The Role of SAHA in Attenuating Liver Fibrosis

by

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Chronic liver disease (CLD) is one of the leading causes of morbidity and mortality globally. There are approximately 1.5 billion cases of CLD worldwide, with 4.5 million cases in the United States alone. Most types of CLD are a result of a wound healing process that leads to extracellular matrix deposition and scar tissue formation, or liver fibrosis. When left untreated, liver fibrosis may progress to cirrhosis or hepatocellular carcinoma, and ultimately liver failure, thereby requiring liver transplantation. Unfortunately, the only treatment available for liver fibrosis is removal of the causative agent. This highlights the importance of finding novel therapeutic targets and repurposing drugs to treat liver fibrosis. Histone deacetylase inhibitors (HDACi) have been reported to be beneficial in diseases of fibrosis. Vorinostat, or suberoylanilide hydroxamic acid (SAHA), a pan HDACi used to treat T-cell lymphoma, was reported to inhibit hepatic stellate cell activation and liver fibrosis. However, the mechanism behind this attenuation has yet to be explored in detail. We previously published that overexpression of a redox regulator, Glutaredoxin-1 (*Glrx*) is protective in liver fibrosis. Therefore, I investigated whether SAHA inhibits hepatic stellate cell activation by upregulating *Glrx*. I first confirmed SAHA's inhibition of both human and mouse hepatic stellate cell activation and attenuation of liver fibrosis in a carbon tetrachloride mouse model. SAHA also significantly upregulated the expression of *Glrx* in both mouse and human hepatic stellate cells. Knockdown of *GLRX* partially attenuated SAHA's inhibitory effect on HSC activation. Based on these results, I conclude that SAHA's inhibition of hepatic stellate cell activation may be *Glrx*-dependent.

Keywords: SAHA, Glutaredoxin-1, hepatic stellate cells, liver fibrosis

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Abbreviations

CLD: Chronic Liver Disease, **NASH:** Non-alcoholic steatohepatitis, **HSCs:** Hepatic stellate Cells, **TGFB:** Transforming growth factor Beta-1, **FXR:** Farnesoid X receptor, **PPAR-γ:** Peroxisome proliferator-activated receptor gamma, **HATs:** Histone acetyl transferases, **HDACs:** Histone deacetylases, **CCL4:** Carbon tetrachloride, **SAHA:** Suberoylanilide hydroxamic acid, **Grxs:** Glutaredoxins, **Glrx:** Glutaredoxin-1, **ALT**: alanine aminotransferase; **AST**: aspartate aminotransferase

Preface

First and foremost, I would like to thank my advisor Dr. Wen Xie for his guidance, encouragement, patience when the progress is slow, and for continuing to challenge me to learn and work outside of my comfort zone. I would also like to thank the members of my committee Dr. Xiaochao Ma and Dr. Da Yang for agreeing to be on my committee and for providing valuable suggestions and advice at the Center for Pharmacogenetics.

From the lab, I would like to thank Meishu Xu, who was always so kind to me and never hesitated to help when I needed it. I would also like to thank the members of my lab: Hung-chun Tung, Xinran Cai, Jingyuan Wang, Jongwon-Kim, and Pengfei Xu for teaching me the basics of many different techniques and for helping me with my experiments.

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1.0 Introduction

1.1 Background on Liver Fibrosis

1.1.1 Epidemiology and background

Chronic liver disease (CLD) is a global issue affecting approximately 1.5 billion individuals worldwide (1), accounting for 2 million deaths each year, which represents 2% of the adult population (2). CLD is defined as continuous deterioration of the liver for a period that lasts over six months. Liver fibrosis, a type of CLD, is the outcome of a wound healing process resulting in extracellular matrix deposition and scar tissue formation. The liver is a regenerative organ, and upon liver injury, the liver begins to regenerate rapidly, creating scar tissue. Over time, this destruction and regenerative cycle begins to impact liver function. CLD in the beginning stages is reversible, but established fibrosis is not, and if the cause is not addressed, liver fibrosis may progress to cirrhosis or hepatocellular carcinoma, both of which may require liver transplantation (3).

1.1.2 Pathophysiology of Liver Fibrosis

The pathogenesis of liver fibrosis is complex involving an interplay of many different cell types including the hepatic stellate cells (HSCs), myofibroblasts, hepatocytes, and inflammatory cells. The pathophysiology of liver fibrosis usually differs depending on the exact cause. For example, cholestatic liver injury, which is simulated by the bile-duct ligation mouse model, results in the accumulation of bile and subsequent inflammation and biliary fibrosis (4). On the other

hand, in the case of non-alcoholic steatohepatitis (NASH), which is caused by excessive accumulation of cholesterol and diet-associated obesity, the hepatocytes are impacted first (5), followed by the release of proinflammatory and profibrogenic cytokines, such as TGFB, IL-6, IL-1β (6, 7). Furthermore, alcoholic liver disease associated liver fibrosis also involves the hepatocytes, where direct liver toxicity is induced by the increased production of acetaldehyde (8). In both NASH and alcoholic liver disease, the release of inflammatory mediators leads to the activation of hepatic stellate cells (HSCs). Although other cell types play a role in the pathogenesis in liver fibrosis, the activated HSCs and myofibroblasts are the main source of collagen deposition in the extracellular matrix (9, 10).

1.1.3 The Hepatic Stellate Cells (HSCs)

Under normal conditions, the HSCs are quiescent and reside in the perisinusoidal space, storing retinol (11, 12). However, upon insult to the liver, HSCs become activated, losing their retinoid lipid droplets, and acquiring different characteristics. The activated HSCs are a main source of myofibroblasts, which are not present in healthy livers(13). Myofibroblasts produce type 1 collagens and contribute to the deposition of extracellular matrix and scar tissue formation. Moreover, in response to fibrogenic stimuli, such as transforming growth factor Beta-1 (TGFB), myofibroblasts upregulate the expression of fibrogenic markers, such as α-smooth muscle actin (13, 14). Upon removing the causative agent, approximately 50% of activated HSCs undergo apoptosis, while others de-differentiate back to the quiescent phenotype. However, these de-differentiated or “inactivated” HSCs tend to become activated quicker than naïve quiescent HSCs (15).

1.1.4 Treatment of Liver Fibrosis

Removing the causative agent is the main form of treatment and can usually reverse early-stage liver fibrosis. Other pharmacological agents have been developed to suppress the inflammatory response associated with liver fibrosis. Pan-caspase inhibitors (16), Farnesoid X receptor (FXR) inhibitors (17), and Peroxisome proliferator- activated receptor gamma (PPAR-γ) have been found to inhibit hepatocyte apoptosis (16), such as in the case of NASH or alcoholic liver disease. Targeting pro-inflammatory and profibrogenic cytokines, such as TGFB, have been proposed but are associated with many severe side effects. However, inhibition of molecules associated with the TGFB signaling pathway, such as CD105, a surface receptor on activated HSCS, have been reported to attenuate fibrosis in mice (18).

Moreover, inhibiting Il-1B mediated inflammatory response and chemokine receptors CCR1, CCR5 (19), and CCR2 (20) have been suggested as an alternative approach to targeting pro-inflammatory cytokines. Many pharmacological agents have been suggested and developed for the treatment of liver fibrosis; however, none of them have been approved and many have not advanced the clinical trials. This highlights the need for identifying new potential targets or repurposing drugs for treatment of liver fibrosis.

1.2 Histone Deacetylase Inhibitors (HDACi)

1.2.1 Background on Histone Deacetylases (HDACs)

Histone deacetylases (HDACs) are a group of enzymes that regulate gene transcription by catalyzing the removal of lysine groups from histone residues. The histone acetyl transferases (HATs) loosen the binding between histones and DNA, allowing the DNA to engage in gene transcription while HDACs make the DNA and histone complex more compact, resulting in the inhibition of gene transcription (21-23). There are 18 different HDACs, and they are divided into 4 classes. Class I, II, IV are the zinc dependent HDACs while class III HDACs, or the sirtuins, are nicotinamide adenine dinucleotide-dependent HDACs (22). Moreover, HDACs not only deacetylate histone proteins, they also deacetylate non-histone proteins, which are associated with various biological processes (24).

1.2.2 HDACs in Liver Fibrosis

Recent studies have linked HDACs to different diseases of fibrosis, including liver fibrosis (25). Knockdown of Class I HDACs decreased the expression of the fibrogenic marker, *Lox* (26). Furthermore, HDACs 1 and 2 have been found to be upregulated upon HSC activation (27), and many different HDACs were upregulated in a carbon tetrachloride (CCL4) liver fibrosis mouse model (28). In line with these studies, fibrotic human livers have been found to have a higher HDAC 9 expression (29). Mechanistically, HDACs have been linked to the TGFB-SMAD pathway, a key signaling pathway involved in the development of liver fibrosis. Activation of the TGFB signaling pathway prevents HSC apoptosis, upregulates transcription of fibrogenic genes,

and increases deposition of collagen in the extracellular matrix (30, 31). Moreover, HDAC1 and HDAC2 have been found to inhibit negative modulators of the TGFB signaling pathway, the BAMBI gene (32) and SMAD7 (28), respectively. HDACs 4 and 9 have also been linked to this pathway, as well, and have been found to upregulate fibrogenic gene expression (29, 33). Of the Class III HDACS, the sirtuins, SIRT1 is the most well-characterized, with many studies demonstrating that SIRT1 is downregulated upon HSC activation (34-36). Moreover, SIRT1 knockdown sensitizes mice to CCL4-induced liver fibrosis (34), while overexpression inhibits HSC activation (35). For this reason, HDAC inhibition has been investigated as a potential target to treat liver fibrosis.

1.2.3 HDAC Inhibition in Liver Fibrosis

HDAC inhibitors are compounds that bind zinc atoms to inhibit different HDACs, and many of them are being used to treat cancers, different mood disorders, and inflammatory conditions (37). Moreover, many HDAC inhibitors have been tested in different models of liver fibrosis. Trichostatin-A, a pan-HDAC inhibitor, has been found to inhibit primary rat HSC activation (38), and in a different study exploring the mechanism behind this, Kaimori et. al showed that Trichostatin-A can inhibit the formation of the SMAD3-SMAD4 complex (39). Furthermore, a class I selective HDAC inhibitor, valproic acid, an anticonvulsant, was shown to inhibit HSC activation, CCL4-induced liver fibrosis (26), and fibrogenic gene expression (40). Mechanistically, valproic acid inhibited SMAD2/SMAD3 phosphorylation (41), and in a different study, it was found to impact the expression of different micro RNAs (42). Another class specific HDAC inhibitor, MC1568, was found to inhibit HSC activation, although it had no significant antifibrogenic effects in the CCL4-induced liver fibrosis mouse model (43).

1.2.4 SAHA in Liver Fibrosis

Suberoylanilide hydroxamic acid (SAHA), or Vorinostat, is a pan-HDAC inhibitor that is used to treat cutaneous T-cell lymphoma. SAHA inhibits both class I and II HDACs, and upon binding, it initiates a series of biological effects, including reduced cell proliferation and migration, cell cycle arrest, and apoptosis (44, 45). Moreover, SAHA has also been investigated for the treatment of liver fibrosis. Wang et. al showed that SAHA can inhibit LX2 cell activation by downregulation of NFKB in an HMGB-dependent manner (46). Another study using the same activated human HSC line suggested that SAHA may inhibit the epithelial to mesenchymal transition and thus the differentiation to myofibroblasts (47). Furthermore, SAHA was found to reduce fibrosis in a CCL4 mouse model (27) and in a bile duct ligation liver fibrosis model using rats (48). Although these studies have demonstrated SAHA's inhibitory effect on HSC activation and liver fibrosis, the mechanism behind its effect has not been clearly defined.

1.3 Glutaredoxins

1.3.1 Background on Glutaredoxins

Glutaredoxins (Grxs) are small thiol proteins from the thioredoxin superfamily. In mammalian cells, Grxs are classified into two classes: the monothiol Grxs and dithiol Grxs, or glutathione dependent Grxs (49). The dithiol Grxs encompass Glutaredoxin-2, which is found in the mitochondria and nucleus, and Glutaredoxin-1 (GLRX), which mainly exists in the cytoplasm but has been reported to be found in small concentrations in the nucleus (50). Grxs are involved

in redox regulation by catalyzing deglutathionylation at specific cysteine residues. Moreover, degluathionylation occurs in presence of excessive glutathione concentrations and a reductive environment. In conditions of oxidative stress, GLRX can shift the reaction towards increased S-glutathionylation, demonstrating that the oxidoreductive process occurs bidirectionally (51). For this reason, investigating the role of GLRX in vivo is complex as genetic deletion has been shown to have both favorable(52) and unfavorable physiological outcomes (53).

1.3.2 Glutaredoxin-1 (GLRX) in Different Diseases

Mice lacking GLRX are demonstrated to be relatively normal and comparable to wild-type mice in terms of life span and susceptibility to oxidative stress (54, 55). Moreover, Shao et. al explored the role of *Glrx* in fatty liver and found that knockdown of *Glrx* can increase mice susceptibility to fatty liver. Furthermore, *Glrx* knockdown inhibited the histone deacetylase, SIRT1, leading to upregulation of lipid synthesis (53). On the other hand, *Glrx* ablation was found to have a protective role by reducing alveolar macrophage activation in a lipopolysaccharide-induced lung inflammation mouse model (56). More recently, Anathy et. al showed that idiopathic pulmonary fibrotic lung tissues had lower levels of *GLRX* compared to non-fibrotic tissues, and *Glrx* knockdown increased collagen deposition in a bleomycin-induced lung fibrosis mouse model (57). In line with this study, we previously published that *Glrx* knockdown sensitizes mice to liver fibrosis, while upregulation inhibits HSC activation (58).

1.4 Overview of Thesis

SAHA inhibits HSC activation and liver fibrosis, but the mechanism behind its inhibitory effect remains relatively unclear. In a gene profile study testing different HDAC inhibitors on bladder carcinoma cell lines, SAHA was found to upregulate *GLRX*(59). Moreover, we previously established that *Glrx* is protective in liver fibrosis. Therefore, my aim is to investigate the mechanism behind SAHA's attenuation of liver fibrosis. I confirmed that SAHA can upregulate *Glrx* while inhibiting HSC activation and liver fibrosis. Moreover, early data suggests that SAHA's protective effect may be *Glrx*-dependent.

2.0 Materials and Methods

2.1 Cell Culture, Drug Treatment, Transfections, and Determination of IC50

5 mg SAHA was purchased from Sigma-Aldrich (**CAS Number: 149647-78-9**) and dissolved in 1.89 ml of DMSO to form 10mM stock. Activated human hepatic stellate cells (LX2) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin and maintained in an atmosphere of 5% CO_2 at 37 °C. The cytotoxicity of SAHA was determined by the Cell-Counting Kit (CCK8) analysis as reported in a previous study (46). LX2 cells were treated with SAHA at a concentration of 2μM for 24 hours and control cells were treated with 0.1% DMSO. In figure 5A and 5B, LX2 cells were transfected with Sicontrol (Silencer Select, Thermofisher) or SiGLRX (s5841 Silencer Select, Thermofisher) using Lipofectamine RNAiMAX. In figure 5C, primary mouse HSCs were infected with adenovirus expressing either ShCtrl or ShGlrx. In figure 5D, primary mouse HSCs were infected with adenovirus expressing either CreCtrl or Cre.

2.1.1 Primary human and mouse liver cells

Primary mouse HSCs were isolated from 10-12 week old C57BL/6 male mice, as previously described (60). The inferior vena cava was cannulated and perfused with Hank's buffered salt solution containing 0.1M Hepes and 0.2 M EGTA for 10 minutes at a rate of 5ml/ min. L-15 medium containing 0.5M calcium chloride, 0.1M Hepes, and 20 mg/ml Liberase TM (Roche, Indianapolis, IN) was perfused at a rate of 5 ml/ min for 10 minutes. The liver was minced

and filtered through a 100 μM cell strainer into a 50 ml falcon tube. The filtered cell containing media was was centrifuged (500 rpm for 3 mins). The supernatant was collected and centrifuged (700g for 7 minutes) and a gradient using 15% Nycodenz was formed. The HSCs were collected at the interface and plated in 6-well plates. The purity of HSCs was confirmed by vitamin A autofluorescence. Primary human hepatic stellate cells were isolated from the supernatant received from the Pittsburgh Liver Research Center. Primary Hepatocytes were cultured in HepatoZYME-SFM after attachment in William E medium for 2 hours.

2.1.2 Immunofluorescence

Cells were washed with 1x PBS for three times before fixation with methanol for 10 minutes at room temperature. Slides were then blocked with 5% BSA and permeabilized with 0.25% TritonX for 30 minutes. Slides were then incubated with primary antibodies against GLRX (1:500; ab45953, Abcam) and α-SMA (1:250; A2547, Sigma-Aldrich) overnight at 4°C. The slides were then washed and incubated with the secondary antibodies (ab150077, ab97035, Abcam) for 1 hour at 37°C before being mounted and visualized.

2.1.3 RNA Extraction and RT PCR

RNA was extracted using TRIzol reagent from Invitrogen. Chloroform was added to extract the RNA, and further extraction was done using isopropanol and ethanol. RNA pellet was dissolved in DEPC water, and 1 mcg of RNA was used for reverse transcription. Reverse transcription was performed using SuperScript™ III Reverse Transcriptase kit from Invitrogen.

SYBR Green-based real-time polymerase chain reaction (PCR) was performed with the ABI 7300 RealTime PCR System.

List of Primers

Table 1: List of Primers

Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')
Cyclophilin	GGAGATGGCACAGGAGGAA	GCCCGTAGTGCTTCAGCTT
Glrx	AACAACACCAGTGCGATTCA	ATCTGCTTCAGCCGAGTCAT
Col1a1	ACTGCAACATGGAGACAGGTCAGA	ATCGGTCATGCTCTCTCCAAACCA
Col1a2	GAGGACTTGTTGGTGAGCCT	CTCACCCTTGTTACCGGATT
Col3a1	CTGTAACATGGAAACTGGGGAA	CCATAGCTGAACTGAAAACCACC
Rbp	TGTGGACTTCAACGGGTACTGG	TTGTCTGGCTTCAGCAAGTTGG
Timp1	GGTGTGCACAGTGTTTCCCTGTT T	TCCGTCCACAAACAGTGAGTGTCA
CYCLOPHILIN	CCTAAAGCATACGGGTCCTGGCA	CACATGCTTGCCATCCAACCACT
GLRX	GCCCAAGAGATCCTCAGTCA	CCCGTGAGCTGTTGCAAATA
ACTA2	AAGAGGAATCCTGACCCTGAA	TGGTGATGATGCCATGTTCT
COL1A1	CGGTGTGACTCGTGCAGC	ACAGCCGCTTCACCTACAGC
COL1A2	TCAAACTGGCTGCCAGCAT	CAAGAAACACGTCTGGCTAGG
COL3A1	CCCAGGGAAAGATGGCCCAA	CTCACCAGGGCTACCACGAG
TGFB	CGCTAAGGCGAAAGCCCTCAATTT	ACAATTCCTGGCGATACCTCAGCA
FN1	GCACCTGATGGTGAAGAAGA	GGAATAGCTGTGGACTGGGT

2.1.4 Western Blot

Cells were lysed and protein was extracted using RIPA lysis buffer. Pierce™ BCA Protein Assay Kit was used to assess protein concentration and to dilute samples accordingly. 30 mcg of protein were separated on 12% SDS PAGE gel and transferred onto polyvinyldifluoride (PVDF) membranes (Millipore). The membrane was then blocked for 1 hour at room temperature in 5% non-fat milk. The membrane was washed several times and then incubated with the primary antibodies overnight at 4°C. The next day, the membrane was washed and probed against with the

secondary antibodies. Bands were visualized using ECL western blotting substrate. ImageJ was used to quantify the bands.

2.2 Animal Experiments/ In- vivo experimental design:

8-week-old male C57bl/6 mice were used. For the carbon tetrachloride liver fibrosis mouse model, mice were injected with CCL4 (1µl/ g 1:3 dilution in corn oil) or corn oil twice weekly for 4 weeks. Mice were treated with SAHA (50 mg/kg/day) or vehicle (2% DMSO+30% PEG 300+5% Tween 80 +saline) by gavage daily for 4 weeks.

2.2.1 Histology

The animals were terminated 72 h after the final CCl_4 injection. Liver tissues were fixed in 10% formalin phosphate for 24 hours and then dehydrated. Dehydrated tissues were embedded in paraffin blocks and cut in 4 µm sections. Dewaxing and rehydration were performed before staining. Sirius Red Staining was done as previously described (58). Antigen retrieval was done by incubating the sections in 10 mM citric acid solution (pH 6.0) at 95°C for 20 minutes. Sections were then placed in 0.3% H2O2 solution for 30 minutes. The sections were then blocked using goat serum for 1 hour followed by incubation with the primary antibodies (anti-α-SMA) overnight at 4°C. The next day, the samples were incubated with the secondary antibody for 1 hour followed by ABC reagents for 30 minutes. Visualization was done using DAB Peroxidase (HRP) Substrate

Kit (Vector Laboratories, Burlingame, CA) followed by dehydration and mounting using Permount™ mounting medium. Quantification of staining was done using ImageJ software.

2.2.2 ALT/AST

Serum levels of ALT and AST were measured using commercial assay kits from Stanbio Laboratory.

3.0 Results

3.1 SAHA inhibits Human Hepatic Stellate Cell Activation

To investigate the mechanism behind SAHA's protective role in liver fibrosis, I first confirmed the phenotype that was previously reported (46, 48, 61). Wang et al. demonstrated that SAHA inhibits activation of LX2 cells, an activated human hepatic stellate cell line. I first sought out to determine the dose necessary to achieve the inhibition while minimizing cell toxicity. I performed a CCK8 analysis using increasing doses of SAHA and two different durations, 24 and 48 hours. As reported, I confirmed the IC50 to be at 48 hours using a concentration of 2.5 μM (Figure 1A). Once I confirmed the appropriate dose, I treated the LX2 cells with SAHA, and analyzed the expression of several genes associated with fibrogenesis (Figure 1B).

α-SMA is a definitive marker for hepatic stellate cell activation and myofibroblast formation, and SAHA was able to downregulate ACTA2, the gene that encodes α-SMA. SAHA was also found to reduce the expression of COL1A1, COL1A2, and COL3A1 (Figure 1B), genes that encode type I and type III collagens. Collagen generation and deposition in the extracellular matrix contributes to the progression of fibrogenesis and is a hallmark of HSC activation. I also performed a histological analysis using immunofluorescence to confirm SAHA's reduction of the expression of α-SMA (Figure 1C).

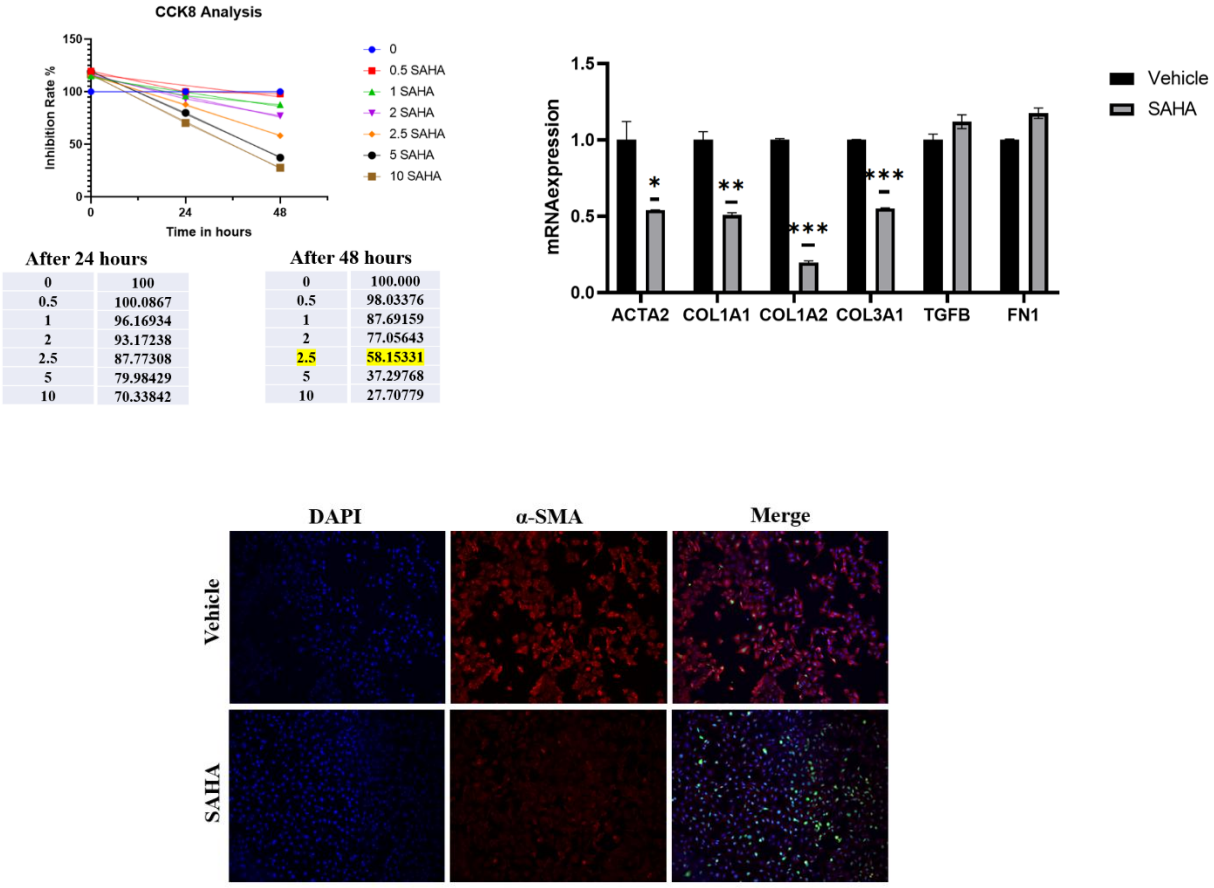


Figure 1: SAHA inhibits human HSC activation: (A) CCK8 analysis on LX2 cells treated with different micromolar concentrations of SAHA. (B) mRNA expression of fibrogenic genes in SAHA treated LX2 cells. (C) Immunofluorescence staining of α-SMA in LX2 cells treated with SAHA 2 μM for 24 hours. Data are means ± SD. *$P < 0.05$ and **$P < 0.01$ and ***P<0.001. NS, statistically not significant.

3.2 SAHA Inhibits Mouse Hepatic Stellate Cell Activation and Liver Fibrosis

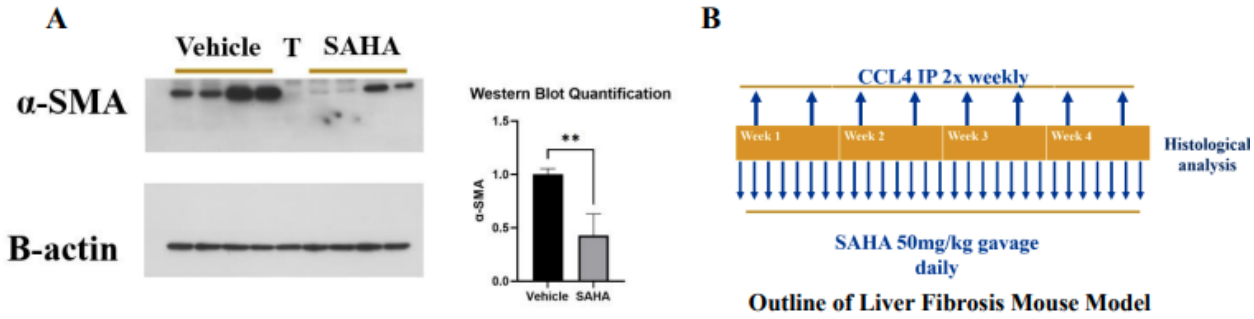
Although LX2 cells are derived from normal human hepatic stellate cells, they are immortalized and are always activated, which may not fully recapitulate physiologically the activation process of hepatic stellate cells (62). Therefore, I isolated primary mouse HSCs, which are initially quiescent, but upon culture, can undergo transdifferentiation to the myofibroblast or

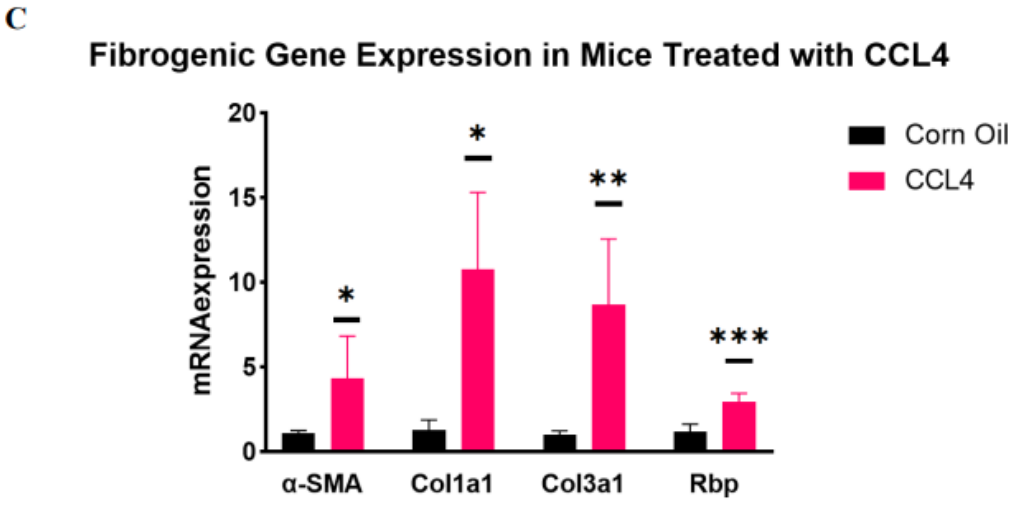
"activated" phenotype. To confirm that SAHA inhibited mouse HSC activation at a protein level, a western blot was performed. Using 4 representative samples, SAHA was able to significantly inhibit α-SMA protein expression compared to the vehicle treated control (Figure 2A).

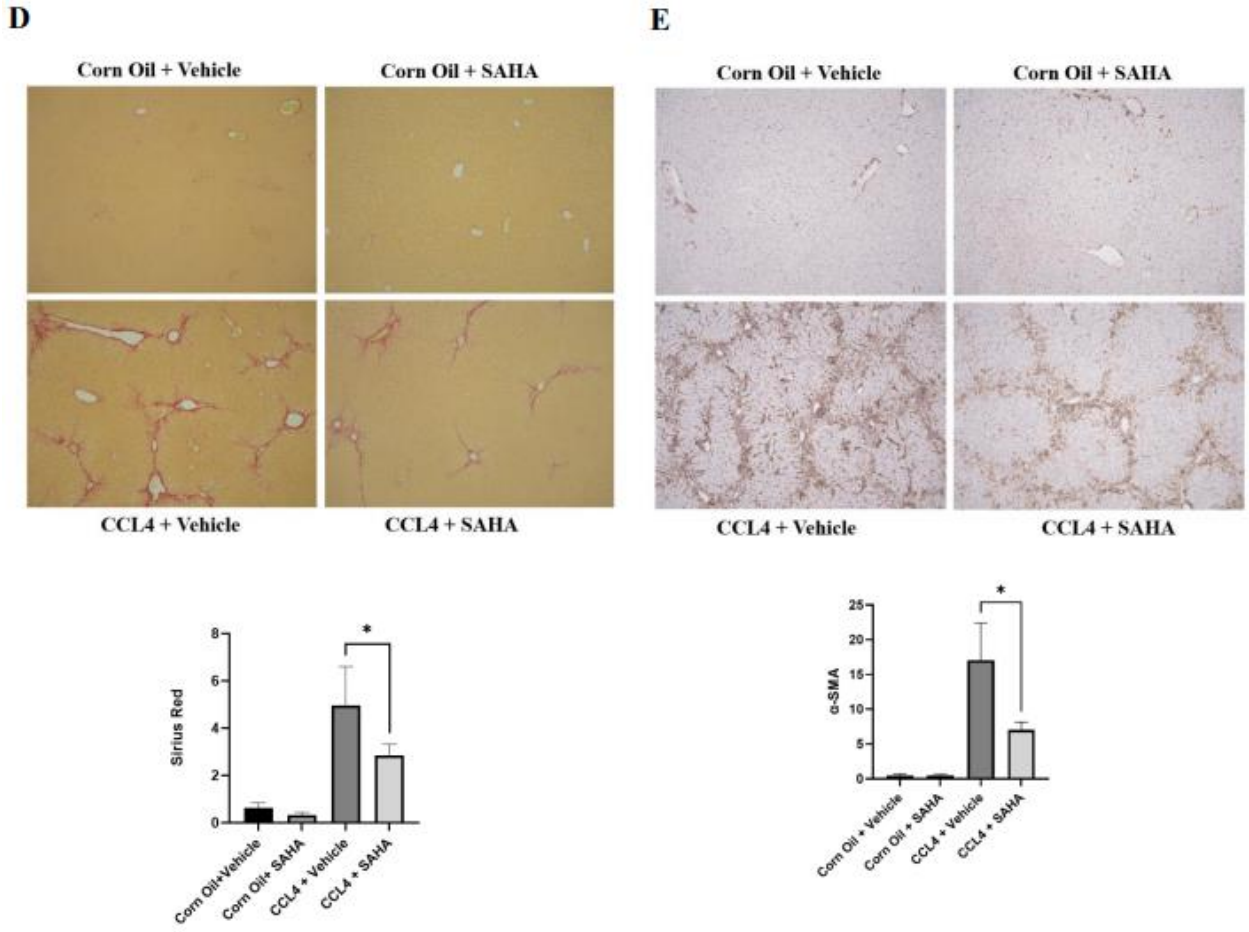
To confirm the preventative role of SAHA in liver fibrosis, I conducted studies on an in vivo liver fibrosis mouse model. The carbon tetrachloride mouse model is one of the most common and reliable experimental liver fibrosis mouse models. Within 4 weeks, significant collagen deposition occurs with the establishment of fibrosis, and if treatment is prolonged to 8 weeks, fibrosis may progress to cirrhosis (63). I injected the mice with carbon tetrachloride and co-treated them with or without SAHA for 4 weeks. Three days after the final injection, the mice were euthanized, and the liver tissues and serum were collected for analysis (Figure 2B). Establishment of fibrosis was confirmed by increased fibrogenic gene expression (Figure 2C), as well as significant α-SMA staining (Figure 2D) and collagen deposition demonstrated by Sirius red staining (Figure 2E). Mice treated with SAHA had significantly lower α-SMA (Figure 2D) and Sirius red staining (Figure 2E) demonstrating that SAHA had a protective effect in this liver fibrosis model.

We also collected serum to analyze the levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) which are usually increased upon liver injury. Although the liver enzymes were not significantly increased after CCL4 treatment, this may be due to the time that was allowed for recovery before tissue harvesting. Moreover, serum ALT and AST increase 3 hours after injecting with CCL4 and reach peak levels by the 24-hour mark. After that, the levels begin to decline dramatically until normalization (64). Furthermore, SAHA was able to decrease serum AST compared to the vehicle treated mice; however, this was not significant

(Figure 2F). Therefore, our results confirm that SAHA can inhibit hepatic stellate cell activation and liver fibrosis, prompting us to explore the mechanism behind this inhibition.






D
Corn Oil + Vehicle
Corn Oil + SAHA
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E
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Sirius Red
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Figure 2: SAHA inhibits mouse HSC activation and Liver Fibrosis: (A) Protein expression of α-SMA in primary mouse HSCs treated with SAHA 2 μM for 48 hours with quantifications on the right. (B) Eight-week-old male WT mice were subjected to the CCl4 model and treated with vehicle (n = 4) or SAHA (50 mg/kg) (n = 4) by daily gavage for 4 weeks. (C) mRNA expression of fibrogenic genes. (D) Sirius red staining with quantifications below. (E) α-SMA staining with quantifications below. Data are means ± SD. *P < 0.05 and **P < 0.01 and ***P<0.0001

3.3 SAHA upregulates Glutaredoxin-1

After confirming the phenotype, I moved into investigating the possible mechanism behind SAHA's attenuation. In a study exploring the gene expression profiles of different HDAC inhibitors, SAHA was found to increase the expression of GLRX in different bladder cancer cell lines (59). Based on our previous work, we have found that GLRX is protective in liver fibrosis (58). Therefore, I explored whether SAHA can affect the expression of GLRX in the context of liver fibrosis. A 6-fold increase in GLRX mRNA expression was observed upon SAHA treatment in LX2 cells (Figure 3A). Moreover, based on our previous study, GLRX is found to be decreased upon hepatic stellate cell activation (58). SAHA was able to significantly upregulate Glrx in both primary mouse hepatic stellate cells (Figure 3B), and primary human hepatic stellate cells isolated from patients with fibrotic livers (Figure 3C). The upregulation in gene expression prompted quantification at a protein level. SAHA treated LX2 cells have significantly higher protein levels of GLRX compared to the vehicle control (Figure 3D). Surprisingly, trehalose, a sugar that has antifbrotic properties was also able to induce the expression of GLRX, but to a lower extent compared to SAHA (Figure 3D).

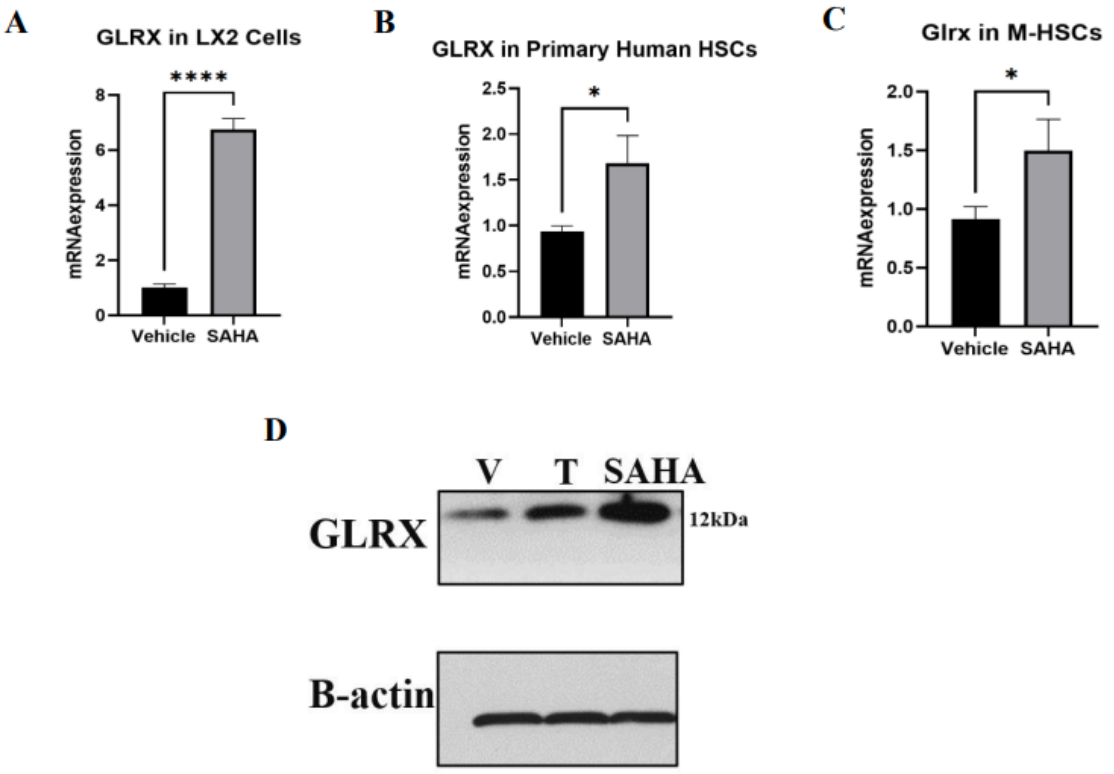


Figure 3: SAHA Upregulates Glutaredoxin-1:: (A) mRNA expression of GLRX in LX2 cells treated with SAHA. (B) mRNA expression of GLRX in primary HSCs treated with SAHA. (C) mRNA expression of GLRX in primary mouse HSCs treated with SAHA. (D) protein expression of GLRX in SAHA treated LX2 cells. Data are means ± SD. *$P < 0.05$ and ****P<0.0001.

3.4 SAHA upregulates GLRX in a dose-dependent manner

After observing the upregulation in GLRX, I determined the effect of increasing doses of SAHA on GLRX and hepatic stellate cell activation. To visualize the upregulation, I performed immunoflurescence on primary mouse hepatic stellate cells treated with increasing concentrations of SAHA. SAHA steadily decreased α-SMA expression while dramatically increasing GLRX immunoflurescence staining in both mouse primary hepatic stellate cells (Figure 4A) as well as

LX2 cells (Figure 4B). Moreover, I measured the gene expression of GLRX in LX2 cells treated with increasing concentrations of SAHA; SAHA upregulated GLRX in a dose-dependent manner (Figure 4C).

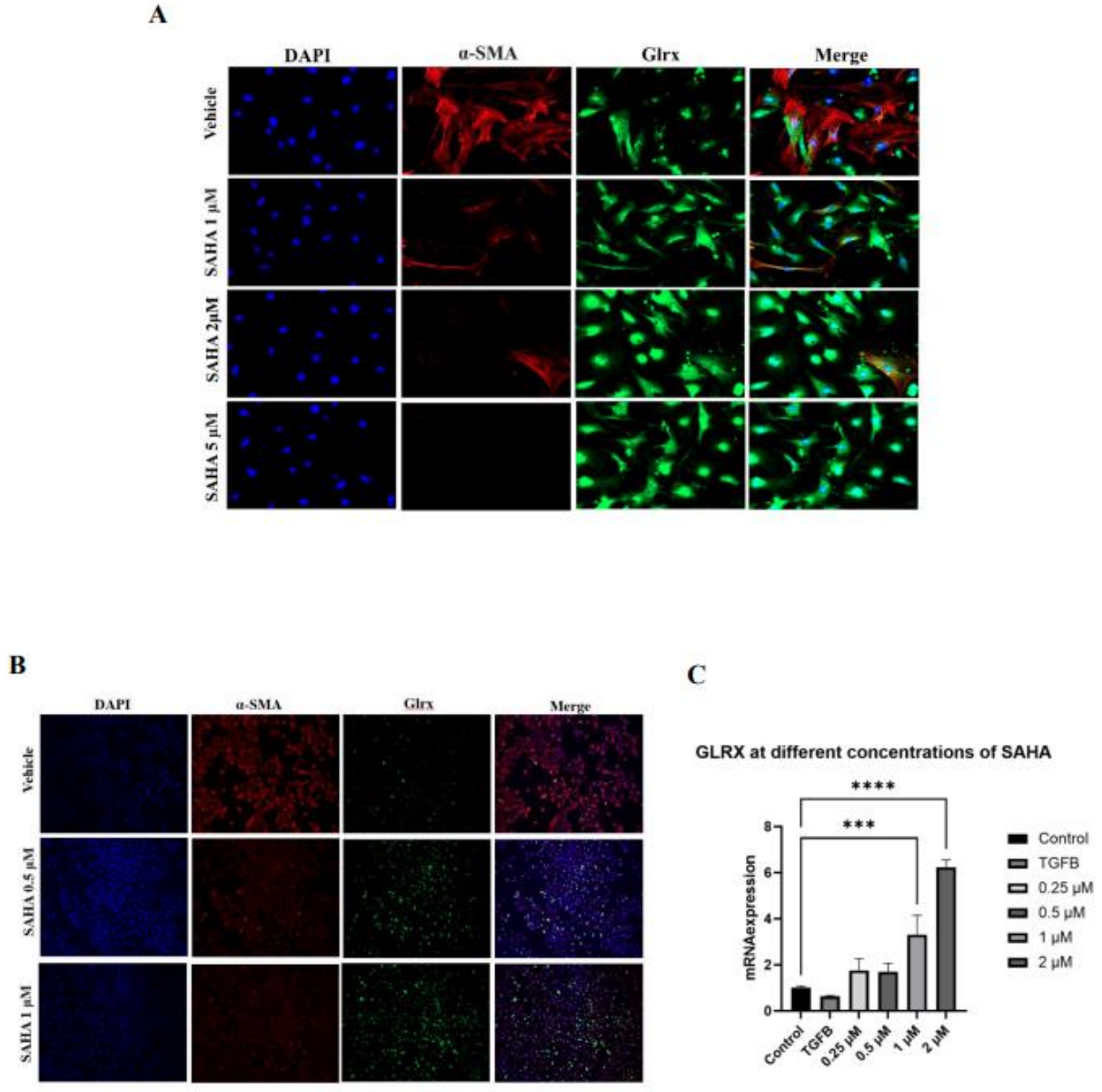
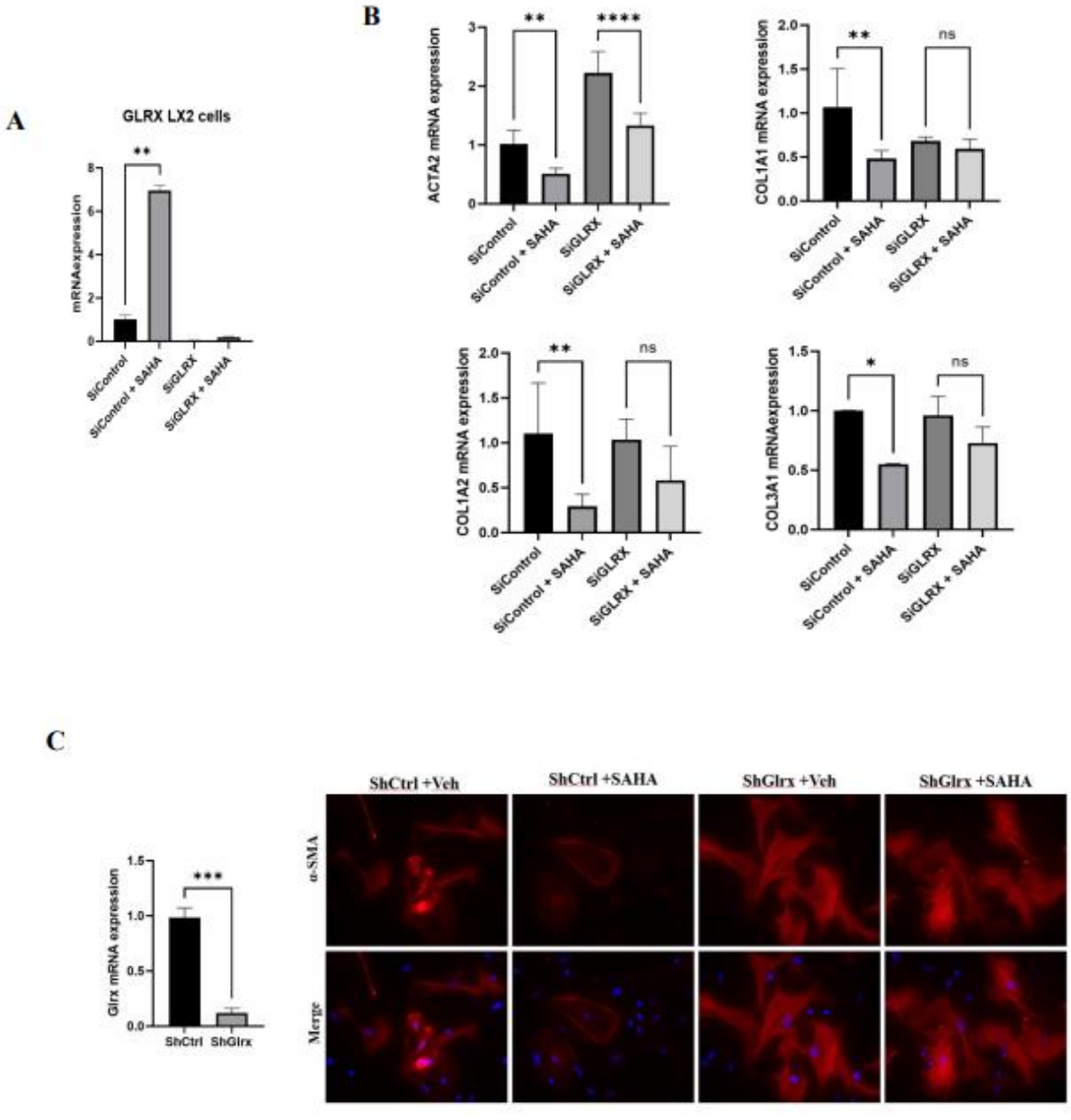


Figure 4: SAHA Upregulates Glutaredoxin-1 in a dose-dependent manner : (A) Immunofluorescence of α-SMA and Glrx in primary mouse HSCs treated with SAHA. (B) Immunofluorescence of α-SMA and Glrx in LX2 cells treated with SAHA. (C) mRNA expression of GLRX in LX2 cells treated with TGFB (5ng/ml) or increasing doses of SAHA. Data are means ± SD. ***P<0.001 and ****P<0.0001.

3.5 SAHA mediates its Inhibitory Effect Partially through Glrx

To understand whether SAHA inhibits hepatic stellate cell activation in a Glrx-dependent manner, I first knocked down GLRX in LX2 cells using SiRNA. I verified the knockdown efficiency by RT-PCR (Figure 5A). As expected, SAHA was able to reduce the expression of the fibrogenic markers in the SiControl group. Upon knockdown of GLRX, the inhibitory effect of SAHA on LX2 cells was partially attenuated (Figure 5B). Moreover, I also isolated primary mouse HSCs from wild-type mice and infected them with adenovirus expressing ShRNA to knockdown Glrx. A knockdown efficiency of approximately 90% was achieved, and I performed immunofluorescence (Figure 5C). Knockdown of GLRX increases α-SMA by immunofluorescence, and treatment with SAHA is unable to attenuate the increase. Furthermore, I performed the same experiment using a different system by isolating mouse HSCs from $Glrx^{fl/fl}$ and infecting them with Cre-virus. Although the knockdown efficiency was only 60% at a protein level, a similar trend was observed with the attenuation of SAHA's inhibitory effect (Figure 5D). Taken together, the data may suggest that SAHA's inhibitory effect is Glrx-dependent.


A
GLRX LX2 cells
mRNAexpression
SiControl
SiControl + SAHA
SiGLRX
SiGLRX + SAHA
B
ACTA2 mRNA expression
COL1A1 mRNA expression
COL1A2 mRNA expression
COL3A1 mRNAexpression
C
Glrx mRNA expression
ShCtrl
ShGlrx
ShCtrl +Veh
ShCtrl +SAHA
ShGlrx +Veh
ShGlrx +SAHA
α-SMA
Merge

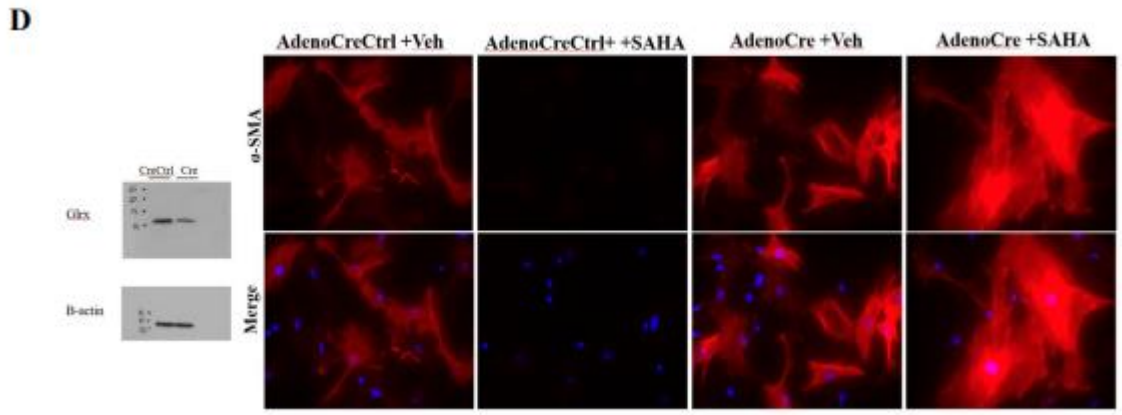


Figure 5: SAHA mediates its Inhibitory Effect Partially through GLRX: (A) mRNA expression of GLRX in LX2 cells treated with Sicontrol or SiGLRX. (B) mRNA expression of fibrogenic genes in LX2 cells treated with Sicontrol or SiGLRX. (C) mRNA expression of *Glrx* in primary mouse HSCs infected with ShCtrl or ShGlrx and immunofluorescence of α-SMA in primary mouse HSCs infected with Ad-ShCtrl or Ad-ShGlrx and treated with SAHA. (D) protein expression of *Glrx* in primary mouse HSCs isolated from $Glrx^{fl/fl}$ mice and infected with Adeno-CreCtrl or Adeno-Cre and immunofluorescence of α-SMA in primary mouse HSCs infected with Adeno-CreCtrl or Adeno-Cre and treated with SAHA. Data are means ± SD. *$P < 0.05$, **$P < 0.01$, ***P<0.001, and ****P<0.0001. NS, statistically not significant.

4.0 Discussion/ Conclusion

Understanding the mechanisms behind pharmacological attenuation of liver fibrosis will provide a platform for identifying potential therapeutic targets or repurposing drugs to treat liver fibrosis. I first confirmed that SAHA inhibits HSC activation and liver fibrosis consistent with previous studies (27, 46, 48). I then explored the mechanism by determining its effect on a redox regulator, GLRX. SAHA upregulated both RNA and protein expression of GLRX significantly. SAHA's upregulation of *GLRX* was more pronounced in activated human HSCs compared to primary mouse HSCs. This is demonstrated by our previous study where we showed that *Glrx* expression is high in quiescent HSCs and decreases upon HSC activation. Moreover, in the in-vivo liver fibrosis model, I found that *Glrx* is reduced upon CCL4 treatment and a trend towards upregulation of *Glrx* by SAHA (data not shown).

Furthermore, our previous study demonstrated that Glrx is protective in liver fibrosis (58). I also showed that SAHA's upregulation of Glrx is protective as when I knocked down *GLRX*, SAHA lost part of its inhibitory effect. Although *GLRX* knockdown efficiency was >90% at an RNA level, knockdown at a protein level was modest indicating the presence of basal levels of GLRX. This suggests that SAHA's attentuation of the inhibitory effect might be more pronounced upon ablation of *GLRX*. However, it is impossible to conclude this without isolating primary mouse HSCs from whole body *Glrx* or HSC specific *Glrx* knockout mice. Moreover, I demonstrated that Class I and Class II HDAC inhibition is linked to the redox regulator, *Glrx*, which is comparable to a study linking a class III HDAC, SIRT1, to *Glrx* (53).

Some limitations include the use of a pan-HDAC inhibitor to investigate a particular mechanism. Although SAHA's inhibition of HSC activation is correlated with a pronounced

upregulation of *GLRX*, it is difficult to exclude other mechanisms that may be involved in attenuating liver fibrosis. Therefore, testing selective HDAC inhibitors is required to fully understand the specific isoforms involved in the upregulation of *GLRX*.

Another limitation is the use of all male cohorts to examine the effect of SAHA in liver fibrosis. Female mice have been shown to experience less severe fibrosis compared to their male counterparts (65). Additionally a recent study demonstrated that myeloid HDAC2 inhibition reduced artherogenesis in male mice but not female mice (66). Therefore, a future study could involve the use of both genders to examine whether SAHA's inhibitory effect and upregulation of *Glrx* is sustained with female mice.

Moreover, other future directions include using HSC specific *Glrx* knockout mice to examine whether SAHA's inhibition of CCL4-induced liver fibrosis is *Glrx*-dependent. Although we have previously showed that whole body *Glrx* knockout sensitizes mice to liver fibrosis, it is difficult to exclude the crosstalk between different tissues and the contribution of hepatocyte ablation of *Glrx*. Therefore, I also aim to investigate whether HSC specific *Glrx* knockout can sensitize mice to liver fibrosis.

Subsequently, conditions related to fibrosis continue to be a complex issue involving an interplay of different molecular players that have yet to be properly addressed. Although removing the causative agent may reverse early-stage fibrosis, established fibrosis may not be reversible. Moreover, many therapeutic targets have been investigated and developed, but none have been approved for the treatment of liver fibrosis. Therefore, finding new targets and understanding mechanisms of existing drugs remain key to establish potential therapies for diseases of fibrosis. In this study, I showed that SAHA can inhibit HSC activation and liver fibrosis while upregulating *Glrx* with preliminary data demonstrating that SAHA's inhibitory effect may be *Glrx*-dependent.

Further investigation is required to determine if SAHA's protective role in vivo is *Glrx*-dependent and the potential signaling axis involved.

References

1. Moon AM, Singal AG, Tapper EB. Contemporary Epidemiology of Chronic Liver Disease and Cirrhosis. Clin Gastroenterol Hepatol. 2020;18(12):2650-66.

2. Asrani SK, Devarbhavi H, Eaton J, Kamath PS. Burden of liver diseases in the world. J Hepatol. 2019;70(1):151-71.

3. Poynard T, Mathurin P, Lai CL, Guyader D, Poupon R, Tainturier MH, et al. A comparison of fibrosis progression in chronic liver diseases. J Hepatol. 2003;38(3):257-65.

4. Hirschfield GM, Heathcote EJ. Cholestasis and cholestatic syndromes. Curr Opin Gastroenterol. 2009;25(3):175-9.

5. Kim JY, Garcia-Carbonell R, Yamachika S, Zhao P, Dhar D, Loomba R, et al. ER Stress Drives Lipogenesis and Steatohepatitis via Caspase-2 Activation of S1P. Cell. 2018;175(1):133-45.e15.

6. Maricic I, Marrero I, Eguchi A, Nakamura R, Johnson CD, Dasgupta S, et al. Differential Activation of Hepatic Invariant NKT Cell Subsets Plays a Key Role in Progression of Nonalcoholic Steatohepatitis. J Immunol. 2018;201(10):3017-35.

7. Malaguarnera M, Di Rosa M, Nicoletti F, Malaguarnera L. Molecular mechanisms involved in NAFLD progression. J Mol Med (Berl). 2009;87(7):679-95.

8. Teschke R. Alcoholic Liver Disease: Alcohol Metabolism, Cascade of Molecular Mechanisms, Cellular Targets, and Clinical Aspects. Biomedicines. 2018;6(4).

9. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest. 2005;115(2):209-18.

10. Friedman SL. Mechanisms of hepatic fibrogenesis. Gastroenterology. 2008;134(6):1655-69.

11. Geerts A. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. Semin Liver Dis. 2001;21(3):311-35.

12. Senoo H, Kojima N, Sato M. Vitamin A-storing cells (stellate cells). Vitam Horm. 2007;75:131-59.

13. Iwaisako K, Jiang C, Zhang M, Cong M, Moore-Morris TJ, Park TJ, et al. Origin of myofibroblasts in the fibrotic liver in mice. Proc Natl Acad Sci U S A. 2014;111(32):E3297-305.

14. Koyama Y, Wang P, Liang S, Iwaisako K, Liu X, Xu J, et al. Mesothelin/mucin 16 signaling in activated portal fibroblasts regulates cholestatic liver fibrosis. J Clin Invest. 2017;127(4):1254-70.

15. Kisseleva T, Cong M, Paik Y, Scholten D, Jiang C, Benner C, et al. Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. Proc Natl Acad Sci U S A. 2012;109(24):9448-53.

16. Canbay A, Feldstein A, Baskin-Bey E, Bronk SF, Gores GJ. The caspase inhibitor IDN-6556 attenuates hepatic injury and fibrosis in the bile duct ligated mouse. J Pharmacol Exp Ther. 2004;308(3):1191-6.

17. Zhang S, Wang J, Liu Q, Harnish DC. Farnesoid X receptor agonist WAY-362450 attenuates liver inflammation and fibrosis in murine model of non-alcoholic steatohepatitis. J Hepatol. 2009;51(2):380-8.

18. Meurer SK, Alsamman M, Sahin H, Wasmuth HE, Kisseleva T, Brenner DA, et al. Overexpression of endoglin modulates TGF-β1-signalling pathways in a novel immortalized mouse hepatic stellate cell line. PLoS One. 2013;8(2):e56116.

19. Seki E, De Minicis S, Gwak GY, Kluwe J, Inokuchi S, Bursill CA, et al. CCR1 and CCR5 promote hepatic fibrosis in mice. J Clin Invest. 2009;119(7):1858-70.

20. Seki E, de Minicis S, Inokuchi S, Taura K, Miyai K, van Rooijen N, et al. CCR2 promotes hepatic fibrosis in mice. Hepatology. 2009;50(1):185-97.

21. Eberharter A, Becker PB. Histone acetylation: a switch between repressive and permissive chromatin. Second in review series on chromatin dynamics. EMBO Rep. 2002;3(3):224-9.

22. Chen PJ, Huang C, Meng XM, Li J. Epigenetic modifications by histone deacetylases: Biological implications and therapeutic potential in liver fibrosis. Biochimie. 2015;116:61-9.

23. Seto E, Yoshida M. Erasers of histone acetylation: the histone deacetylase enzymes. Cold Spring Harb Perspect Biol. 2014;6(4):a018713.

24. Joanna F, van Grunsven LA, Mathieu V, Sarah S, Sarah D, Karin V, et al. Histone deacetylase inhibition and the regulation of cell growth with particular reference to liver pathobiology. J Cell Mol Med. 2009;13(9b):2990-3005.

25. Yoon S, Kang G, Eom GH. HDAC Inhibitors: Therapeutic Potential in Fibrosis-Associated Human Diseases. Int J Mol Sci. 2019;20(6):1329.

26. Mannaerts I, Nuytten NR, Rogiers V, Vanderkerken K, van Grunsven LA, Geerts A. Chronic administration of valproic acid inhibits activation of mouse hepatic stellate cells in vitro and in vivo. Hepatology. 2010;51(2):603-14.

27. Wang Y, Zhao L, Jiao FZ, Zhang WB, Chen Q, Gong ZJ. Histone deacetylase inhibitor suberoylanilide hydroxamic acid alleviates liver fibrosis by suppressing the transforming growth factor-β1 signal pathway. Hepatobiliary Pancreat Dis Int. 2018;17(5):423-9.

28. Li X, Wu XQ, Xu T, Li XF, Yang Y, Li WX, et al. Role of histone deacetylases(HDACs) in progression and reversal of liver fibrosis. Toxicol Appl Pharmacol. 2016;306:58-68.

29. Yang Y, Bae M, Park YK, Lee Y, Pham TX, Rudraiah S, et al. Histone deacetylase 9 plays a role in the antifibrogenic effect of astaxanthin in hepatic stellate cells. J Nutr Biochem. 2017;40:172-7.

30. Dudás J, Kovalszky I, Gallai M, Nagy JO, Schaff Z, Knittel T, et al. Expression of decorin, transforming growth factor-beta 1, tissue inhibitor metalloproteinase 1 and 2, and type IV collagenases in chronic hepatitis. Am J Clin Pathol. 2001;115(5):725-35.

31. Xu F, Liu C, Zhou D, Zhang L. TGF-β/SMAD Pathway and Its Regulation in Hepatic Fibrosis. J Histochem Cytochem. 2016;64(3):157-67.

32. Liu C, Chen X, Yang L, Kisseleva T, Brenner DA, Seki E. Transcriptional repression of the transforming growth factor β (TGF-β) Pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI) by Nuclear Factor κB (NF-κB) p50 enhances TGF-β signaling in hepatic stellate cells. J Biol Chem. 2014;289(10):7082-91.

33. Qin L, Han YP. Epigenetic repression of matrix metalloproteinases in myofibroblastic hepatic stellate cells through histone deacetylases 4: implication in tissue fibrosis. Am J Pathol. 2010;177(4):1915-28.

34. Li M, Hong W, Hao C, Li L, Xu H, Li P, et al. Hepatic stellate cell-specific deletion of SIRT1 exacerbates liver fibrosis in mice. Biochim Biophys Acta Mol Basis Dis. 2017;1863(12):3202-11.

35. Wu Y, Liu X, Zhou Q, Huang C, Meng X, Xu F, et al. Silent information regulator 1 (SIRT1) ameliorates liver fibrosis via promoting activated stellate cell apoptosis and reversion. Toxicol Appl Pharmacol. 2015;289(2):163-76.

36. Ma JQ, Sun YZ, Ming QL, Tian ZK, Yang HX, Liu CM. Ampelopsin attenuates carbon tetrachloride-induced mouse liver fibrosis and hepatic stellate cell activation associated with the SIRT1/TGF-β1/Smad3 and autophagy pathway. Int Immunopharmacol. 2019;77:105984.

37. Claveria-Cabello A, Colyn L, Arechederra M, Urman JM, Berasain C, Avila MA, et al. Epigenetics in Liver Fibrosis: Could HDACs be a Therapeutic Target? Cells. 2020;9(10).

38. Niki T, Rombouts K, De Bleser P, De Smet K, Rogiers V, Schuppan D, et al. A histone deacetylase inhibitor, trichostatin A, suppresses myofibroblastic differentiation of rat hepatic stellate cells in primary culture. Hepatology. 1999;29(3):858-67.

39. Kaimori A, Potter JJ, Choti M, Ding Z, Mezey E, Koteish AA. Histone deacetylase inhibition suppresses the transforming growth factor beta1-induced epithelial-to-mesenchymal transition in hepatocytes. Hepatology. 2010;52(3):1033-45.

40. Aher JS, Khan S, Jain S, Tikoo K, Jena G. Valproate ameliorates thioacetamide-induced fibrosis by hepatic stellate cell inactivation. Hum Exp Toxicol. 2015;34(1):44-55.

41. Watanabe T, Tajima H, Hironori H, Nakagawara H, Ohnishi I, Takamura H, et al. Sodium valproate blocks the transforming growth factor (TGF)-β1 autocrine loop and attenuates the TGF-β1-induced collagen synthesis in a human hepatic stellate cell line. Int J Mol Med. 2011;28(6):919-25.

42. Lu P, Yan M, He L, Li J, Ji Y, Ji J. Crosstalk between Epigenetic Modulations in Valproic Acid Deactivated Hepatic Stellate Cells: An Integrated Protein and miRNA Profiling Study. Int J Biol Sci. 2019;15(1):93-104.

43. Mannaerts I, Eysackers N, Onyema OO, Van Beneden K, Valente S, Mai A, et al. Class II HDAC Inhibition Hampers Hepatic Stellate Cell Activation by Induction of MicroRNA-29. PLoS One. 2013;8(1):e55786.

44. Grant S, Easley C, Kirkpatrick P. Vorinostat. Nature Reviews Drug Discovery. 2007;6(1):21-2.

45. Bubna AK. Vorinostat-An Overview. Indian J Dermatol. 2015;60(4):419-.

46. Wang W, Yan M, Ji Q, Lu J, Ji Y, Ji J. Suberoylanilide hydroxamic acid suppresses hepatic stellate cells activation by HMGB1 dependent reduction of NF-κB1. PeerJ. 2015;3:e1362.

47. Özel M, Baskol M, Akalın H, Baskol G. Suberoylanilide Hydroxamic Acid (SAHA) Reduces Fibrosis Markers and Deactivates Human Stellate Cells via the Epithelial-Mesenchymal Transition (EMT). Cell Biochem Biophys. 2021;79(2):349-57.

48. Park KC, Park JH, Jeon JY, Kim SY, Kim JM, Lim CY, et al. A new histone deacetylase inhibitor improves liver fibrosis in BDL rats through suppression of hepatic stellate cells. British Journal of Pharmacology. 2014;171(21):4820-30.

49. Holmgren A. Hydrogen donor system for Escherichia coli ribonucleoside-diphosphate reductase dependent upon glutathione. Proc Natl Acad Sci U S A. 1976;73(7):2275-9.

50. Lundberg M, Johansson C, Chandra J, Enoksson M, Jacobsson G, Ljung J, et al. Cloning and expression of a novel human glutaredoxin (Grx2) with mitochondrial and nuclear isoforms. J Biol Chem. 2001;276(28):26269-75.

51. Mannervik B, Axelsson K. Role of cytoplasmic thioltransferase in cellular regulation by thiol-disulphide interchange. Biochemical Journal. 1980;190(1):125-30.

52. Murdoch CE, Shuler M, Haeussler DJF, Kikuchi R, Bearelly P, Han J, et al. Glutaredoxin-1 up-regulation induces soluble vascular endothelial growth factor receptor 1, attenuating

post-ischemia limb revascularization. The Journal of biological chemistry. 2014;289(12):8633-44.

53. Shao D, Han J, Hou X, Fry J, Behring JB, Seta F, et al. Glutaredoxin-1 Deficiency Causes Fatty Liver and Dyslipidemia by Inhibiting Sirtuin-1. Antioxid Redox Signal. 2017;27(6):313-27.

54. Ho YS, Xiong Y, Ho DS, Gao J, Chua BH, Pai H, et al. Targeted disruption of the glutaredoxin 1 gene does not sensitize adult mice to tissue injury induced by ischemia/reperfusion and hyperoxia. Free Radic Biol Med. 2007;43(9):1299-312.

55. Matsui R, Ferran B, Oh A, Croteau D, Shao D, Han J, et al. Redox Regulation via Glutaredoxin-1 and Protein S-Glutathionylation. Antioxid Redox Signal. 2019;32(10):677-700.

56. Aesif SW, Anathy V, Kuipers I, Guala AS, Reiss JN, Ho YS, et al. Ablation of glutaredoxin-1 attenuates lipopolysaccharide-induced lung inflammation and alveolar macrophage activation. Am J Respir Cell Mol Biol. 2011;44(4):491-9.

57. Anathy V, Lahue KG, Chapman DG, Chia SB, Casey DT, Aboushousha R, et al. Reducing protein oxidation reverses lung fibrosis. Nat Med. 2018;24(8):1128-35.

58. Xi Y, Li Y, Xu P, Li S, Liu Z, Tung HC, et al. The anti-fibrotic drug pirfenidone inhibits liver fibrosis by targeting the small oxidoreductase glutaredoxin-1. Sci Adv. 2021;7(36):eabg9241.

59. Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. Gene Expression Profiling of Multiple Histone Deacetylase (HDAC) Inhibitors: Defining a Common Gene Set Produced by HDAC Inhibition in T24 and MDA Carcinoma Cell Lines. Molecular Cancer Therapeutics. 2003;2(2):151.

60. Mederacke I, Dapito DH, Affò S, Uchinami H, Schwabe RF. High-yield and high-purity isolation of hepatic stellate cells from normal and fibrotic mouse livers. Nature Protocols. 2015;10(2):305-15.

61. Wang Y, Zhao L, Jiao F-Z, Zhang W-B, Chen Q, Gong Z-J. Histone deacetylase inhibitor suberoylanilide hydroxamic acid alleviates liver fibrosis by suppressing the transforming growth factor-β1 signal pathway. Hepatobiliary & Pancreatic Diseases International. 2018;17(5):423-9.

62. Weiskirchen R, Weimer J, Meurer SK, Kron A, Seipel B, Vater I, et al. Genetic Characteristics of the Human Hepatic Stellate Cell Line LX-2. PLoS One. 2013;8(10):e75692.

63. Scholten D, Trebicka J, Liedtke C, Weiskirchen R. The carbon tetrachloride model in mice. Lab Anim. 2015;49(1 Suppl):4-11.

64. Wang PY, Kaneko T, Tsukada H, Nakano M, Nakajima T, Sato A. Time courses of hepatic injuries induced by chloroform and by carbon tetrachloride: comparison of biochemical and histopathological changes. Arch Toxicol. 1997;71(10):638-45.

65. Yang JD, Abdelmalek MF, Pang H, Guy CD, Smith AD, Diehl AM, et al. Gender and menopause impact severity of fibrosis among patients with nonalcoholic steatohepatitis. Hepatology. 2014;59(4):1406-14.

66. Wang L, Ahn YJ, Asmis R. Inhibition of myeloid HDAC2 upregulates glutaredoxin 1 expression, improves protein thiol redox state and protects against high-calorie diet-induced monocyte dysfunction and atherosclerosis. Atherosclerosis. 2021;328:23-32.