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Research Paper

Serum response factor (SRF) promotes ROS generation and hepatic stellate cell activation by epigenetically stimulating NCF1/2 transcription

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ABSTRACT

Activation of hepatic stellate cells (HSC) is a hallmark event in liver fibrosis. Accumulation of reactive oxygen species (ROS) serves as a driving force for HSC activation. The regulatory subunits of the NOX complex, NCF1 ($p47^{phox}$) and NCF2 ($p67^{phox}$), are up-regulated during HSC activation contributing to ROS production and liver fibrosis. The transcriptional mechanism underlying NCF1/2 up-regulation is not clear. In the present study we investigated the role of serum response factor (SRF) in HSC activation focusing on the transcriptional regulation of NCF1/2. We report that compared to wild type littermates HSC-conditional SRF knockout (CKO) mice exhibited a mortified phenotype of liver fibrosis induced by thioacetamide (TAA) injection or feeding with a methionine-and-choline deficient diet (MCD). More importantly, SRF deletion attenuated ROS levels in HSCs *in vivo*. Similarly, SRF knockdown in cultured HSCs suppressed ROS production *in vitro*. Further analysis revealed that SRF deficiency resulted in repression of NCF1/NCF2 expression. Mechanistically, SRF regulated epigenetic transcriptional activation of NCF1/NCF2 by interacting with and recruiting the histone acetyltransferase KAT8 during HSC activation. In conclusion, we propose that SRF integrates transcriptional activation of NCF1/NCF2 and ROS production to promote liver fibrosis.

1. Introduction

Liver fibrosis is generally considered a host defense mechanism to mitigate injury and aid wound healing. Whereas controlled resolution of fibrosis leads to restoration of liver structure and function excessive fibrosis contributes to disruption of normal hepatic anatomy and is often associated with end-stage liver diseases such as hepatocellular carcinoma and cirrhosis [1]. Regardless of its etiology, liver fibrosis is invariably mediated by activation of myofibroblast, a unique population of cells capable of both producing extracellular matrix fibers and performing muscle-like contraction [2]. Recent studies harnessing state-of-the-art fate-mapping techniques have demonstrated that hepatic stellate cells (HSCs) represent the major source of activated myofibroblasts in the fibrotic liver [3]. The mechanism underlying trans-differentiation of HSCs to myofibroblasts is being actively investigated and hotly debated.

Reactive oxygen species (ROS) accumulation is considered a paradigm during host defense response [4]. Coincidently, current evidence suggests that ROS serves as a pivotal factor contributing to HSC activation [5]. Cellular ROS levels are modulated by, among others, the NADPH oxidase (NOX) family of proteins. Several members, including both catalytic and accessory subunits, have been reported to play a role in HSC activation. For instance, mice with a deficiency in $p47^{phox}$ (encoded by *NCF1*), an organizer for the NOX2 complex, are resistant to liver fibrosis owing to delayed HSC activation [6]. Another regulatory subunit for the NOX2 complex $p67^{phox}$ (encoded by *NCF2*) has also been

Abbreviations: Serum response factor, SRF; hepatic stellate cell, HSC; reactive oxygen species, ROS; thioacetamide, TAA; chromatin immunoprecipitation, ChIP; quantitative PCR, qPCR; NADPH oxidase, NOX; neutrophil cytosolic factor, NCF; lysine (K) acetyltransferase 8, KAT8

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implicated in liver fibrosis and HSC activation [7]. Levels of both $p47^{phox}$ and $p67^{phox}$ are up-regulated in activated HSCs presumably due to transcriptional activation although the underlying mechanism is not clear [8].

Serum response factor (SRF) is a transcription factor playing key roles in the pathogenesis of human diseases. Previous studies have demonstrated that SRF is essential for myofibroblast trans-differentiation in multiple different tissues. Nanoparticle-mediated delivery of SRF inhibitor or small interfering RNA (siRNA) has been used to antagonize conjunctival fibrosis [9–11] and lung fibrosis [12]. SRF protein levels are up-regulated during HSC activation *in vitro* [13]. Furthermore, it has been reported that the lncRNA HOTTIP promotes HSC activation by augmenting SRF expression [14]. In addition, You *et al* have shown that SRF knockdown significantly attenuates α-SMA expression in the immortalized HSC cell line SJ1 [15]. However, it remains an open question whether HSC-specific SRF deletion is sufficient to block liver fibrosis *in vivo*. Nor is it clear whether SRF may promote HSC maturation via regulating ROS production. Here we report that conditional SRF knockout in HSCs ameliorates liver fibrosis and ROS production in mice. Mechanistically, SRF regulates ROS levels in HSCs by epigenetically activating NCF1/2 transcription both *in vitro* and *in vivo*. Therefore, screening for small-molecule SRF inhibitors may yield novel therapeutic solutions against liver fibrosis.

2. Methods

2.1. Animal studies

All the animal protocols were reviewed and approved by the intramural Ethics Committee on Humane Treatment of Experimental Animals. Specific deletion of SRF in hepatic stellate cells was achieved by crossing the the $Srf^{flox/flox}$ strain [16] to the *GFAP*-Cre strain [17,18]. To induce liver fibrosis, 6-8 week-old, male mice were injected peritoneally with TAA (100 mg/kg body weight) or saline every other day for 2 weeks [19]. Alternatively, the mice were fed a methionine-and-choline deficient (MCD) diet or a control diet for 8 weeks as previously described [20].

2.2. Cell isolation, viral infection, and transient transfection

Immortalized human hepatic stellate cells (LX-2) were maintained in DMEM supplemented with 10% FBS. Primary hepatic stellate cells were isolated and maintained as previously described [21]. RNA targeting SRF (GAUGGAGUUCAUCGACAACAA) was transfected with Lipofectamine RNAiMax (Thermo) per vendor's recommendation. GFP-tagged SRF expression construct [22], HA-tagged KAT8 expression construct [23], NCF1/p47 promoter-luciferase constructs [24], and NCF2/p67 promoter luciferase constructs [25] have been previously described. Transient transfection was performed with Lipofectamine 2000. Briefly, cells were plated in 12-well culture dishes (~60,000 cells/well). The next day, equal amounts (0.1 μg) of reporter construct and effector construct were transfected into each well. DNA content was normalized by the addition of an empty vector (pcDNA3). For monitoring transfection efficiency and for normalizing luciferase activity, 0.02 μg of GFP construct was transfected into each well. Cells were harvested 48 h after transfection and reporter activity was measured using a luciferase reporter assay system (Promega) as previously described [26]. All experiments were repeated at least three times.

2.3. Protein extraction, immunoprecipitation, and Western blot

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100) with freshly added EDTA-free protease inhibitor tablet (Roche)as previously described [27].Specific antibodies or pre-immune IgGs (P.I.I.) were added to and incubated with cell lysates overnight before being absorbed by Protein A/G-plus Agarose beads (Santa Cruz). Precipitated immune complex was released by boiling with 1X SDS electrophoresis sample buffer. Alternatively, FLAG-conjugated beads (M2, Sigma) were added to and incubated with lysates overnight. Precipitated immune complex was eluted with 3X FLAG peptide (Sigma). Proteins were separated by 8% polyacrylamide gel electrophoresis with pre-stained markers (Bio-Rad) for estimating molecular weight and efficiency of transfer to blots. Proteins were transferred to nitrocellulose membranes (Bio-Rad) in a Mini-Trans-Blot Cell (Bio-Rad). The membranes were blocked with 5% milk powder in Tris-buffered saline buffer (0.05% Tween 20, 150 mM NaCl, 100 mM Tris-HCl pH7.4) at 4 °C overnight. Western blot analyses were performed with anti-SRF (sc-13029, Santa Cruz), anti-β-actin (A1978, Sigma), anti-α-SMA (ab5694, Abcam), anti-NCF1 (ab795, Abcam), anti-NCF2 (15551-1, Proteintech), anti-KAT8 (13842-1, Proteintech), and anti-α-SMA (ab5694, Abcam) antibodies. The protein blots were visualized using ECL reagent (PerkinElmer Life Sciences) on a Kodak image station (PerkinElmer Life Sciences).

2.4. RNA isolation and real-time PCR

RNA was extracted with the RNeasy RNA isolation kit (Qiagen). Reverse transcriptase reactions were performed as previously described using a SuperScript First-strand Synthesis System (Invitrogen) [28]. Data were normalized with 18S rRNA as an internal control according to manufacturer's protocol and expressed as fold change over the control group. All experiments were repeated at least three times.

2.5. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed essentially as described before [29–35]. Briefly, chromatin in control and treated cells were cross-linked with 1% formaldehyde. Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with protease inhibitor tablet and PMSF. DNA was fragmented into ~500 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-acetyl H3 (06-599, Millipore), anti-acetyl H4 (06-598, Millipore), anti-acetyl H3K27 (17-683, Millipore), anti-H4K16 (13534, Cell Signaling), anti-p300 (sc-585, Santa Cruz), anti-SRF (5147, Cell Signaling), and anti-KAT8 (13842-1, Proteintech) antibodies. Precipitated genomic DNA was amplified by real-time PCR with primers that span the target promoters or a control promoter (GAPDH). Serially diluted genomic DNA extracted from normal cells/tissues was used to generate a standard curve to calculate the amount of DNA being precipitated by a particular antibody. A total of 10% of the starting material is also included as the input. Data are then normalized to the input and expressed as fold changes compared to the control group. All experiments were repeated at least three times.

2.6. Histology

Histological analyses were performed essentially as described before [36–38]. Briefly, paraffin sections were stained with picrosirius red (Sigma) or Masson's trichrome (Sigma) according to standard procedures. Pictures were taken using an Olympus IX-70 microscope.

2.7. DHE and DCFH-DA staining

DHE and DCFH-DA stainings were performed essentially as previously described [39]. Frozen liver sections or cells were stained with DHE (10 μM) or DCFH-DA (10 μM) at 37 °C for 30 min. Fluorescence was visualized by co-focal microscopy (LSM 710, Zeiss). Quantifications were performed with Image J. 3 slides were stained from each individual mouse and ~5 fields counted per slide.

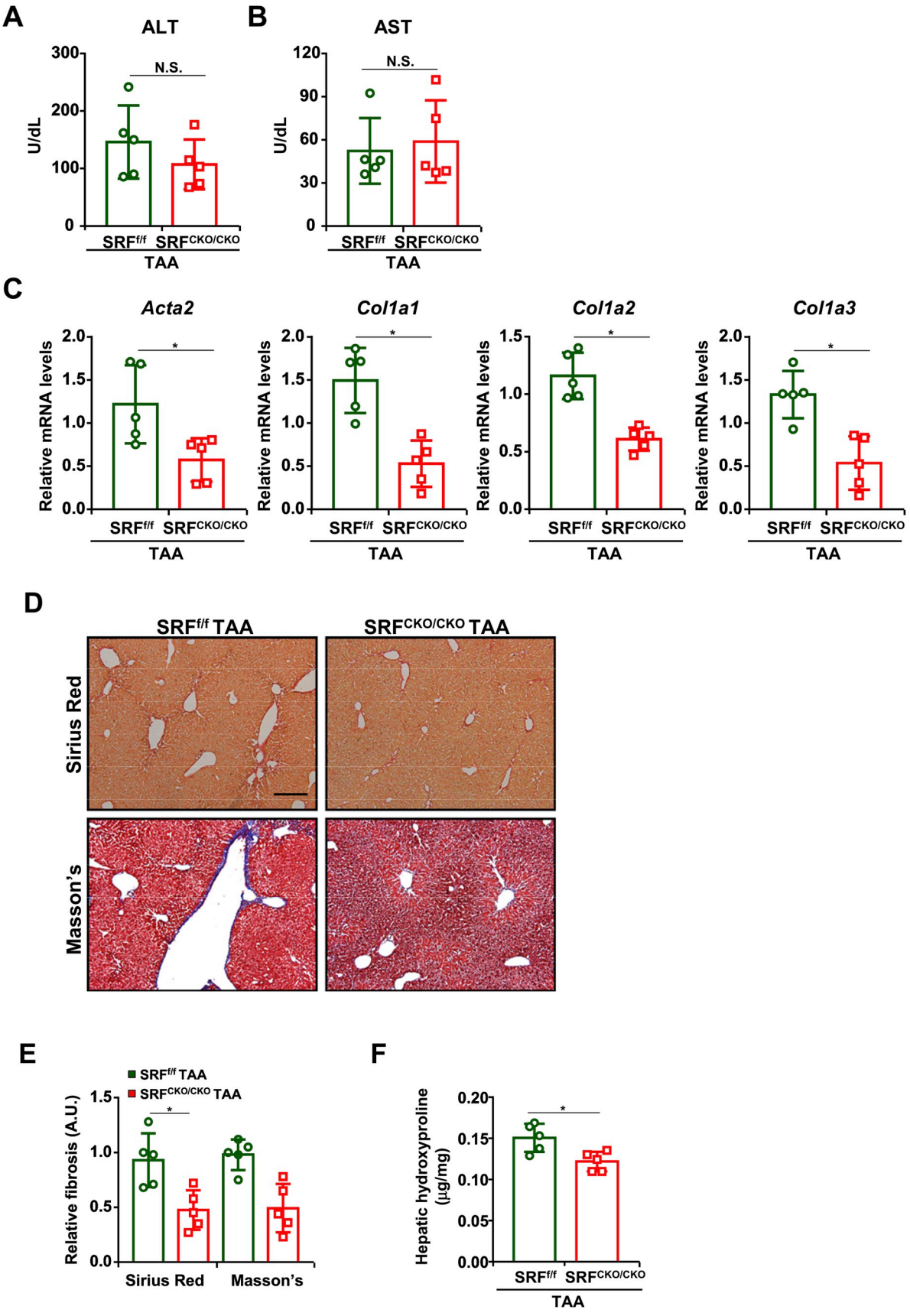

Fig. 1. ***HSC-specific SRF deficiency attenuates TAA-induced liver fibrosis in mice.*** WT and CKO mice were injected with TAA to induce liver fibrosis as described in Methods. (**A, B**) Plasma ALT and AST levels. (**C**) Pro-fibrogenic gene expression was measured by qPCR. (**D, E**) Picrosirus red and Masson's trichrome staining. (**F**) Hepatic hydroxyl proline levels. N = 5 mice for each group. Error bars represent SD (*p < 0.05, 2-tailed student's t-test).

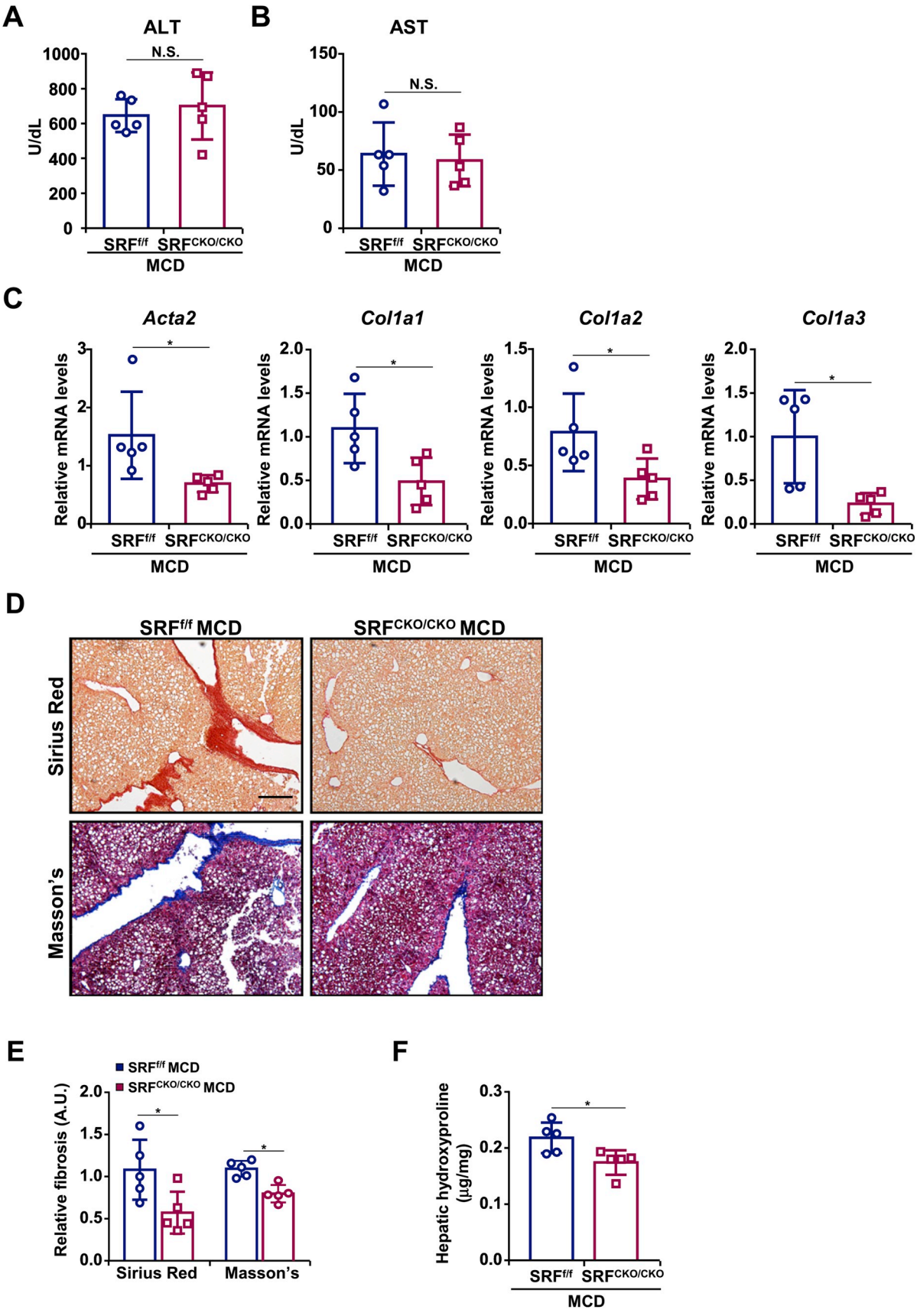

Fig. 2. ***HSC-specific SRF deficiency attenuates MCD-induced liver fibrosis in mice.*** WT and CKO mice were fed with a MCD diet to induce liver fibrosis as described in Methods. (**A, B**) Plasma ALT and AST levels. (**C**) Pro-fibrogenic gene expression was measured by qPCR. (**D, E**) Picrosirus red and Masson's trichrome staining. (**F**) Hepatic hydroxyl proline levels. N = 5 mice for each group. Error bars represent SD (*p < 0.05, 2-tailed student's t-test).

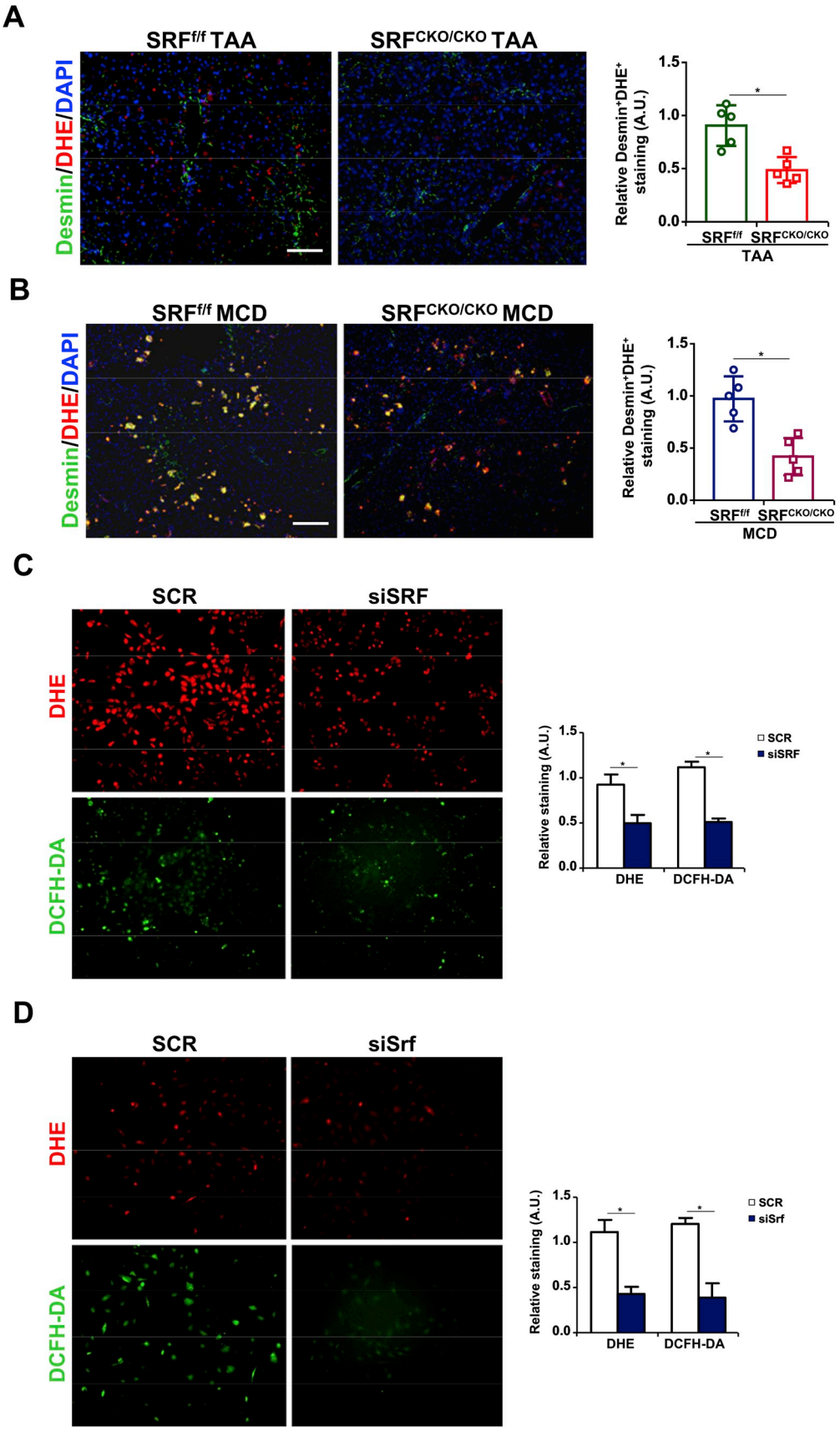

Fig. 3. ***SRF regulates ROS levels in hepatic stellate cells.*** (**A**) Liver fibrosis was induced by TAA in WT and CKO mice as described in Methods. Cryosections were co-stained with DHE and anti-desmin. N = 5 mice for each group. Error bars represent SD (*p < 0.05, 2-tailed student's t-test). (**B**) Liver fibrosis was induced by MCD feeding in WT and CKO mice as described in Methods. Cryosections were co-stained with DHE and anti-desmin. N = 5 mice for each group. Error bars represent SD (*p < 0.05, 2-tailed student's t-test). (**C**) LX-2 cells were transfected with siRNA targeting SRF or scrambled siRNA (SCR) followed by staining with DHE and DCFH. Data represent averages of three independent experiments and error bars represent SEM (*$p < 0.05$, 2-tailed student's t-test). (**D**) Primary mouse HSCs were transfected with siRNA targeting SRF or SCR followed by staining with DHE and DCFH. Data represent averages of three independent experiments and error bars represent SEM (*$p < 0.05$, 2-tailed student's t-test).

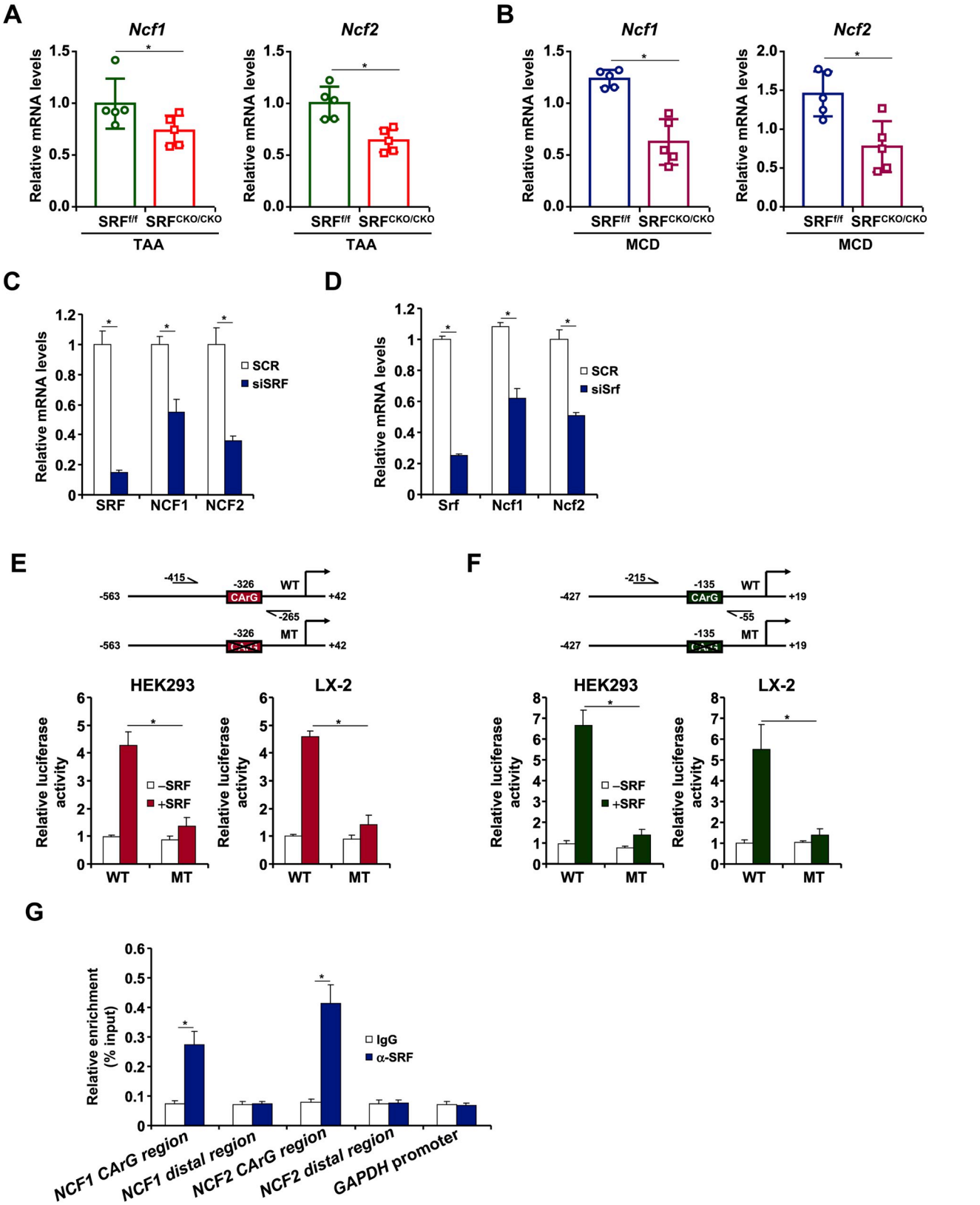

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Fig. 4. ***SRF activates NCF1/NCF2 transcription in hepatic stellate cells***. (**A**) WT and CKO mice were induced to develop liver fibrosis by TAA injection. Hepatic levels of NCF1 and NCF2 were examined by qPCR. (**B**) WT and CKO mice were induced to develop liver fibrosis by MCD diet. Hepatic levels of NCF1 and NCF2 were examined by qPCR. (**C**) LX-2 cells were transfected with siRNA targeting SRF or SCR. Expression levels of NCF1 and NCF2 were examined by qPCR. (**D**) Primary mouse HSCs were transfected with siRNA targeting SRF or SCR. Expression levels of NCF1 and NCF2 were examined by qPCR. (**E**) Wild type (WT) or mutant (MT) NCF1 promoter-luciferase construct was transfected into HEK293 and LX-2 cells with or without SRF. Luciferase activities were normalized by GFP fluorescence and protein concentration. (**F**) Wild type (WT) or mutant (MT) NCF2 promoter-luciferase construct was transfected into HEK293 and LX-2 cells with or without SRF. Luciferase activities were normalized by GFP fluorescence and protein concentration. (**G**) Nuclear lysates were extracted from LX-2 cells and ChIP assays were performed with anti-SRF or IgG. Data represent averages of three independent experiments and error bars represent SEM (*$p < 0.05$, 2-tailed student's t-test).

2.8. Statistical analysis

One-way ANOVA with post-hoc Scheffe analyses were performed using an SPSS package. Unless otherwise specified, p values smaller than 0.05 were considered statistically significant (*).

3. Results

3.1. HSC-specific SRF deficiency attenuates liver fibrosis in mice

To directly probe the effect of SRF on liver fibrosis, HSC-conditional SRF knockout mice (CKO) were obtained by crossing $Srf^{f/f}$ mice to *GFAP*-Cre mice. When both WT and CKO mice were induced to develop liver fibrosis by TAA injection, no difference in liver injury was observed as assessed by plasma ALT (Fig. 1A) and AST (Fig. 1B) levels. Quantitative PCR (Fig. 1C) analyses showed that hepatic expression levels of pro-fibrogenic genes were down-regulated in CKO mice. Picrosirius red and Masson's trichrome stainings (Fig. 1D and E) showed that collagenous tissues were reduced in CKO mice. Hydroxylproline quantification confirmed that the pro-fibrogenic response was dampened in the CKO mice compared to the WT mice (Fig. 1F).

We then verified these observations in a second model of liver fibrosis in which the mice were fed an MCD diet for 8 weeks. Again, WT and CKO mice exhibited comparable liver injury as evidenced by plasma ALT (Fig. 2A) and AST (Fig. 2B) levels. QPCR (Fig. 2C), picrosirius red staining, Masson's trichrome staining (Fig. 2D and E), and hydroxylproline quantification (Fig. 2F) all pointed to mitigation of liver fibrosis as a result of HSC-specific SRF deficiency. Combined, these data support an essential role for SRF in HSC activation and liver fibrosis *in vivo*.

3.2. SRF regulates ROS levels in hepatic stellate cells

Because ROS accumulation contributes to HSC activation, we performed the following experiments in an attempt to establish a relationship between SRF and ROS production in HSCs. Immunofluorescence staining showed that the number of $Desmin^{+}DHE^{+}$ cells were significantly down-regulated in the CKO livers compared to the WT livers in both the TAA model (Fig. 3A) and the MCD model (Fig. 3B), suggesting that SRF deficiency may be associated with reduced ROS production in HSCs. Depletion of SRF with siRNA in human immortalized HSCs (LX-2) significantly down-regulated ROS levels as assessed by both DHE and DCFH-DA stainings (Fig. 3C). Similarly, SRF knockdown suppressed ROS accumulation in primary mouse HSCs undergoing spontaneous activation *in vitro* (Fig. 3D). Collectively, these data suggest that SRF may play an essential role regulating ROS production in hepatic stellate cells.

3.3. SRF activates NCF1/NCF2 transcription in hepatic stellate cells

ROS production can be mediated by the NOX family of enzymes consisting of both catalytic and structural subunits. Of interest, qPCR analysis revealed that SRF deficiency resulted in a decrease in expression levels of NCF1/$p47^{Phox}$ and NCF2 /$p67^{Phox}$, two organizers for the NOX2 complex, in the livers following TAA administration (Fig. 4A). Likewise, SRF deletion also caused a down-regulation of both NCF1 and NCF2 in the MCD-fed livers (Fig. 4B). We therefore hypothesized that SRF may be necessary to activate NCF1/NCF2 transcription in hepatic stellate cells thereby contributing to HSC activation. Indeed, depletion of SRF with siRNA in LX-2 repressed NCF1/NCF2 mRNA expression (Fig. 4C). Likewise, SRF silencing in primary mouse HSCs resulted in repression of NCF1/NCF2 expression (Fig. 4D).

We next attempted to probe whether SRF could directly activate NCF1/NCF2 transcription. Examination of human NCF1 promoter and human NCF2 promoter uncovered a CArG box for each within the proximal region: the NCF1 promoter contains a CArG box situated -326 relative to the transcription start site (TSS, Fig. 4E) whereas the NCF2 promoter contains a CArG box at -135 relative to TSS (Fig. 4F). Overexpression of SRF strongly augmented activities of both the NCF1 promoter (Fig. 4E) and the NCF2 promoter (Fig. 4F); mutation of the CArG box abrogated SRF-induced activation without altering basal activity. ChIP assays confirmed that SRF directly bound to the CArG boxes, but not the more distant regions, of NCF1/NCF2 promoters (Fig. 4G). Taken together, these data support a role for SRF as a direct transcriptional activator of NCF1/NCF2 in HSCs.

3.4. SRF recruits KAT8 to activate NCF1/NCF2 transcription

We then tackled the epigenetic mechanism whereby SRF contributes to NCF1/NCF2 transcription. ChIP assays showed that SRF knockdown significantly reduced levels of histone H3 acetylation (Fig. 5A) and H4 acetylation (Fig. 5B), typically associated with transcriptional activation, surrounding the NCF1/NCF2 promoters but not the GAPDH promoter. Further examination of individual lysine residues revealed that a reduction in acetylation of H3K27 (Fig. 5C) and H4K16 (Fig. 5D) on the NCF1/NCF2 promoters. Consistently, SRF knockdown suppressed the occupancy of p300, an H3K27 acetyltransferase [40], on the NCF1/NCF2 promoters (Fig. 5E). SRF deficiency also dampened the occupancy of KAT8, a dedicated H4K16 acetyltransferase [41], on the NCF1/NCF2 promoter (Fig. 5F).

The reliance of p300 on SRF for promoter binding was anticipated because an interaction between SRF and p300 had been demonstrated previously [42,43]. We decided to focus on the potential interplay between SRF and KAT8 in regulating NCF1/NCF2 transcription. Co-immunoprecipitation assays showed that ectopically expressed SRF and KAT8 interacted with each other in HEK293 cells (Fig. 5G). More important, endogenous SRF and KDAT8 formed a complex in LX-2 cells (Fig. 5H). We next performed Re-ChIP assays to examine the interaction between SRF and KAT8 on target promoters. As shown in Fig. 5I, an SRF-KAT8 complex was readily detectable on the NCF1 promoter and the NCF2 promoter but not on the GAPDH promoter in LX-2 cells. Re-ChIP assay similarly confirmed the SRF-KAT8 interaction on the NCF1/NCF2 promoter in activated primary mouse HSCs (Fig. 5J). Therefore, we conclude that SRF may contribute to NCF1/NCF2 transcription by recruiting histone acetyltransferases to the gene promoters.

3.5. KAT8 is essential for HSC activation by regulating NCF1/NCF2 transcription

Finally, we evaluated the role of KAT8 in HSC activation. In LX-2 cells, KAT8 knockdown by siRNA led to marked decrease in the expression of pro-fibrogenic genes, including α-SMA and collagen type I,

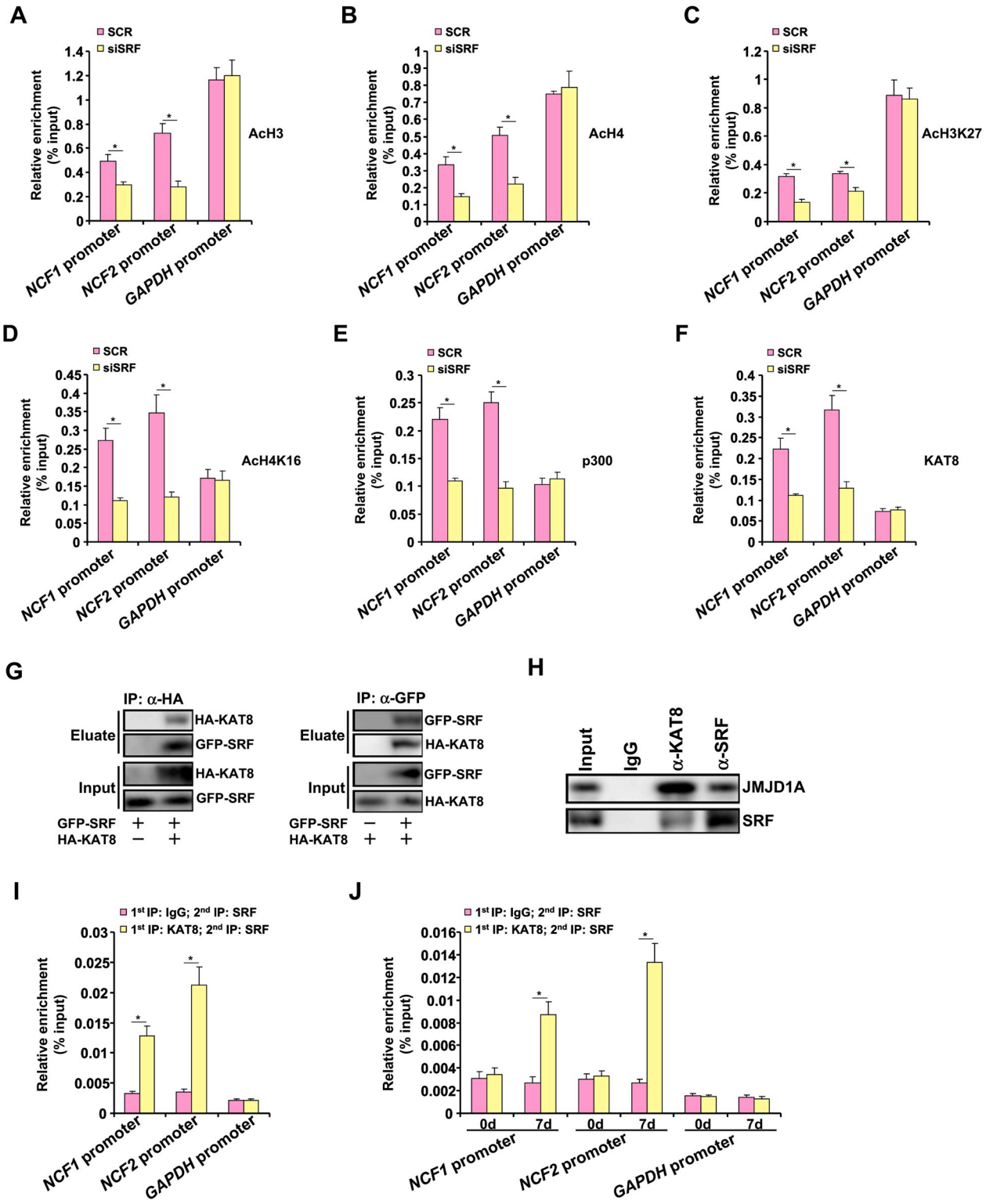

Fig. 5. ***SRF recruits KAT8 to activate NCF1/NCF2 transcription.*** (**A-F**) LX-2 cells were transfected with siRNA targeting SRF or SCR. ChIP assays were performed with indicated antibodies. (**G**) HA-tagged KAT8 and GFP-tagged SRF were transfected into HEK293 cells. Immunoprecipitation was performed with anti-HA or anti-GFP. (**H**) Nuclear lysates were extracted from LX-2 cells. Immunoprecipitation was performed with indicated antibodies. (**I**) Nuclear lysates were extracted from LX-2 cells and Re-ChIP assays were performed with indicated antibodies. (**J**) Primary mouse HSCs were isolated and nuclear lysates were extracted at 7d after activation. Re-ChIP assays were performed with indicated antibodies. Data represent averages of three independent experiments and error bars represent SEM (*$p < 0.05$, 2-tailed student's t-test).

at both mRNA (Fig. 6A) and protein (Fig. 6B) levels. Down-regulation of pro-fibrogenic gene expression was accompanied by a concomitant reduction of NCF1/NCF2 expression (Fig. 6A and B). ChIP assay confirmed that there was diminished enrichment of acetyl H4K16 surrounding the NCF1/NCF2 promoters following KAT8 knockdown. Of note, KAT8 depletion did not alter the levels of acetyl H4K16

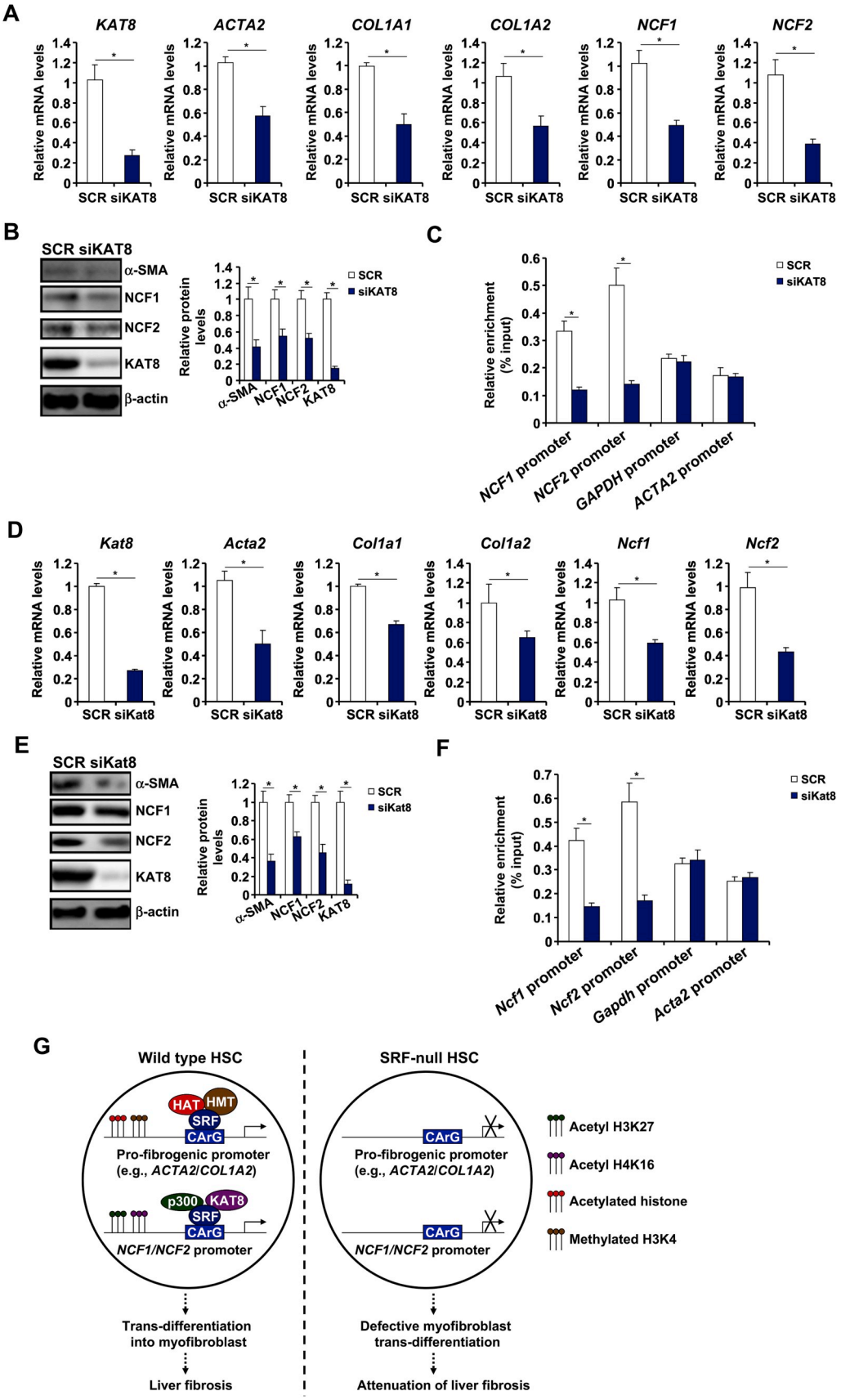

Fig. 6. ***KAT8 is essential for HSC activation by regulating NCF1/NCF2 transcription.*** (**A-C**) LX-2 cells were transfected with siRNA targeting KAT8 or SCR. Gene expression levels were examined by qPCR and Western. ChIP assays were performed with anti-acetyl H4K16. (**D-F**) Primary mouse HSCs were transfected with siRNA targeting KAT8 or SCR. Gene expression levels were examined by qPCR and Western. ChIP assays were performed with anti-acetyl H4K16. Data represent averages of three independent experiments and error bars represent SEM (*$p < 0.05$, 2-tailed student's t-test). (**G**) A schematic model.

surrounding the ACTA2 (encoding α-SMA) promoter, suggesting that KAT8 may regulate pro-fibrogenic gene expression indirectly. Similar observations were made in activated primary mouse HSCs in which KAT8 knockdown reduced the expression of NCF1/NCF2 and in parallel suppressed acetylation of H4K16 on the NCF1/NCF2 promoters (Fig. 6D-F).

4. Discussion

Trans-differentiation of hepatic stellate cells to myofibroblasts is considered the linchpin in the pathogenesis of liver fibrosis [2]. Serum response factor, by virtue of programming cell-specific transcriptional events, represents a key regulator of HSC maturation [44]. Previous investigations have focused on the regulatory role of SRF, along with its co-factor myocardin-related transcription factors A (MRTF-A), in the induction of pro-fibrogenic genes (e.g., *ACTA2* and *COL1A1*) during HSC maturation. Here we detail a novel mechanism whereby SRF integrates NCF1/NCF2 trans-activation and ROS production to promote liver fibrosis (Fig. 6G).

We show here that SRF directly binds to the proximal NCF1/NCF2 promoters to activate transcription. NCF1 and NCF2 are two regulatory subunits of the NOX family of ROS-producing proteins that have been previously implicated in HSC maturation and liver fibrosis [6,7]. Of note, other NOX proteins have also been reported to play a role in this process. For instance, deficiencies of NOX1, NOX2, or NOX4 in mice are associated with dampened fibrogenic response with a concomitant defect in HSC activation [45–48]. We have previously shown that MRTF-A is essential for the induction of NOX1 and NOX2 in macrophages [23,49]. Of note, there is no conserved SRF binding site (CArG box) present in either the proximal NOX1 promoter or the proximal NOX2 promoter; instead, MRTF-A relies on its interaction with other sequence-specific transcription factors such as AP-1 and NF-κB to activate NOX transcription. Therefore, although both SRF deficiency, as reported here, and MRTF-A deficiency, as reported previously [19,50,51], can lead to defective HSC maturation and consequently dampened liver fibrosis, there is not necessarily a co-independence between these two factors in the process. Further, mounting evidence suggests that SRF activity and/or expression can be modulated by intracellular ROS levels. For instance, Westendorf [52] has shown that SRF-dependent transcription, as assessed by serum response element (SRE) driven reporter activity, is enhanced by Rac1, a small GTPase that plays a key role in HSC activation [53]. On the other hand, two separate reports have independently provided evidence that NOX4-mediated ROS production directly up-regulates SRF expression (via a transcriptional mechanism) in embryonic stem cells [54] and promotes SRF nuclear accumulation (via a post-translational modification mechanism) in vascular smooth muscle cells, respectively [55]. Therefore, our data solidify the interplay between SRF and ROS during HSC maturation and suggest that targeting ROS may be beneficial in the treatment of liver fibrosis.

The epigenetic underpinnings of SRF-mediated transcriptional regulation appear to be an underexplored area of investigation. Here we show that SRF may interact with and recruit the histone acetyltransferase KAT8 to activate NCF1/NCF2 transcription. Of interest, it has previously been demonstrated that KAT8 can contribute to the transcriptional activation of several NOX genes, including NOX1 [20,23], NOX2 [49], and NOX4 [56] in a wide range of different cells. Moreover, a co-expression pattern of KAT8 and NOX4 has been noted in the liver in a mouse model of hepatocellular carcinoma [57], which is often preceded by excessive liver fibrosis. In contrast, there are also reports to suggest that KAT8 may be involved in the elimination/clearance of intracellular ROS. For instance, it has recently been observed by Yin *et al* that conditional deletion of KAT8 in oocyte causes female infertility in mice, which is accompanied by a collective down-regulation of antioxidant genes [58]. In addition, the Akhtar group has demonstrated that deletion of KAT8 in the heart disrupts mitochondrial homeostasis, which secondarily causes excessive ROS generation although it remains to be determined whether specific genes involved in ROS production/cleansing may be regulated by KAT8 directly [59]. Of intrigue, KAT8 has been shown to regulate autophagy [60]. Whereas autophagy and ROS form extensive crosstalk [61], it is not known at this point whether KAT8 serves as a mediator between these two processes and, if so, how it may contribute to HSC maturation and liver fibrosis. Our data that SRF may rely on KAT8 to activate NCF1/NCF2 transcription and promote ROS production nonetheless add to the accumulating body of evidence that KAT8 may be intimately intertwined in the regulation of cellular ROS generation. Additional studies are warranted before a rationalized decision to target KAT8 can be made in the intervention of liver fibrosis.

Our data add to a growing body of evidence that illustrates the regulation of HSC activation and liver fibrosis by ROS. It was first reported by Casini *et al* that neutrophil-derived ROS leads to up-regulation of collagen synthesis in HSCs [62]. ROS has since been suggested as a common link between pro-fibrogenic stimuli, including transforming growth factor (TGF) [63], high glucose [64], platelet derived growth factor (PDGF) [65], homocysteine [66], advanced glycation end product (AGE) [67], and hepatitis virus [68], and HSC activation. Consistent with this notion, studies conducted both in cell culture and in experimental animals of chronic liver disease have shown that antioxidants can be effective in antagonizing HSC activation and liver fibrosis [69,70]. For instance, the NOX1/NOX4 specific inhibitor GKT137831 has been demonstrated to exert a protective effect in several different models of liver fibrosis in mice [71,72]. Of intrigue, the SRF inhibitor CCG-1423, which has been documented to attenuate ROS production in macrophages [23,49], possesses potent anti-fibrogenic abilities although it remains obscure whether the anti-fibrogenic effects are achieved through manipulation of ROS production [73–75]. Another question that remains unanswered is whether SRF in non-HSC compartments (e.g., myeloid cells) could promote liver fibrosis via ROS production because ROS can be generated in hepatocytes [72], neutrophils [62], endothelial cells [31], and macrophages [76] within the liver. Moreover, excessive ROS generation may provoke senescence or apoptosis of HSCs thus dampening, instead of inducing, ECM accumulation and eventually causing resolution of liver fibrosis [77]. How the pro- and anti-fibrogenic roles of ROS are balanced and, more importantly, whether SRF contributes to this layer of regulation await further investigation. Future studies that address these lingering issues will ultimately clarify the intricate relationship between ROS and liver fibrosis to provide novel insights for the development of therapeutic strategies to reduce fibrosis in patients suffering from chronic liver diseases involving excessive production of extracellular matrix.

In summary, we present novel evidence here to support an instrumental role for SRF in ROS-driven maturation of hepatic stellate cells and liver fibrosis. Small compound KAT8 inhibitors are available although their efficacy and specificity have not been determined *in vivo* [78]. On the other hand, sequence-specific transcription factors such as SRF are notoriously difficult to drug in the past. The newly identified SRF-KAT8 axis may be considered as a potential target in the development of novel anti-fibrogenic strategies.

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