nature portfolio

Peer Review File

The Role of Forkhead Box M1-Methionine Adenosyltransferase 2A/2B Axis in Liver Inflammation and Fibrosis

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Summary
In the submitted manuscript authors aim to test the hypothesis that there is a crosstalk between Forkhead Box M1 (FOXM1) and Methionine Adenosyltransferase 2A/2B (MAT1A, MAT1B) and that the crosstalk drives liver inflammation and fibrosis. The authors confirmed that the expression of all three were upregulated in hepatocytes, HSCs and KC after bile duct ligation or carbon tetrachloride (CCl4) treatment. Furthermore, FDI-6, an inhibitor of FOXM1, not only attenuated the development of CCl4-induced liver injury and fibrosis but also reversed progression of liver fibrosis while lowering FOXM1/MAT2A/2B expression. FOXM1, MAT2A, and MAT2B were shown to interact and positively regulate each other and that their deletion in hepatocytes, stellate cells, or Kupffer cells equally protected the liver from BDL induced fibrosis. Furthermore, the authors demonstrated that extracellular vesicles produced by each cell type elicited protective effects as observed in each knockout model. Collectively, this study presents compelling evidence for a key role of the FOXM1, MAT2A, and MAT2B axis in fibrosis. While the presented work is expected to be of interest to readers, revisions to improve clarity in the methodology and study designs are required.

Comments:
• It is odd that the first section of the results primarily discusses supplemental materials. Usually, supplemental materials are used to complement the introduced work, yet it seems here that this is important data and therefore should be included in the main text.
• The prevention and treatment models are poorly described. For example, if FDI-6 is used for prevention, why is FDI-6 also found in the treatment portion of the plot? Did the prevention include two independent treatments with FDI-6? This needs to be better described. It would make much more sense to show treatment first then prevention.
• Because of the organization of the manuscript (methods at the end), the result section needs to include key experimental variables. For example, for MAT2A/FOXM1 activity we find out later that it is a MAT2A or FOXM1 reporter and not endogenous expression. The figures also have no units making it difficult to interpret. Diagrams on top of the plots showing FOXM1 binding motifs don't match the x-axis so it is also confusing what those diagrams mean and what the X values represent (no label).
• The conclusion that "FOXM1, MAT2A and MAT2B … in EVs can influence the expression of these protein in other cell types" is not well supported. Figure 8 is poorly described, and the authors should provide more study design details. Which cells came from a BDL model vs sham model. What is just the EVs or also the treated cells that originated from a BDL model? If the EV treated cells came from a BDL model how did the authors distinguishing cells that were highly expressing the proteins instead of the EVs proteins. Did EVs from FOXM1 knockout cells have factors that degraded MAT2A and MAT2B or was there no protein to the taken up from the EVs?
• Numerous measurements are presented. It would improve clarity if the source of these measurements in their results were specified. Using this approach throughout the Results section is suggested.
• The upregulation of FOXM1, MAT2A and MAT2B in hepatocytes, HSCs, and KCs is reported but in the Introduction "MAT2A and MAT2B are expressed in extrahepatic tissues and non-parenchymal cells of the liver". Are MAT2A and MAT2B also expressed in hepatocytes? Please clarify.

• Figure 1A: Please present a better Western blot for β-actin as it appears to decrease over time? Does that influenced the normalization of FOXM1 MAT2A and MAT2B expression?
• The absence of scale bars on immunofluorescence images and liver sections, along with the presence of random scale bars other liver sections, is notable. Scale bars play a crucial role in facilitating comparisons between pictures within or across figures. It is considered best practice to ensure that all images in a figure share the same scale for easy and accurate visual assessment. Please improve images accordingly.

Reviewer #2 (Remarks to the Author):

Bing et al. in this manuscript describe the pivotal role of the FOXM1/MAT2A/2B axis in fibrogenesis in several models, using variety of complementary in vivo and in vitro methods. Also, the efficacy of a FOXM1 inhibitor in fibrosis was assessed.
The paper focuses on the crosstalk between these proteins and cell specific knock-out models of FOXM1 are generated to decipher its role in vivo. It was interesting to see how KO cells communicate negative signals to other cells modulating their signaling cascades however, these studies were not well developed. In addition, there were several technical issues, as outlined below.

Comments:
• In Fig. 1A the WB quality, and in 1B the quality of IFC images needs to be improved. Some images appear stretched.
• Suppl 1-2: Make sure the selected areas and the magnifications correspond.
• Supplementary Fig. 1A: why is FOXM1 highlighted in the BD cells in CCl4? Is this a real positive signal?
• Improve the image quality in Supplementary Fig. 1B-C.
• In the figure legends please include the n numbers
• In Suppl Fig. 2, MAT2β IHC the PBC IHC is not very convincing.
• In the figure legend of Figure 2 and Supplementary figure 3 it is not obvious whether the statistics of the CCl4+FDI-6 (treatment group) was compared to the CCl4+DMSO (treatment group) or CCl4 +DMSO (prevention group). To say that the drug reverses already existing fibrosis the CCl4+FDI-6 (treatment group) has to be compared to the CCl4+DMSO (prevention group, 3-week CCl4 treatment). This has to be clarified in the graph and the legend.
• It would be interesting to see the hierarchy of FOXM1/MAT2A/2B interactions in the different models.
• How deletion of FOXM1 in hepatocytes improved biliary fibrosis? Related to this, how do we explain that Foxm1KC-/- mice have lower protein (Fig. 6h) and mRNA level of Foxm1, Mat2a, and Mat2b in hepatocytes and HSCs compared to Flox controls?
• Please correct this statement: “HSCs activate and release ECM”.
• The EV studies are interesting, but they do not entirely explain how EVs from KO cells regulate other cells lowering the same targets. What is the cargo, and how are these EVs recognized? Does FDI-6 impact EV release/communication to other cells?

Reviewer #3 (Remarks to the Author):

This manuscript elucidates the mechanism through which the FOXM1/MAT2A/2B axis contributes to liver inflammation and fibrosis, presenting novel insights for the treatment of fibrotic liver injury. The author extensively demonstrated the interplay between FOXM1, MAT2A and MAT2B using various advanced technologies including ChIP, EMSA, and supershift assay. Meanwhile, the authors also investigated the feasibility of FOXM1 knockdown to improve liver injury in a BDL model by targeting multiple liver cell types. However, this manuscript did not provide evidence of how FOXM1 facilitates communication between different cells through extracellular vesicles containing FOXM1. The specific mechanism by which FOXM1 alleviates liver inflammation and fibrosis was not clearly explained, and more research is needed to understand its effect on related injury indicators and its underlying mechanism of action. The authors should further clarify which cells are most critical for the role of FOXM1. Above are several key issues that require further clarification. Additionally, there are areas in the manuscript that could be further optimized to enhance the paper's quality.

1. In animal experiments, the author selected 14 days after BDL surgery for research, at which time severe fibrosis appeared in the liver. However, the cell experiments used LX2 under normal condition, which shows that the degree of liver fibrosis in vivo and in vitro is inconsistent in the results of Figure 1. HSC cells were derived from WT mice, why not from mice with liver fibrosis?
2. The homeostasis between MAT1A and MAT2A primarily exists in normal liver cells. Studies have indicated that the target genes of FOXM1 include MAT1A, and there is a homeostatic relationship between MAT2A and MAT1A (1). Does FOXM1 regulate MAT2A indirectly through MAT1A? Also, is there a transition from MAT1A to MAT2A in fibrotic injury?
3. MAT1A promotes the synthesis of S-adenosylmethionine, while MAT2A inhibits the synthesis. Current research has demonstrated that supplementing with S-adenosylmethionine can improve liver fibrosis (2). Does the effect of FOXM1 in promoting liver fibrosis occur by ultimately altering S-adenosylmethionine supplementation?
4. In Figure 1, the BDL model was utilized to investigate the changes in FOXM1/MAT2A/MAT2B, while in Figure 2, the CCl4 model was employed to study the anti-liver injury effect of FMI-6. Although both models involve fibrotic damage, the underlying mechanisms are different. Could these two models impact the relevant findings of this study?
5. FDI-6 has been shown to reduce the proliferation of LX2 cells (Figure 1G) and migration (Figure 1H). Can FDI-6 inhibit HSC proliferation and migration in pathological conditions?
6. The results of each sample in Figure 1A are not clear. The immunofluorescence of Figure 1B is unclear, and the images of MATα2 and FOXM1 appear almost identical. Is this a normal occurrence? All the pictures in the article do not have a scale bar.
7. The data for HSCs in the sham group is missing in Figure 1C. If HSCs in the sham group are cultured normally for 5 days, will the mRNA levels of Foxm1, Mat2a, and Mat2b change accordingly?
8. The most significant decrease in FOXM1, MATα2, and MAT2β in hepatocytes can be observed after the administration of FDI-6, as depicted in Figure 2A. Does this suggest that the role of the FOXM1/MAT2A/MAT2B axis in liver fibrosis is primarily mediated through hepatocytes? In Figure 2C, there are only three samples in some groups. Is there any sample loss or other situation?
9. Why were cholangiocytes selected for investigating the crosstalk in the FOXM1/MAT2A/MAT2B axis, instead of HSCs, hepatocytes, or KCs?

10. Part of the WB image in the paper is marked with marker position, while some are not marked. The WB format should be unified.
11. In LX2, the predicted FOX region on the MAT2A promoter is located in regions -264 to 272 and -16 to 22, while the primers are designed to cover -270 to +60. Only a portion of the FOX region is included. Could this experimental design lead to false positive results after administering FDI-6 and SI-FOXM1?
12. In Figure 3G, the content of MAT2β re-ChIP after FOXM1 ChIP remains consistent regardless of different siRNA treatments, while MATα2 re-ChIP after FOXM1 ChIP shows lower content after knocking down MAT2A compared to knocking down MAT2B and FOXM1. Although the contents of MAT2β re-ChIP after FOXM1 ChIP increase after overexpression of different genes, there is no significant difference between them. On the other hand, MATα2 re-ChIP after FOXM1 ChIP shows the highest increase after overexpression of FOXM1 chip. What could explain this phenomenon? Does it imply that only MAT2A stabilizes FOXM1 expression, while MAT2B is not a stable FOXM1 protein?
13. The results of Figure 3K demonstrated direct interaction between FOXM1 and MATα2 and MAT2β but cannot demonstrated that FOXM1 and MATα2 and MAT2β activate the transcription of each other.
14. In addition to inhibiting the transcription of FOXM1, FDI-6 can also bind to FOXM1 protein and inhibit the transcription of FOXM1 activating genes (3). Does FDI-6 bind to both MAT2A and MAT2B proteins, thereby directly affecting the transcription of their genes?
15. BDL surgery first caused cholangiocytes injury and Figure 4H showed significant changes of FOXM1, MATa2 and MAT2β expression in cholangiocytes. Why was Foxm specifically silenced in HSC cells and KC cells only, but not in cholangiocytes?
16. In Figure 4, knocking out Foxm1 in hepatocytes can also inhibit bile duct response. Does this mean that knocking down Foxm1 in cholangiocytes can also alleviate bile duct reaction? Or does the low expression of Foxm1 in hepatocytes inhibit the bile duct response by releasing a stimulating factor through some extracellular vesicle?
17. As shown in Figure 4, why did the protein content of Foxm1 in HSCs decrease when Foxm1 was knocked out in hepatocytes, but the mRNA content did not change?
18. As shown in Figure 5, the specific knockdown of Foxm1 in HSCs did not affect Foxm1, mat2a, and mat2b in hepatocytes, cholangiocytes, and KCs cells. Does it mean that Foxm1 cells mainly achieve intercellular communication through hepatocytes instead of HSCs
19. After knocking out Foxm1 in various liver cells, it was observed that not all of these cells were able to decrease the Foxm1 content in other cells. The question arises as to why different cells are targeted by extracellular vesicles. Although extracellular vesicles can be secreted by various cells, not all cells could absorb them. What is the underlying reason for this selective absorption?
20. The results in Figure 4 show that Foxm1Hep-/- mice have lower BDL-induced injury and fibrosis, while LX2 cells and macrophages were selected in the in vitro experiment in Figure 7, why not simultaneously study hepatocytes?
21. As shown in Figure 8E, ductular proliferation and inflammation can be reduced in Foxm1Hep-/- mice, but only the number of F4/80 and CK19 positive cells were detected, so the proof lacks credibility.
22. The experiment in Figure 8 also needs to directly detect the contents of EVs secreted by liver cells, thus effectively proving that liver cells secrete EVs containing FOXM1/MATα2/MAT2β.

References

1. Li Y, Lu LQ, Tu J, Zhang J, Xiong T, Fan W, Wang JH, et al. Reciprocal Regulation Between Forkhead Box M1/NF-κB and Methionine Adenosyltransferase 1A Drives Liver Cancer. Hepatology 2020;72:1682-1700.

2. Pascale RM, Simile MM, Calvisi DF, Feo CF, Feo F. S-Adenosylmethionine: From the Discovery of Its Inhibition of Tumorigenesis to Its Use as a Therapeutic Agent. Cells 2022;11.
3. Gormally MV, Dexheimer TS, Marsico G, Sanders DA, Lowe C, Matak-Vinkovic D, Michael S, et al. Suppression of the FOXM1 transcriptional programme via novel small molecule inhibition. Nature Communications 2014;5.

Reviewer #4 (Remarks to the Author):

In this work, Bing Yang and the colleagues focused the mechanism that how FOXM1/ MAT2A/ MAT2B axis regulates the liver fibrosis. They first found the upregulation of FOXM1/ MAT2A/ MAT2B in human and animal fibrotic liver sample with different etiologies. They proved FOXM1 inhibitor FDI-6 could prevent and reverse the liver fibrosis in CCl4-induced model. In mechanism, the authors proved that FOXM1/MAT2A/MAT2B could co-occupied the FOX elements in promoters of themselves and upregulated their expression in transcript level. In view of complicated contribution of HSCs, KCs or other non-parenchymal cells in liver fibrosis, the authors established the organ and cell-specific knockout animal model and build the BDL-induced liver fibrosis model. Through analysis of primary cells from gene-editing model, the authors found the cell-cell crosstalk of FOXM1/MAT2A/MAT2B through EVs. The work has some strengths: 1) found the reciprocal positive regulation of FOXM1/MAT2A/MAT2B through sequential ChIP experiment which could be a novel therapeutic target in hepatic fibrosis, inflammatory or malignancy; 2) thus far, few research reported the FOXM1/MAT2A/MAT2B function in EVs. The authors comprehensively analyzed the contribution of non-parenchymal cells to liver fibrosis in microenvironment and highlighted the significance of FOXM1/MAT2A/MAT2B-contained EVs in this process.
Questions
1. How to explain the different change of FOXM1, MATα2 and MAT2β in KCs and cholangiocytes with HSCs and hepatocytes. (the capacity to receive the EVs is different among them?) If so, the mechanism diagram should be revised.
2. The authors didn't provide the morphological evidence for EVs (like TEM or particle size analysis), and how to prove that they are not contaminated by exosomes? No FOXM1 or MAT protein-contained EVs can be find in immunofluorescence results in Figure 1B and Figure 1E.
3. The histological feature of CCl4-induced liver fibrosis is the diffuse HSCs activation in the hepatic lobule while BDL mainly induce the cholestatic liver fibrosis which begins from the portal area. Why the authors choose BDL model to study the cell-cell EVs crosstalk but used CCl4 model to study liver fibrosis prevention and treatment?
4. In introduction, "MAT2A and MAT2B are expressed in extrahepatic tissues and non-parenchymal cells of the liver, such as HSCs and Kupffer cells (KCs)", but in Figure 4H, 5H and 6H, if the protein loading mass is equal, the MAT2A and MAT2B levels are comparable in hepatocytes with HSCs and KCs.
5. According to data of Figure 4H, 5H and 6H, the KCs can`t be regulated by EVs from HSCs or hepatocytes. In addition, the FOXM1, MATα2 and MAT2β of cholangiocytes only changed in hepatocyte-specific knockdown group, which is different from hepatocytes, HSCs or KCs. The authors should explain the underlying mechanism and may provide more related data in this aspect.

6. Although the authors used the FOXM1 OV and MAT protein knockdown cells to prove the mechanisms that FOXM1 functions in transcription promotion of FOXM1/MAT2A/MAT2B axis in Figure 7E, the authors didn`t provide any other data to strengthen the importance of FOXM1-MAT2A-MAT2B complex. MAT2A and MAT2B seem to be dispensable in this work, because only FOXM1 can initiate and promote the regulation axis.
7. In Figure 2E, the authors presented low-resolution fluorescence images showing separate co-localization of MATα2 and MAT2β with FOXM1. However, the limited image resolution compromised the effectiveness of co-localization visualization. Additionally, high-resolution co-localization fluorescence images of MATα2, MAT2β, and FOXM1, confirming their formation as a complex within the cellular context, were lacking.
8. In Figure 7, the authors employed TGF-β treatment to stimulate HSC activation and evaluated downstream SMAD3 signaling. However, studies investigating the TGF-β/SMAD3 pathway typically assess the phosphorylation level of SMAD3 rather than solely examining changes in SMAD3 protein levels. It is recommended to include Western blot results for phosphorylated SMAD3 as a supplementation.
9. Why the same signal pathway can function in different cell phenotype? What is the underlying mechanism?
10. In Figure 1 and Figure 2, the authors focus on the HCS activation, but study the KCs and other cells in subsequent research, which makes readers confused.
11. Why to chose FDI-6 to inhibit the FOXM1? Can FDI-1 inhibit the binding between FOXM1, MAT2A and MAT2B?
12. Figure 1E shows that FDI-6 treatment reduced cytoplasmic and nuclear staining of FOXM 1, MATα2, and MAT2β in LX 2 cells; in particular, the expression of MAT2β changed to cytoplasmic expression; what is the potential significance of this change in intracellular localization of protein expression, and what is the reference value of this study?
13. In this study, extracellular vesicles were used as mediators of cell-to-cell interactions, and the vesicles were supposed to be non-selective, but as the results show, not all cells responded to this change, how can this phenomenon be explained?
Minor flaws
1. Supplementary Figure 1b FOXM1 staining BDL D14 magnificent figure seem to be a wrong indication.
2. There is no scale bar in some of figures such as Figure 1E and B and Figure 2A.
3. Could the author explain why used Tublin as cytoplasm control instead of Actin for Western blotting analysis in Figure 1F?
4. The data of sequential-ChIP confirm the co-occupation of MAT2 and FOXM1 protein on FOX elements, but more details of the experiment should be provided (like beads used, condition of antigen-antibody complex formation).
5. In Figure 3L, using FOXM1 antibody to performed the IP experiments is irrational, since the FOXM1 have already been knockout.
6. The magnification times are different among the panels in Figure 4A and Figure 5A.
7. TNF-α and IL-6 are exocrine proteins should be measured by ELISA.
8. Figure 1B isolates HSC in the mouse model on day 5, and the text describes day 7.
9. Figure 1C vertical axis title (mRNA in HSC fold change of Day1) is wrong.
10. In Figures 4-6A, the authors performed staining for multiple markers and conducted statistical analysis to demonstrate the impact of FOXM1 on fibrosis. However, there was a lack of statistical analysis for the staining results of SMA (smooth muscle actin). It is recommended to include supplementary

statistical analysis for SMA staining.
11. In Figure 7D, there is a mosaic artifact observed on the band of β-ACTIN in the last lane. It is recommended to resubmit all the original image to address.

Response to review of NCOMMS-23-45202

We are most grateful for the editors' and the reviewers' helpful review of our manuscript. We have responded to the comments made by the reviewers, performed additional experiments and revised the manuscript as suggested. These are detailed in the response to the referee comments.

Reviewer #1:

1. It is odd that the first section of the results primarily discusses supplemental materials. Usually, supplemental materials are used to complement the introduced work, yet it seems here that this is important data and therefore should be included in the main text.
Response: We understand the reviewer's point of view, but we are limited by space so have elected to prioritize data that appear in the main versus supplemental text.

2. The prevention and treatment models are poorly described. For example, if FDI-6 is used for prevention, why is FDI-6 also found in the treatment portion of the plot? Did the prevention include two independent treatments with FDI-6? This needs to be better described. It would make much more sense to show treatment first then prevention.
Response: We apologize for the confusion. In the prevention arm FDI-6 was administered from the start of CCl_4 treatment for three weeks. In the treatment group, FDI-6 was started after liver fibrosis was already established (after three weeks of CCl_4) and continued with CCl_4 for two additional weeks.

3. Because of the organization of the manuscript (methods at the end), the result section needs to include key experimental variables. For example, for MAT2A/FOXM1 activity we find out later that it is a MAT2A or FOXM1 reporter and not endogenous expression. The figures also have no units making it difficult to interpret. Diagrams on top of the plots showing FOXM1 binding motifs don't match the x-axis so it is also confusing what those diagrams mean and what the X values represent (no label).
Response: We apologize for the lack of clarity. The numbers on the x-axis are promoter constructs. We have revised the manuscript to make them clearer.

4. The conclusion that "FOXM1, MAT2A and MAT2B … in EVs can influence the expression of these protein in other cell types" is not well supported. Figure 8 is poorly described, and the authors should provide more study design details. Which cells came from a BDL model vs sham model. What is just the EVs or also the treated cells that originated from a BDL model? If the EV treated cells came from a BDL model how did the authors distinguishing cells that were highly expressing the proteins instead of the EVs proteins. Did EVs from FOXM1 knockout cells have factors that degraded MAT2A and MAT2B or was there no protein to the taken up from the EVs?
Response: We apologize for the confusion. EVs were obtained from different cell types isolated from BDL livers (in order to increase the expression of these proteins in the EVs). Cells treated with EVs were from different cell-type specific knockouts not subjected to BDL. We think the reason that cells treated with EVs from different knockouts after BDL have lower expression of MATα2 and MAT2β is because the expression of these two proteins is lower in the EVs as compared to EVs from WT cells (see Supplemental Figure 14g in the revised version).

5. Numerous measurements are presented. It would improve clarity if the source of these

measurements in their results were specified. Using this approach throughout the Results section is suggested.
Response: We have revised the description in the text to improve clarity.

6. The upregulation of FOXM1, MAT2A and MAT2B in hepatocytes, HSCs, and KCs is reported but in the Introduction “MAT2A and MAT2B are expressed in extrahepatic tissues and non-parenchymal cells of the liver”. Are MAT2A and MAT2B also expressed in hepatocytes? Please clarify.
Response: Yes, MAT2A and MAT2B are induced in hepatocytes after BDL as shown in Figure 4H-I and Supplemental Figure 1b. They are also induced in human liver fibrosis/cirrhosis (Supplemental Figure 1a).

7. Figure 1A: Please present a better Western blot for β-actin as it appears to decrease over time? Does that influenced the normalization of FOXM1 MAT2A and MAT2B expression?
Response: We have replaced the western blot with one that has similar β-actin intensity. The results remain the same. FOXM1 is induced first (one day after BDL), followed by MATα2 and MAT2β (day 2 after BDL).

8. The absence of scale bars on immunofluorescence images and liver sections, along with the presence of random scale bars other liver sections, is notable. Scale bars play a crucial role in facilitating comparisons between pictures within or across figures. It is considered best practice to ensure that all images in a figure share the same scale for easy and accurate visual assessment. Please improve images accordingly.
Response: Thank you for this comment. We have added scale bars on all immunofluorescence images and IHCs.

Reviewer #2

1. In Fig. 1A the WB quality, and in 1B the quality of IFC images needs to be improved. Some images appear stretched.
Response: We have replaced Fig. 1A and 1B to better quality images.

2. Suppl 1-2: Make sure the selected areas and the magnifications correspond.
Response: We have checked, and they correspond. We removed the insets (magnified bile ducts) since they do not add additional information to the paper.

3. Supplementary Fig. 1A: why is FOXM1 highlighted in the BD cells in CCl4? Is this a real positive signal?
Response: We have removed them to avoid confusion. The induction occurs mainly in hepatocytes on IHC.

4. Improve the image quality in Supplementary Fig. 1B-C.
Response: We have replaced them with better quality images.

5. In the figure legends please include the n numbers
Response: We have included the n in all revised legends.

6. In Suppl Fig. 2, MAT2β IHC the PBC IHC is not very convincing.
Response: We have replaced it with a better quality image.

7. In the figure legend of Figure 2 and Supplementary figure 3 it is not obvious whether the

statistics of the CCl4+FDI-6 (treatment group) was compared to the CCl4+DMSO (treatment group) or CCl4 +DMSO (prevention group). To say that the drug reverses already existing fibrosis the CCl4+FDI-6 (treatment group) has to be compared to the CCl4+DMSO (prevention group, 3-week CCl4 treatment). This has to be clarified in the graph and the legend.
Response: We apologize for this confusion; in the revised version we have compared the groups as indicated and the FDI-6+CCl_4 in the treatment group had significantly lower liver injury (AST, ALT), inflammation (F4/80) and fibrosis (hydroxyproline, COL1A1 expression) as compared to the CCl_4+DMSO prevention group.

8. It would be interesting to see the hierarchy of FOXM1/MAT2A/2B interactions in the different models.
Response: We thank the reviewer for this suggestion. Since the CCl_4 model did not include a time course, we are not able to address this question. However, in the case of BDL, FOXM1 is the first to be induced, but induction of MAT2A and MAT2B can positively feedback to further induce FOXM1.

9. How deletion of FOXM1 in hepatocytes improved biliary fibrosis? Related to this, how do we explain that Foxm1KC-/- mice have lower protein (Fig. 6h) and mRNA level of Foxm1, Mat2a, and Mat2b in hepatocytes and HSCs compared to Flox controls?
Response: The most likely explanation is the cross-talk between hepatocytes and HSCs via extracellular vesicles (EVs). We were surprised to find that KC-specific KOs have lower expression of these three proteins in hepatocytes and HSCs after BDL as compared to flox+BDL, and we tested the hypothesis that these three proteins are present in EVs, which can be taken up by other cells. Our results show that indeed, hepatocytes, HSCs and KCs all release EVs and all three proteins are found in the EVs. We think they are taken up by the other cells and since they transcriptionally activate each other, a high amount of any of them will increase the other two. KCs can also release proinflammatory cytokines that can activate FOXM1 and MAT2A.

10. Please correct this statement: “HSCs activate and release ECM”.
Response: Thanks for catching this mistake, we have corrected the English.

11. The EV studies are interesting, but they do not entirely explain how EVs from KO cells regulate other cells lowering the same targets. What is the cargo, and how are these EVs recognized? Does FDI-6 impact EV release/communication to other cells?
Response: Please see response to reviewer 1, #4. These EVs contain all three proteins (higher from WT as compared to KOs) as shown in Supplemental Figure 14g. EVs are internalized by cells. Since FDI-6 lowers the expression of all three proteins, it would be expected that EVs from FDI-6 treated cells will have lower levels of these proteins.

Reviewer #3:

This manuscript elucidates the mechanism through which the FOXM1/MAT2A/2B axis contributes to liver inflammation and fibrosis, presenting novel insights for the treatment of fibrotic liver injury. The author extensively demonstrated the interplay between FOXM1, MAT2A and MAT2B using various advanced technologies including ChIP, EMSA, and supershift assay. Meanwhile, the authors also investigated the feasibility of FOXM1 knockdown to improve liver injury in a BDL model by targeting multiple liver cell types. However, this manuscript did not provide evidence of how FOXM1 facilitates communication between different cells through extracellular vesicles containing FOXM1. The specific mechanism by which FOXM1 alleviates

liver inflammation and fibrosis was not clearly explained, and more research is needed to understand its effect on related injury indicators and its underlying mechanism of action. The authors should further clarify which cells are most critical for the role of FOXM1. Above are several key issues that require further clarification. Additionally, there are areas in the manuscript that could be further optimized to enhance the paper's quality.

Response: Since FOXM1, MAT2A and MAT2B transcriptionally activate each other, high level of any of the three would raise the expression of the other two. Furthermore, FOXM1 is known to auto-regulate and increases NF-kB expression, which also activates MAT2A. Since FOXM1 has been shown to be important in fibrosis and inflammation, we established the cell-type specific knockouts to address the question of which cell type is most critical for the role of FOXM1. We were surprised to find that they essentially had the same phenotype in terms of liver injury markers and fibrosis. They differ in ductular proliferation (only albumin-Cre had less) and F4/80 (HSC knockouts were unchanged). This led us to the finding that they release EVs and treatment with these EVs influenced the level of these three proteins. EVs released from these cells after BDL all contained more FOXM1, MATα2, and MAT2β as compared to controls, and EVs can be internalized by other cell types to raise their expression.

1. In animal experiments, the author selected 14 days after BDL surgery for research, at which time severe fibrosis appeared in the liver. However, the cell experiments used LX2 under normal condition, which shows that the degree of liver fibrosis in vivo and in vitro is inconsistent in the results of Figure 1. HSC cells were derived from WT mice, why not from mice with liver fibrosis?

Response: LX2 cells are already activated human HSCs (https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0075692). If the second question is why HSCs from WT mice were used in Fig. 1C and not those with liver fibrosis, it is because we were examining whether culture activated HSCs would show induction of these three proteins and whether FDI-6 would block their induction.

2. The homeostasis between MAT1A and MAT2A primarily exists in normal liver cells. Studies have indicated that the target genes of FOXM1 include MAT1A, and there is a homeostatic relationship between MAT2A and MAT1A (1). Does FOXM1 regulate MAT2A indirectly through MAT1A? Also, is there a transition from MAT1A to MAT2A in fibrotic injury?

Response: We showed FOXM1 and MAT1A exert reciprocal negative regulation on each other in hepatocytes (reference #2). However, MAT1A is expressed only in hepatocytes and cholangiocytes in the liver. The fact that FOXM1 is regulating MAT2A in LX2 cells, which do not express MAT1A, tells us that FOXM1 directly regulate MAT2A. We and others have shown that in fibrotic injury MAT1A is downregulated and MAT2A is induced.

3. MAT1A promotes the synthesis of S-adenosylmethionine, while MAT2A inhibits the synthesis. Current research has demonstrated that supplementing with S-adenosylmethionine can improve liver fibrosis (2). Does the effect of FOXM1 in promoting liver fibrosis occur by ultimately altering S-adenosylmethionine supplementation?

Response: MAT1A and MAT2A encode α1 and α2, which are catalytic subunits of MAT isoenzymes. They both catalyze the biosynthesis of S-adenosylmethionine. However, they have different kinetic and regulatory properties (MAT2A-encoded isoenzyme has lower K_m for methionine but is subject to feedback inhibition by S-adenosylmethionine) so that cells expressing MAT1A have higher steady state level of S-adenosylmethionine (reviewed in Physiological Reviews 92:1515-1542, 2012). It is possible that FOXM1 promotes liver fibrosis in part by lowering S-adenosylmethionine level since a switch from MAT1A to MAT2A would result in lower level as we showed (Hepatology 24:1090–1097, 1996). However, FOXM1 also

activatesing NF-kB, chemokine CCL2, and many cell cycle genes so it is likely to promote liver fibrosis by multiple mechanisms.

4. In Figure 1, the BDL model was utilized to investigate the changes in FOXM1/MAT2A/MAT2B, while in Figure 2, the CCl4 model was employed to study the anti-liver injury effect of FDI-6. Although both models involve fibrotic damage, the underlying mechanisms are different. Could these two models impact the relevant findings of this study?
Response: We used different models of liver fibrosis (peri-central in CCl_4 vs. peri-portal in BDL) to make sure this axis was important in more than one model.

5. FDI-6 has been shown to reduce the proliferation of LX2 cells (Figure 1G) and migration (Figure 1H). Can FDI-6 inhibit HSC proliferation and migration in pathological conditions?
Response: LX2 cells are activated human HSCs, so our data suggest the answer is yes.

6. The results of each sample in Figure 1A are not clear. The immunofluorescence of Figure 1B is unclear, and the images of MATα2 and FOXM1 appear almost identical. Is this a normal occurrence? All the pictures in the article do not have a scale bar.
Response: We apologize for not including scale bars and have now included them in all of the relevant images. Regarding the comment on the almost identical images of MATα2 and FOXM1, they are indeed the same cells under different stains, as visualized in the merged images.

7. The data for HSCs in the sham group is missing in Figure 1C. If HSCs in the sham group are cultured normally for 5 days, will the mRNA levels of Foxm1, Mat2a, and Mat2b change accordingly?
Response: Figure 1C is culture activated HSCs, a well-established in vitro model for studying HSC activation. At day 1, HSCs are still quiescent, and they become activated by day 5. The HSCs we used were from normal wild type mice so that we can study normal HSCs undergoing culture activation. In this model the mRNA levels of *Foxm1*, *Mat2a* and *Mat2b* increased during activation and were suppressed by FDI-6. Western blotting confirmed changes also occurred at the protein level.

8. The most significant decrease in FOXM1, MATα2, and MAT2β in hepatocytes can be observed after the administration of FDI-6, as depicted in Figure 2A. Does this suggest that the role of the FOXM1/MAT2A/MAT2B axis in liver fibrosis is primarily mediated through hepatocytes? In Figure 2C, there are only three samples in some groups. Is there any sample loss or other situation?
Response: We can't say that hepatocytes mediate the effect because deletion of FOXM1 in three different cell types all exerted protective effects. Since hepatocytes make up most of the liver, it is deceiving to conclude based on liver IHC. Regarding Fig. 2C, the reviewer is correct. We only had enough samples from 3 mice in some groups.

9. Why were cholangiocytes selected for investigating the crosstalk in the FOXM1/MAT2A/MAT2B axis, instead of HSCs, hepatocytes, or KCs?
Response: We began the studies using albumin-cre and found protection in ductular proliferation, which prompted us to investigate cholangiocytes. However, later experiments found ductular proliferation did not seem to be important (for instance, both HSC- and KC-specific knockouts had the same ductular proliferation as their flox controls). We did study the crosstalk in HSCs and KCs (Figure 7). We didn't use hepatocytes to study the crosstalk because normal hepatocytes express very low levels of MAT2A or MAT2B.

10. Part of the WB image in the paper is marked with marker position, while some are not marked. The WB format should be unified.
Response: Thank you for pointing that out, we have corrected them and added markers to all.

11. In LX2, the predicted FOX region on the MAT2A promoter is located in regions -264 to 272 and -16 to 22, while the primers are designed to cover -270 to +60. Only a portion of the FOX region is included. Could this experimental design lead to false positive results after administering FDI-6 and SI-FOXM1?
Response: Thank you for pointing this out and we apologize that there was an error in the figure. The FOX elelment is located at -264 to -271 and the promoter construct we used was -271/+60, which includes both FOX binding sites. We used this construct because the magnitude of inhibition was the greatest with this construct as compared to -571/+60 or -1329/+60 (Figure 3A).

12. In Figure 3G, the content of MAT2β re-ChIP after FOXM1 ChIP remains consistent regardless of different siRNA treatments, while MATα2 re-ChIP after FOXM1 ChIP shows lower content after knocking down MAT2A compared to knocking down MAT2B and FOXM1. Although the contents of MAT2β re-ChIP after FOXM1 ChIP increase after overexpression of different genes, there is no significant difference between them. On the other hand, MATα2 re-ChIP after FOXM1 ChIP shows the highest increase after overexpression of FOXM1 chip. What could explain this phenomenon? Does it imply that only MAT2A stabilizes FOXM1 expression, while MAT2B is not a stable FOXM1 protein?
Response: We examined this data again and there were no significant differences between MATα2 and MAT2β re-ChIP after FOXM1 ChIP under different conditions, which are summarized in Figure 3H. Since the three proteins directly interact (and we previously showed MATα2 and MAT2β stabilize each other), we don't think one is more stable than the other.

13. The results of Figure 3K demonstrated direct interaction between FOXM1 and MATα2 and MAT2β but cannot demonstrated that FOXM1 and MATα2 and MAT2β activate the transcription of each other.
Response: Please see Figure 3d-f, where we show clearly that they regulate each other at the promoter level, as silencing any of the three lowered the promoter activity of the other two. Figure 3j shows these proteins bind to the FOX element on EMSA+supershift and Supplemental Figure 7 confirms the reciprocal positive regulation of these three at the protein level in HSCs.

14. In addition to inhibiting the transcription of FOXM1, FDI-6 can also bind to FOXM1 protein and inhibit the transcription of FOXM1 activating genes (3). Does FDI-6 bind to both MAT2A and MAT2B proteins, thereby directly affecting the transcription of their genes?
Response: Since the two MAT proteins are structurally different from FOXM1, it is unlikely that FDI-6 would bind to them. However, since FDI-6 binds to FOXM1, it is possible that it may interfere with FOXM1 binding to MATα2 and MAT2β. To address this, we have performed additional experiments and found that indeed, FDI-6 also lowered the interaction between FOXM1 and MATα2 and MAT2β. This new result is included in revised Supplemental Figure 3f-g.

15. BDL surgery first caused cholangiocytes injury and Figure 4H showed significant changes of FOXM1, MATa2 and MAT2β expression in cholangiocytes. Why was Foxm specifically silenced in HSC cells and KC cells only, but not in cholangiocytes?
Response: Thank you for the question. We focused on the three cell types because 1) hepatocytes are the dominant cells and albumin-cre also deletes FOXM1 in cholangiocytes, and

2) FOXM1 is known to be involved in fibrosis and inflammation, making HSCs and KCs more important.

16. In Figure 4, knocking out Foxm1 in hepatocytes can also inhibit bile duct response. Does this mean that knocking down Foxm1 in cholangiocytes can also alleviate bile duct reaction? Or does the low expression of Foxm1 in hepatocytes inhibit the bile duct response by releasing a stimulating factor through some extracellular vesicle?
Response: ~~A~~lbumin-Cre also deletes FOXM1 in cholangiocytes, but ductular proliferation was not protected in the HSC and KC-specific knockouts, suggesting that it is not an important marker for liver injury and fibrosis under these experimental conditions. Note that in the experiments where KO cells were treated with EVs from different cell types, the EVs were obtained from cells isolated from BDL livers (in order to have higher levels). Cells treated with EVs from KO cells had lower expression of these three proteins as compared to cells treated with EVs from WT cells (Figure 8A-F). This is likely due to the finding that after BDL, WT cells release EVs that contain more FOXM1, MATα2 and MAT2β (Supplemental Figure 14g).

17. As shown in Figure 4, why did the protein content of Foxm1 in HSCs decrease when Foxm1 was knocked out in hepatocytes, but the mRNA content did not change?
Response: Densitometry for Figure 4H is shown in Supplemental Figure 11c, which includes western blots from 3 independent experiments and the expression of the three proteins in HSCs was not significantly different in the sham groups.

18. As shown in Figure 5, the specific knockdown of Foxm1 in HSCs did not affect Foxm1, mat2a, and mat2b in hepatocytes, cholangiocytes, and KCs cells. Does it mean that Foxm1 cells mainly achieve intercellular communication through hepatocytes instead of HSCs.
Response: Please see response to 17. When multiple experiments were analyzed, there were no significant differences in Foxm1 expression in HSCs in Hep specific Foxm1 KOs under the sham condition.

19. After knocking out Foxm1 in various liver cells, it was observed that not all of these cells were able to decrease the Foxm1 content in other cells. The question arises as to why different cells are targeted by extracellular vesicles. Although extracellular vesicles can be secreted by various cells, not all cells could absorb them. What is the underlying reason for this selective absorption?
Response: We were intrigued by this result as well. One consideration is the spatial relationship of these cells. For instance, hepatocytes and HSCs are in close contact with each other but KCs are in the sinusoids, separated from the other two cell types by sinusoidal endothelial cells. However, KCs can influence the other cell types via release of proinflammatory cytokines. We have added this speculation in the revised discussion.

20. The results in Figure 4 show that Foxm1Hep-/- mice have lower BDL-induced injury and fibrosis, while LX2 cells and macrophages were selected in the in vitro experiment in Figure 7, why not simultaneously study hepatocytes?
Response: We focused on HSCs and macrophages because FOXM1 has been implicated in fibrosis and inflammation. Normal hepatocytes have very little expression of FOXM1/MAT2A/MAT2B.

21. As shown in Figure 8E, ductular proliferation and inflammation can be reduced in Foxm1Hep-/- mice, but only the number of F4/80 and CK19 positive cells were detected, so the proof lacks credibility.

Response: New Figure 8G is just a summary of the main findings. CK19 positive cells correlated well with ductular proliferation and F4/80 is a marker for macrophages. Ductular proliferation turned out not to be a driver of the liver injury since HSC and KC-specific knockouts had no change in this parameter. Regarding inflammation, we are not only using the F4/80 number as proof that the FOXM1-MAT2A/2B axis is important. Figures 7D-E show clearly that FOXM1 overexpression increased the expression of TNF-α and IL-6, which was attenuated by silencing either MAT2A or MAT2B.

22. The experiment in Figure 8 also needs to directly detect the contents of EVs secreted by liver cells, thus effectively proving that liver cells secrete EVs containing FOXM1/MATα2/MAT2β.
Response: This was done and shown in Supplemental Figure 14g.

Reviewer #4:

In this work, Bing Yang and the colleagues focused the mechanism that how FOXM1/ MAT2A/ MAT2B axis regulates the liver fibrosis. They first found the upregulation of FOXM1/ MAT2A/ MAT2B in human and animal fibrotic liver sample with different etiologies. They proved FOXM1 inhibitor FDI-6 could prevent and reverse the liver fibrosis in CCl4-induced model. In mechanism, the authors proved that FOXM1/MAT2A/MAT2B could co-occupied the FOX elements in promoters of themselves and upregulated their expression in transcript level. In view of complicated contribution of HSCs, KCs or other non-parenchymal cells in liver fibrosis, the authors established the organ and cell-specific knockout animal model and build the BDL-induced liver fibrosis model. Through analysis of primary cells from gene-editing model, the authors found the cell-cell crosstalk of FOXM1/MAT2A/MAT2B through EVs. The work has some strengths: 1) found the reciprocal positive regulation of FOXM1/MAT2A/MAT2B through sequential ChIP experiment which could be a novel therapeutic target in hepatic fibrosis, inflammatory or malignancy; 2) thus far, few research reported the FOXM1/MAT2A/MAT2B function in EVs. The authors comprehensively analyzed the contribution of non-parenchymal cells to liver fibrosis in microenvironment and highlighted the significance of FOXM1/MAT2A/MAT2B-contained EVs in this process.
Response: We are grateful for the reviewer's positive comments.

Questions
1. How to explain the different change of FOXM1, MATα2 and MAT2β in KCs and cholangiocytes with HSCs and hepatocytes. (the capacity to receive the EVs is different among them?) If so, the mechanism diagram should be revised.
Response: Thank you for pointing this out. Indeed, although EVs can influence the expression of FOXM1 in KCs when treated in vitro, this does not appear to be the case in vivo. One consideration is the spatial relationship of these cells. For instance, hepatocytes and HSCs are in close contact with each other but KCs are in the sinusoids, separated from the other two cell types by sinusoidal endothelial cells. We have added this speculation in the revised discussion. We have also revised the summary diagram in Figure 8G.

2. The authors didn't provide the morphological evidence for EVs (like TEM or particle size analysis), and how to prove that they are not contaminated by exosomes? No FOXM1 or MAT protein-contained EVs can be find in immunofluorescence results in Figure 1B and Figure 1E.
Response: We agree and characterization of the EVs using Nanosight have been added to Supplemental Figure 14h. Figure 1B and 1E are immunofluorescent images of the cells so we cannot visualize the EVs under such low magnification.

3. The histological feature of CCl4-induced liver fibrosis is the diffuse HSCs activation in the hepatic lobule while BDL mainly induce the cholestatic liver fibrosis which begins from the portal area. Why the authors choose BDL model to study the cell-cell EVs crosstalk but used CCl4 model to study liver fibrosis prevention and treatment?
Response: The reason we chose the BDL model to study cell-cell EVs crosstalk is because it takes much less time to establish the BDL model and there is less toxicity to the animals. These two models were compared in Acta Cir Bras. 2012 (8):589-94.

4. In introduction, “MAT2A and MAT2B are expressed in extrahepatic tissues and non-parenchymal cells of the liver, such as HSCs and Kupffer cells (KCs)”, but in Figure 4H, 5H and 6H, if the protein loading mass is equal, the MAT2A and MAT2B levels are comparable in hepatocytes with HSCs and KCs.
Response: This is an excellent point. In order to enhance signals, western blots of hepatocytes were exposed for much longer periods as compared to blots for HSCs or KCs. On IHC hepatocytes have little to no expression of these proteins. To illustrate the difference between hepatocytes and the nonparenchymal cells of the liver, we have added real-time PCR comparing the expression of MAT2A and MAT2B in the three different cell types. As shown in Supplemental Figure 1e, hepatocytes have much lower expression of *Mat2a* and *Mat2b* as compared to HSCs and KCs.

5. According to data of Figure 4H, 5H and 6H, the KCs can’t be regulated by EVs from HSCs or hepatocytes. In addition, the FOXM1, MATα2 and MAT2β of cholangiocytes only changed in hepatocyte-specific knockdown group, which is different from hepatocytes, HSCs or KCs. The authors should explain the underlying mechanism and may provide more related data in this aspect.
Response: Albumin-Cre also deletes FOXM1 in cholangiocytes (Hepatology 2015;61:2100-2103). In vivo the cholangiocytes do not appear to be influenced by the EVs released by the other three cell types. At present we are not sure of the underlying mechanisms, but one speculation is how the different cells receive their blood supply. Bile duct epithelial cells receive their blood supply from the hepatic artery (Compr Physiol 2013; 3(1): doi:10.1002/cphy.c120019). It is possible that hepatocytes release EVs mainly into sinusoidal blood under our experimental conditions. We have added this to the discussion.

6. Although the authors used the FOXM1 OV and MAT protein knockdown cells to prove the mechanisms that FOXM1 functions in transcription promotion of FOXM1/MAT2A/MAT2B axis in Figure 7E, the authors didn`t provide any other data to strengthen the importance of FOXM1-MAT2A-MAT2B complex. MAT2A and MAT2B seem to be dispensable in this work, because only FOXM1 can initiate and promote the regulation axis.
Response: Transcriptional regulation of the three genes is shown in Figure 3. Silencing FOXM1, MAT2A and MAT2B individually resulted in comparable lowering of the three promoters. We also provided evidence using ChIP and Seq-ChIP that MAT2A or MAT2B overexpression enhanced FOXM1 binding to the promoter, and the opposite was true of MAT2A or MAT2B knockdown (Fig. 3G-H). Lastly, these proteins directly interact and bind to the FOX elements on EMSA (Fig. 3I-J). These data show clearly MAT2A and MAT2B are important components of this axis and are not dispensable. Indeed, FOXM1’s profibrogenic and proinflammatory effects were significantly attenuated if MAT2A or MAT2B was silenced (Fig. 7).

7. In Figure 2E, the authors presented low-resolution fluorescence images showing separate co-localization of MATα2 and MAT2β with FOXM1. However, the limited image resolution

compromised the effectiveness of co-localization visualization. Additionally, high-resolution co-localization fluorescence images of MATα2, MAT2β, and FOXM1, confirming their formation as a complex within the cellular context, were lacking.
Response: We have repeated and replaced Figure 1E with higher-resolution images. In Figure 3L using liver lysates from different conditions, we provided evidence that the three proteins interact using co-IP and the interaction was enhanced after BDL.

8. In Figure 7, the authors employed TGF-β treatment to stimulate HSC activation and evaluated downstream SMAD3 signaling. However, studies investigating the TGF-β/SMAD3 pathway typically assess the phosphorylation level of SMAD3 rather than solely examining changes in SMAD3 protein levels. It is recommended to include Western blot results for phosphorylated SMAD3 as a supplementation.
Response: We thank the reviewer for this comment and have done as suggested and added p-SMAD3 to the western blots in Fig. 7a-c.

9. Why the same signal pathway can function in different cell phenotype? What is the underlying mechanism?
Response: FOXM1 is a transcription factor that regulates many genes important in inflammation and growth as an oncogene. It is induced in hepatocytes, HSCs and KCs in fibrotic liver injuries. Others have shown hepatocyte-specific FOXM1 transgenic mice spontaneously develop liver inflammation, injury, fibrosis and HCC (reference #3). What we are finding in our current work is that FOXM1 requires MAT2A and MAT2B to exert its full effects and the three form a complex that can feedforward to perpetuate the fibrotic and inflammatory process.

10. In Figure 1 and Figure 2, the authors focus on the HCS activation, but study the KCs and other cells in subsequent research, which makes readers confused.
Response: We apologize for the confusion and have revised the text to make the flow easier to follow. We started with HSC activation because of its known effect in liver fibrosis (FOXM1 transgenic mice develop spontaneous liver fibrosis). Since it is known to activate NF-kB, we decided to study KCs. We added hepatocytes because on IHC FOXM1 expression is greatly induced in hepatocytes following BDL and CCl_4 treatments.

11. Why to chose FDI-6 to inhibit the FOXM1? Can FDI-1 inhibit the binding between FOXM1, MAT2A and MAT2B?
Response: This is an excellent question and we have done additional experiments to address this. FDI-6 also lowered the interaction between FOXM1 and MATα2 and MAT2β (see revised Supplemental Figure 3f-g).

12. Figure 1E shows that FDI-6 treatment reduced cytoplasmic and nuclear staining of FOXM 1, MATα2, and MAT2β in LX 2 cells; in particular, the expression of MAT2β changed to cytoplasmic expression; what is the potential significance of this change in intracellular localization of protein expression, and what is the reference value of this study?
Response: In Fig. 1E both cytoplasmic and nuclear levels of MAT2β appear lower after FDI-6 treatment. However, western blots of cytoplasmic and nuclear fractions from BDL livers shown in Fig. 1f (densitometry shown in Supplemental Fig. 3c-d), the drop in MAT2β was not as much as the other two proteins and did not reach statistical significance. This suggests FOXM1 and MATα2 likely played a more dominant role on changes in gene expression.

13. In this study, extracellular vesicles were used as mediators of cell-to-cell interactions, and the vesicles were supposed to be non-selective, but as the results show, not all cells responded to this change, how can this phenomenon be explained?
Response: Thank you for the excellent question and as mentioned in the response above, one consideration is the spatial relationship of these cells. For instance, hepatocytes and HSCs are in close contact with each other but KCs are in the sinusoids, separated from the other two cell types by sinusoidal endothelial cells. We also speculate whether the blood supply might influence in vivo access of cells to the EVs in the circulation. For instance, bile duct epithelial cells receive their blood supply from the hepatic artery (Compr Physiol 2013; 3(1): doi:10.1002/cphy.c120019). It is possible that hepatocytes release EVs mainly into sinusoidal blood under our experimental conditions. We have added these in the revised discussion.

Minor flaws
1. Supplementary Figure 1b FOXM1 staining BDL D14 magnificent figure seem to be a wrong indication.
Response: We have reviewed the images to make sure they are correct.

2. There is no scale bar in some of figures such as Figure 1E and B and Figure 2A.
Response: We have added scale bars to all images.

3. Could the author explain why used Tublin as cytoplasm control instead of Actin for Western blotting analysis in Figure 1F?
Response: This project has taken a long time to complete and different individuals have been involved and used different housekeeping controls. Both are acceptable as housekeeping control for the cytosol.

4. The data of sequential-ChIP confirm the co-occupation of MAT2 and FOXM1 protein on FOX elements, but more details of the experiment should be provided (like beads used, condition of antigen-antibody complex formation).
Response: We have expanded the Methods section on ChIP and Seq-ChIP.

5. In Figure 3L, using FOXM1 antibody to performed the IP experiments is irrational, since the FOXM1 have already been knockout.
Response: We included it as a negative control.

6. The magnification times are different among the panels in Figure 4A and Figure 5A.
Response: We have reviewed all panels to make sure the descriptions in the legends are correct.

7. TNF-α and IL-6 are exocrine proteins should be measured by ELISA.
Response: This is an excellent point and we have repeated the experiment using RAW cells (to avoid sacrificing more mice to get Kupffer cells, since the results are the same). The new data is now shown in Supplemental Figure 13e.

8. Figure 1B isolates HSC in the mouse model on day 5, and the text describes day 7.
Response: Thank you for catching the error. We have corrected the mistake.

9. Figure 1C vertical axis title (mRNA in HSC fold change of Day1) is wrong.
Response: We have changed it to mRNA levels, fold of Day 1.

10. In Figures 4-6A, the authors performed staining for multiple markers and conducted

statistical analysis to demonstrate the impact of FOXM1 on fibrosis. However, there was a lack of statistical analysis for the staining results of SMA (smooth muscle actin). It is recommended to include supplementary statistical analysis for SMA staining.
Response: We have added this to the revised panel g of Figures 4-6, with the raw data included in the source file.

11. In Figure 7D, there is a mosaic artifact observed on the band of β-ACTIN in the last lane. It is recommended to resubmit all the original image to address.
Response: We have replaced the image.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

none

Reviewer #2 (Remarks to the Author):

The authors answered the questions, and the manuscript is much improved. I have no further comments.

Reviewer #4 (Remarks to the Author):

We believe the author has provided appropriate responses and experimental supplements to the previous questions. We acknowledge the author's answers and currently have no further questions. We respect the editorial decision of the journal.