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Peer Review File

Alleviation of liver fibrosis by inhibiting a non-canonical ATF4-regulated enhancer program in hepatic stellate cells

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Yang et al. suggest that ATF4 may directly mediate EMT responses and the expression of key genes in hepatic stellate cells in response to TGFb stimulation. Several questions and concerns as follow could challenge the conclusions of this study:
1) Why multiple bands for ATF4 in Fig 3c after ATF4-oe?
2) Could CCl4 affect ATF4 expression in HSCs?
3) Fig 4f data indicate liver injury was minimal in HSC-ATF4KO mice after CCl4 treatment. What could be the mechanisms underlying this phenotype?
4) What could be the mechanisms underlying the HSC fibrosis phenotypes observed from CCl4 HSC-ATF4KO mice?
5) Based on the data in Fig 7a-g, it is hard to understand whether the liver phenotypes were mainly derived from the effect of ISRIB on HSCs. Because eIF2a also plays a profound regulation of the functions of other hepatic cells such as hepatocytes. An ATF4 inhibitor should be used.
6) In addition to ATF4, eIF2a activation can affect many responses, which also contribute to liver fibrosis phenotypes. Authors should demonstrate the role of ATF4 in the effect of eIF2a inhibitor. Thus, the conclusion in lines 50-51 in the abstract is overstated.
7) In the human liver scRNAseq data, ATF4 abundance in HSCs between cirrhosis vs healthy?
8) Ref 42 suggests that ATF4 directly regulates the expression of TRIB3 which is critical for HSC activation. Did the authors find a similar interaction between ATF4 and TRIB3? Please discuss the findings between these two papers.
9) There is not much connection between the data from endothelial cells and HSCs. In addition, no vivo data to support the observation from in vitro assays.

Reviewer #2

(Remarks to the Author)
The study by Yang, Qi, Lu et al. show, by using cell lines, that ATF4 can control the expression of genes induced during the EMT transition, being a gene-expression program that contributes to hepatic stellate cell (HSC) activation. Authors used CHIP seq to identify the mechanism by which ATF4 controls the expression of pro-fibrotic genes. Then, authors performed in vivo studies to find that mice with ATF4 deleted in HSC are protected from CCL4-induced liver fibrosis, while mice treated with ISRIB also showed a decrease in CCL4 and bile-duct ligation-induced fibrosis. The findings are innovative and interesting, showing that ATF4 might not just be involved in pro-survival or cell-death responses, but also in determining cell fate. The major concern is that some of the conclusions are not supported by the data shown:

1) Authors propose that ATF4-dependent fibrosis in the liver is dependent on Eif2-alpha activation to increase ATF4 transcriptional activity in stellate cells. However, the experiments that demonstrate dependency on EIF2alpha are performed in mice with ISRIB delivered by intraperitoneal injections. Therefore, it is a possibility that less fibrosis is observed in vivo because ISRIB is preventing cell death in hepatocytes or the release of activating signals from hepatocytes, preventing HSC activation.

2) Related to point 1), it is unlikely that the ECM proteins produced by activated HSC can escape the blockage of protein translation induced by EIF2alpha phosphorylation. Thus, it is likely that EMT and HSC activation are increasing ATF4 activity by other mechanisms. Authors need to measure ATF4 phosphorylation and its translocation to the nucleus in the in

vitro models of EMT, as well as in TGFb-induced HSC activation, together with EIF2alpha phosphorylation, in the presence and absence of ISRIB. In my opinion, it is likely that in these models, ATF4 will be activated in an EIF2alpha-independent manner.

3)Authors conclude that ATF4 regulates an epigenomic signature of EMT. However, the authors only found that ATF4 binding sites coincide with sites of H3K27ac. It could be the opposite conclusion, that changes in the epigenome are what determines where ATF4 binds. Therefore, such a model places ATF4 translocation to the nucleus as the key regulatory point to determine the type of ATF4 program activated, together with epigenetic marks. To reinforce this conclusion, authors would need to perform the same experiments in the EMT cell model itself. Otherwise, the epigenetic marks themselves might not be the shared mechanism by which ATF4 promotes EMT transition and HSC activation. With the current data, it is still a possibility that the mechanism is different, despite that some of the ATF4-dependent genes overlap.

Reviewer #3

(Remarks to the Author)
The manuscript presents a compelling exploration of ATF4 as a therapeutic target in liver fibrosis, a significant pathological condition with progression potential to cirrhosis. The authors have elucidated a non-canonical role of ATF4 in regulating an epigenetic program in hepatic stellate cells (HSCs) that promotes epithelial-mesenchymal transition (EMT) and fibrogenesis, independent of the classical endoplasmic reticulum (ER) stress response. Through methodical experimentation, the study demonstrates that ATF4 depletion in HSCs considerably mitigates liver fibrosis in a mouse model. The modulation of ATF4 activity by TGFβ and the subsequent impact on EMT and fibrotic gene expression provide valuable insights into the molecular underpinnings of liver fibrosis. Moreover, the therapeutic efficacy of a small molecule inhibitor targeting p-eIF2α to hinder ATF4 function suggests a viable approach to treat this disease.

Despite these strengths, the manuscript would benefit from addressing the following key points:
1. The manuscript should establish a correlation between ATF4 expression/activity and fibrosis severity, potentially through comparison with clinical fibrosis indices like the Fib4 score.
2. The therapeutic potential of ATF4 inhibition should be validated across a broader spectrum of hepatic fibrosis models to ensure the generalizability of the findings.
3. An investigation into the tissue specificity of the eIF2α-ATF4 pathway inhibition is necessary, with particular attention to potential off-target effects in critical metabolic tissues such as skeletal muscle, white adipose tissue (WAT), and brown adipose tissue (BAT), as well as in the skin.
4. The authors are encouraged to include a schematic diagram detailing the method used for creating HSC-specific ATF4 knockout mice.
5. The claim of ATF4-specific deletion in HSCs should be substantiated with additional evidence, possibly by single-cell RNA sequencing analysis, FACS sorting of HSC and non-HSC populations, or other confirmatory techniques.
6. In Figure 7A, the quantification of ATF4 expression within HSCs must be validated to bolster the proposed mechanism.
7. The single-cell RNA sequencing data presented in Figure 7H require a more thorough analysis. Additional visualizations such as HSC cell population distribution, ATF4, and other fibrogenic gene expressions adjusted to support the model would be insightful. Trajectory analysis could further elucidate the role of ATF4 in the fibrotic process.

Minor Concern
1. The manuscript should include q-values for each gene set enrichment analysis (GSEA) result presented in Figure 1 to provide a complete statistical context.

In summary, the study is methodologically sound and presents a potentially transformative approach to liver fibrosis therapy. Addressing the above points will significantly enhance the manuscript's impact and clinical relevance.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors have made substantial changes and provided additional data which addresses my questions. No additional comments.

Reviewer #2

(Remarks to the Author)
I want to congratulate the authors for addressing most of the points raised in the first round. However, there is one point that has not been successfully addressed. The authors only measured the effects of ISRIB on apoptosis in hepatocytes. However, CCl4 induces other forms of cell death that are more pro-inflammatory than apoptosis, such as necrosis and pyroptosis. The fact that only TUNEL and cleaved caspase were performed to address the point of cell death raises concerns about discarding the role of hepatocyte death in HSC activation.

Reviewer #3

(Remarks to the Author)

First, I would like to express my gratitude for your respectful consideration of my comments and your efforts in addressing them.

Regarding my first comment, "The manuscript should establish a correlation between ATF4 expression/activity and fibrosis severity, potentially through comparison with clinical fibrosis indices like the Fib4 score," although a formal correlation assay was not conducted, I agree with your assessment that the observed trends suggest statistical significance. I believe that performing a correlation assay, such as calculating Spearman's rho, could have potentially resulted in stronger trends and improved p-values.

For my second comment, "The therapeutic potential of ATF4 inhibition should be validated across a broader spectrum of hepatic fibrosis models to ensure the generalizability of the findings," no additional experiments were performed. Instead, you evaluated the LX-2 cells in vitro model using PDGF-BB to induce HSC activation (Extended Data Fig. 2f, g). While Western blotting showed ATF4 expression reduced to a level comparable to the control, the expression of target genes remained elevated in qRT-PCR. I find it challenging to conclude that the generalizability I requested has been achieved based solely on the Western blot and qRT-PCR results of three genes in the in vitro model.

Regarding my third and fourth comments, I have no further concerns.

In my fifth comment, I requested a comparison between "HSCs and non-HSCs populations." However, the experiments were only performed comparing WT and HSC-Atf4-KO, and the more detailed comparison I requested was not addressed.

For my sixth comment, while it was noted that additional experimental data were provided in Extended Data Fig. 5a, I was unable to locate this figure. I presume that Extended Data Fig. 5b contains the relevant results instead.

For my seventh comment, the response was well-done, and I have no further comments.

The minor comments were also well addressed.

While a considerable amount of additional experimentation was performed, I feel that many of my key comments were not sufficiently addressed. Particularly, despite providing explicit guidance on the experiments and analyses I believed would strengthen the study, these were not executed as requested, which I find disappointing. My comments were aimed at enhancing the robustness and credibility of the study's findings, and thus I am somewhat dissatisfied with the authors' response.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Congratulations to the authors. I am still surprised that ISRIB does not change non-HSC cell death in the liver, but the data is convincing.

Reviewer #3

(Remarks to the Author)
Thank you for your thorough revisions. The authors have sufficiently addressed all my concerns, now, and incorporated the necessary changes to a satisfactory level. I have no further comments or concerns at this stage. Congratulations on your excellent work!

A point-by-point response letter to the Reviewers' remarks

Reviewer #1 (Remarks to the Author):

Yang et al. suggest that ATF4 may directly mediate EMT responses and the expression of key genes in hepatic stellate cells in response to TGFβ stimulation. Several questions and concerns as follow could challenge the conclusions of this study:

We are extremely grateful for your efforts in evaluating our manuscript and providing important critiques to help strengthen our story. Following your suggestions, we have thoroughly revised the manuscript and hope you will agree that our conclusions are now well-supported by the presented data.

1) Why multiple bands for ATF4 in Fig 3c after ATF4-oe?

There were actually two reasons that led to such a phenomenon on the western blots for ATF4. First, the exogenously expressed ATF4 is 3xFLAG-tagged, therefore migrating slower than the endogenous ATF4 protein and showing a different band with slightly larger molecular weight. Second, in our experience, the commercial anti-ATF4 antibody, particularly in certain batches, has a moderate specificity. While the ATF4 protein itself represents the most intense band that could be captured and visualized, multiple other bands with less intensity could also be seen in the blot. Since we have ATF4-knockdown cells in our experiments (as a negative control), we were able to precisely recognize the ATF4 band on the western blots.

2) Could CCl_4 affect ATF4 expression in HSCs?

This is truly important information that could strengthen the conclusion of our story. In **Extended Data Fig. 5b of the revised manuscript**, we found that the protein level of ATF4 was increased significantly upon CCl_4 treatment, which could be effectively reversed by ISRIB treatment. Consistent with the change of ATF4 in HSCs, the key fibrotic genes were induced by CCl_4 and repressed by the treatment of ISRIB in HSCs (**Fig. 7b of the revised manuscript**).

Related to this question, we now also show in the revised manuscript that the protein level of ATF4 was increased in the HSCs of human livers with severe cirrhosis, further confirming the connection between liver injury and the increased expression of ATF4 in HSCs (**Fig. 7k of the revised manuscript**).

3) Fig 4f data indicate liver injury was minimal in HSC-ATF4-KO mice after CCl_4 treatment. What could be the mechanisms underlying this phenotype?

Thanks for your question. We consider that reduced fibrosis is the primary factor contributing to decreased liver injury after CCl_4 treatment in HSC-ATF4-KO mice.

1) The blood sample used in Fig. 4f was from animals that were treated with CCl_4 for five weeks and washed out for three days prior to sample collection. Therefore, the change of serological enzymes observed in Fig. 4f represented the extent of liver injury after a five-week treatment of CCl_4 but not an immediate effect of CCl_4-induced acute liver damage.

2) Consistent with many other reports, we found that a five-week treatment of CCl_4 caused significant liver injury in wild-type animals due to the induction of liver fibrosis (Salhab et al., ***Gut***, 2022; Yang et al., ***Hepatology****, 2021;* Gao et al., ***J Hepatol***, 2020). It is known that during the process of CCl_4-triggered liver damage and recovery, HSCs are activated and produce an excessive amount of extracellular matrix (ECM), leading to fibrosis and the formation of scar tissue. The scar tissue replaces hepatocytes and distorts the liver's internal structure, thereby disrupting intrahepatic blood flow and reducing the blood supply to the remaining hepatocytes. The insufficient, abnormal blood flow triggers hepatocyte death and causes liver injury. In short, fibrosis triggered by long-term CCl_4 treatment was the main reason leading to sustained liver injury.

3) However, in the HSC-ATF4-KO mice, we found that loss-of-ATF4 expression in HSCs significantly represses HSC activation as well as ECM production (**Fig. 4b-f of the revised manuscript**), therefore inhibiting liver fibrosis caused by CCl_4. As a result, liver injury caused by CCl_4 is also mitigated in the HSC-ATF4-KO mice.

4) What could be the mechanisms underlying the HSCs fibrosis phenotypes observed from CCl_4 HSC-ATF4 KO mice?

We appreciate your question since this is a key finding of our manuscript that we are happy to explain further and elaborate. It is known that in response to liver damage (e.g., triggered by CCl_4), HSCs are activated from their quiescent state and start proliferating and secreting a huge amount of ECM proteins, including collagens. Therefore, activation of HSCs is the main driver of liver fibrosis. By using both HSC cell lines and primary HSCs, we provided extensive evidence to show that ATF4 is required for HSC activation, therefore promoting liver fibrosis *in vivo*.

1) Using TGFβ treatment, the most classical way to induce HSC activation, we have shown in the initial and revised manuscripts that ATF4 positively regulates LX-2 activation and inhibition of ATF4 by shRNAs or a chemical inhibitor of its upstream factor represses LX-2 activation. (**Fig. 3a, b, e, f, and h of the revised manuscript**). To strengthen this conclusion, in the revised manuscript, we went on to examine if ATF4 is also involved in PDGF-mediated HSC activation. Consistently, inhibition of ATF4 translation also significantly mitigates the expression of HSC activation genes under PDGF treatment (**Extended Data Fig. 2f, g of the revised manuscript**).

2) To confirm the role of ATF4 in HSC activation *in vivo*, we isolated HSCs from wild-type and HSC-ATF4-KO mice and found that expression of HSC activation markers is dramatically reduced

upon ATF4 depletion in HSCs (**Extended Data Fig. 3f of the revised manuscript**). These results are consistent with the findings of HSC cell lines.

3) On the mechanistic level, we found that in response to the signal of HSC activation, ATF4 can be re-directed to the regulatory regions of essential HSC activation genes, thereby driving their expression and mediating the transition of LX-2 cells from a quiescent state to an active one (**Fig. 1 and Fig. 6 of the revised manuscript**).

Together, these data indicate that ATF4 plays a vital role in HSC activation. As a result, tissue-specific loss-of-ATF4 in HSCs effectively alleviates fibrosis development in mice treated with CCl_4.

5) Based on the data in Fig 7a-g, it is hard to understand whether the liver phenotypes were mainly derived from the effect of ISRIB on HSCs. Because eIF2α also plays a profound regulation of the functions of other hepatic cells such as hepatocytes. An ATF4 inhibitor should be used.

6) In addition to ATF4, eIF2α activation can affect many responses, which also contribute to liver fibrosis phenotypes. Authors should demonstrate the role of ATF4 in the effect of eIF2α inhibitor. Thus, the conclusion in lines 50-51 in the abstract is overstated.

We appreciate your critical comments on the part where we aimed to demonstrate the translational implications of our study. Since the above two questions (**comments #5 and #6**) are both related to the use of ISRIB in alleviating fibrosis, we would like to address them together.

1. Cell-type specificity of ISRIB

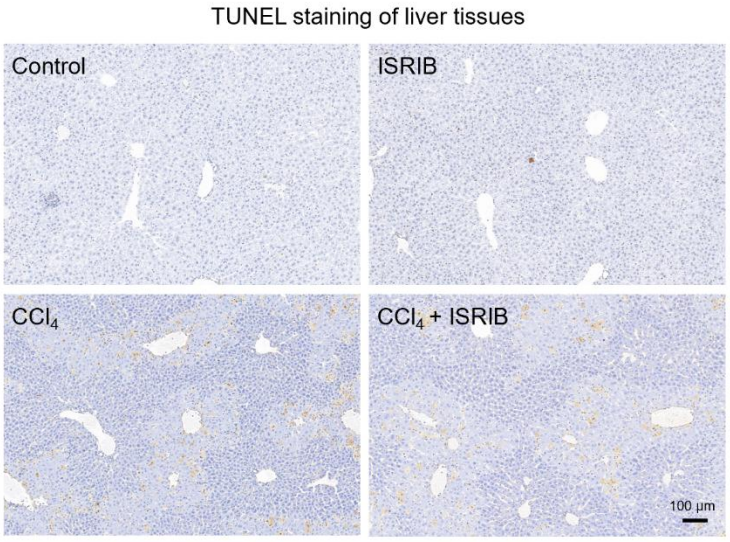
While it is truly an important issue of the cell-type specificity of ISRIB, our data suggest that HSCs are the major cell type that mediates the anti-fibrotic effect of ISRIB in liver damage-caused fibrosis. As a chemical with encouraging safety properties, ISRIB has been used in many contexts and is effective in repressing the activity of p-eIF2α in different tissues (Sidrauski et al., ***Elife***, 2013; Wong et al., ***Elife****, 2018;* Chou et al., ***Proc Natl Acad Sci U S A***, 2017; Baird et al., ***Mol Cell***, 2020).

As for cells in the liver, it is not surprising that the administration of ISRIB would affect both hepatocytes and non-parenchymal cells, including HSCs. However, it has been well-established that HSC is the major cell type producing ECM proteins therefore responsible for fibrosis development (Mederacke et al., ***Nat Commun***, 2013; Tsuchida & Friedman, ***Nat. Rev. Gastroenterol. Hepatol.***, 2017). Consistent with these previous reports, we found that the expression level of key fibrotic genes, including *COL1A1*, *COL1A2*, and *FN1*, was much more abundant in LX-2 cells compared to AML12, a hepatic cell line (**Extended Data Fig. 2h, i of the revised manuscript**). More importantly, by use of primary HSCs and hepatocytes collected from the CCl_4- and ISRIB-treated animals, we confirmed that expression of these key fibrotic genes was much higher in primary HSCs compared to hepatocytes under fibrotic conditions and that ISRIB treatment could effectively reduce the expression of fibrotic genes in HSCs (**Fig. 7b of the revised manuscript**). These striking

differences between HSCs and hepatocytes indicated that HSCs is the major cell type producing pro-fibrotic proteins and responding to ISRIB treatment.

In addition, we agree with you that the eIF2α pathway is important for the function of hepatocytes, e.g., the survival of hepatocytes during ER stress or metabolic complications. However, we found that in the condition of acute liver damage caused by CCl_4, treatment of ISRIB does not affect the apoptosis of liver cells as gauged by the expression of apoptotic marker cleaved PARP (**Extended Data Fig. 5d of the revised manuscript**). These phenomena could be validated by the TUNEL assay (**Fig. 1 of this letter**). These data suggested that the anti-fibrotic effects of ISRIB may not be mediated by promoting hepatocyte survival.

Fig. 1 Effects of ISRIB on CCl_4-triggered apoptosis in hepatocytes



Together, we hope you can concur that the anti-fibrotic effects of ISRIB are primarily through targeting HSCs.

2. The role of ATF4 in mediating the effect of ISRIB

We do agree with you that eIF2α, as a multi-functional protein, could affect fibrosis via diverse mechanisms. For example, it has been reported that in certain metabolic stress conditions, like high-fructose diet, phosphorylation of eIF2α is required for the survival of hepatocytes (Sung Hoon Back et al., ***Nutr Metab***, 2017). By regulating the antioxidant and autophagy pathways, phosphorylation of eIF2α represses liver cell death, thereby protecting animals from high-fructose diet-triggered fibrosis. However, as mentioned in the last section, we found that under the condition of acute liver damage caused by CCl_4, disruption of p-eIF2α by ISRIB does not affect liver cell death (**Extended Data Fig. 5d of the revised manuscript; Fig. 1 of this letter**), suggesting an underlying mechanism beyond the role of p-eIF2α in cell death control.

Considering our findings that ATF4 is required for HSC activation, we proposed inhibition of p-eIF2α by ISRIB may repress HSC activation and fibrosis development via targeting ATF4, the most important downstream factor of p-eIF2α. To further confirm the essentiality of ATF4 in the

effect of p-eIF2α inhibitor, we overexpressed ATF4 in the LX-2 cells and conducted TGFβ and ISRIB treatment. The exogenously overexpressed ATF4 does not contain the 5' UTR region of the endogenous gene and, therefore, is resistant to ISRIB-mediated inhibition. While the TGFβ-induced ECM protein expression, including *FN1*, *COL1A1*, and *COL1A2*, was repressed by ISRIB treatment in the control LX-2 cells, overexpression of ATF4 rendered the LX-2 cells resistant to ISRIB treatment (**Extended Data Fig. 2h, i of the revised manuscript**). This rescue experiment strongly suggested that ATF4 is an essential factor in mediating the effects of ISRIB in suppressing HSC activation.

While ATF4 is an important target in liver fibrosis therapy, ATF4-specific inhibitors are currently unavailable. This is not surprising since it is a common problem that most of the transcription factors are difficult to target. As a proof-of-concept study, we turned to p-eIF2α, the upstream factor of ATF4, to examine if targeting p-eIF2α could be a viable strategy to alleviate fibrosis. Very excitingly, we found that ISRIB was indeed effective in treating fibrosis in multiple animal models. Combining the rescue experiment we provided in the revised manuscript, we hope you can agree that inhibition of p-eIF2α represents a promising strategy to tackle the problem of liver fibrosis via targeting ATF4.

Lastly, we were regretful to make an overstatement in the abstract of the original manuscript and have toned down the language in the revised manuscript. We truly appreciate your valuable comments on this issue.

7) In the human liver scRNAseq data, ATF4 abundance in HSCs between cirrhosis vs healthy?

This is indeed a critical question. Per your suggestion, we examined the expression of ATF4 in the scRNAseq data and observed that the RNA level of ATF4 was significantly increased in the HSCs from cirrhotic livers compared to healthy controls (**Fig. 7i of the revised manuscript**). Despite the change of ATF4 RNA expression between cirrhotic and healthy HSCs, it was previously known that the regulation of ATF4 mainly occurred on the protein level, where p-eIF2α promotes ATF4 translation. Therefore, it holds significance to further interrogate ATF4 on the protein level, or its transcriptional activity. Using the same scRNA-seq data, we assessed the function of ATF4 by examining the RNA expression of a set of ATF4 target genes, which could faithfully represent the activity of ATF4 in different conditions. By doing so, we found that the activity of ATF4 in promoting HSCs genes is dramatically increased in the HSCs from cirrhotic patients compared to healthy controls (**Fig. 7j of the revised manuscript**).

8) Ref 42 suggests that ATF4 directly regulates the expression of TRIB3 which is critical for HSC activation. Did the authors find a similar interaction between ATF4 and TRIB3? Please discuss the findings between these two papers.

In short, we did not find such a connection between ATF4 and TRIB3 during HSC activation or fibrosis as Ref 42 reported. We found that, in the TGFβ-triggered HSC activation model, loss of ATF4 expression did not alter the expression of TRIB3 in LX-2 cells. Consistently, we did not find any change in TRIB3 expression in the primary ATF4-KO HSCs compared to the WT controls under the treatment of CCl_4. However, we did notice that under the treatment of Thapsigargin, a classical ER stressor, loss of ATF4 expression can reduce the expression of TRIB3 by half. Consistent with this pattern of gene expression, we found from the ChIP-seq data that ATF4 strongly bound to the promoter region of TRIB3 under the condition of ER stress, but it merely exhibited weak binding to TRIB3 under the condition of HSC activation. These findings demonstrated that, as a classical stress response factor, TRIB3 is regulated by ATF4 mainly under classical stress-inducing conditions. These findings exemplify our conclusion that the process of HSC activation can reshape the transcriptome of ATF4.

We also would like to point out a critical problem of Ref 42 that could undermine their conclusion. In Ref 42, the authors used an AAV8-mediated viral infection strategy to deliver gene targeting in liver tissue, with a goal to target gene expression in HSCs and study the underlying mechanism. However, it is well established that the AAV8 virus mostly infects hepatocytes but not HSCs, in the *in vivo* settings (Sands et al., ***Methods Mol Biol***, 2011; Hoet al., ***Am J Physiol Gastrointest Liver Physiol***, 2008; Cabanes-Creus et al., ***Mol Ther Methods Clin Dev***, 2021). As a result, the conclusion of this paper is not convincing for readers familiar with the AAV8 system or hepatocyte targeting. In addition, for readers who are unfamiliar with this field, the results of Ref.42 might be misleading and can result in follow-up experiments using this problematic AAV8 strategy to manipulate HSCs. On the contrary, we utilized a genetic model that is highly specific in our manuscript. We employed HSC-specific ATF4 knockout mice driven by the LRAT promoter, which clearly and rigorously demonstrated that cell-type specific deletion of ATF4 in HSCs effectively inhibits the progression of liver fibrosis.

9) There is not much connection between the data from endothelial cells and HSCs. In addition, no vivo data to support the observation from in vitro assays.

We appreciate your comments that could potentially broaden the scope of our study, and we would like to address them from the following aspects.

1. The potential role of endothelial cells in ATF4-regulated HSC functions

In the process of liver fibrosis, multiple types of cells are involved in the progression of the disease, including parenchymal cells, immune cells, endothelial cells, and HSCs. Among these different types of cells, activation of HSCs is the most direct cause of collagen production and fibrosis. Therefore, in our current manuscript, we primarily explored the regulatory mechanisms of HSC activation and discovered that ATF4 plays an important role in this process.

In addition to HSCs, other types of cells, such as endothelial cells, are important players in the process of liver fibrosis. Many groups have reported that endothelial cells can regulate HSC activation through various mechanisms. For example, it was reported that adipocyte fatty acid binding protein (A-FABP) is induced specifically in liver sinusoidal endothelial cells (LSECs) during liver fibrosis. LSEC-derived A-FABP acts on HSCs in a paracrine manner to enhance the transactivation of TGFβ1 by activating the JNK/c-Jun signaling pathway (Wu et al., ***Adv Sci,*** 2021). Another study revealed that EC-specific deletion of Mlkl mitigated the activation of the Smad pathway, disrupting the pro-fibrotic crosstalk between endothelial cells and HSCs (Yan et al., ***Int J Mol Sci,*** 2023). Therefore, EC cells are important modulators of HSC activation during liver fibrosis.

In our study, we identified ATF4 as a key factor required for HSC activation. While largely known as a master regulator in stress response, we surprisingly found that ATF4 promotes the activation and transdifferentiation of HSCs via stress-independent regulation of the EMT program, a key pathway in cell state determination. While it would be interesting to further examine if ATF4 is also involved in the regulation of HSC activation by endothelial cells, it is beyond the scope of our current study. In fact, in a separate project, we have been studying the role of ATF4 in regulating the crosstalk between HSCs and other types of cells in the liver, including endothelial cells. It is hopeful that we can address this question with this ongoing work.

2. The in vivo data to support the in vitro findings

In this study, we investigated the impact of ATF4 on HSC activation from both *in vivo* and *in vitro* perspectives and further explored its influence on liver fibrosis by use of different mouse models.

On the *in vitro* level, we primarily used the HSC cell line LX-2 to show that ATF4 is required for HSC activation. We applied lentivirus-based overexpression and gene knockdown techniques, as well as a chemical inhibitor of p-eIF2α (upstream factor of ATF4), to systemically manipulate the expression and activity of ATF4 *in vitro*, and tested their effects on HSC activation (**Fig. 3 of the revised manuscript**).

To corroborate these *in vitro* findings, we developed an HSC-ATF4-KO mouse model to further study the role of HSC-ATF4 in the development of liver fibrosis. We found that ATF4 depletion in HSCs can effectively reduce the progression of liver fibrosis (**Fig. 4 of the revised manuscript**). These are important ***in vivo*** **data** to support our findings *in vitro*. In addition, intervening with ATF4 translation by a chemical inhibitor can also effectively impede the progression of liver fibrosis in various mouse models, providing additional evidence that ATF4 regulates fibrosis *in vivo*. To further strengthen the link between the *in vivo* finding and the *in vitro* cell line studies, during this revision, we conducted IHC staining for ATF4 in fibrotic liver tissues from patients and found that the Fib-4 scores in the ATF4-high cohort were significantly higher than those of the ATF4-low cohort (**Fig. 7k of the revised manuscript**), providing **human data** that demonstrate a strong correlation between ATF4 expression in HSCs and fibrosis progression.

Therefore, we hope you can concur with us that we have sufficient *in vivo* data to support our findings from the *in vitro* studies.

3. The connection between the findings of mammary epithelial cells and HSCs.

While we did not mention the role of ATF4 in endothelial cells, we did initiate our story with the findings that ATF4 is required for EMT induction in mammary epithelial cells. We would also like to briefly outline our understanding of the connection between mammary epithelial cells and HSCs.

Firstly, the hypothesis and subsequent validation that ATF4 is required for HSC activation were derived from our initial findings that ATF4 is required for EMT induction in the mammary epithelial cells (HMLE). In other words, ATF4 is an important player in regulating the EMT molecular program in both mammary epithelial cells and HSCs. The consistency of ATF4's function across these two distinct cellular contexts strongly underscores its broad role in cell fate determination, which extends beyond its traditional function in stress response.

Although ATF4 facilitates the EMT program in various contexts, the resulting phenotypes may differ depending on the specific environment. This is not unexpected, as the EMT program itself plays complex and multifaceted roles that vary with tissue type and disease state. In this manuscript, we have concentrated on ATF4's role in HSC activation and liver fibrosis, given that HSC activation is central to liver fibrosis. In mammary epithelial cells, however, the function of ATF4 must be different. In fact, in a separate study, we observed that ATF4 influences cell differentiation of primary mammary epithelial cells, thereby impacting mammary gland development. While these findings are intriguing, we believe they fall outside the scope of the current manuscript.

Reviewer #2 (Remarks to the Author):

The study by Yang, Qi, Lu et al. show, by using cell lines, that ATF4 can control the expression of genes induced during the EMT transition, being a gene-expression program that contributes to hepatic stellate cell (HSC) activation. Authors used CHIP seq to identify the mechanism by which ATF4 controls the expression of pro-fibrotic genes. Then, authors performed *in vivo* studies to find that mice with ATF4 deleted in HSC are protected from CCl_4-induced liver fibrosis, while mice treated with ISRIB also showed a decrease in CCl_4 and bile-duct ligation-induced fibrosis. The findings are innovative and interesting, showing that ATF4 might not just be involved in pro-survival or cell-death responses, but also in determining cell fate. The major concern is that some of the conclusions are not supported by the data shown:

We truly appreciate your great efforts in reviewing our manuscript and providing insightful suggestions and critiques. It is very encouraging to learn that our findings were considered innovative and interesting. With the new experiments conducted during revision, we hope you can agree with us that our conclusion can now be fully supported by the data provided in the revised manuscript.

1) Authors propose that ATF4-dependent fibrosis in the liver is dependent on Eif2-alpha activation to increase ATF4 transcriptional activity in stellate cells. However, the experiments that demonstrate dependency on eIF2alpha are performed in mice with ISRIB delivered by intraperitoneal injections. Therefore, it is a possibility that less fibrosis is observed *in vivo* because ISRIB is preventing cell death in hepatocytes or the release of activating signals from hepatocytes, preventing HSC activation.

We agree with you that it is very important to examine other potential mechanisms underlying the anti-fibrotic effects of ISRIB. Cell death of hepatocytes is the very beginning, triggering step of liver fibrosis. Macrophages can sense and uptake the damaged and dead hepatocytes and, in turn, release signaling molecules, like TGFβ, to activate HSCs and initiate the process of fibrosis. It has been recently reported that the mitochondrial fragments, like mtDNA, from the injured hepatocytes can directly activate HSCs and promote liver fibrosis (An et al., ***Nat Commun***, 2020). Therefore, the extent of hepatocyte death is considered an important parameter in liver fibrosis.

To test if ISRIB treatment prevents HSC activation via directly modulating cell death in hepatocytes, we conducted acute CCl_4 and ISRIB treatment to see if cell death in the liver tissue was different with or without ISRIB treatment. By doing so, we can test the effects of ISRIB on hepatocyte survival prior to the occurrence of fibrosis. By measuring the expression of cleaved PARP, an apoptotic marker, we found while CCl_4 caused very dramatic apoptosis in the liver, treatment of ISRIB did not change the level of cleaved PARP, indicating that inhibition of p-eIF2α did not prevent cell death of hepatocytes under acute liver damage (**Extended Data Fig. 5d of the revised manuscript**). These findings could be further confirmed by measurement of apoptosis in the

liver tissue using the TUNEL assay (**Fig. 1, page 4 of this letter**). These data suggested that the anti-fibrotic effects of ISRIB may not be mediated by promoting hepatocyte survival. Considering the data that ISRIB can directly inhibit HSC activation *in vitro* (**Fig. 3b, h of the revised manuscript**), we hope you can concur with us that the anti-fibrotic effects of ISRIB are primarily through targeting HSCs.

2) Related to point 1), it is unlikely that the ECM proteins produced by activated HSCs can escape the blockage of protein translation induced by eIF2αlpha phosphorylation. Thus, it is likely that EMT and HSC activation are increasing ATF4 activity by other mechanisms. Authors need to measure ATF4 phosphorylation and its translocation to the nucleus in the in vitro models of EMT, as well as in TGFβ-induced HSC activation, together with eIF2αlpha phosphorylation, in the presence and absence of ISRIB. In my opinion, it is likely that in these models, ATF4 will be activated in an eIF2αlpha-independent manner.

We appreciate your important comments on the mechanisms of how ATF4 is regulated under EMT or HSC activation. We would like to address your questions from the following aspects:

Firstly, we would like to clarify the role of p-eIF2α in HSC activation and ECM production. We agree with you that in canonical ER stress conditions, eIF2α is hyper-phosphorylated, thereby inhibiting the translation of many proteins, including ECM proteins. In other words, under stress conditions, the expression of ECM proteins cannot escape the blockage of protein translation induced by eIF2α phosphorylation. However, under the process of EMT induction, we have previously shown that in cancer cells, TGFβ treatment, as well as other EMT-inducing conditions, can modestly increase the level of p-eIF2α and, at the same time, induce the expression of ECM proteins including FN1 and Collagens (Feng et al., ***Cancer Discovery,*** 2014).

[Redacted]

To better illustrate our argument, we designed a set of experiments to compare the response of LX-2 cells to ER stress inducer (Thapsigargin) and HSC activation inducer (TGFβ) and summarized the results in **Fig. 2 of this letter**. We found that, in contrast to the pronounced induction of p-eIF2α and ATF4 during ER stress, the increase in these two proteins during HSC activation is noticeable but relatively modest (**Fig. 2a of this letter**). At the same time, the expression of Col1A1 was

blocked by Thapsigargin treatment but apparently induced upon TGFβ treatment (**Fig. 2a of this letter**). To further confirm the regulatory effect of p-eIF2α on ECM proteins, we showed in **Fig. 3b, h of the revised manuscript** that inhibition of p-eIF2α by ISRIB repressed the expression of ECM protein production. In addition, overexpression of ATF4 can overcome the repressive effects of ISRIB, further confirming the regulatory role of p-eIF2α-ATF4 in ECM production and HSC activation (**Extended Data Fig. 2h, i of the revised manuscript**).

On the mechanistic level, we have shown in the ChIP-seq analysis that ATF4 has completely different binding patterns on the genome under different conditions. Under classical ER stress, ATF4 primarily binds to protein folding and stress response genes. However, under the condition of HSC activation, ATF4 primarily binds to many essential EMT genes, including ECM proteins like Col1A1, therefore directly promoting the expression of ECM proteins.

In addition to the p-eIF2α-dependent translational control, ATF4 can also be regulated by other mechanisms, including phosphorylation and subcellular localization (Li et al., ***Cell Discov***, 2023; Gao et al., ***EMBO Mol Med***, 2021). To check if the phosphorylation of ATF4 was altered during HSC activation, we pulled down the ATF4 protein by an α-ATF4 antibody and then probed for the phosphorylated form of ATF4 by using α-pSer/Thr and α-pTyr antibodies. While we found an increase in both p-Ser/Thr and p-Tyr levels of ATF4 upon TGFβ treatment, this increase was relatively proportional to the increase of total ATF4, suggesting that the change of p-ATF4 was mainly due to the alteration of ATF4 translation (**Fig. 2b of this letter**). Most importantly, the increased p-ATF4 was reduced upon ISRIB treatment, also demonstrating that the increased phosphorylation of ATF4 depends on increased translation. In addition to phosphorylation, we also checked the subcellular localization of ATF4 in the HSC activation model. We found the majority, if not all, of the ATF4 protein was localized in the nuclear fraction (**Fig. 2c of this letter**). While the regulation of ATF4 trafficking may be important in other cell contexts (Gao et al., ***EMBO Mol Med***, 2021; Du et al., ***Int J Med Sci***, 2021), it appears not a major mechanism of regulation for ATF4 in our models.

Although the total amount of p-ATF4 at serine, threonine, or tyrosine does not change dramatically upon HSC activation, we cannot completely rule out the possibility that certain specific modifications of ATF4, like S245, S166, T173, etc., might be changed during HSC activation. The potential change of ATF4 phosphorylation at specific sites, even if not affecting its subcellular localization in our models, would still be possible to affect the transcriptional activity of ATF4. It would be a very interesting question for further exploration in a separate study.

3) Authors conclude that ATF4 regulates an epigenomic signature of EMT. However, the authors only found that ATF4 binding sites coincide with sites of H3K27ac. It could be the opposite conclusion, that changes in the epigenome are what determines where ATF4 binds. Therefore, such a model places ATF4 translocation to the nucleus as the key regulatory point to determine the type of

ATF4 program activated, together with epigenetic marks. To reinforce this conclusion, authors would need to perform the same experiments in the EMT cell model itself. Otherwise, the epigenetic marks themselves might not be the shared mechanism by which ATF4 promotes EMT transition and HSC activation. With the current data, it is still a possibility that the mechanism is different, despite that some of the ATF4-dependent genes overlap.

This is truly an important question that we should address. In the initial manuscript, we have shown that in response to the signal of HSC activation, the epigenetic landscape in LX-2 cells is changed, and ATF4 preferentially binds to the newly formed super-enhancer (SE) regions in the activated LX-2 cells to drive the expression of essential EMT genes, thereby promoting HSC activation and mediating the liver fibrosis phenotype. In fact, our understanding of how ATF4 is involved in the epigenomic signature of EMT is in agreement with your perspective — we believe that the change of epigenome is a determinant of ATF4's activity during HSC activation, according to the following aspects:

1) We have found in the initial manuscript that in the absence of TGFβ, the triggering signal of HSC activation, overexpression of ATF4 alone led to a very mild increase of the expression of pro-EMT and HSC activation genes (**Fig. 3c, d of the revised manuscript**). In comparison, we found that overexpression of ATF4 can strongly potentiate the function of TGFβ to induce the expression of pro-EMT and fibrotic genes (**Fig. 3c, d, g of the revised manuscript**). Therefore, we proposed that epigenetic alteration caused by TGFβ is required for ATF4's function.

2) To further delineate the functional connection between the change of epigenome and ATF4's transcriptional activity, we applied two chemical inhibitors, JQ1 and C646, to interfere with the epigenome in the TGFβ-treated LX-2 cells. JQ1 is one of the most potent inhibitors of the activity of super-enhancers (Lovén, ***Cell*** 2013). While TGFβ induced the binding of ATF4 to the super-enhancer region of the *COL1A1* and *LOXL2* genes, treatment of JQ1 effectively abrogated the interaction between ATF4 and its target regions (**Fig. 6b of the revised manuscript**). Consistently, treatment of JQ1 repressed the expression of ATF4-target genes during HSC activation (**Fig. 6c, d of the revised manuscript**). In addition, we applied C646, an inhibitor of H3K27ac transferase p300, to examine if the genome binding of ATF4 depends on the TGFβ-triggered epigenomic modification. Similar to the JQ1 treatment, C646 also effectively reduced the binding of ATF4 to its target regions on the *COL1A1* and *LOXL2* genes (**Extended Data Fig. 4f of the revised manuscript**). According to these new data, we proposed that in response to the activation signal of HSCs, the epigenome of HSCs undergoes remodeling, creating the necessary conditions for ATF4 binding and subsequent transcriptional activation governed by ATF4.

3) Consistent with this proposed mechanism, we found in the HMLE-EMT model that the binding of ATF4 to the fibrotic genes also depends on the formation and activity of the super-enhancer (**Fig. 3 of this letter**). In addition, the epigenetic modification in the fibrotic genes showed similar changes as in the HSCs (**Fig. 4 of this letter**). Despite these shared mechanisms in

fibrotic genes, we noticed that the overall pattern of H3K27ac modification was quite different in the HMLE cells compared to the HSCs. For example, some mammary development-related genes were changed only in the HMLE cells (data not shown). This is not surprising since the formation of

[Redacted]

super-enhancers is an important player in cell identity determination (Lovén, ***Cell*** 2013). Although the tissue specificity of this regulation is very interesting, it is beyond the scope of our manuscript and will be described in a separate study where we elaborate on the function of ATF4 in shapin gmammary development and mammary epithelial cell differentiation.

Reviewer #3 (Remarks to the Author):

The manuscript presents a compelling exploration of ATF4 as a therapeutic target in liver fibrosis, a significant pathological condition with progression potential to cirrhosis. The authors have elucidated a non-canonical role of ATF4 in regulating an epigenetic program in hepatic stellate cells (HSCs) that promotes epithelial-mesenchymal transition (EMT) and fibrogenesis, independent of the classical endoplasmic reticulum (ER) stress response. Through methodical experimentation, the study demonstrates that ATF4 depletion in HSCs considerably mitigates liver fibrosis in a mouse model. The modulation of ATF4 activity by TGFβ and the subsequent impact on EMT and fibrotic gene expression provide valuable insights into the molecular underpinnings of liver fibrosis. Moreover, the therapeutic efficacy of a small molecule inhibitor targeting p-eIF2α to hinder ATF4 function suggests a viable approach to treat this disease. Despite these strengths, the manuscript would benefit from addressing the following key points:

We are grateful for your considerate and thoughtful comments on our manuscript! With the new experiments we conducted during revision, we are confident that all your questions have been addressed by the new data provided in the revised manuscript.

1. The manuscript should establish a correlation between ATF4 expression/activity and fibrosis severity, potentially through comparison with clinical fibrosis indices like the Fib4 score.

We appreciate your very important suggestions! It is truly a critical issue to further strengthen the clinical relevance of our study. In this revised manuscript, we conducted IHC staining for ATF4 in a set of 15 fibrotic liver tissues from patients and divided them into ATF-low and ATF4-high cohorts according to the ATF4 staining intensity in the HSCs. We found that the Fib-4 scores in the ATF4-high cohort are significantly higher than those of the ATF4-low cohort (**Fig. 7k of the revised manuscript**). These results clearly demonstrated that the expression of ATF4 in HSCs is correlated with the severity and progression of fibrosis and cirrhosis.

2. The therapeutic potential of ATF4 inhibition should be validated across a broader spectrum of hepatic fibrosis models to ensure the generalizability of the findings.

We appreciate your suggestion to help strengthen the conclusion of our study. In the initial submission, we applied two approaches, namely CCl_4 treatment and bile duct ligation, to mimic hepatic fibrosis development. These two methods encompass both ***exogenous*** chemical-induced liver damage and ***endogenous*** cholestasis-induced liver injury, representing the most typical etiology of liver fibrosis development. A great many high-profile studies used these two methods in their research (Hackstein et al., ***J Hepatol***, 2023; Kong et al., ***Gut***, 2024; Yang et al., ***Hepatology***, 2021). As a proof-of-concept study, we hope you can concur with us that our findings, based on these two

classical strategies, are sufficient to argue that inhibition of ATF4 translation is a valid and potential way to overcome liver fibrosis in mouse models. This being said, we completely agree with you that validating the effects of ATF4 inhibition across a broader spectrum of hepatic fibrosis models is an extremely important task in our future study. In fact, we have been planning to apply additional pre-clinical models like rats and rabbits to test the effects of ATF4 inhibition on liver fibrosis further, with the goal of pushing forward to clinical investigation. We sincerely hope that the publication of the current work will be a strong support for us, a lab in its infancy, to acquire additional resources for these proposed studies.

While we have two *in vivo* models to test the effect of ISRIB in liver fibrosis, we do notice that currently, we have only one way to induce HSC activation i*n vitro*. To strengthen this part, we applied another reagent, namely PDGF-BB, to induce HSC activation in the LX-2 cells. We found while PDGF-BB induced ATF4 translation as well as HSC activation, treatment with ISRIB effectively suppressed PDGF-BB-induced LX-2 activation, further supporting the notion that inhibiting ATF4 upregulation represses HSC activation (**Extended Data Fig. 2f, g of the revised manuscript**). We hope you can agree with us that, with the data from two *in vitro* models and two *in vivo* models, ATF4 is an important target in HSC activation and liver fibrosis.

3. An investigation into the tissue specificity of the eIF2α-ATF4 pathway inhibition is necessary, with particular attention to potential off-target effects in critical metabolic tissues such as skeletal muscle, white adipose tissue (WAT), and brown adipose tissue (BAT), as well as in the skin.

Indeed, potential off-target effects were issues that we should have tested in our initial submission. Thank you very much for your great suggestion. Per your suggestion, we have examined the expression of ATF4 in WAT, BAT, and the skin. We found that CCl4 does not affect the ATF4 expression in these tissues, and meanwhile, ISRIB did not affect the basal level of ATF4 expression in these tissues (**Extended Data Fig. 5e of the revised manuscript**).

4. The authors are encouraged to include a schematic diagram detailing the method used for creating HSC-specific ATF4 knockout mice.

We have added a schematic diagram accordingly in the revised manuscript (**Fig. 4a of the revised manuscript**).

5. The claim of ATF4-specific deletion in HSCs should be substantiated with additional evidence, possibly by single-cell RNA sequencing analysis, FACS sorting of HSCs and non-HSCs populations, or other confirmatory techniques.

We agree that it is important to confirm the ATF4-specific deletion in HSCs. We were regretful that in the initial manuscript, we had only shown the HSC-ATF4-KO on RNA levels. In the revised manuscript, we managed to collect enough primary HSCs from mice to validate ATF4-KO on protein levels. Currently, we have evidence from both transcriptional and translational levels that ATF4 is deleted in HSCs (**Extended Data Fig. 3d of the revised manuscript**).

6. In Figure 7A, the quantification of ATF4 expression within HSCs must be validated to bolster the proposed mechanism.

We truly appreciate your important comment. We should have tested the expression of ATF4 in HSCs for Fig. 7A. Unfortunately, despite our efforts, we previously had difficulties obtaining enough HSCs for protein level analysis and measurement. During this revision, we improved our techniques and managed to collect sufficient protein samples from HSCs. In **Extended Data Fig. 5a of the revised manuscript**, we showed that the protein level of ATF4 in HSCs was dramatically increased upon CCl_4 treatment, while ISRIB treatment repressed the induction of ATF4 in HSCs.

7. The single-cell RNA sequencing data presented in Figure 7H require a more thorough analysis. Additional visualizations such as HSCs cell population distribution, ATF4, and other fibrogenic gene expressions adjusted to support the model would be insightful. Trajectory analysis could further elucidate the role of ATF4 in the fibrotic process.

Per your suggestions, we have conducted additional analysis and modified our manuscript accordingly:

1) In **Extended Data Fig. 6a of the revised manuscript**, we added a series of new plots showing the strategy of how we managed to isolate the HSC population for further analysis.

2) By comparing the HSCs from the cirrhotic and healthy liver samples, we found that the RNA level of ATF4 was increased in HSCs from cirrhotic livers (**Fig. 7i of the revised manuscript**). To further confirm the change of ATF4 on the protein level, we examined the expression of ATF4-target genes, which can faithfully represent the transcriptional activity of ATF4. By conducting GSEA using the ATF4-regulated genesets derived from the LX-2 cells, we found that the ATF4-regulated genes under TGFβ treatment (ATF4-TGFβ) were significantly upregulated in the HSCs of cirrhotic patients compared to healthy donors (**Fig. 7j of the revised manuscript**). In contrast, the canonical ATF4-regulated genes triggered by ER stress (ATF4-Tg) were not enriched in the context of cirrhosis (**Fig. 7j of the revised manuscript**). These results not only showed increased activity of ATF4 in the HSCs but also highlighted the context-dependent role of ATF4 in liver fibrosis.

3) We examined the relation of ATF4 in fibrosis by investigating the evolutionary relationship between quiescent and active HSCs (qHSCs and aHSCs) using pseudotime trajectory analysis (**Extended Data Fig. 6b of the revised manuscript**). We found that the ATF4-TGFβ genes were

gradually induced during the pseudo-activation of HSC (from qHSC to aHSC, **Extended Data Fig. 6c of the revised manuscript**), suggesting that the transcriptional activity of ATF4 increased during HSC activation in patients.

Minor Concern

1. The manuscript should include q-values for each gene set enrichment analysis (GSEA) result presented in Figure 1 to provide a complete statistical context.

We had revised the Fig. 1 and other figures that have GSEA data accordingly. Thank you very much for the valuable comments.

In summary, the study is methodologically sound and presents a potentially transformative approach to liver fibrosis therapy. Addressing the above points will significantly enhance the manuscript's impact and clinical relevance.

A point-by-point response letter to the Reviewers' remarks

Reviewer #1 (Remarks to the Author):
Authors have made substantial changes and provided additional data which addresses my questions. No additional comments.

We are grateful for the important suggestions provided by the Reviewer to help strengthen our manuscript.

Reviewer #2 (Remarks to the Author):
I want to congratulate the authors for addressing most of the points raised in the first round. However, there is one point that has not been successfully addressed. The authors only measured the effects of ISRIB on apoptosis in hepatocytes. However, CCl4 induces other forms of cell death that are more pro-inflammatory than apoptosis, such as necrosis and pyroptosis. The fact that only TUNEL and cleaved caspase were performed to address the point of cell death raises concerns about discarding the role of hepatocyte death in HSC activation.

This is indeed a critical question that we should further address. In the revised manuscript, we have further examined if necrosis or pyroptosis of hepatocytes was affected by ISRIB.
For necrosis, we examined the expression of p-RIP3 and found that while p-RIP3 was induced upon CCl_4 treatment, ISRIB treatment did not affect the level of p-RIP3 (**Supplementary Fig. 5d of the revised manuscript**). In addition, the morphological change of hepatocytes upon CCl_4 treatment confirmed that ISRIB did not affect the area of the necrotic zone (**Supplementary Fig. 5e of the revised manuscript**). In the TUNEL images, we can find that the necrotic areas near the central vein, indicated by the light-stained cells with swelling morphology and nuclear shrinkage, were not changed upon ISRIB treatment (**Supplementary Fig. 5e of the revised manuscript**). In fact, the observations from the TUNEL assay also greatly align with your perspective—apoptotic cell death is not the major type of cell death, as the TUNEL-positive cells took up only a minor fraction in the cell death zone around the central vein. Therefore, the results from the TUNEL assay, as well as the expression of protein markers, indicated that neither apoptosis nor necrosis was changed upon ISRIB treatment.
For pyroptosis, we examined the expression of cleaved GSDMD and cleaved Caspase-1, markers for pyroptotic cell death. We did not find any expression of the cleaved GSDMD and cleaved Caspase-1 (**Supplementary Fig. 5f of the revised manuscript**), indicating that acute CCl_4 treatment did not induce pyroptosis in the liver.
Together, we hope you can concur with us that hepatocyte death is not changed by ISRIB.

Reviewer #3 (Remarks to the Author):
First, I would like to express my gratitude for your respectful consideration of my comments and your efforts in addressing them.

Regarding my first comment, "The manuscript should establish a correlation between ATF4 expression/activity and fibrosis severity, potentially through comparison with clinical fibrosis indices like the Fib4 score," although a formal correlation assay was not conducted, I agree with your

assessment that the observed trends suggest statistical significance. I believe that performing a correlation assay, such as calculating Spearman's rho, could have potentially resulted in stronger trends and improved p-values.

Thank you very much for the important suggestion. We have revised our analysis accordingly using the Spearman correlation analysis. The results greatly aligned with your perspective—a stronger trend between ATF4 expression and the Fib-4 score was observed, and the significance was also more pronounced (**Fig. 7l of the revised manuscript**).

For my second comment, "The therapeutic potential of ATF4 inhibition should be validated across a broader spectrum of hepatic fibrosis models to ensure the generalizability of the findings," no additional experiments were performed. Instead, you evaluated the LX-2 cells in vitro model using PDGF-BB to induce HSC activation (Extended Data Fig. 2f, g). While Western blotting showed ATF4 expression reduced to a level comparable to the control, the expression of target genes remained elevated in qRT-PCR. I find it challenging to conclude that the generalizability I requested has been achieved based solely on the Western blot and qRT-PCR results of three genes in the in vitro model.

We sincerely apologize for not having addressed your comments properly. In the revised manuscript, we applied another hepatic fibrosis model, namely thioacetamide (TAA) treatment, to further examine if ATF4 inhibition could generally repress liver fibrosis. According to the newly collected data, we found that ISRIB treatment can inhibit liver fibrosis caused by TAA.

1) By measuring the expression of Vimentin, αSMA, and collagens, we found the ISRIB treatment repressed the expression of fibrotic markers on both transcriptional and translational levels (**Fig. 7f and Supplementary Fig. 6b of the revised manuscript**), consistent with the results from the CCl_4- and BDL-related experiments (**Fig. 7a, b, e; Supplementary Fig. 5a and Supplementary Fig. 6a of the revised manuscript**).

2) By visualizing the expression of collagen proteins *in situ*, we found that ISRIB significantly reduced the fibrotic scar caused by collagen aggregation in the liver (**Supplementary Fig. 6d, f of the revised manuscript**), which was also consistent with the results from the CCl_4 and BDL models.

3) Lastly, by quantifying the content of collagen, we found that ISRIB significantly reduced collagen production upon TAA treatment (**Fig. 7h of the revised manuscript**), which was also consistent with the CCl_4 and BDL models (**Fig. 7d, g of the revised manuscript**).

Collectively, these data showed that ISRIB could effectively inhibit fibrotic progression caused by TAA treatment. Therefore, we hope you can concur with us that the therapeutic potential of ATF4 inhibition is consistent across multiple fibrotic models, highlighting the generalizability of our findings.

Regarding my third and fourth comments, I have no further concerns.

In my fifth comment, I requested a comparison between "HSCs and non-HSCs populations." However, the experiments were only performed comparing WT and HSC-Atf4-KO, and the more detailed comparison I requested was not addressed.

We are regretful for not having addressed your comment properly. To verify the tissue specificity of ATF4-KO, we isolated protein samples from the non-HSC fraction in the liver, as well as additional peripheral tissues, including spleen, adipose tissue and skin, to examine the expression of ATF4. We found that, in all the tissues tested, the level of ATF4 was not changed in the HSC-*Atf4*-KO animals compared to the WT animals (**Supplementary Fig. 6d, e of the revised manuscript**). Combining the data that ATF4 was dramatically reduced in the HSC fraction in the liver, we hope you can agree that ATF4-KO is a tissue-specific phenomenon in the HSC-*Atf4*-KO animals.

For my sixth comment, while it was noted that additional experimental data were provided in Extended Data Fig. 5a, I was unable to locate this figure. I presume that Extended Data Fig. 5b contains the relevant results instead.

We apologize for the confusion. Indeed, Extended Data Fig. 5b (currently **Supplementary Fig. 5b of the revised manuscript**) contains the results that addressed the question from your sixth comment.

For my seventh comment, the response was well-done, and I have no further comments.

The minor comments were also well addressed.

While a considerable amount of additional experimentation was performed, I feel that many of my key comments were not sufficiently addressed. Particularly, despite providing explicit guidance on the experiments and analyses I believed would strengthen the study, these were not executed as requested, which I find disappointing. My comments were aimed at enhancing the robustness and credibility of the study's findings, and thus I am somewhat dissatisfied with the authors' response.

We are extremely grateful for the valuable comments provided by the Reviewer, and at the same time, we regret that some of the critiques have not been fully addressed during the first round of revision. With the new data provided in the current revised manuscript, we are confident that all the questions from the Reviewer have been fully addressed.