the surface of the liver in ISG15cKO mice (figure 2B). Although the serum levels of ALT and AST still showed no difference, ISG15cKO mice exhibited a more significant increase in liver hydroxyproline levels compared with those in WT littermates (figure 2C,D). We also observed an accumulation of activated HSC marker α SMA and collagen deposition markers Col1 α 1 in ISG15cKO mice by WB analysis (figure 2E). The mRNA levels of fibrosis-related genes (α SMA, Col1 α 1, connective tissue growth factor CTGF) and inflammatory genes (IL1 β , C-C motif chemokine ligand 5 (CCL5), colony stimulating factor 1(CSF1)) were substantially upregulated in the livers of 18-month-old ISG15cKO mice (figure 2F). Furthermore, histological analysis using H&E, Sirius red, Masson staining, α SMA/F4/80/Ly6G staining and Ishak scoring revealed mild collagen deposition and HSC activation in ISG15cKO mice at 9 months of age (figure 2G). With ageing, ISG15cKO mice displayed a massive diffuse ECM and HSC initiation at 12 months and 18 months, respectively (figure 2G), and inflammatory infiltration became stronger at 18 months (online supplemental figure S4A). These findings demonstrate that ISG15 deficiency in HSCs induces spontaneous liver fibrosis at 18 months of age in mice.

ISG15 deletion in HSCs promotes experimental mouse liver fibrosis

To further assess the role of HSC-specific ISG15 in liver fibrosis progression, we investigated the fibrosis degree of ISG15cKO mice under three experimental conditions: CCl₄, BDL treatment and choline-deficient L-amino-defined high-fat diet (CDAHFD)–induced metabolic dysfunction-associated steatohepatitis model. Following an 8-week CCl₄ injection, ISG15cKO mice exhibited elevated serum ALT/AST levels and hepatic hydroxyproline content compared with controls (figure 3A–C). Histopathological analysis (H&E/Sirius Red/Masson staining) and immunostaining (α SMA, F4/80 and Ly6G) revealed exacerbated collagen deposition, fibrotic septa formation and inflammatory infiltration in ISG15cKO livers (figure 3D and online supplemental figure S4B). WB confirmed upregulated α SMA and Col1 α 1 protein expression (figure 3E), while qPCR assays demonstrated the upregulation of profibrotic and inflammatory genes in the livers of ISG15cKO mice (figure 3F). Three weeks after BDL treatment, ISG15cKO mice exhibited increased hepatic hydroxyproline and serum ALT/AST levels (figure 3G–I), accompanied by aggravated collagen deposition, α SMA expression, liver injury and inflammatory cell infiltration compared with WT controls (figure 3J and online supplemental figure S4C). Profibrotic and proinflammatory genes were also significantly upregulated in ISG15cKO mice (figure 3K,L). Similarly, after 6 weeks of CDAHFD, the ISG15cKO mice showed more obvious collagen/lipid deposition and inflammatory responses (online supplemental figure S5). Collectively, these models collectively demonstrate ISG15 deficiency in HSCs promotes experimental fibrosis progression.

Intracellular ISG15 is both essential and sufficient to inhibit HSC activation, proliferation and ECM production

ISG15 may function through intracellular ISGylation or as an extracellular cytokine.²¹ To investigate this, we stimulated primary human HSCs with varying concentrations of exogenous ISG15 and assessed its effects on proliferation, activation and ECM production. We found that only specific concentrations of extracellular ISG15 (100 ng/mL) influenced α SMA expression (online supplemental figure S6A,B). Furthermore, ELISA analysis of cell supernatants after IFN β stimulation and serum from fibrotic mice revealed ISG15 concentrations consistently below 1 ng/mL—significantly lower than the levels required for HSC activation (online supplemental figure S6C). Therefore, we propose that ISG15 likely regulates HSC function primarily through intracellular ISGylation. To delineate the HSC-specific function of ISGylation, we generated different cell line models with varying levels of ISGylation in human immortalised HSC line LX-2 cells and primary human HSCs: ISG15KO (ISG15 knockout using CRISPR/Cas9), shUBE2L6 (UBE2L6 knockdown using shRNA), and siUSP18 (USP18 knockdown using siRNA) in LX-2; siISG15, siUBE2L6 and siUSP18 (ISG15, UBE2L6 and USP18 knockdown using siRNA) in primary human HSCs. These cell lines allowed us to test the molecular and cellular characteristics of cells devoid of ISG15 (ISG15KO and siISG15), decreased levels of ISGylation (shUBE2L6 and siUBE2L6), and enhanced levels of ISGylation (siUSP18). ISG15 deficiency increased proliferation in both ISG15KO LX-2 (online supplemental figure S6D) and siISG15 primary HSCs (online supplemental figure S6E), paralleled by upregulated α SMA (myofibroblast transdifferentiation marker) and ECM components (vimentin, Col1 α 1) in these models (online supplemental figure S6F,G). Reduced ISGylation (shUBE2L6/siUBE2L6)

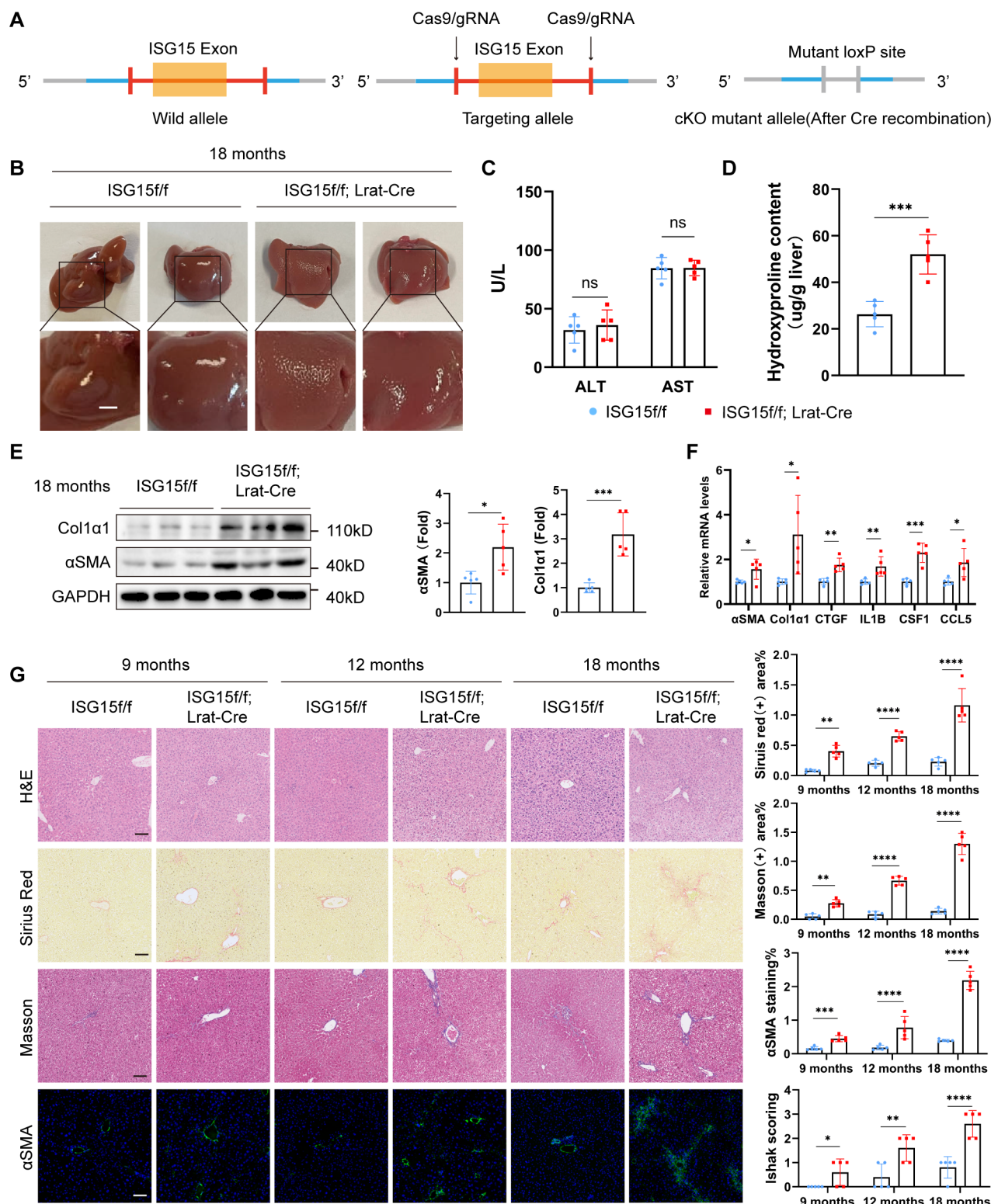


Figure 2 ISG15 deficiency in HSCs develops liver fibrosis in 18-month-old male mice. (A) Schematic representation of the creation of ISG15cKO mice by CRISPR-Cas9 gene targeting. (B) Representative pictures of livers from 18-month-old WT (ISG15f/f) and ISG15cKO male mice (ISG15f/f; Lrat-Cre). Scale bar, 2 mm. (C) Serum levels of hepatotoxicity indicators ALT and AST in mice (n=5/group). (D) Collagen content in the livers was determined as hydroxyproline content (n=5/group). (E) The protein levels of αSMA, Col1α1 in the liver tissues of WT and ISG15cKO mice (n=5/group). (F) Expression levels of fibrosis-related and proinflammatory genes in the liver (n=5/group). (G) Macroscopic examination, H&E, Sirius red, Masson, αSMA staining and semiquantitative Ishak scoring in the liver sections from WT mice and ISG15cKO mice (n=5/group, scale bar, 50 μm). For bar graphs, data are means ± SEM. *p < 0.05, **p < 0.01. ALT, alanine aminotransferase; AST, aspartate; αSMA, α-smooth muscle actin; BDL, bile duct ligation; CCL4, carbon tetrachloride; HSC, hepatic stellate cell; ISG15, interferon-stimulated gene 15 aminotransferase; ISG15cKO, HSC-specific deletion of ISG15; WT, wild type.

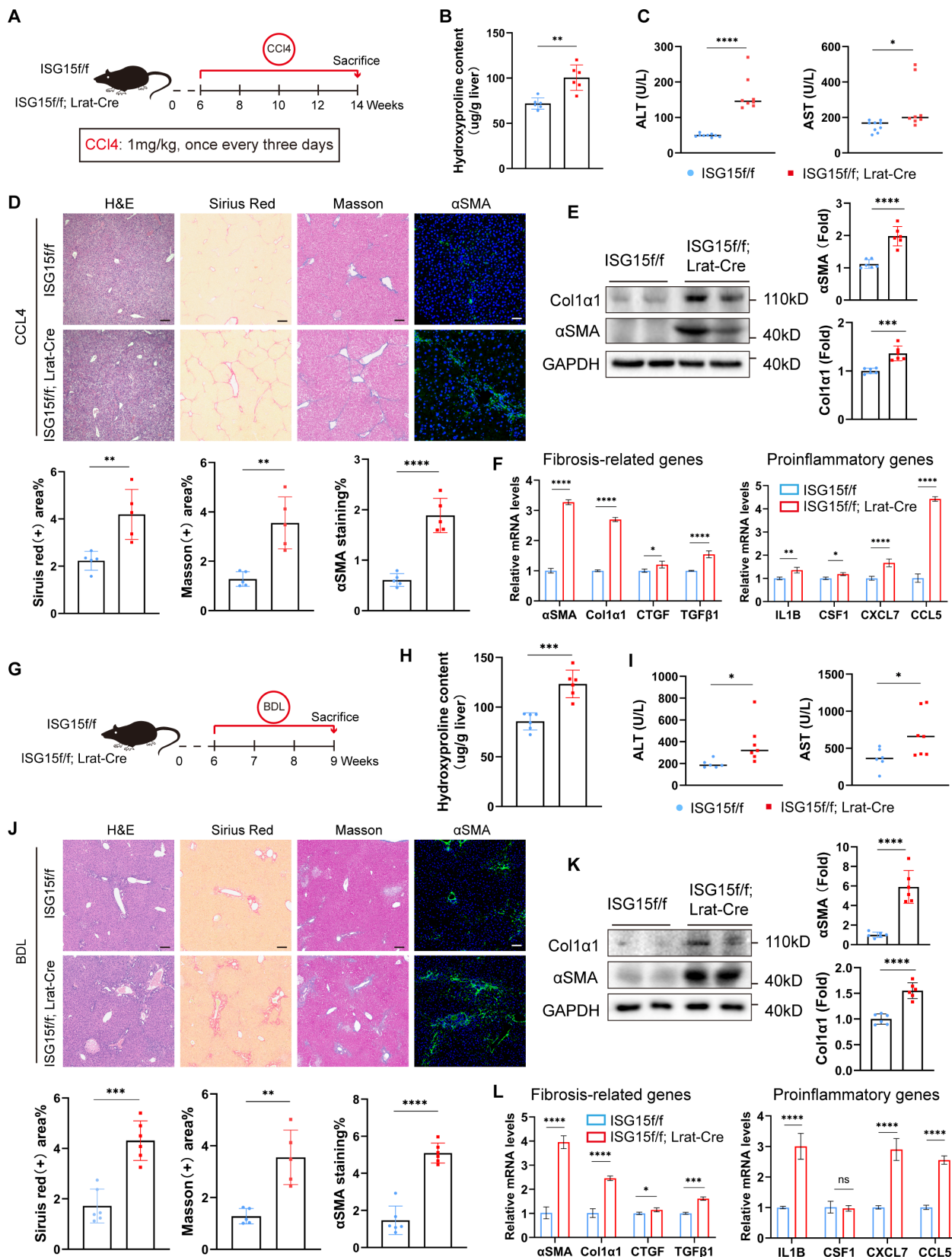


Figure 3 Genetic deletion of ISG15 in HSCs promotes CCl₄-induced and BDL-induced mouse liver fibrosis. (A, G) The experimental approach of the animal model establishment. (B, H) Hydroxyproline content (Hyp) in liver tissues from WT and ISG15cKO male mice (n=6/group). (C, I) Serum levels of hepatotoxicity indicators ALT and AST in mice (n=6/group). (D, J) Macroscopic examination, H&E, Sirius red, Masson and αSMA staining in the liver sections from WT mice and ISG15cKO male mice (n=5/group, scale bar, 50 μm). (E, K) Expression levels of fibrosis-related and proinflammatory genes in the liver. (F, L) The protein levels of αSMA, Col1α1 in the liver tissues of WT and ISG15cKO mice (n=6). For bar graphs, data are means±SEM *p<0.05, **p<0.01. ALT, alanine aminotransferase; AST, aspartate; αSMA, α-smooth muscle actin; BDL, bile duct ligation; CCl₄, carbon tetrachloride; HSC, hepatic stellate cell; ISG15, interferon-stimulated gene 15 aminotransferase; ISG15cKO, HSC-specific deletion of ISG15; WT, wild type.

similarly enhanced proliferation and ECM protein levels across both cell types (online supplemental figure S6D–G). Conversely, ISGylation augmentation (siUSP18) suppressed HSC proliferation (online supplemental figure S6D,E) while downregulating fibronectin, Col1 α 1, vimentin and α SMA in LX-2 and primary HSCs (online supplemental figure S6F,G). These findings establish that ISG15 deficiency drives HSC activation, proliferation and ECM production, with reduced ISGylation levels sufficient to trigger these profibrotic phenotypes.

ISG15 deficiency in HSCs promotes TGF β 2/SMAD2 signalling

To investigate how ISG15 deficiency promotes HSC activation, proliferation and ECM production, WT and ISG15KO LX-2 HSCs were used to perform a whole proteomics analysis and showed distinct segregation via principal component analysis (figure 4A). Among 481 differentially expressed genes (DEGs) (7.41% of proteome), 226 were downregulated and 254 upregulated (figure 4B). Next, the genetic effects of ISG15 on the main signalling pathways involved in HSC activation were investigated. Gene Set Enrichment Analysis highlighted enrichment of inflammatory pathways (eg, cell adhesion, cytokine-cytokine receptor interactions) in ISG15-deficient LX-2 (figure 4C and online supplemental figure S7). Therefore, we focused on all differentially expressed inflammatory-related proteins such as TNFSF4, IL1B and IL7R (figure 4D). Note that TGF β 2 was the second upregulated protein in ISGKO LX-2 HSCs (figure 4E). A marked increase of TGF β 2 in ISGKO and shUBE2L6 LX-2 HSCs was confirmed at protein and secretion levels by WB and ELISA, respectively (figure 4E,F), while ISGylation enhancement by siUSP18 could decrease the protein and secretion levels of TGF β 2 (figure 4F,G). Furthermore, IFN- β -induced ISGylation reduced TGF β 2 expression in LX-2 HSCs (online supplemental figure S8A), counteracted by the knockout of ISG15 (online supplemental figure S8B). The TGF β family comprises three isoforms: TGF β 1, TGF β 2 and TGF β 3, who are encoded by separate genes and have different receptor-binding domains; however, they are processed similarly and could perform similar functions.²² To rule out the possible accompanying role of TGF β 1 and TGF β 3, we detected their expressions. Surprisingly, TGF β 1/TGF β 3 secretion remained unchanged in ISG15KO/shUBE2L6 LX-2 HSCs (online supplemental figure S8C). Considering that Smad2 signalling pathways acted as canonical downstream of the TGF β family,^{23,24} we checked the expression of p-smad2/smard2 in the ISGylation-modulated cell lines and found the activation of smad2 signalling in ISGylation-modulated LX-2 HSCs (figure 4H). TGF β 2 siRNA in LX-2 and primary human HSCs attenuated ISG15 deficiency-induced HSC activation phenotypes (figure 4I–K). These findings demonstrated that ISG15 and ISGylation deficiency could activate TGF β 2/SMAD2 signalling to drive HSC proliferation, activation and ECM production.

Inhibition of TGF β 2 signalling suppressed ISG15 deficiency-mediated CCl4-induced mouse liver fibrosis

To validate the role of TGF β 2 in ISG15 deficiency-mediated liver fibrosis *in vivo*, we applied anti-TGF β 2 antibody to CCl4-treated mice (online supplemental figure S9A). As shown in online supplemental figure S9B, ISG15 deficiency in HSCs elevated α SMA and Col1 α 1 protein levels, which were blocked by TGF β 2 neutralisation. In parallel, ISG15 deficiency-induced serum ALT/AST levels and hepatic hydroxyproline content were also attenuated in the anti-TGF β 2 group (online supplemental figure S9C,D). In line with these results, the anti-TGF β 2 group significantly attenuated hepatic fibrosis in CCl4-treated mice as

determined by H&E, Sirius red staining, Masson's trichrome and α SMA staining (online supplemental figure S9E). These data indicated that interfering TGF β 2 signalling can mediate ISG15 deficiency-mediated CCl4-induced mouse liver fibrosis.

ISG15 regulates CREB1 activity and inhibits TGF β 2 transcription

To explore how ISG15/ISGylation regulates TGF β 2 expression, we first assessed TGF β 2 transcription. The results of qRT-PCR experiments showed that ISG15 and enhanced ISGylation reduce TGF β 2 expression at mRNA level in LX-2 HSCs, while reduced ISGylation increased it (figure 5A). Dual-luciferase reporter assay using a TGF β 2 promoter construct (pGL3-TGF β 2-pro) revealed increased transcriptional activity in ISG15KO and shUBE2L6 LX-2 HSCs (figure 5B). These findings led us to propose that ISG15 interacts with TGF β 2 transcription factors to regulate its transcription negatively. Therefore, we then tested TGF β 2-related transcription factors including RFX1,²⁵ SP1²⁶ and CREB1.²⁷ ISG15 knockout did not alter the expression of RFX1, SP1 or CREB1, but increased p-CREB1 in LX-2s (figure 5C and online supplemental figure S10A). More importantly, the nuclear translocation of CREB1, which was indispensable for its functional activity as a transcription factor to activate TGF β 2 gene,²⁷ was enhanced by ISG15/ISGylation deficiency and suppressed by ISGylation enhancement (figure 5D). Finally, the upregulated expression of p-CREB1 and TGF β 2 was confirmed in primary HSCs from our 18-month-old WT/ISG15cKO mice and CCl4/BDL-induced fibrotic mice, suggesting the crucial role of CREB1 in the regulation of TGF β 2 transcription by ISG15 (figure 5E and online supplemental figure S10B).

To examine the biological effect of ISG15 on CREB1 activity, ChIP assay was used to evaluate the binding of CREB1 on the TGF β 2 promoter. As shown in figure 5F, the results revealed enhanced CREB1 binding to the TGF β 2 promoter in ISG15KO and shUBE2L6 LX-2 HSCs, while siUSP18 (enhancing ISGylation) suppressed this interaction. We next identified the target site of CREB1 on the TGF β 2 promoter by ISG15. It has been reported that the promoter regions of TGF β 2 at nucleotides (nt) -74/-67 relative to the transcription sites, a cAMP response element-like (CRE-L) sequence (GCACGTCA), is important for TGF β 2 transcription regulation.²⁷ Considering one of the CREB family, cAMP-responsive element binding protein 3 like 3, could target and bind the CRE-L sequence,²⁸ it was possible that CREB1 could recognise the CRE-L sequence. Therefore, we substituted two mutations within CRE-L and found a marked decrease in the promoter activity (figure 5G). These findings provided direct evidence that ISG15 could regulate the CREB1 activity of binding on the TGF β 2 promoter regions.

To further demonstrate the role of ISGylation on TGF β 2 transcription, we mutated the C-terminal diglycine LRLRG motif required for ligation to the target lysine residue to the LRLRAA motif⁹ and overexpressed ISG15/ISG15AA in LX-2 HSCs. ISG15AA restored p-CREB1 levels and CREB1 nuclear localisation suppressed by ISG15 overexpression (figure 5H,I). Importantly, ISG15AA completely restored CREB1 binding on the TGF β 2 promoter (figure 5J). These data demonstrate that ISGylation—via its C-terminal diglycine—suppresses CREB1 binding to the TGF β 2 promoter, thereby inhibiting TGF β 2 transcription.

ISG15 binds and ISGylates CREB1

To uncover how ISG15 modulates CREB1, we performed immunoprecipitation-mass spectrum analysis to identify

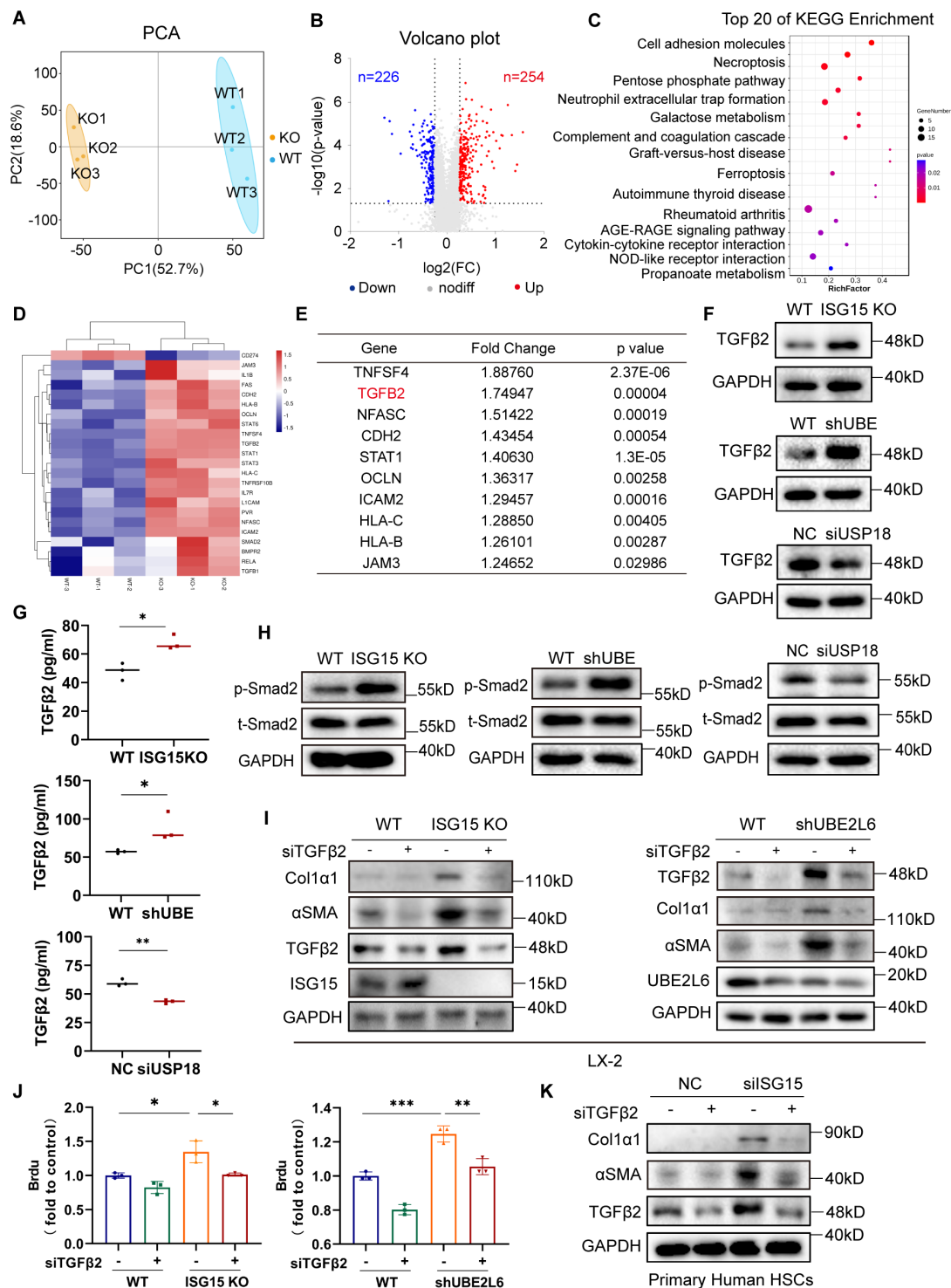


Figure 4 ISG15 deficiency in HSCs promotes TGFβ2/SMAD2 signalling (A) PCA of proteomics analysis data from WT and ISGKO LX-2 HSCs. $n=3$ per group. (B) Volcano plot (red, upregulation; blue, downregulation) of DEGs based on the proteomics analysis data using WT and ISGKO LX-2 HSCs. ($n=3$ each; DEGs with adjusted value of $p<0.05$). (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of proteomics analysis data from WT and ISGKO LX-2 HSCs. (D) The heatmap of all differentiated inflammatory-related proteins in the proteomics analysis data from WT and ISGKO LX-2 HSCs. (E) Expression of top 10 genes in the inflammatory-related proteins based on proteomics analysis data. (F) Western blot was used to detect TGFβ2 expression in LX-2 HSCs (WT, ISG15KO, shUBE2L6 and siUSP18) and (G) ELISA assay was performed to determine the secretin level of TGFβ2 in the conditioned media. (H) Western blot analysis of p-Smad2 and Smad2 in cells. LX-2 HSCs (WT, ISG15KO, shUBE2L6) were transfected with the siRNA against TGFβ2 for 48 hours. The expression levels of TGFβ2, α-SMA and Col1α1 were determined by Western blot (I), and the proliferation activity was detected by BrdU proliferation assay (J). Primary human HSCs (NC, siISG15) were transfected with the siRNA against TGFβ2 for 48 hours. The expression levels of TGFβ2, α-SMA and Col1α1 were determined by Western blot (K). For bar graphs, data are means±SEM. * $p<0.05$, ** $p<0.01$. HSC, hepatic stellate cell; ISG15, interferon-stimulated gene 15 aminotransferase; ISG15cKO, HSC-specific deletion of ISG15; PCA, principal component analysis; shUBE2L6, UBE2L6 knockdown using shRNA; siUSP18, USP18 knockdown using siRNA; UBE2L6, ubiquitin-conjugating enzyme E2 L6; USP18, ubiquitin-specific protease 18; WT, wild type.

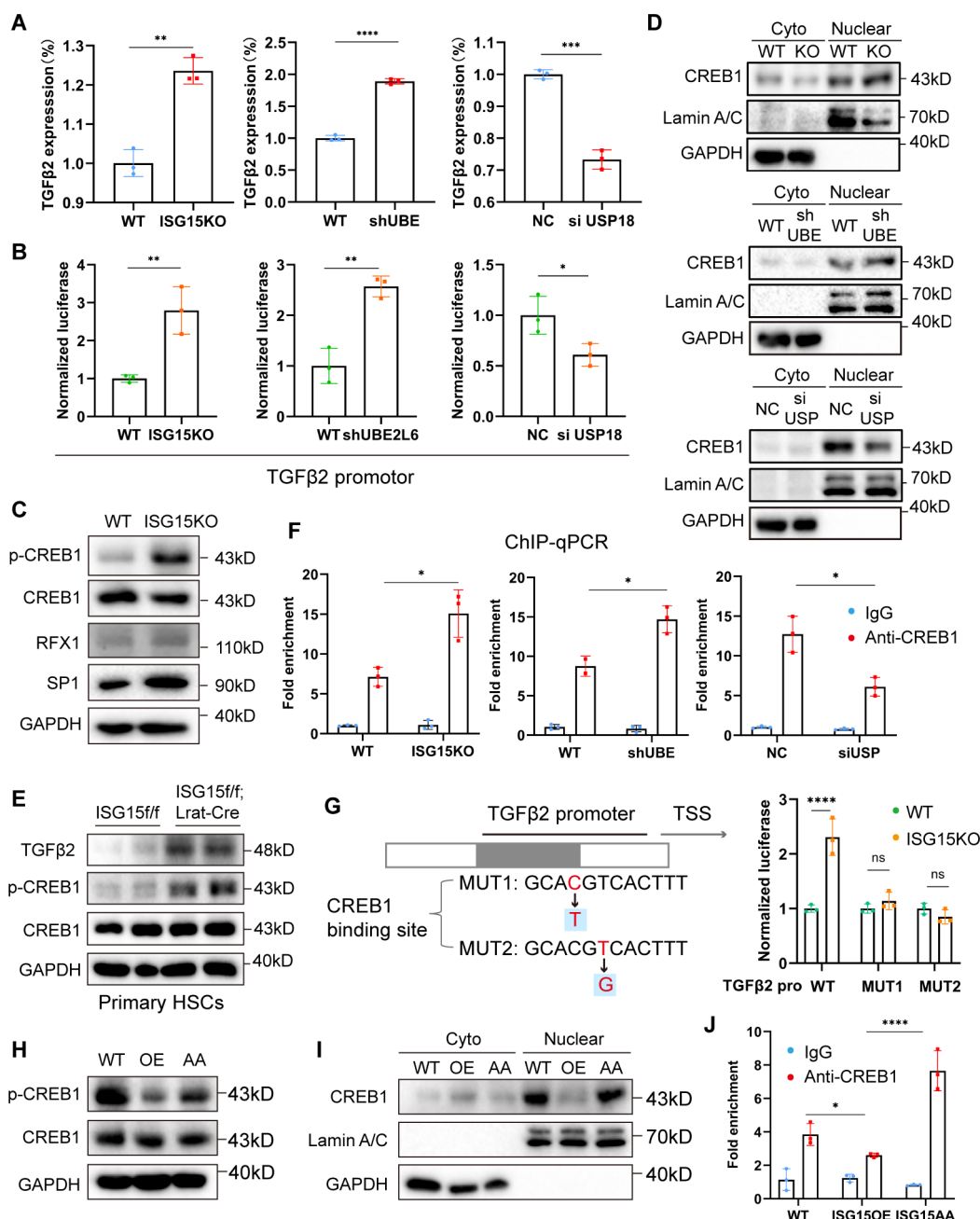


Figure 5 ISG15/ISGylation regulated CREB1 activity and inhibited TGFβ2 transcription. (A) The mRNA expression of TGFβ2 in LX-2 HSCs (WT, ISG15KO, shUBE2L6 and siUSP18) was determined by qRT-PCR. (B) The luciferase assay showed the effect of ISG15/ISGylation on luciferase activity of TGFβ2 promoter in LX-2 HSCs. (C) Western blot data showed that ISG15 knockout increased p-CREB1 expression, but not the expression levels of CREB1, SP1 and RFX1. (D) Western blot was performed to detect CREB1 expression in the nucleus and cytoplasm in LX-2 HSCs (WT, ISG15KO, shUBE2L6 and siUSP18). (E) The expression levels of p-CREB1 and TGFβ2 were determined in primary HSCs from 18-month-old WT and ISG15cKO mice by western blot. (F) The enrichment of CREB1 in the TGFβ2 promoter and its modulation by ISG15/ISGylation was examined by ChIP-qPCR. (G) The diagram illustrating the mutation design of TGFβ2 promoter (left panel). The right panel showed the luciferase activity in LX-2 HSCs transfected with the plasmid expressing TGFβ2-promoter WT, TGFβ2-promoter Mut1 or TGFβ2-promoter Mut2, respectively. (H) Western blot was performed to detect p-CREB1 expression in LX-2 HSCs (WT, ISG15OE, ISG15AA). (I) Western blot was performed to detect CREB1 expression in the nucleus and cytoplasm in LX-2 HSCs (WT, ISG15OE, ISG15AA). (J) The enrichment of CREB1 in the TGFβ2 promoter in LX-2 HSCs (WT, ISG15OE, ISG15AA) was examined by ChIP-qPCR. For bar graphs, data are means±SEM. **p*<0.05, ***p*<0.01. HSC, hepatic stellate cell; ISG15, interferon-stimulated gene 15 aminotransferase; ISG15cKO, HSC-specific deletion of ISG15; shUBE2L6, UBE2L6 knockdown using shRNA; siUSP18, USP18 knockdown using siRNA; UBE2L6, ubiquitin-conjugating enzyme E2 L6; USP18, ubiquitin-specific protease 18; WT, wild type.

potential ISG15-interacting proteins in LX-2s. Briefly, we immunoprecipitated ISG15 from LX-2s expressing FLAG-ISG15 and then identified these immunoprecipitates through mass spectrometry. Surprisingly, results identified CREB1 as a binding

candidate (figure 6A,B). Co-IP in HEK293T cells confirmed HA-CREB1 interacts with FLAG-ISG15 (figure 6C), but not with FLAG-tagged ISG15AA, which lacks the C-terminal diglycine required for ligation to the target lysine residue (figure 6D).

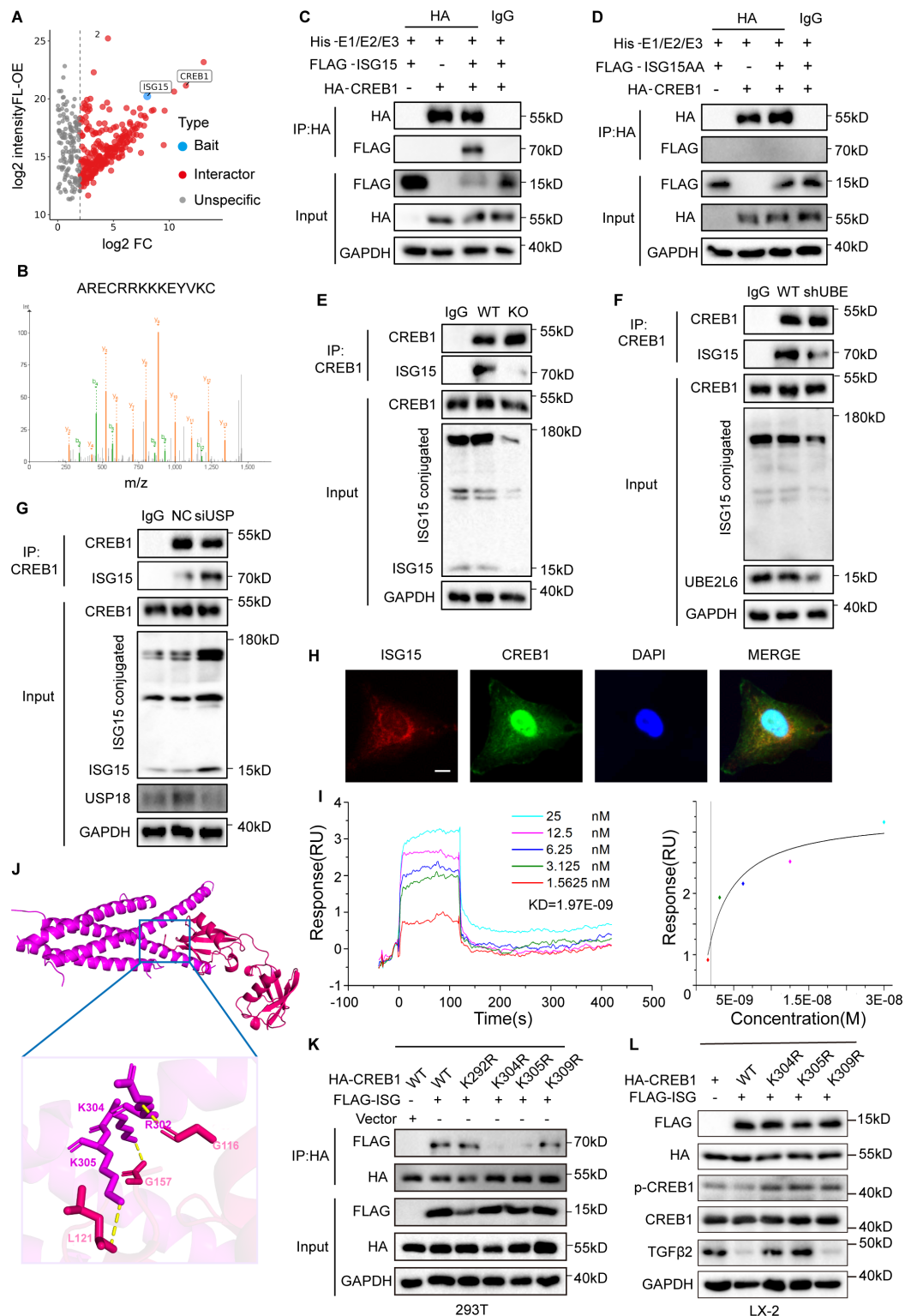


Figure 6 ISG15 binds and ISGylates CREB1. (A) Proteins associated with ISG15 were identified by mass spectrometry. (B) The CREB1 peptides identified by mass spectrometry were shown. (C,D) HEK293T cells were transfected with the indicated plasmids. Thirty-six hours after transfection, cell lysates were immunoprecipitated with an anti-HA antibody and then immunoblotted with the indicated antibodies. (E–G) Endogenous co-immunoprecipitation of CREB1 and ISG15 in LX-2 cells (WT, ISG15KO, shUBE2L6 and siUSP18). (H) Confocal assays confirmed that CREB1 and ISG15 were mainly co-localised (scale bar: 10 μ m, 63 $\times$ objective). (I) The surface plasmon resonance (SPR) assay revealing the binding between ISG15 and CREB1. (J) The homology modelling structure of CREB1 protein complexed with ISG15. (K) HEK293T cells were transfected with the indicated plasmids. Thirty-six hours after transfection, cell lysates were immunoprecipitated with an anti-HA antibody and then immunoblotted with the indicated antibodies. (L) LX-2 cells were transfected with the indicated plasmids. Thirty-six hours after transfection, TGF β 2, CREB1 and p-CREB1 expression were measured. ISG15, interferon-stimulated gene 15 aminotransferase; ISG15cKO, HSC-specific deletion of ISG15; shUBE2L6, UBE2L6 knockdown using shRNA; siUSP18, USP18 knockdown using siRNA; UBE2L6, ubiquitin-conjugating enzyme E2 L6; USP18, ubiquitin-specific protease 18; WT, wild type.

Endogenous ISG15-CREB1 interaction in LX-2s was diminished by ISG15KO/UBE2L6 knockdown and enhanced by USP18 knockdown (figure 6E–G), with spatial colocalisation observed via confocal imaging of LX-2s (figure 6H). Surface plasmon resonance (SPR) assay revealed ISG15 can directly bind to CREB1 with a K_d of 1.97×10^{-9} (figure 6I). Leveraging protein data bank to provide the 3D structure of CREB1 and ISG15, we found evidence of a direct interaction between ISG15 and CREB1 by online software GRAMM. Molecular docking showed that the Lys-304 and Lys-305 of CREB1 may interact with C-terminal diglycine (Gly-157) of ISG15 most likely with the highest binding free energy (-427 kcal/mol) (figure 6J). Therefore, combining all the sites provided in figure 6B,J, we next generated four CREB1 mutant alleles bearing mutations on K292, K304, K305 and K309, respectively, and tested whether they could be ISGylated. Results showed K304/K305 mutations abolished ISG15 binding with CREB1 (figure 6K). ISGylation can affect protein expression or protein function, but in figure 5 ISG15 does not influence the protein expression of CREB1. And the modification of K304 is related to the capacity of CREB nuclear localisation.²⁹ Accordingly, we investigated whether ISGylation affects the subcellular localisation and functional properties of CREB1. From figure 6L, ISGylation suppressed TGF β 2/p-CREB1 expression, which was rescued by K304/K305 CREB1 mutations. These results demonstrate ISG15 covalently modifies CREB1 at Lys-304/305 to suppress its nuclear activity.

Overexpression of ISG15 in HSCs ameliorates CCl₄-induced liver fibrosis

To determine whether ISG15 overexpression in HSCs protects liver fibrosis, we specifically overexpressed ISG15 in HSCs of WT and ISG15cKO mice through tail vein injection of adeno-associated virus (AAV) harbouring FLAG-ISG15 (AAV-ISG15) or AAV-Control (AAV-CON) with an HSC-specific reporter glial fibrillary acidic protein in a CCl₄-induced liver fibrosis model (figure 7A). Successful HSC-targeted ISG15 expression was confirmed by Desmin co-staining and WB analysis of primary HSCs (figure 7B,C). As expected, the re-expression of ISG15 in ISG15cKO mice significantly alleviated liver injury, fibrosis and inflammation, as indicated by improvements in serum ALT/AST, H&E, Sirius Red, Masson, α SMA, F4/80 and Ly6G immunostaining and the levels of profibrotic/proinflammatory genes (figure 7D–H, online supplemental figure S11A). Notably, WT mice with HSC-specific ISG15 overexpression exhibited a mild alleviation of liver fibrosis compared with those injected with AAV-CON (figure 7D–H). These results suggest that the AAV-mediated restoration of HSC-specific ISG15 protects against CCl₄-induced liver fibrosis in mice.

A CREB1 inhibitor blocked ISG15 deficiency-induced liver fibrosis in CCl₄-treated mice

Although overexpressing ISG15 or targeting TGF β 2 could act as the potential treatment in liver fibrosis, there were no suitable clinical drugs for TGF β 2 inhibition or ISG15 overexpression. TGF β 2 is a member of the TGF β family; however, systemic TGF β inhibition risks side effects like autoinflammation.²² And the application of delivering viral vectors to overexpress genes into humans is also not yet mature. Thus, we pivoted to CREB1 as an upstream regulator. The clinical significance of p-CREB1 expression in cirrhosis was verified through IHC. IHC revealed elevated p-CREB1 in the liver sections obtained from patients with cirrhosis in cohort 2 versus those human patients without cirrhosis, colocalising with α SMA-positive activated HSCs

(figure 8A), supporting the clinical importance of p-CREB1 in cirrhosis and the possibility of taking CREB1 as a target for anti-cirrhosis drugs.

Therefore, we chose 666–15, an inhibitor of CREB1 activity, to confirm whether the drug works well in vivo. This drug (666–15) was administered intraperitoneally at a dose of 5 mg/kg twice a week (figure 8B). As shown in figure 8C,D 666–15 significantly decreased ISG15 deficiency-induced serum ALT/AST levels and the hepatic hydroxyproline content. Consistently, the treatment group showed reduced liver injury, fibrosis and inflammation assessed by H&E, Sirius red, Masson, α SMA, F4/80, Ly6G staining and profibrotic/proinflammatory genes (figure 8E,F, online supplemental figure S11B). In parallel, ISG15 deficiency-induced hepatic protein levels of α SMA and Col1 α 1 were attenuated in the 666–15 group (figure 8G). In a word, these data establish CREB1 inhibition as a viable therapeutic strategy for ISG15 deficiency-mediated liver fibrosis.

DISCUSSION

This study identified ISG15 as a novel regulator of liver fibrosis progression. We revealed significant ISG15 downregulation in HSCs from the human fibrotic liver and fibrosis mice. Mechanistically, ISG15 deficiency in HSCs significantly promoted the progression of hepatic fibrosis via decreasing the ISGylation on Lys-304/305 of CREB1 to enhance CREB1 transcription activity, thus facilitating CREB1 binding to the TGF β 2 promoter and driving its transcription (figure 8H). These results provide important insights into the role of ISG15 and ISGylation in liver fibrosis.

ISG15, a downstream effector of IFN signalling primarily induced by type I IFNs and microbial infections,⁷ is widely associated with immune cells including macrophages and T cells to modulate immune-related diseases.^{30–32} Emerging evidence highlights its functional relevance beyond immune contexts.⁷ For example, ISG15/ISGylation governs cancer stemness in anaplastic thyroid carcinoma,³³ drives hepatocellular carcinoma progression via ISGylated p53,³⁴ and participates in hepatocyte differentiation from pluripotent stem cells.³⁵ While ISGylation has been implicated in cardiac vascular fibrosis and kidney injury,^{11 12} its role in liver fibrosis remains underexplored. Notably, our results showed that ISG15 in HSCs inhibited liver fibrosis. Compared with the normal liver tissues, ISG15 expression in whole liver lysates from fibrotic liver tissues was slightly decreased. The most significant difference occurred in non-parenchymal cell HSCs but not in hepatocytes. HSC-specific ISG15 deletion exacerbated liver fibrosis by promoting HSC activation, proliferation and ECM deposition—consistent with reports showing ISG15 knockdown elevates vimentin/fibronectin in renal cells.³⁶ These data collectively establish ISG15/ISGylation as antifibrotic regulators. In addition, our ageing model for spontaneous hepatic fibrosis has some limitations: in older mice, the concurrent metabolic disturbances (eg, insulin resistance) and abnormal immune response may synergistically exacerbate fibrogenesis with ISG15-specific effects.^{37 38} Therefore, future studies will benefit from multiomics analysis and other cell type-specific ISG15 deletion (eg, in hepatocytes vs macrophages) to quantify microenvironmental heterogeneity and resolve its potential spatial regulation of fibrogenesis.

TGF β 2 is a key profibrogenic mediator driving HSC trans-differentiation and ECM accumulation.^{22 39} TGF β 2 binds to its receptor and phosphorylates SMAD2, triggering many biological processes.^{40 41} We demonstrate ISG15 deficiency activates TGF β 2/SMAD2 signalling in HSCs, with TGF β 2 neutralising antibody

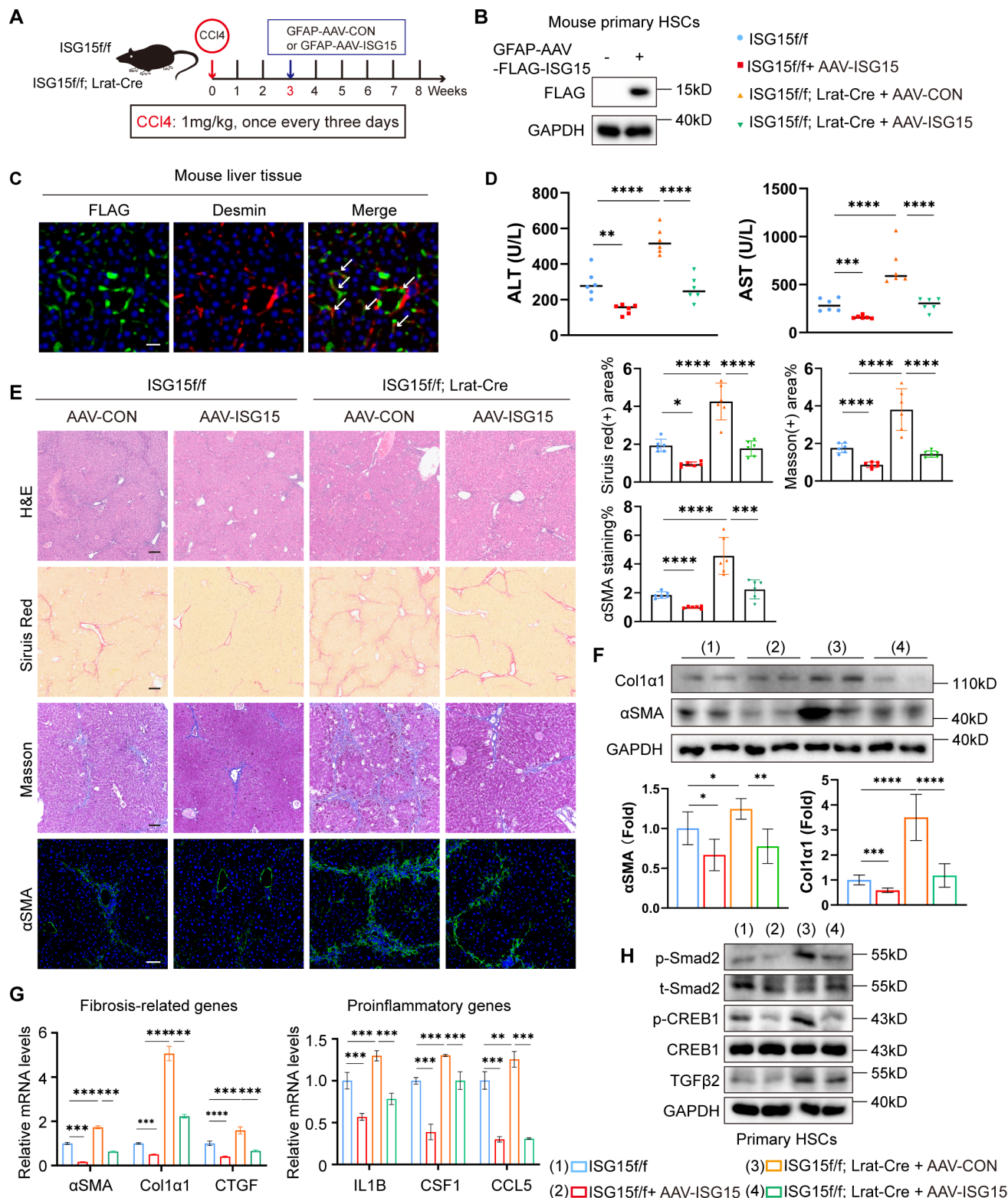


Figure 7 Re-overexpression of ISG15 in HSCs ameliorates CCl₄-induced liver fibrosis. (A) The experimental approach of the animal model establishment. (B) Immunoblots of Flag-ISG15 in primary HSCs isolated from male mice after AAV injection. (C) Dual immunofluorescence staining for the Flag-ISG15 and Desmin was performed. Scale bar: 200 μ m. (D) Serum levels of hepatotoxicity indicators ALT and AST in mice (n=6/group). (E) Macroscopic examination, H&E, Sirius red, Masson and α SMA staining in the liver sections from four group mice (n=6/group, scale bar, 50 μ m). (F) The protein levels of α SMA, Col1 α 1 in the liver tissues of four group mice (n=6/group). 1: ISG15f/f+GFAP-AAV-CON; 2: ISG15f/f+GFAP-AAV-ISG15; 3: ISG15f/f; Lrat-Cre+GFAP-AAV-CON; 4: ISG15f/f; Lrat-Cre+GFAP-AAV-ISG15. (G) Expression levels of fibrosis-related and proinflammatory genes in the liver (n=3/group). (H) The expression levels of Smad2, p-Smad2, CREB1, p-CREB1 and TGF β 2 were determined in primary HSCs from mice after AAV injection by western blot. For bar graphs, data are means $\pm$ SEM. *p<0.05, **p<0.01. AAV, adeno-associated virus; AAV-CON, AAV-Control; ALT, alanine aminotransferase; AST, aspartate; α SMA, α -smooth muscle actin; CCl₄, carbon tetrachloride; HSC, hepatic stellate cell; ISG15, interferon-stimulated gene 15 aminotransferase; GFAP, glial fibrillary acidic protein.

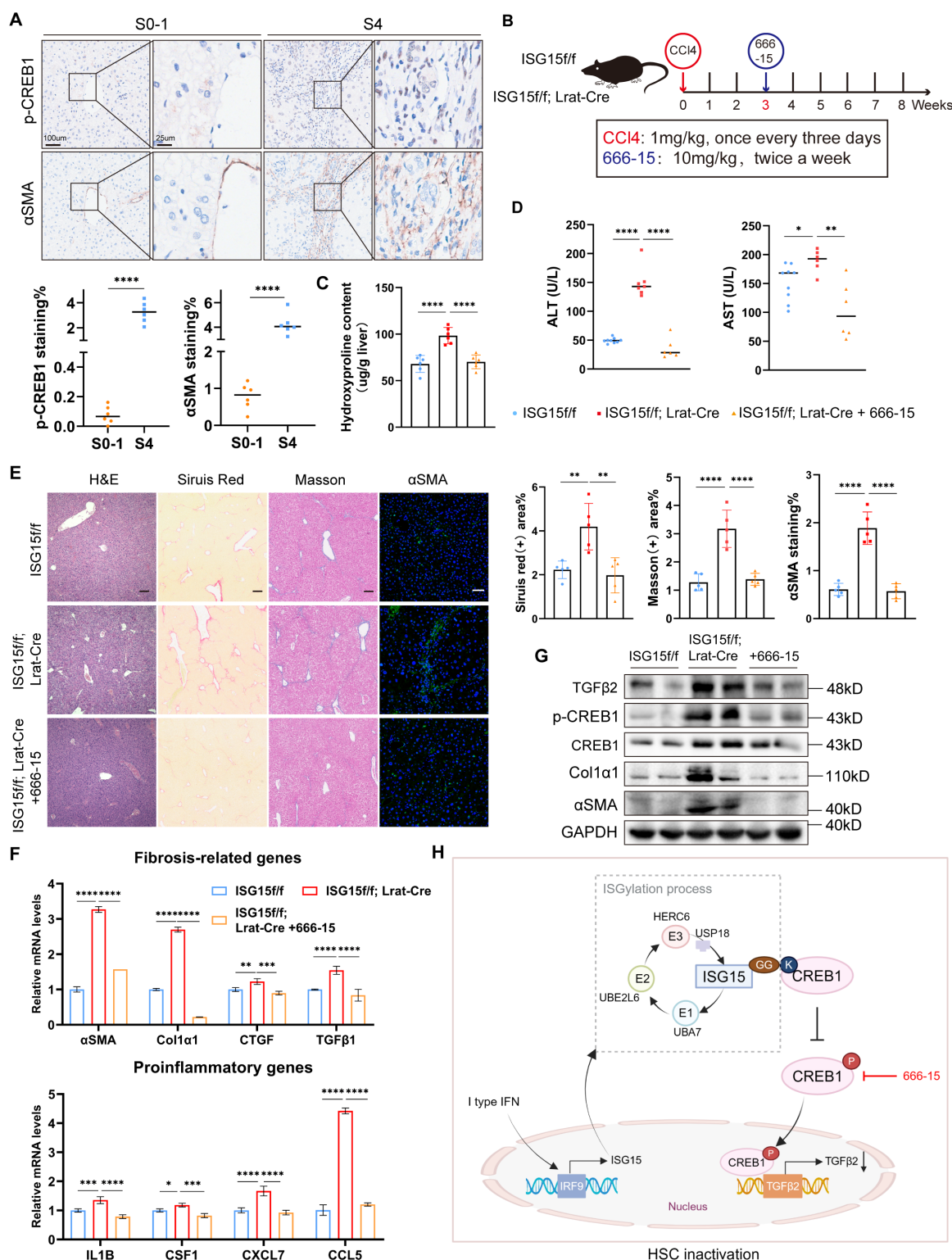


Figure 8 Effects of CREB1 inhibitor treatment in ISG15 deficiency-induced liver fibrosis in CCl4-treated mice. (A) Representative images and expression pattern of p-CREB1 in liver tissues from cohort 2 patients (cirrhosis: Scheurer Fibrosis Score 4, S4, n=13; non-cirrhosis: Scheurer Fibrosis Score 0–1, S0-1, n=8). (B) Schematic illustration of experimental design for inhibiting CREB1 by 666–15 in fibrotic mouse liver from WT and ISG15cKO male mice induced by CCl4. 666–15 (10 mg/kg, intraperitoneally twice a week) was started from week 3 of the CCl4 treatment. (C) Hydroxyproline content (Hyp) in liver tissues. (D) Serum levels of hepatotoxicity indicators ALT and AST in mice. (E) Representative images of H&E, Sirius red, Masson and αSMA staining in the liver sections from different groups of mice (n=5, scale bar, 50μm). (F) Expression levels of fibrosis-related and proinflammatory genes in the livers. (G) The protein levels of αSMA, Col1α1, CREB1, p-CREB1 and TGFβ2 in the liver tissues of mice (n=6). (H) Underlying mechanisms of ISG15 deficiency in HSCs promote HSC activation and fibrosis via the CREB1/TGFβ2 axis. For bar graphs, data are means±SEM. *p<0.05, **p<0.01. ALT, alanine aminotransferase; AST, aspartate; αSMA, α-smooth muscle actin; CCl4, carbon tetrachloride; HSC, hepatic stellate cell; IFN, interferon; ISG15, interferon-stimulated gene 15 aminotransferase; ISG15cKO, HSC-specific deletion of ISG15; UBE2L6, ubiquitin-conjugating enzyme E2 L6; USP18, ubiquitin-specific protease 18; WT, wild type.

reversing fibrotic progression. This confirms ISG15 deficiency aggravates liver fibrosis via TGF β 2 upregulation. Noteworthy, although TGF β 1 is extensively studied in fibrosis, severe side effects of pan-TGF β inhibition have shifted focus to other isoforms (TGF β 2, TGF β 3), each encoded by distinct genes.^{42,43} These three subtypes have distinct expression profiles, patterns of functional domain activation and activation thresholds, conferring biological specificity.²² Unlike TGF β 1, TGF β 2 activation bypasses conventional integrin binding.^{22,44} Isoform expression varies across cellular and disease contexts:⁴⁴ in hepatopulmonary fibrosis, TGF β 2 is significantly highly expressed in patients with more advanced fibrotic stages of disease, while TGF β 1 was comparable across disease severity.²² Although the specific mechanisms are unclear, certain transcription factors regulate TGF β 2 transcription via promoter binding,^{28,45} suggesting its generation mechanism is distinct from TGF β 1. In our study, ISG15 deficiency in HSCs induced TGF β 2 expression rather than TGF β 1 and TGF β 3, with CREB1 binding its promoter, indicating the specificity to ISG15 loss. In addition, from the perspective of translation, although one study indicates that ISG15 could regulate TGF β 1 expression in renal injury, a receptor for the TGF β family,¹² our proteomics showed unchanged TGF β 1. Therefore, whether ISG15 modulates the protein stability of other TGF β -related molecules to drive differential isoform expression in hepatic fibrosis warrants investigation.

Mechanistically, ISG15 knockout increased p-CREB1 levels and CREB1 nuclear translocation in HSCs. CREB1 inhibitor 666–15 abolished the effect of ISG15 deficiency-induced fibrosis and inflammation in CCl₄-treated mice. We identified CREB1 binding on the CRE-L sequence of the TGF β 2 promoter, enhanced by ISG15 deficiency. Although ISG15/UBE2L6-dependent ISGylation typically regulates protein stability via proteasomal competition,^{34,46} we observed unchanged CREB1 expression with enhanced p-CREB1 activity—indicating ISGylation modulates CREB1 function, not stability. Analogous mechanisms occur in breast cancer (Akt signaling)⁴⁷ and adipose thermogenesis (LDHA suppression).⁹ We further replaced the ligation amino acid GG to AA in the LRLRGG motif of ISG15, and this replacement altered TGF β 2 promoter binding. SPR assay and molecular docking analysis revealed direct ISG15-CREB1 interaction ($K_d=1.97\times10^{-9}$), with Lys-304/305 critical for binding. The binding of ISG15 to CREB1 disrupted the interaction of CREB1 with the TGF β 2 promoter, thus repressing TGF β 2 transcription. As for how the CREB1 nuclear allocation influences p-CREB1 and whether ISGylated CREB1 leads to spatial conception changes, we will try to explore in subsequent experiments.

From a clinical perspective, despite extensive antifibrotic drug trials, no therapy has achieved clinical approval.⁴⁸ Our findings demonstrated the CREB1 inhibitor 666–15 alleviates CCl₄-induced liver fibrosis and inflammation, suggesting the possibility of 666–15 acting as a therapeutic strategy to treat cirrhosis. And its treatment possibility has been validated in mammary tumours and polycystic kidney disease.^{49,50} Current evidence suggests 666–15 lacks overt toxicity in normal cells/animals.^{51,52} Whether 666–15 can be used as a clinical therapy for liver fibrosis and its safe dosage requires further investigation.

In conclusion, the present study demonstrated that decreased ISG15/ISGylation induced HSC activation, proliferation and ECM production via directly targeting and repressing transcriptional activity of CREB1 to inhibit TGF β 2 expression during the development of liver fibrosis. This study not only elucidates the ISG15-CREB1-TGF β 2 axis as a novel fibrogenic pathway but also proposes CREB1 inhibition as a translatable therapeutic strategy.

Correction notice This article has been corrected since it published Online First. Figure 7E has been corrected and replaced.

Contributors All the authors have approved and agreed to publish this manuscript. JZ is the guarantor. YY designed the outline of the manuscript, and wrote and reviewed the manuscript. HL and JZ edited the manuscript with important intellectual content. JL, DR, Xun L and MC: acquisition and analysis of data. Xin L and BZ revised the manuscript. XC and QC discussed the topic and outlines of the manuscript and reviewed the text. All authors contributed to the article and approved the submitted version.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the Ethics Committee of Tongji Hospital (HUST, Wuhan, China) (TJ-IRB20231140). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. Data are available upon reasonable request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<https://proteomecentral.proteomexchange.org>) via the iProX partner repository with the data set identifier PXD057170. Other data from this article are available from the corresponding authors upon request.

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