


Original research

Histone methyltransferase Suv39h1 regulates hepatic stellate cell activation and is targetable in liver fibrosis

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► Additional supplemental material is published online only. To view, please visit the journal online (<http://dx.doi.org/10.1136/gutjnl-2023-329671>).

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Received 8 February 2023
Accepted 15 December 2023

ABSTRACT

Objective Liver fibrosis is a prelude to a host of end-stage liver diseases. Hepatic stellate cells (HSCs), switching from a quiescent state to myofibroblasts, are the major source for excessive production of extracellular matrix proteins. In the present study, we investigated the role of Suv39h1, a lysine methyltransferase, in HSC-myofibroblast transition and the implication in liver fibrosis.

Design HSC-specific or myofibroblast-specific Suv39h1 deletion was achieved by crossbreeding the *Suv39h1^{fl/fl}* mice to the *Lrat-Cre* mice or the *Postn-Cre^{ERT2}* mice. Liver fibrosis was induced by CCl₄ injection or bile duct ligation.

Results We report that Suv39h1 expression was universally upregulated during HSC-myofibroblast transition in different cell and animal models of liver fibrosis and in human cirrhotic liver tissues. Consistently, Suv39h1 knockdown blocked HSC-myofibroblast transition in vitro. HSC-specific or myofibroblast-specific deletion of Suv39h1 ameliorated liver fibrosis in mice. More importantly, Suv39h1 inhibition by a small-molecule compound chaetocin dampened HSC-myofibroblast transition in cell culture and mitigated liver fibrosis in mice. Mechanistically, Suv39h1 bound to the promoter of heme oxygenase 1 (HMOX1) and repressed HMOX1 transcription. HMOX1 depletion blunted the effects of Suv39h1 inhibition on HSC-myofibroblast transition in vitro and liver fibrosis in vivo. Transcriptomic analysis revealed that HMOX1 might contribute to HSC-myofibroblast transition by modulating retinol homeostasis. Finally, myofibroblast-specific HMOX1 overexpression attenuated liver fibrosis in both a preventive scheme and a therapeutic scheme.

Conclusions Our data demonstrate a previously unrecognised role for Suv39h1 in liver fibrosis and offer proof-of-concept of its targetability in the intervention of cirrhosis.

INTRODUCTION

Challenged with a myriad of injurious stimuli that encompass hepatotoxins, hepatitis viruses, cholestasis, ischaemia and excessive influx of nutrients/metabolites, the liver initiates a reparative response aiming to contain the injuries and restore hepatic mass/function. This process, which is characterised by an initial spike in immune cell infiltration and inflammation in the liver followed

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Liver fibrosis, when aberrantly regulated, contributes to cirrhosis and hepatocellular carcinoma.
- ⇒ Myofibroblasts, the major effector cell type in liver fibrosis, are primarily derived from hepatic stellate cells (HSCs).

WHAT THIS STUDY ADDS

- ⇒ Suv39h1 expression is upregulated during HSC-myofibroblast transition in mice and in humans.
- ⇒ C/EBPβ mediates Suv39h1 transactivation during HSC-myofibroblast transition.
- ⇒ Suv39h1 deletion from HSCs or myofibroblasts attenuates liver fibrosis in mice.
- ⇒ Suv39h1 inhibition by the small-molecule compound chaetocin mitigates liver fibrosis in mice.
- ⇒ HMOX1 is a novel transcriptional target of Suv39h1 that mediates the antifibrotic effect of Suv39h1 deletion/inhibition in vitro and in vivo.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ Suv39h1 is a master regulator of HSC-myofibroblast transition and liver fibrosis.
- ⇒ Small-molecule compounds that target Suv39h1 may provide new therapeutic solutions to treat liver fibrosis.

by migration and maturation of myofibroblasts at the site of injury to produce extracellular matrix (ECM) proteins, shares striking similarity to wound healing.¹ Therefore, liver fibrosis is considered an integral part of the host defence mechanism to safeguard hepatic architecture and function from demise.² Typically, the fibrogenic response is terminated or resolved once the injurious stimuli recede; prolonged and excessive liver fibrosis inevitably leads to adverse remodelling of the hepatic anatomy and consequently irretrievable loss of hepatic function. Indeed, liver fibrosis is present in virtually all forms of chronic liver diseases and precedes cirrhosis and hepatocellular carcinoma.³ Despite decades of vigorous research efforts, no pharmacotherapy has been developed specifically for the intervention of liver fibrosis to date indicating major gaps in our understanding of the underlying pathobiology.⁴



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To cite: Kong M, Zhou J, Kang A, et al. *Gut* Epub ahead of print: [please include Day Month Year]. doi:10.1136/gutjnl-2023-329671

Regardless of aetiology, ECM-producing myofibroblasts are the chief effector cell type contributing to liver fibrosis.⁵ First described by Gabbiani and Majno,⁶ myofibroblasts represent a morphologically and phenotypically distinct cell lineage displaying strong capacity of proliferation, migration, muscle-like contraction and synthesising ECM proteins. Absent from the liver under physiological conditions, myofibroblasts quickly emerge following the onset of liver injuries to mediate fibrogenesis. The origin(s) from which myofibroblasts are derived during liver fibrosis was a subject of extensive debate and investigation. An elegant study published by the Schwabe group demonstrates that hepatic stellate cells (HSCs), specifically and faithfully labelled by lecithin retinol acyltransferase (*Lrat*), are the predominant, if not the sole, precursor to myofibroblasts in an exhaustive array of animal models.⁷ The notion that hepatic myofibroblasts exclusively originate from HSCs has been challenged recently by a study using single-cell transcriptomic techniques, which appears to suggest that portal fibroblasts, at least under certain conditions, could serve as the progenitor to myofibroblasts.⁸ It is noteworthy that neither the Mederacke *et al* study⁷ nor the Yang *et al* study⁸ traced the fate of hepatic myofibroblasts dynamically leaving open the possibility that different hepatic cell lineages may contribute to the pool of myofibroblasts at different points during the entire course of liver fibrosis.

HSCs in quiescent state primarily function as a reservoir for lipids and vitamin A but can transition to myofibroblasts when prompted by a sea of pro-fibrogenic growth factors and cytokines (eg, TGF- β) during liver fibrosis. HSC-myofibroblast transition is hallmarked by overhaul of cellular transcriptome. Multiple studies have recorded variations in chromatin accessibility at different stages of HSC-myofibroblast transition alluding to transcriptional reprogramming.^{9–11} In mammalian cells, transcriptional regulation is intimately linked to the epigenetic machinery consisting of histone and DNA-modifying enzymes, non-coding RNAs and chromatin-remodelling proteins.¹² Initially isolated and characterised by the Jenuwein laboratory, suppressor of variegation 3–9 homolog 1 (*Suv39h1*, also termed KMT1A) is the first histone lysine methyltransferase specialised in H3K9 trimethylation.^{13–15} Although *Suv39h1* is considered an essential regulator of heterochromatin establishment and maintenance, a plethora of studies have documented its role in regulating euchromatin-borne transcription.^{16–18} In the present study, we investigated the involvement of *Suv39h1* in HSC-myofibroblast transition and liver fibrosis focusing on the mechanism and translational potential. Our data as summarised here suggest that *Suv39h1* plays an indispensable role for HSC-myofibroblast transition and can be targeted for the intervention of liver fibrosis.

METHODS

Animals

All animal protocols were reviewed and approved by the intramural China Pharmaceutical University Committee on Ethical Treatment of Experimental Animals. Global *Suv39h1* knockout (KO) mice were generated by the Jenuwein laboratory at The Max Planck Institute of Immunobiology and Epigenetics (MPI-IE) in Freiburg and have been described previously.¹⁵ The *Suv39h1*^{fl/fl} mice were generated by introducing the *LoxP* sites to flank exon 2 and exon 3 of the *Suv39h1* alleles (Cyagen). The *Lrat*-Cre mice (#069595) and the *Postn*-Cre^{ERT2} mice (#029645) were purchased from the Jackson Laboratory. HSC conditional *Suv39h1* KO mice were generated by crossing *Suv39h1*^{fl/fl} mice to the *Lrat*-Cre mice.⁷ Myofibroblast conditional *Suv39h1* KO

mice were generated by crossing *Suv39h1*^{fl/fl} mice to the *Postn*-Cre^{ERT2} mice.¹⁹ Liver fibrosis was induced in 6–8-week-old male mice using one of the following two protocols: (1) the mice were subjected to bile duct ligation (BDL) or the sham procedure for 2 weeks; or (2) the mice were injected with carbon tetrachloride (CCl₄, 1.0 mL/kg body weight as 50% vol/vol) or corn oil thrice a week for 4 weeks as previously described.^{20,21} To induce Cre expression in the *Postn*-Cre^{ERT2} mice, tamoxifen was injected peritoneally (50 mg/kg) for five consecutive days followed by maintaining the mice on a TAX-containing diet (Cat#: TD.130855, Inotiv) until the day when the mice were sacrificed. In certain experiments, recombinant adeno-associated virus 6 (AAV6)²² carrying heme oxygenase 1 (HMOX1) cDNA downstream of the *Postn* promoter²³ was injected into C57/B6 mice intravenously at a dose of 1×10^{11} viral genome (vg).

Cell culture

Human immortalised HSCs (LX-2, ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal bovine serum (FBS). LX-2 cells were serum-starved overnight prior to the addition of TGF- β (2 ng/mL). Primary murine HSCs were isolated and seeded on collagen-coated culture plates for 8 days during which time the cells underwent spontaneous activation as previously described.²⁴ Primary hepatocytes, Kupffer cells and liver sinusoidal endothelial cells (LSECs) were isolated as previously described.²⁵ Primary human HSCs were purchased from Lonza and maintained in the culture media supplied by the vendor. Small interfering RNAs were purchased from Dharmacon. Transient transfections were performed with Lipofectamine 2000. Briefly, siRNAs and the Lipofectamine reagent were separately diluted in serum-free OptiMEM (Thermo Fisher) and then mixed for 15 min. The oligonucleotide-Lipofectamine complex was added to the cells dropwise. Luciferase activities were assayed 24–48 hours after transfection using a luciferase reporter assay system (Promega) as previously described.²⁶

Protein extraction and Western blot

Whole cell lysates were obtained by resuspending cell pellets in radioimmunoprecipitation (RIPA) buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100) with freshly added protease inhibitor (Roche) as previously described.^{27,28} Western blot analyses were performed with anti- α -SMA (Abcam, ab7817), anti-collagen type I (Proteintech, 14 695–1), anti-*Suv39h1* (Cell Signaling Tech, 8729), anti-HMOX1 (Proteintech, 10701-1) and anti- β -actin (Sigma, A2228) antibodies. For densitometrical quantification, densities of target proteins were normalised to those of β -actin. Data are expressed as relative protein levels compared with the control group which is arbitrarily set as 1.

Statistical analysis

One-way analysis of variance with post-hoc Scheffé analyses were performed by SPSS software (IBM SPSS V.18.0, Chicago, Illinois, USA). Unless otherwise specified, values of $p < 0.05$ were considered statistically significant.

RESULTS

Suv39h1 upregulation by C/EBP β accompanies HSC activation

Our first goal was to examine the (dynamic) changes of *Suv39h1* expression when quiescent HSCs transition into myofibroblasts. Two different animal models of liver fibrosis were exploited, carbon tetrachloride (CCl₄) injection and BDL surgery, for this purpose. When primary HSCs were isolated and examined,

it was determined that Suv39h1 expression was significantly higher in the (activated) HSCs isolated from the fibrotic livers than in the (quiescent) HSCs isolated from the control livers in both models (figure 1A and B and online supplemental figure S1). Next, primary HSCs were isolated from normal C57B/L mice and allowed to undergo spontaneous activation in vitro. As shown in figure 1C and D, Suv39h1 expression was progressively increased along with the myofibroblast marker α -SMA (*Acta2*) when quiescent HSCs became activated. Finally, when human immortalised HSCs (LX-2) were treated with TGF- β , the most potent stimulator of myofibroblast maturation, Suv39h1 expression was again upregulated along with α -SMA (figure 1E and F). In addition, Suv39h1 levels were detected to be higher in the HSCs isolated from the cirrhotic human liver specimens than from the non-cirrhotic liver specimens (figure 1G). Importantly, a positive correlation between Suv39h1 and myofibroblast marker genes was identified in the human livers (figure 1H). Additionally, a positive correlation between Suv39h1 and fibrosis grade was evident in patients with cirrhosis (figure 1I).

We then made efforts to elucidate the transcriptional mechanism underlying Suv39h1 upregulation during HSC activation. Ingenuity pathway analysis suggested that suppressor of Ty 5 homolog (SUPT5H), proliferation-associated 2G4 (PA2G4), fifth ewing variant (FEV) and CCAAT enhancer binding protein beta (CEBPB or C/EBP β) might be the upstream regulators of Suv39h1 transcription (online supplemental figure S2A). Knockdown experiments verified that depletion of CEBPB, but not SUPT5H, or PA2G4, or FEV, down-regulated Suv39h1 expression in activated primary murine HSCs and in TGF- β treated LX-2 cells (online supplemental figure S2B, S2C). When a reporter construct driven by the Suv39h1 promoter (−2000/+125) was transfected into LX-2 cells, TGF- β treatment significantly augmented the reporter activity (online supplemental figure S4A). However, when inward deletions introduced to the reporter construct extended beyond −500 relative to the transcription start site, TGF- β treatment no longer elicited an induction (online supplemental figure S3A). Indeed, a conserved C/EBP-binding motif was identified between −1000 and −500 of the Suv39h1 promoter; mutation of this motif completely abrogated the TGF- β response (online supplemental figure S3B). Chromatin immunoprecipitation (ChIP) showed that association of C/EBP β with the Suv39h1 promoter was markedly enhanced in activated HSCs compared with quiescent HSCs (online supplemental figure S3C). Of note, C/EBP β mRNA expression was markedly upregulated and C/EBP β protein became predominantly located to the nucleus following TGF- β stimulation in LX-2 cells (online supplemental figure S4). In addition, higher C/EBP β expression was detected in the HSCs isolated from the mice induced to develop liver fibrosis than from the control mice (online supplemental figure S5). Interestingly, C/EBP β silencing did not alter the expression of other H3K9 methyltransferases (online supplemental figure S6). Combined, these data demonstrate that upregulation of Suv39h1 expression, likely mediated by C/EBP β , is synonymous with HSC transdifferentiation.

Global Suv39h1 deficiency attenuates liver fibrosis in mice

We next sought to determine the causal relationship between Suv39h1 and liver fibrosis. Primary HSCs isolated from germline Suv39h1 knockout (KO) mice displayed an inefficient pattern of transdifferentiation into myofibroblasts compared with those isolated from wild-type mice as evidenced by decreased expression of myofibroblast marker genes (figure 2A and B) and reduced proliferation rate (figure 2C). It was also found that WT

HSCs became more activated (expressed more profibrogenic markers) when cocultured with the CCl₄-treated hepatocytes than with the vehicle-treated hepatocytes. However, KO HSCs were less activated than the WT HSCs under either condition when cocultured with hepatocytes (online supplemental figure S7). Similarly, Suv39h1 knockdown decreased the expression of myofibroblast marker genes and dampened proliferation in human primary HSCs (online supplemental figure S8). Notably, trimethylated H3K9 was lower in the KO cells than the WT cells, whereas trimethylated H3K4 and trimethylated H3K27 remained intact (online supplemental figure S9). Suv39h1 deficiency, however, did not reciprocally affect C/EBP β expression or localisation (online supplemental figure S10A and B). Nor did it influence the levels of other H3K9 methyltransferases (online supplemental figure S10C). It was further observed that TGF- β expression and signalling (as measured by SMAD3 phosphorylation) were comparable between the KO cells and the WT cells (online supplemental figure S11). Interestingly, intracellular lactate levels were lower in activated KO cells compared with activated WT cells indicative of dampened anaerobic glycolysis, a hallmark of HSC-myofibroblast transition (online supplemental figure S12A). This was possibly due to the downregulation of HK2 and PFKF, two key enzymes involved in glycolysis (online supplemental figure S12B).

When WT and KO mice were injected with CCl₄ to induce liver fibrosis, it was found that plasma ALT (figure 2D) and AST (figure 2E) levels were lower in the KO mice than in the WT mice suggesting that Suv39h1 deficiency might alleviate liver injury. qPCR (figure 2F) and Western blotting (figure 2G) showed a decrease in expression of profibrogenic genes in the KO livers compared with the WT livers. In addition, Picrosirius Red/Masson's trichrome staining (figure 3H) and hydroxyproline quantification (figure 2I) confirmed that there were fewer collagenous tissues in the KO livers than in the WT livers. The effect of global Suv39h1 deletion on liver fibrosis was further validated in the BDL model (online supplemental figure S13): Suv39h1 deletion invariably attenuated liver fibrosis. On the other hand, immunohistochemical staining revealed that there were fewer F4/80⁺ macrophages and Ly6G⁺ neutrophils in the KO livers than in the WT livers (online supplemental figure S14A). There was also a concomitant downregulation of proinflammatory mediators in the KO livers compared with the WT livers (online supplemental figure S14B). Expression of C/EBP β and H3K9 methyltransferases was not significantly altered (online supplemental figure S14C). Taken together, these data suggest that Suv39h1 might play an essential role for HSC transdifferentiation and liver fibrosis.

HSC-specific or myofibroblast-specific Suv39h1 deficiency attenuates liver fibrosis in mice

Next, we asked whether conditional deletion of Suv39h1 in HSCs would likewise lead to alteration of liver fibrosis in mice. First, exon 2 and exon 3 of the Suv39h1 allele, which encode the chromo domain essential for methyltransferase activity,¹⁵ were floxed by gene targeting (figure 3A). Next, the *Suv39h1*^{fl/fl} mice were bred with the *Lrat*-Cre mice to generate HSC-specific Suv39h1 KO mice. To verify the specificity and efficiency of Suv39h1 deletion, primary hepatocytes, HSCs, Kupffer cells, and LSECs were isolated; the purity of each type was confirmed by qPCR using primers that detect lineage-specific markers (online supplemental figure S15A). Suv39h1 expression was reduced in primary HSCs but remained unchanged in primary hepatocytes, Kupffer cells and LSECs isolated from the *Suv39h1*^{fl/fl}; *Lrat*-Cre

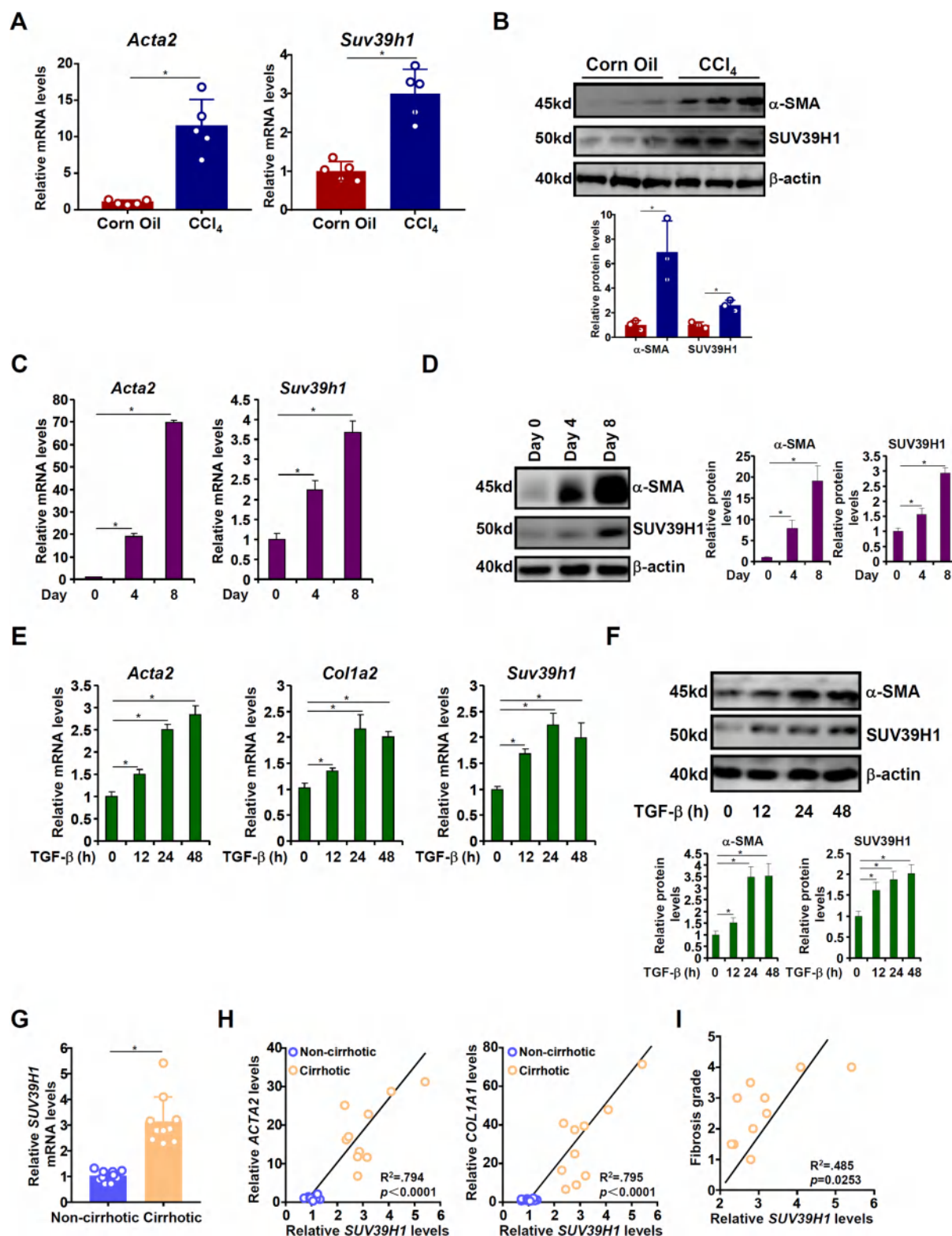


Figure 1 Suv39h1 upregulation accompanies HSC activation. (A, B) C57B/6 mice were injected with carbon tetrachloride or corn oil for 4 weeks. Primary HSCs were isolated and Suv39h1 expression was examined by qPCR and Western blotting. N=3–5 mice for each group. Data are expressed as mean±SD. * $p < 0.05$, two-tailed Student's t-test. (C, D) Primary HSCs were isolated from C57B/6 mice and allowed to undergo spontaneous activation in vitro. The cells were harvested at indicated time points and Suv39h1 expression was examined by qPCR and Western blotting. N=3 biological replicates. Data are expressed as mean±SD. * $p < 0.05$, one-way ANOVA with post-hoc Scheffé. (E, F) LX-2 cells were treated with or without TGF-β (2 ng/mL). The cells were harvested at indicated time points and Suv39h1 expression was examined by qPCR and Western blotting. N=3 biological replicates. Data are expressed as mean±SD. * $p < 0.05$, one-way ANOVA with post-hoc Scheffé. (G–I) HSCs were isolated from human liver biopsy specimens and gene expression was examined by qPCR. N=10 individuals for each group. Data are expressed as mean±SD. * $p < 0.05$, two-tailed Student's t-test. Pearson correlation was performed using Graphpad. ANOVA, analysis of variance; CCl₄, carbon tetrachloride; HSCs, hepatic stellate cells; qPCR, quantitative PCR.

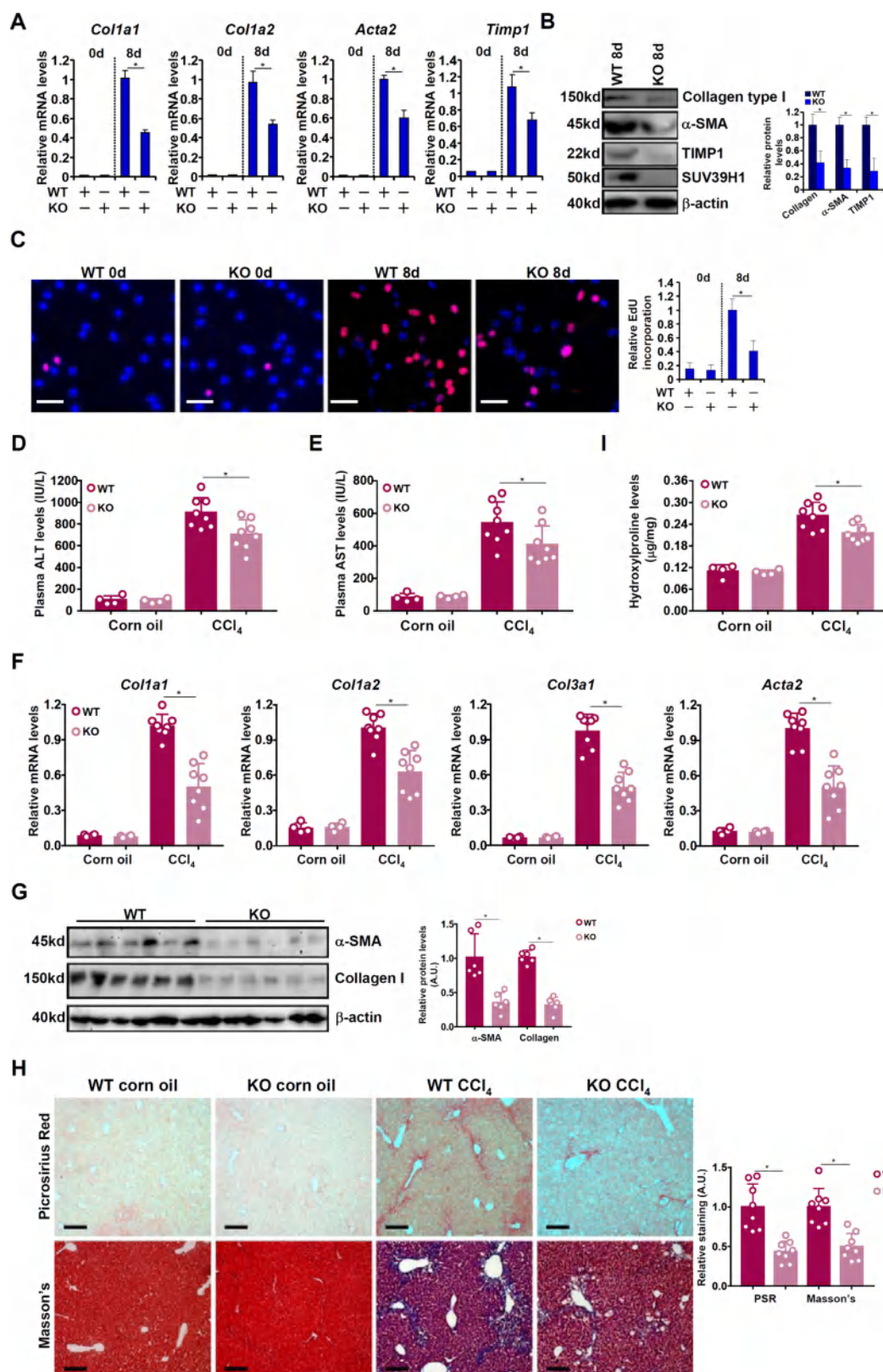


Figure 2 Global Suv39h1 deficiency attenuates liver fibrosis in mice. (A–C) Primary HSCs were isolated from global Suv39h1 KO mice and WT littermates and allowed to undergo spontaneous activation. Myofibroblast marker genes were examined by qPCR (A) and Western blotting (B). Cell proliferation was measured by EdU incorporation (C). N=3 biological replicates. Data are expressed as mean \pm SD. * p <0.05, two-tailed Student's t-test. (D–I) Global Suv39h1 KO mice and WT littermates were subjected to CCl₄ injection for 4 weeks. Plasma alanine transaminase (ALT) (D) and aspartate transaminase (AST) (E) levels. Hepatic profibrogenic genes were examined by qPCR (F) and Western blotting (G). (H) Paraffin sections were stained with picrosirius red and Masson's chrome. (I) Hepatic hydroxyproline levels. n=4–8 mice for each group. Data are expressed as mean \pm SD. * p <0.05, two-tailed Student's t-test. scale bar, 50 μ m. CCl₄, carbon tetrachloride; HSCs, hepatic stellate cells; KO, knockout; qPCR, quantitative PCR; WT, wild-type.

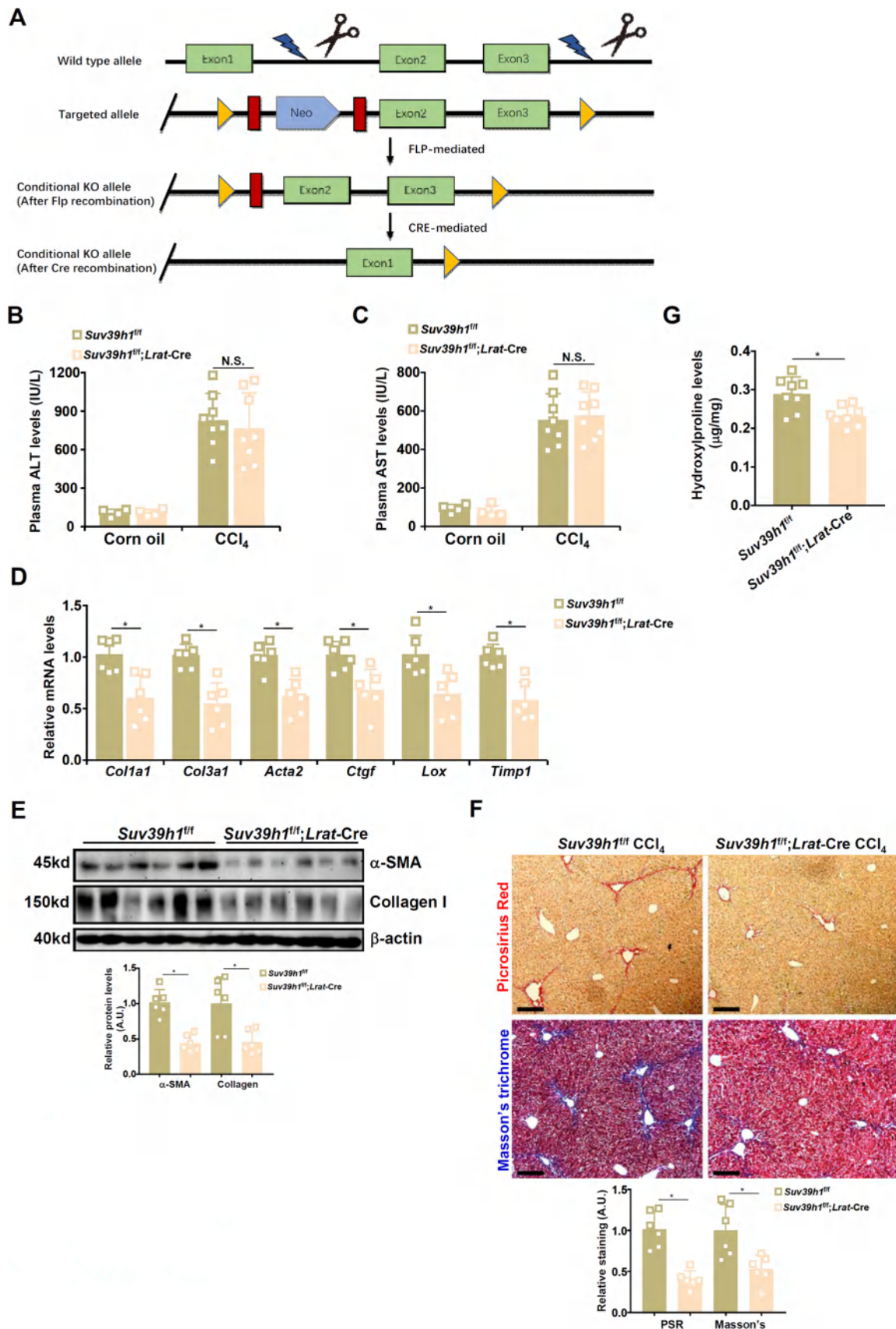


Figure 3 HSC-specific *Suv39h1* deficiency attenuates liver fibrosis in mice. (A) *Suv39h1*-flox strain targeting scheme. (B–G) HSC-specific *Suv39h1* KO mice (*Suv39h1*^{flf}; *Lrat-Cre*) and wild type littermates (*Suv39h1*^{flf}) were subjected to CCl₄ injection for 4 weeks. Plasma ALT (B) and AST (C) levels. Hepatic profibrogenic genes were examined by qPCR (D) and Western blotting (E). (F) Paraffin sections were stained with picrosirius red and Masson's chrome. (G) Hepatic hydroxyproline levels. N=4–8 mice for each group. Data are expressed as mean±SD. *p<0.05, two-tailed Student's t-test. scale bar, 50 μm. CCl₄, carbon tetrachloride; HSCs, hepatic stellate cells; KO, knockout; qPCR, quantitative PCR.

mice compared with the *Suv39h1^{flf}* mice (online supplemental figure S15B). Notably, *Suv39h1* was expressed at higher levels in hepatocytes and HSCs than in Kupffer cells and LSECs but overall *Suv39h1* expression in different hepatic cells appeared to be comparable (online supplemental figure S15B and C).

Reduced levels of trimethylated H3K9 were detected in the primary HSCs isolated from the *Suv39h1^{flf}; Lrat-Cre* mice compared with the control mice (online supplemental figure S16A), whereas expression of C/EBP β and H3K9 methyltransferases was comparable (online supplemental figure S16B). Consistently, HSCs isolated from the *Suv39h1^{flf}; Lrat-Cre* mice had a decreased rate of glycolysis as evidenced by lactate levels (online supplemental figure S16C) and expression of glycolytic enzymes (online supplemental figure S16D). Additionally, HSCs isolated from the *Suv39h1^{flf}; Lrat-Cre* mice were not activated as well as those from the control mice when cocultured primary hepatocytes (online supplemental figure S17).

When the *Suv39h1^{flf}; Lrat-Cre* mice and the *Suv39h1^{flf}* mice were subjected to CCl₄ injection, it was found that liver injury, as assessed by plasma ALT levels (figure 3B) and plasma AST levels (figure 3C), was comparable. However, qPCR (figure 3D), Western blotting (figure 3E) and PicroSirius Red (PSR)/Masson's staining (figure 3F) all pointed to attenuation of ECM production. Hepatic hydroxyproline quantification confirmed a reduction in collagenous tissues in the *Suv39h1^{flf}; Lrat-Cre* livers compared with the *Suv39h1^{flf}* livers (figure 3G). Similar observations were made in the BDL model (online supplemental figure S18). Of note, similar hepatic inflammation was observed in the *Suv39h1^{flf}; Lrat-Cre* livers and the *Suv39h1^{flf}* livers (online supplemental figure S19).

Myofibroblast-specific *Suv39h1* deficiency attenuates liver fibrosis in mice

In order to tackle the question as to whether *Suv39h1* might play an essential role maintaining the myofibroblast phenotype in vivo, the *Suv39h1^{flf}* mice were crossed to the *Postn-Cre^{ERT2}* mice to generate myofibroblast-specific *Suv39h1* KO mice. The *Suv39h1^{flf}; Postn-Cre^{ERT2}* mice and the *Suv39h1^{flf}* mice were injected with CCl₄ to induce liver fibrosis (figure 4A). No difference in liver injury, as measured by plasma ALT (figure 4B) and AST (figure 4C) levels, was detected between the *Suv39h1^{flf}; Postn-Cre^{ERT2}* mice and the *Suv39h1^{flf}* mice. However, liver fibrogenic response, as examined by qPCR (figure 4D) and Western (figure 4E) measurements of myofibroblast marker genes, PSR/Masson's staining of ECM molecules (figure 4F) and hepatic hydroxyproline quantification (figure 4G), was dampened markedly in the *Suv39h1^{flf}; Postn-Cre^{ERT2}* mice compared with the *Suv39h1^{flf}* mice. In a second model of liver fibrosis induced by BDL, it was similarly observed that myofibroblast-specific *Suv39h1* deficiency, while minimally impacting liver injury, ameliorated fibrogenesis (online supplemental figure S20). It was noted that there was no significant difference in hepatic inflammation in *Suv39h1^{flf}; Postn-Cre^{ERT2}* livers and the *Suv39h1^{flf}* livers (online supplemental figure S21).

Suv39h1 inhibition attenuates liver fibrosis

Because both HSC-restricted or myofibroblast-restricted deletion of *Suv39h1* led to mitigation of liver fibrosis, we were prompted to evaluate the possibility that targeting *Suv39h1* with the small-molecule compound chaetocin²⁹ might achieve similar effects. When primary murine HSCs underwent spontaneous activation (figure 5A), the addition of chaetocin partially reversed the transition to myofibroblasts as determined by

myofibroblast marker gene expression (figure 5B and C) and cell proliferation (figure 5D). Of interest, chaetocin treatment did not significantly alter apoptosis as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and expression of proapoptotic molecules (online supplemental figure S22). Additionally, treatment of chaetocin significantly dampened myofibroblast marker gene expression and proliferation in primary human HSCs (figure 5E–G). Next, C57/BL6 mice were given CCl₄ injection to induce liver fibrosis; administration of chaetocin was started 2 weeks after CCl₄ injection (figure 5H). Primary HSCs isolated from these mice revealed that chaetocin treatment significantly downregulated trimethylated H3K9 without altering trimethylated H3K4/K27 (online supplemental figure S23). Chaetocin administration not only ameliorated liver injury, as determined by plasma ALT/AST levels (figure 5I and J), but weakened liver fibrogenesis (figure 5K, L and M). These observations were further validated in an alternative model of BDL-induced liver fibrosis (online supplemental figure S24). Of interest, further analysis revealed that administration of chaetocin dampened hepatic inflammation in both models of liver fibrosis (online supplemental figure S25).

RNA-Seq identifies HMOX1 as a novel *Suv39h1* target in HSCs

In order to determine the transcriptional mechanism whereby *Suv39h1* might programme HSC-myofibroblast transition, two sets of RNA-seq experiments were performed. In one set, human primary HSCs were transfected with *Suv39h1* targeting siRNA. In the other set, human primary HSCs were treated with chaetocin. Both *Suv39h1* knockdown and inhibition markedly alter HSC transcriptome (figure 6A). Pathway analysis indicated that genes involved in HSC-myofibroblast transition, including those regulating cell proliferation, migration and ECM production, were preferentially altered by *Suv39h1* knockdown and inhibition (figure 6B). Notably, 140 genes were identified as being coregulated by *Suv39h1* knockdown and inhibition (figure 6C). Because *Suv39h1* is considered as a repressor of transcription, we focused on the 78 genes being upregulated by *Suv39h1* manipulation; HMOX1 was the most significantly altered among those genes (figure 6D).

qPCR data verified that HMOX1 expression was upregulated by *Suv39h1* knockdown or inhibition (figure 6E). HMOX1 levels were higher in the mice in which *Suv39h1* was deleted or inhibited (online supplemental figure S26). Of interest, *Suv39h1* knockdown or inhibition attenuated reactive oxygen species (ROS) levels in HSCs consistent with a role of HMOX1 in suppressing ROS production (online supplemental figure S27). In addition, it was observed that HMOX1 expression was progressively downregulated during HSC-myofibroblast transition in HSCs isolated from WT mice while staying relatively constant in those isolated from *Suv39h1* KO mice (figure 6F). Further, ChIP assays confirmed that *Suv39h1* abounded the proximal regions, but not the more distal regions, of the HMOX1 promoter during HSC-myofibroblast transition, which led to accumulation of trimethyl H3K9 surrounding the same regions (figure 6G). Importantly, HMOX1 expression was downregulated in the HSCs isolated from the cirrhotic human liver specimens (figure 6H) and inversely correlated with *Suv39h1* expression (figure 6I).

HMOX1 depletion overrides *Suv39h1* deficiency to normalise liver fibrogenesis

The following experiments were performed to further authenticate the functional relationship between *Suv39h1* and HMOX1 in liver fibrosis. In primary HSCs, *Suv39h1*

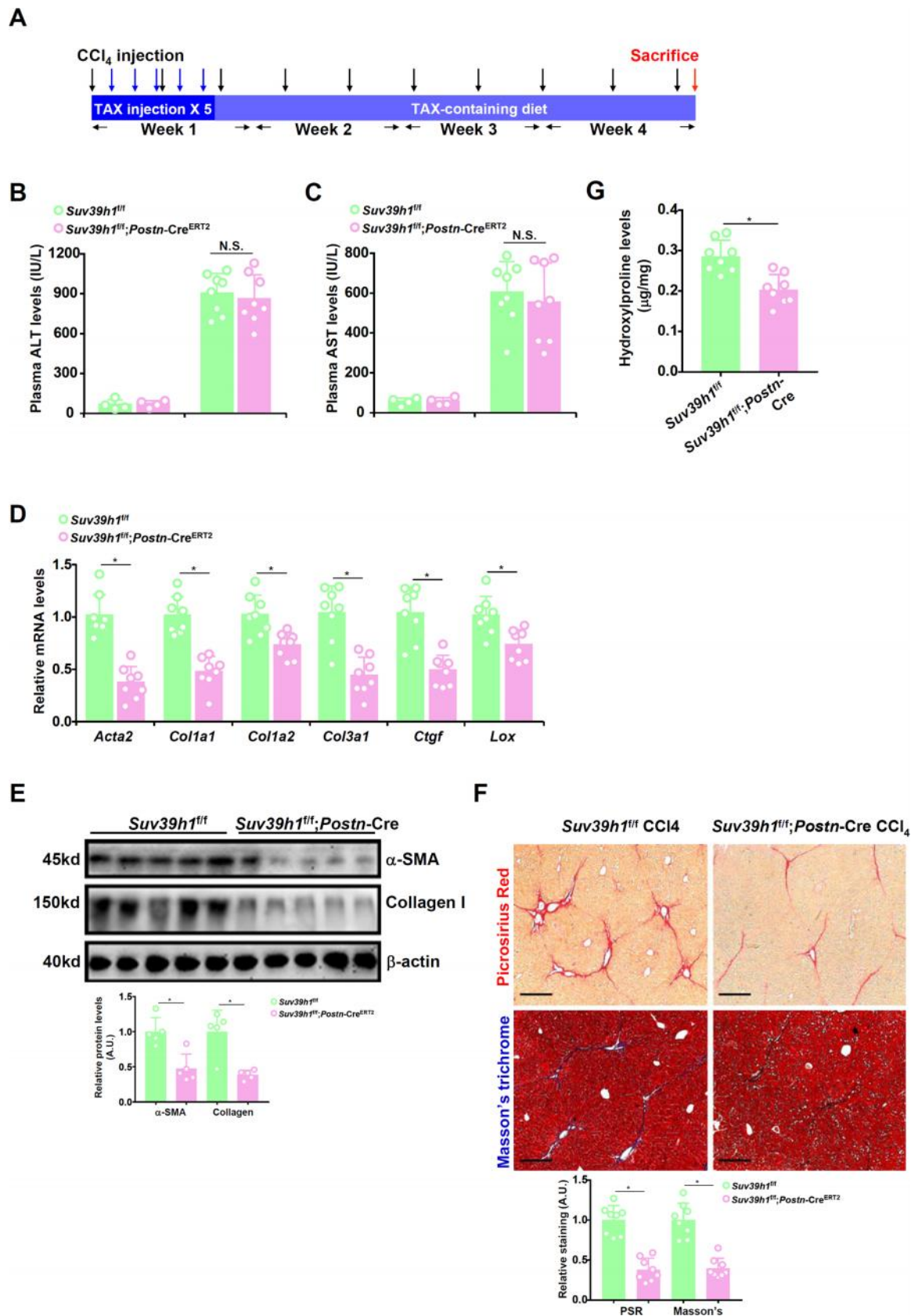


Figure 4 Myofibroblast-specific *Suv39h1* deficiency attenuates liver fibrosis in mice. (A–G) Myofibroblast-specific *Suv39h1* knockout mice (*Suv39h1^{fl/fl}; Postn-Cre^{ERT2}*) and wild-type littermates (*Suv39h1^{fl/fl}*) were subjected to CCl₄ injection procedure or the sham procedure and sacrificed 2 weeks after the surgery. (A) Scheme of protocol. Plasma ALT (B) and AST (C) levels. (D) Hepatic profibrogenic genes were examined by qPCR (D) and Western blotting (E). (F) Paraffin sections were stained with picrosirius red and Masson's chrome. (G) Hepatic hydroxylproline levels. N=4–8 mice for each group. Data are expressed as mean \pm SD. *p<0.05, two-tailed Student's t-test. Scale bar, 50 μ m. CCl₄, carbon tetrachloride; qPCR, quantitative PCR.

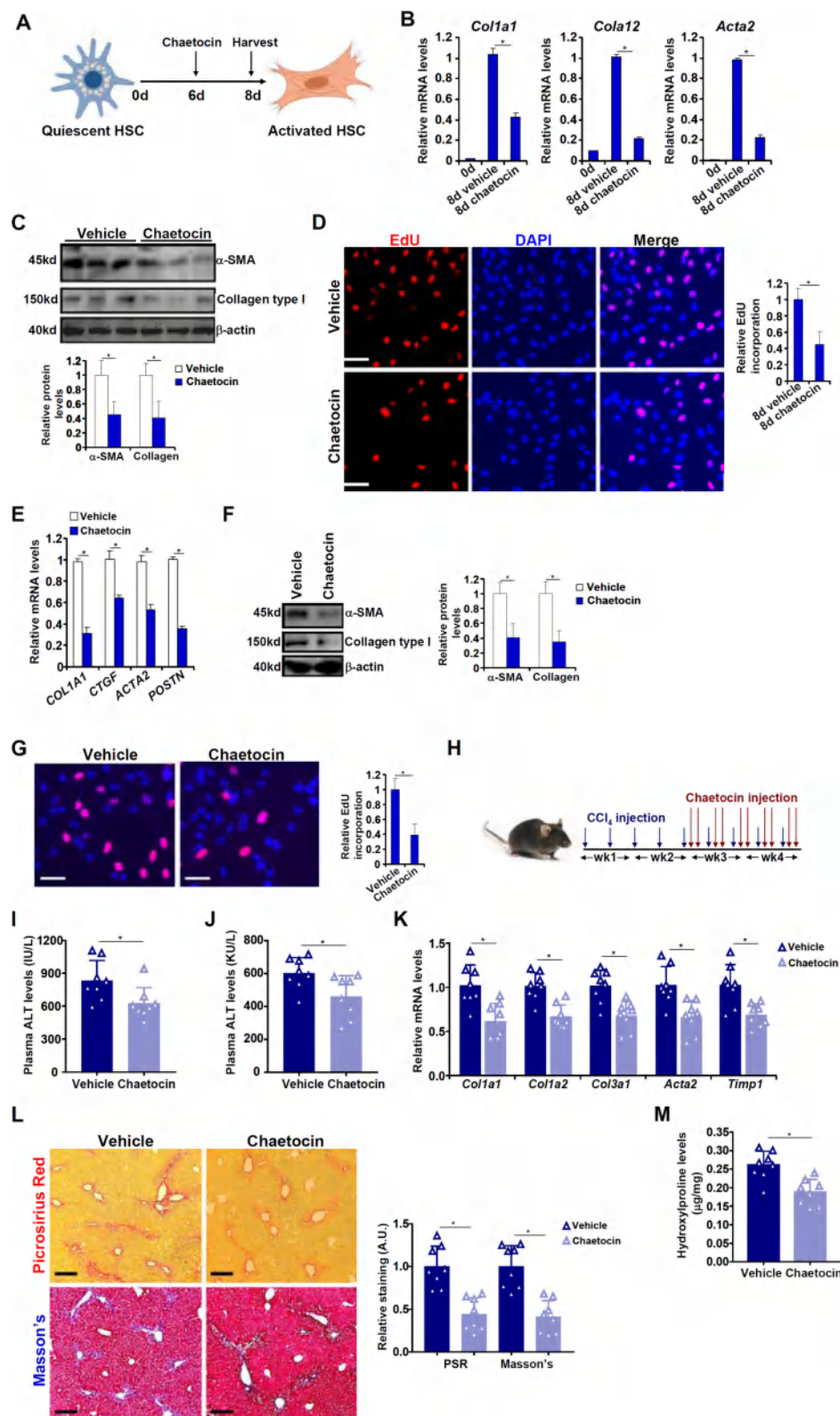


Figure 5 Suv39h1 inhibition attenuates liver fibrosis. (A) Primary murine HSCs were isolated and allowed to undergo spontaneous activation; chaetocin (0.1 μM) was added at 6 days and the cells were harvested at 8 days. (B, C) Myofibroblast marker genes were examined by qPCR and Western blotting. (D) Cell proliferation was evaluated by EdU incorporation. N=3 biological replicates. Data are expressed as mean±SD. *p<0.05, two-tailed Student's t-test. (E–G) Primary human HSCs were treated with or without chaetocin for 48 hours. Myofibroblast marker genes were examined by qPCR (E) and Western blotting (F). Cell proliferation was evaluated by EdU incorporation (G). N=3 biological replicates. Data are expressed as mean±SD. *p<0.05, two-tailed Student's t-test. (H–M) C57/BL6 mice were injected with CCl₄ to induce liver fibrosis followed by chaetocin injection. Scheme of protocol (H). Plasma ALT (I) and AST (J) levels. Hepatic profibrogenic genes were examined by qPCR (K). Paraffin sections were stained with picrosirius red and Masson's chrome (L). Hepatic hydroxyproline levels (M). N=8 mice for each group. Data are expressed as mean±SD. *p<0.05, two-tailed Student's t-test. scale bar, 50 μm. HSCs, hepatic stellate cells; qPCR, quantitative PCR.

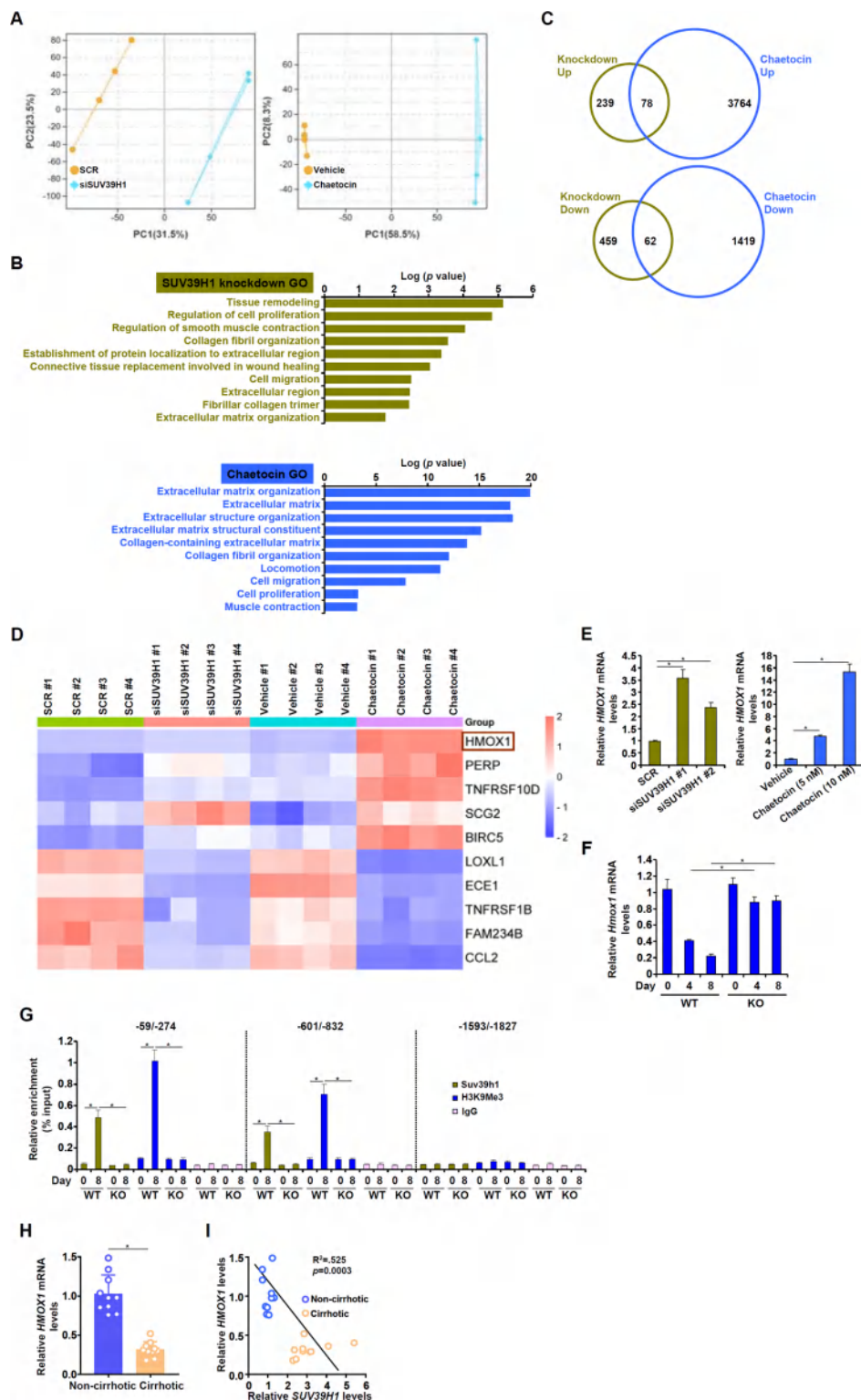


Figure 6 RNA-seq identifies HMOX1 as a novel Suv39h1 target in HSCs. (A–D) Primary human HSCs were transfected with siRNAs targeting Suv39h1 or scrambled siRNA (SCR). Alternatively, primary human HSCs were treated with or without chaetocin (0.1 μ M) for 48 hours. RNA-seq was performed as described in methods. PCA plots (A). GO analysis (B). Venn diagram (C). Heatmap (D). (E) Primary human HSCs were transfected with siRNAs targeting Suv39h1 or scrambled siRNA (SCR). Alternatively, primary human HSCs were treated with or without chaetocin (0.1 μ M) for 48 hours. HMOX1 expression was examined by qPCR. N=3 biological replicates. Data are expressed as mean \pm SD. * p <0.05, one-way ANOVA with post-hoc Scheffé. (F, G) Primary HSCs were isolated from WT and Suv39h1 KO mice and allowed to undergo spontaneous activation for 8D. HMOX1 expression was examined by qPCR (F). ChIP assays were performed with Suv39h1, trimethyl H3K9 or IgG (G). N=3 biological replicates. Data are expressed as mean \pm SD. * p <0.05, one-way ANOVA with post-hoc Scheffé. (H, I) HSCs were isolated from human liver biopsy specimens and gene expression was examined by qPCR. N=10 cases for each group. Data are expressed as mean \pm SD. * p <0.05, two-tailed Student's t-test. Pearson correlation was performed using Graphpad. ANOVA, analysis of variance; ChIP, chromatin immunoprecipitation; HMOX1, heme oxygenase 1; HSCs, hepatic stellate cells; KO, knockout; qPCR, quantitative PCR; WT, wild-type.

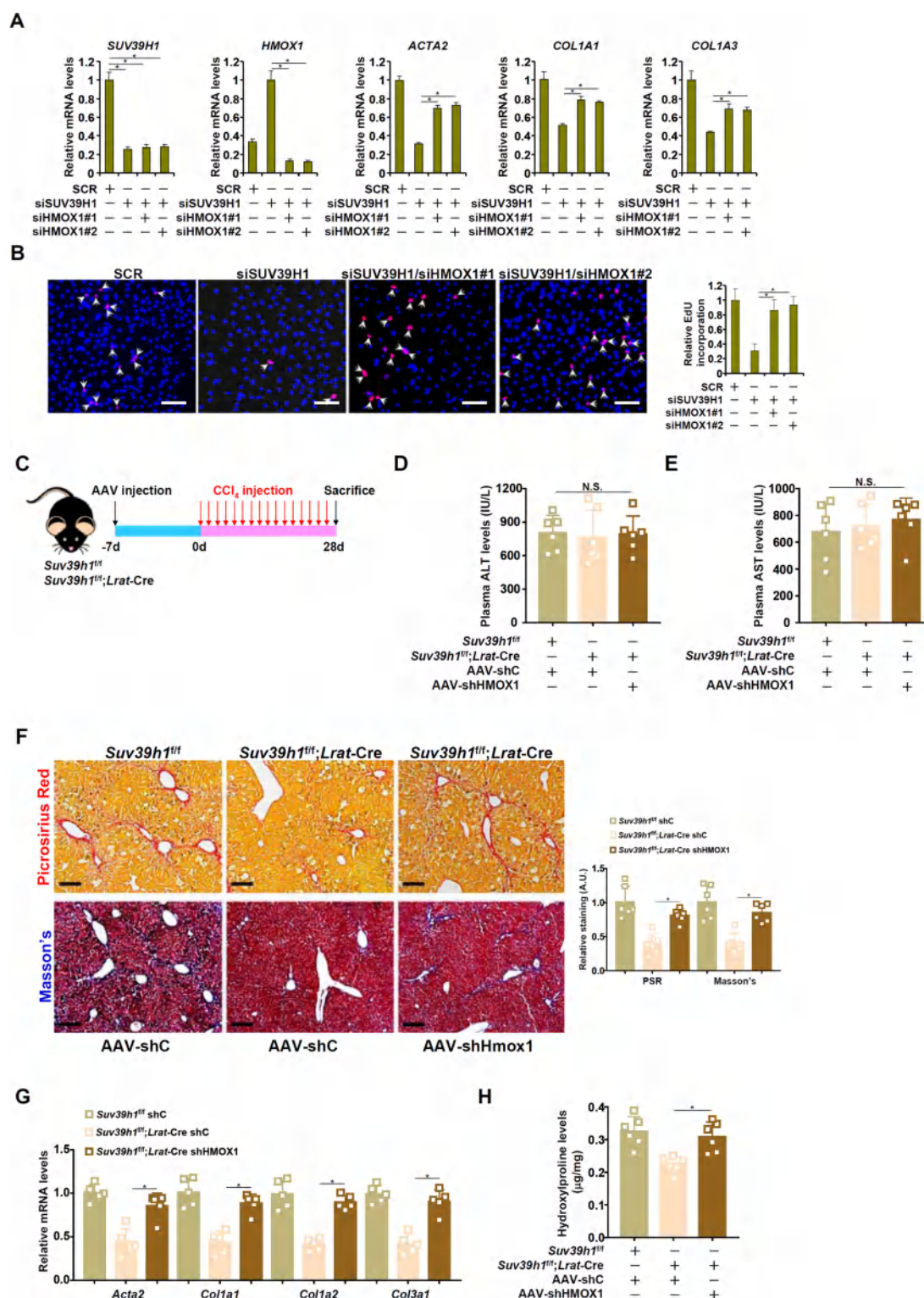


Figure 7 HMOX1 depletion overrides *Suv39h1* deficiency to normalise liver fibrogenesis. (A, B) Primary human HSCs were transfected with indicated siRNAs. Myfibroblast marker genes were examined qPCR. Cell proliferation was evaluated by EdU incorporation. N=3 biological replicates. Data are expressed as mean±SD. *p<0.05, one-way ANOVA with post-hoc Scheffé. (C–H) The *Suv39h1^{fl/fl}*; *Lrat-Cre* mice and the *Suv39h1^{fl/fl}* mice were injected AAV carrying shRNA targeting HMOX1 (shHMOX1) or control shRNA (Shc) followed by CCl₄ injection to induce liver fibrosis. Scheme of protocol (C). Plasma ALT levels (D). Plasma AST levels (E). Picrosirius red and Masson's trichrome staining (F). Expression levels of myfibroblast marker genes were examined by qPCR (G). Hydroxyproline levels (H). N=6 mice for each group. Data are expressed as mean±SD. *p<0.05, one-way ANOVA with post-hoc Scheffé. Scale bar, 50 μm. AAV, adeno-associated virus; ANOVA, analysis of variance; HMOX1, heme oxygenase 1.

knockdown led to repression of myfibroblast marker genes and weakening of cell proliferation. However, simultaneous knockdown of HMOX1 reversed this trend and restored the

expression of myfibroblast marker genes (figure 7A) and cell proliferation (figure 7B). HMOX1 silencing alone, however, did not appreciably alter the expression of myfibroblast

markers or proliferation in HSCs (online supplemental figure S28).

Next, shRNA targeting HMOX1 was placed downstream of the *Postn* promoter and packaged into

AAV6 for efficient myofibroblast delivery²²; the *Suv39h1^{flf}*; *Lrat*-Cre mice and the *Suv39h1^{flf}* mice were injected with AAV followed by CCl₄ injection to induce liver fibrosis (figure 7C). HMOX1 depletion in myofibroblasts, verified by qPCR (online supplemental figure S29, did not alter liver injury as evidenced by plasma ALT (figure 7D) and AST (figure 7E) levels. However, PSR/Masson's staining showed that HMOX1 depletion in the *Suv39h1^{flf}*; *Lrat*-Cre mice significantly elevated liver fibrogenesis to the level observed in the *Suv39h1^{flf}* mice (figure 7F). QPCR measurements of myofibroblast marker genes (figure 7G) and hepatic hydroxyproline quantification (figure 7H) both verified that HMOX1 depletion was able to override Suv39h1 deficiency to normalise liver fibrogenesis. Notably, these observations were replicated in an alternative model of liver fibrosis induced by BDL (online supplemental figure S30). Additionally, HMOX1 knockdown relieved the suppression of HSC-myofibroblast transition *in vitro* (online supplemental figure S31) and liver fibrosis *in vivo* (online supplemental figures S32, S33) by chaetocin treatment.

Myofibroblast-specific HMOX1 overexpression attenuates liver fibrosis

The final series of experiments were performed to tackle the myofibroblast-specific role for HMOX1 in liver fibrosis and the underlying mechanism. HMOX1 overexpression in primary human HSCs repressed the expression of myofibroblast marker genes and reduced cell proliferation (online supplemental figure S34). RNA-seq data revealed that HMOX1 overexpression altered HSC transcriptome resulting in 149 genes being upregulated and 132 genes being downregulated (1.25× fold change and $p < 0.05$). Of note, HMOX1 overexpression was most significantly correlated with the retinol metabolic pathway; several genes thought to stabilise the retinoid droplets were upregulated by HMOX1 overexpression (online supplemental figure S35), indicating that HMOX1 likely contribute to HSC-myofibroblast transition by modulating retinol homeostasis.

Next, myofibroblast-specific HMOX1 overexpression in mice was achieved by the same strategy as HMOX1 knockdown; qPCR data verified that HMOX1 levels were robustly increased in myofibroblasts (activated HSCs), but not in hepatocytes, from mice injected with AAV-HMOX1 compared with those injected with AAV-GFP (online supplemental figure S36). In the first scheme, myofibroblast-specific HMOX1 overexpression virus was injected prior to the induction of liver fibrosis by CCl₄ (figure 8A). HMOX1 overexpression significantly mitigated liver fibrosis without altering liver injury (figure 8B–F). In the second scheme, myofibroblast-specific HMOX1 overexpression virus was injected after the initiation of CCl₄-induced liver fibrosis (figure 8G). Similarly, HMOX1 overexpression significantly mitigated liver fibrosis without altering liver injury (figure 8H–K). The efficacy of myofibroblast-specific HMOX1 overexpression, either before or after the instigation of injurious stimuli, in amelioration of liver fibrosis was further authenticated in the BDL model (online supplemental figure S37, S38).

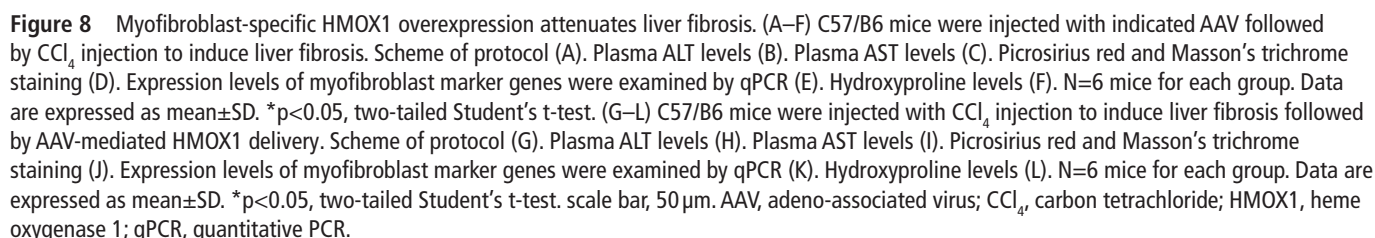
DISCUSSION

Liver fibrosis, for which effective interventional options are lacking, serves as a prelude to cirrhosis and hepatocellular carcinoma. HSC-myofibroblast transition encompasses a wide

spectrum of cellular and molecular processes and is considered the linchpin of liver fibrosis.^{5,30} Here we detail a novel epigenetic mechanism whereby the lysine methyltransferase Suv39h1 links HMOX1 transrepression to HSC-myofibroblast transition and liver fibrosis. We show here that Suv39h1 expression is elevated during HSC-myofibroblast transition *in vitro* and *in vivo*, which likely occurs at the transcription level mediated by C/EBP β . A large body of evidence implicates C/EBP β in liver fibrosis. For instance, C/EBP β levels are upregulated by TGF- β treatment in cultured HSCs.³¹ Consistently, systemic C/EBP β deletion dulls liver fibrogenesis in mice.³² Whereas it remains to be determined whether HSC-specific C/EBP β deficiency would be sufficient to dampen liver fibrosis, it is worth noting that activation of C/EBP β in either hepatocytes³³ or in Kupffer cells³⁴ is similarly associated with (enhanced) liver fibrosis *in vivo* suggesting that C/EBP β may play a profibrogenic role in the liver regardless of cellular origins. Small-molecule inhibitors for C/EBP β with anti-tumour activities have been identified^{35,36}; it would of interest to test these compounds for efficacies as antifibrotic reagents.

Our data suggest that mice with HSC-specific or myofibroblast-specific Suv39h1 deletion are refractory to liver fibrosis. More importantly, administration of the Suv39h1 inhibitor chaetocin potently mitigates liver fibrosis in mice. Of intrigue, Suv39h1 expression has been shown to progressively decrease when pre-adipocytes (fibroblasts) are induced to differentiate into mature adipocytes, a process greatly accelerated by Suv39h1 depletion.³⁷ Because quiescent HSCs are thought to resemble adipocytes and pro-adipogenic regimens have been shown to pivot activated HSCs (myofibroblasts) back to a quiescent phenotype,^{10,38} it is tempting to speculate that Suv39h1 might function as a molecular switch that quantitatively determines the fate of HSCs. Although compelling genetic evidence is provided here to suggest that regulation of HSC-myofibroblast by Suv39h1 is largely cell-autonomous, the contribution of Suv39h1 in other intrahepatic and extrahepatic cell lineages to liver fibrosis cannot be excluded at this point. For instance, HSC-myofibroblast transition and liver fibrosis are acutely influenced by the hepatic immune microenvironment.² Mounting evidence portrays Suv39h1 as a key epigenetic regulator of immunity by programming multiple immune cell functions including homing, differentiation/transdifferentiation and survival.³⁹ Additional studies are warranted to investigate the role of Suv39h1 in various immune cell lineages in liver fibrosis and expand the territory over which Suv39h1 reins to influence liver pathologies.

Transcriptomic analysis leads to the identification of HMOX1 as a novel target for Suv39h1. Although functional studies cement the notion that antifibrotic effects of Suv39h1 deletion/inhibition are mediated through HMOX1, other potential Suv39h1 targets deserve further attention. TNFRSF10D, also known as decoy receptor 2 (DcR2), is a well-established marker of cellular senescence.⁴⁰ During the resolution stage of liver fibrosis, senescence of HSCs is believed to be responsible for the termination of fibrogenic response.⁴¹ It is therefore reasonable to propose that DcR2 upregulation following Suv39h1 deletion or inhibition may trigger senescence and consequently clearance of HSCs leading to attenuation of liver fibrosis. Alternatively, it has long been observed that activated HSCs (myofibroblasts) undergo apoptosis when the liver recovers from injuries.⁴² P53 apoptosis effector related to PMP22 (PERP) is another potential target of Suv39h1, whose upregulation by Suv39h1 deletion or inhibition may account for the antifibrotic phenotypes. Li *et al* have recently reported that attenuation of fibroblast-myofibroblast transition, a process with high resemblance to HSC-myofibroblast transition, in a model of pulmonary hypertension is accompanied by



HMOX1 has been long reported to participate in HSC-myofibroblast transition and liver fibrosis.^{44 45} Because most previous studies rely on pharmaceutical manipulation of HMOX1 activities, a myofibroblast-specific role for HMOX1 is blurred since HMOX1 can transmit an antifibrotic signal from myeloid cells.⁴⁶ Here we provide evidence to show that myofibroblast-restricted HMOX1 overexpression ameliorates liver fibrosis without altering liver injury. Mechanistically, HMOX1 overexpression appears to reverse HSC-myofibroblast transition by modulating the expression of perilipins, a group

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transition cannot be entirely and reliably drawn from the transcriptomic data. Future studies employing multi-omics tools would hopefully shed more light in this regard.

In summary, our data suggest that genetic and pharmaceutical manipulation of Suv39h1 influences HSC-myofibroblast transition and liver fibrosis. There are limitations with this study that dampen its translational potential. First, it is observed that Suv39h1 inhibition by chaetocin leads to more profound transcriptomic alterations than Suv39h1 deletion in HSCs pointing to possible off-target effects of chaetocin. Modified Suv39h1 inhibitors have been reported, although the specificities and efficacies of those compounds are not yet ascertained.⁵⁰ It is essential that the new generation of Suv39h1 inhibitors be tested in models of liver fibrosis to validate its targetability. Second, a subpopulation of HSCs has recently been shown to link fibrogenesis to hepatocarcinogenesis.⁵¹ Because targeting liver fibrosis finds most significant clinical relevance in cirrhosis and hepatocarcinoma, the present study would benefit from additional experimentation in preclinical models of cirrhosis/hepatocarcinoma.

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Funding Wuxi Taihu Lake Talent Plan, Supports for Leading Talents in Medical and Health Profession (to J Zhou)

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 Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. The data that support the findings of this study are available upon reasonable request.

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