nature portfolio

Peer Review File

Dysregulated RNA splicing impairs regeneration in alcohol-associated liver disease

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is a very innovative and comprehensive analysis identifying a role for dysregulated RNA splicing in liver of patients with severe AH and alcohol-cirrhosis. Previous work in the field identified a failure of hepatocytes to regenerate into their fully differentiated forms in sAH; the data presented here put forth a plausible mechanism for this problem related to perturbations in RNA splicing. It is very interesting that many previously observed phenomena in sAH livers and in murine models of ALD, such as abnormal localization of key transcription factors to the nucleus, can be accounted for by the RNA splicing issues identified here. This work will open-up multiple new avenues of research related to ALD.

I have a few suggestions for comment/discussion:
1) Given that the authors have previously published an impact of ESPR KO in a murine model of ALD, this should be stated in multiple places in the manuscript. For example, in Results, at the intro to ESPR2 (about line 164) and then they should indicate that this previous work in a different model is consistent with the current chronic plus multiple binge (Figure 6)
2) Any comment on why the responses studied differ to some degree in AH vs AC, despite the similar exposure to alcohol toxicity.
3) There has been a fair bit of research related to epigenetic changes in sAH--can the authors comment whether the differences in chromatin accessibility seen in their ATAC seq data is related to epigenetic changes?
4) I have some concerns about the use of AML12 cells—as the authors are focused on the differentiation state of hepatocytes, these experiments might be better done in organoids or spheroids. At least, the authors can comment on the limitations of the use of AML12 cells.
5) Regarding the studies in the ESPR2 KO mice: since these mice have already been studied by the authors, the use of the alternative model is somewhat incremental. Is there a hepatocyte specific KO available, or perhaps using AAV to KO ESPR2 in hepatocytes would more directly target hepatocyte mechanisms. What is the background of the KO mice BL6/J or N?

Minor comment:
1) results on line 244/Figure 5 are overstated—data from the AML12 cells is consistent with the hypothesis/data from AH is more appropriate than the “data indicate”

Reviewer #2

(Remarks to the Author)
This manuscript represents a very comprehensive analysis of transcriptomic changes in alcoholic liver disease. Overall I find the data fascinating and the authors model for liver disease compelling. There are a number of issues with the manuscript however, that I think should be addressed. I found it difficult to follow the data analysis as the description in the results and figure legends is VERY sparse. More information is available in the methods but is fairly generic. The only methods were

detail is given is for the zonation analysis. In a manuscript with such an extensive bioinformatic analysis I would expect tables of changes in expression, splicing, accessibility etc. be available as supplementary tables but none of the analyzed data was provided. The raw data as well as the analyzed data should be made available either directly or from a repository.

Many figures appear to show differences in cell populations or gene expression but statistical significance is not given. For example Fig 1c shows increases in T cells and macrophages in SAH and AC but are those changes significant? Fig. 1d shows the distribution of zonation values which look the same but the authors claim they are different. Fig. 1f the authors claim modest increases in TNF and IL1b but I don't see it in the figure. Fig. 1e is a typical Seurat single cell expression plot. Are these the top 6 genes that identify the cell populations from the Seurat analysis or were these curated genes that were picked for their known roles? Do the misregulated processes on the right correspond to the individual gene or to the whole group of genes like an enrichment analysis? Which differentially expressed genes indicated inflammation associated pathways? Just the 6 given for each cell?

The authors investigate the "activity" of TFs by examining chromatin accessibility of targets. As the authors point out later in the paper the correlation of accessibility and gene expression is weak, so this is not really measuring TF activity. The adult liver show decreased accessibility around motifs for the TFs HNF4A, CEBPA and FOXA2 in SAH but not in AC. Why would the AC samples not show a decrease given that disease has progressed further? Similarly, the cell population differences are greater in SAH than AC. If the TF activity is decreased according to the ATACseq, then the genomic localization for the TF should show decreased accessibility, but the opposite is seen. All 3 TFs seem to show increased accessibility in the SAH samples. The discrepancy needs to be addressed. Also the data for HNF1A and B show that AC decreases accessibility but SAH does not (lettering needs to be consistent for these groups). Moving on to the precursor TFs, there is no accessibility around the motifs for SOX9, RELA or E2F1 in 2b and no changes in SAH or AC. The authors conclude that there is an incomplete transition to a progenitor state as there is no increase in SOX9, RELA or E2F1 "activity" despite increases in the proteins. This is based on negative results, i.e. not seeing a change in something. This is a difficult case to make as there could be many different explanations for a null result. Is there any change in genomic accessibility in the SOX9, RELA or E2F1 genes that would mirror the increase in expression? The expression values in Supp Fig 4 are also a concern as the scSeq data contradicts the bulk RNAseq data, e.g. HNF4A ETS2 etc.

How did they pick the three loci in Fig. 3c? Heatmap in 3d is very low resolution as is the other heat map in Fig S5. Fig 5g is Venn diagram showing overlap of SAH and AC altered SEs. Does this figure really say that none of the splicing changes are in common? Where are the tables of splicing changes by rMATS? The authors verify selected SEs by RT-PCR. They need to state which exon or splice site is being described. The nomenclature used is the length of the exon which is not informative. Perhaps the exon number should be given.

There is confusion in the manuscript regarding TCF4. The authors refer to TCF4 but in fact they mean TCF7L2 on chr 10. TCF4 is a separate gene on chromosome 18. The length of the TCF7L2 exon is 73 so this is an out of frame deletion. The authors need to explain what happens to this transcript isoform. The exon is at the C-terminus and causes a different C-terminus. As it is at the end, it might not cause NMD and the protein could be expressed.. Fig 5n makes it look like this exon is in frame. The SLK exon 13 is a known in-frame alternative exon.

No details are given for the Slk ASO. It seems to be very efficient unlike the TCF4 ASO. In the ASO experiments it looks like the overall expression of SLK and TCF4 is decreased. Should be quantified. TCF4 ASO has very little effect on target genes in 5m. Is this because the change in splicing is so weak? Are the changes significant? Can't tell from the heat map. Authors describe the TCF4 exon as containing an ER retention signal. The speckled staining does not look like ER, so mis this really a ER signal?

Fig 6a and b need better description of the tracks. Are the green and blue tracks RNAseq counts? And the top track eCLIP counts?

Figure 7 is not described adequately. What are we looking at in 7A?

Reviewer #3

(Remarks to the Author)
The current study perfoemed bulk and single-nucleus RNA- plus ATAC-seq in healthy liver and livers with end-stage alcohol-related liver disease (ALD). The authors found profound changes in RNA binding protein (RBP) expression, that may cause misregulation RNA splicing. The authors propose that changes in stromal cell populations enrich failing ALD livers, which suppresses ESRP2-driven epithelial splicing program and replaces functional parenchyma with quasi-progenitor-like cells lacking liver-specific functions.

1. The main weakness of the study is that ALD samples come from explant livers, from both advanced cirrhosis and alcohol-related hepatitis (AH) that have not respond to medical therapy. These livers typically show cirrhosis and marked liver failure with a massive ductular reaction and poor hepatocyte regeneration. Whether the results apply to earlier forms of ALD and compensated cirrhosis is unclear. While I understand that explant tissue allows the collection of larger liver tissue, this is a clear limitation of the study. The authors should perform some feasible studies in tissue other than explants to confirm their hypothesis.

2. In fact, the animal model used in this study (i.e. control Lieber-DeCarli diet ad libitum for 5 days and then access for 20 days to the ethanol diet plus two doses of high ethanol content by gavage) does not cause liver failure, jaundice or

advanced fibrosis, all features present in human samples. Although it is known that ethanol per se in unable to cause jaundice and cirrhosis in mice, the authors should confirm these results in an alternative model of more advanced liver disease.

3. Some of the conclusions of the study are too strong and should be tempered. For example, the statement that “altered immune milieu in ALD promotes a quasi-progenitor state of hepatocytes” is not fully proven by the data. Association of biological findings that could be related does not necessarily mean causality. The authors should temper their conclusions throughout the manuscript.

4. The role of inflammation (eg, PMNs) in ALD differs from early forms to severe AH. This should be acknowledged in the manuscript.

5. The authors should discuss the potential limitations of using nuRNAseq instead of scRNAseq, including the lack of mitochondrial DNA, etc.

6. The term “regeneration failure” is not commonly used and it can be confused with “liver failure”. I propose “impairs liver regeneration” or similar.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed all my concerns

Reviewer #2

(Remarks to the Author)
I am happy with the revised manuscript

REVIEWER COMMENTS

We thank the Reviewers for their positive comments and constructive evaluation of our work. We have now performed additional experiments and statistical analyses that address the comments made by the Reviewers; we have also modified and clarified the text of the manuscript accordingly. Our point-by-point responses are listed below.

Reviewer #1 (Remarks to the Author):

This is a very innovative and comprehensive analysis identifying a role for dysregulated RNA splicing in liver of patients with severe AH and alcohol-cirrhosis. Previous work in the field identified a failure of hepatocytes to regenerate into their fully differentiated forms in sAH; the data presented here put forth a plausible mechanism for this problem related to perturbations in RNA splicing. It is very interesting that many previously observed phenomena in sAH livers and in murine models of ALD, such as abnormal localization of key transcription factors to the nucleus, can be accounted for by the RNA splicing issues identified here. This work will open-up multiple new avenues of research related to ALD.

We are delighted by the interest and comments of the Reviewer. Thank you for appreciating our work and for providing thoughtful suggestions and feedback.

I have a few suggestions for comment/discussion:

1) Given that the authors have previously published an impact of ESRP KO in a murine model of ALD, this should be stated in multiple places in the manuscript. For example, in Results, at the intro to ESPR2 (about line 164) and then they should indicate that this previous work in a different model is consistent with the current chronic plus multiple binge (Figure 6)

Thanks for pointing out this omission. We have now cited our previously published ALD-related ESRP2 KO paper[1]; and added the information about our earlier ESRP2 KO work with 10 Day + single binge NIAAA model to the revised manuscript **(line 185; and lines 320-321)**.

2) Any comment on why the responses studied differ to some degree in AH vs AC, despite the similar exposure to alcohol toxicity.

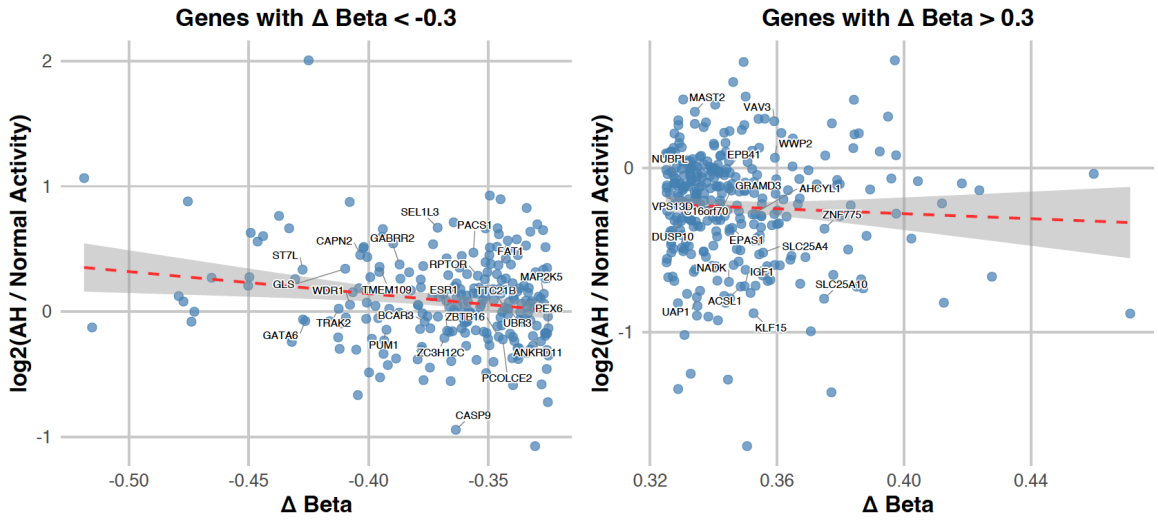
Exposure to alcohol toxicity *differs* in patients with severe acute alcohol-related hepatitis (SAH) and alcohol-related cirrhosis (AC). Typical patients with SAH who require liver transplantation were drinking alcohol excessively until around the time they became sick enough to require hospitalization for acute liver failure. Liver transplantation is offered to a subset of these SAH patients who develop *rapidly progressive hepatic decompensation and multiple organ failure despite discontinuing alcohol* and receiving maximal medical care. Thus, at the time of liver transplantation, SAH patients have typically been "off" alcohol for only a couple of weeks and liver replacement is necessary to survive *acute* liver failure[2-4]**.** In contrast, hepatic decompensation ensues gradually (i.e., over months to years) in patients with AC who are deemed eligible for future liver transplantation. Transplant eligibility criteria typically mandate a minimum 6-month period of documented sobriety before transplantation. So, AC patients have typically been "off" alcohol for months to years at the time of transplantation and require a new liver to survive *slowly progressive hepatic decompensation (and secondary failure of other organs)* that have evolved *despite prolonged abstinence from alcohol*[5,6]. Hence, although hepatic

dysfunction progresses despite discontinuation of alcohol in both SAH and AC patients, the speed and intensity of decompensation is dramatically different in the two groups (stated another way, SAH patients are "*rapid progressors*" and AC patients are "*slow progressors*" to liver failure). As alcohol-related liver disease generally improves as the duration of sobriety increases, this difference in disease progression in SAH and AC patients is due, in part, to differences in proximity to alcohol exposure. However, more research is needed to determine the basis for inherent inter-individual differences in the susceptibility to alcohol-induced liver failure. Such research must also address persistent knowledge gaps about the basis for *intra*-individual variations in susceptibility to hepatic decompensation as individuals with slowly progressive AC can suffer a superimposed episode of SAH, leading to "acute-on-chronic" liver failure despite seemingly steady levels of alcohol intake.

3) There has been a fair bit of research related to epigenetic changes in sAH--can the authors comment whether the differences in chromatin accessibility seen in their ATAC seq data is related to epigenetic changes?

We thank the Reviewer for raising this important question. We used a published dataset for genome methylation from Unaffected and SAH patient liver samples[7] to compare gene loci that show a significant change in methylation status (SAH vs. Unaffected), with their respective genome accessibility in our snATAC-seq dataset **(See below)**. We observed a weak pattern of negative correlation (Pearson corr –0.45), wherein hypomethylated genes (delta_beta < -0.3) showed increased accessibility in SAH samples and hypermethylated genes showed reduced accessibility.

The weak correlation might stem from the fact that the methylation data is based on bulk tissue measurements while the accessibility data is cell type specific. Future work analyzing epigenetic patterns of specific cell types in disease conditions is likely to shed more light on the relationships between epigenetic changes and genome accessibility in SAH livers.



4) I have some concerns about the use of AML12 cells—as the authors are focused on the differentiation state of hepatocytes, these experiments might be better done in organoids or spheroids. At least, the authors can comment on the limitations of the use of AML12 cells.

Thank you for raising this concern. We agree that AML12 cells come with certain limitations. We have acknowledged these limitations in the revised manuscript **(lines 359-367)**.

5) Regarding studies in the ESPR2 KO mice: since these mice have already been studied by the authors, the use of the alternative model is somewhat incremental. Is there a hepatocyte specific KO available, or perhaps using AAV to KO ESPR2 in hepatocytes would more directly target hepatocyte mechanisms. What is the background of the KO mice BL6/J or N?

We appreciate the Reviewer's suggestion to use a hepatocyte-specific KO model for ESPR2. Our study employed whole-body ESPR2 KO mice on a C57BL/6J background, which successfully recapitulated many features of human SAH, including splicing dysregulation, TGF-β-mediated inflammation, and impaired regenerative capacity. Earlier work by us and others showed that ESRP2 expression is restricted to mature epithelial cells and thus, ESRP2 is ~ exclusively expressed by hepatocytes in the adult liver[8,9]. However, we acknowledge that hepatocyte-specific KO models (e.g., AAV-based targeting of ESPR2 in hepatocytes) could further isolate the role of ESPR2 in liver parenchymal cells and minimize confounding systemic effects. We plan to explore these hepatocyte-targeted approaches in future studies to build on the findings from the whole-body KO model and provide additional mechanistic insights into ESPR2's role in liver pathology. Thank you for this valuable suggestion.

Minor comment:

1) results on line 244/Figure 5 are overstated data from the AML12 cells is consistent with the hypothesis/data from AH is more appropriate than the "data indicate"

Thank you for pointing this out. As suggested, we have toned down this conclusion in the revised manuscript **(line 266)**.

Reviewer #2 (Remarks to the Author):

This manuscript represents a very comprehensive analysis of transcriptomic changes in alcoholic liver disease. Overall, I find the data fascinating and the authors model for liver disease compelling.

We appreciate the Reviewer's interest and comments, and we share the enthusiasm about the comprehensive data analyses and compelling findings presented in our manuscript.

1. There are a number of issues with the manuscript however, that I think should be addressed. I found it difficult to follow the data analysis as the description in the results and figure legends is VERY sparse. More information is available in the methods but is fairly generic. The only methods where detail is given is for the zonation analysis. In a manuscript with such an extensive bioinformatic analysis I would expect tables of changes in expression, splicing, accessibility etc. be available as supplementary tables but none of the analyzed data was provided. The raw data as well as the analyzed data should be made available either directly or from a repository.

We thank the reviewer for this comment, and we are committed to making the vast amount of data and analysis generated in this manuscript easily accessible to readers.

We have deposited the data generated in this manuscript into publicly accessible GEO/SRA server with the accession number **GSE281574**. This submission includes all raw data and metadata corresponding to the multi-omics experiments. Further, we have included the processed Seurat file as an RDS object, which is a package containing all processed data used for deriving conclusions, such as RNA-seq counts and ATAC-seq based accessibilities for SAH, AC and Unaffected individuals. This file can be downloaded and be readily used to plot gene-expressions, UMAP-plots and accessibilities. This file also contains metadata that includes cell type annotations. We apologize that while the GEO accession number was added in the initial submission, it was not mentioned in the manuscript text. We have now added a "data availability" section to the manuscript and included the GEO accession number. Bulk RNA sequencing data is deposited under the accession number **GSE143318**.

We have also added detailed steps in the Methods section to describe how multiome data was processed. This explains steps including obtaining RNA-seq and ATAC-seq matrices, cutoffs used, batch correction procedure, doublet removal, dimensionality reduction, cell clustering, cell type identification and motif identification.

Further, we have now included the following excel sheets in the **Source Data** file with processed data that corresponds to analysis we have performed in the manuscript:

1. All alternative splicing events identified from SAH patients relative to Unaffected controls. The excel sheet is grouped into sheets with different types of alternative splicing events.
2. All alternative splicing events identified from AC patients relative to Unaffected controls. The excel sheet is grouped into sheets with different types of alternative splicing events.
3. Alternative splicing datasets from ethanol-fed WT C57Bl/6 and ESRP2 KO mice compared to pair-fed WT C57Bl/6 mice.
4. Excel sheet containing raw gene expression counts calculated from human bulk RNA-seq data.
5. Top gene markers enriched in SAH livers compared to Unaffected livers for each cell type. The table shows data obtained from each cell type for SAH vs Unaffected liver comparison using 'findmarkers' module in Seurat. Genes that satisfy Avg. log fold change| > 1 and Adj. Pvalue < 0.05 were filtered to obtain the top candidates.
6. Gene ontology analysis performed using top differential genes marking each cell type in SAH livers.

2. Many figures appear to show differences in cell populations or gene expression but statistical significance is not given. For example, Fig 1c shows increases in T cells and macrophages in SAH and AC but are those changes significant?

We thank the Reviewer for pointing out the lack of statistics shown for various analysis. We agree, and in response, we have added several statistical tests in the revised manuscript.

Chi-squared test was performed on cell numbers obtained from SAH, AC and Unaffected livers. This analysis gave a p value <0.0001 implying statistically significant change in cell populations depending on conditions investigated.

2a. Fig. 1d shows the distribution of zonation values which look the same but the authors claim they are different.

In the updated manuscript we performed Kolmogorov-Smirnov (K-S) test in **Fig. 1d** to demonstrate statistically significant change in diseased livers compared to Unaffected controls. This is also demonstrated using Quantile-Quantile plots shown in **Supplementary Fig. 2a**. Detailed descriptions are provided in legends and methods sections.

2b. Fig. 1f the authors claim modest increases in TNF and IL1b but I don't see it in the figure.

We have now updated heatmap in **Fig. 1F** to also show average gene TPM values that were used to generate the figure. We can see that the average TPM for IL1B increases from 2.66 in Unaffected individuals to 3.99 in livers. Similarly, TPM for TNF increases from 0.02 in Unaffected individuals to 0.05 in SAH livers. Average TPM values for TGFB1 is 36.26 in SAH livers compared to 13.28 in Unaffected livers. We have also provided raw counts in the **Source Data** file that we obtained from bulk human RNA-seq data, from which TPM values were derived.

3. Fig. 1e is a typical Seurat single cell expression plot. Are these the top 6 genes that identify the cell populations from the Seurat analysis or were these curated genes that were picked for their known roles? Do the misregulated processes on the right correspond to the individual gene or to the whole group of genes like an enrichment analysis? Which differentially expressed genes indicated inflammation associated pathways? Just the 6 given for each cell

We used 'findmarkers' module in Seurat analysis to determine genes that distinctly mark each cell type in SAH compared to Unaffected individuals. The dot plot in **Fig. 1e** shows top 6 genes obtained from this analysis for each cell type. Further, the entire set of genes that were found to be significantly different (|Avg. log fold change| > 1, Adj. Pvalue < 0.05) were used for gene ontology analysis, hence correspond to more than the 6 genes shown in heatmap.

We thank the Reviewer for this question as this provides us a chance to present our data better. We have now included tables showing outputs from Seurat analysis showing SAH-specific marker genes for each cell type (see **Source Data**). Inflammation-associated pathways are represented by genes such as C-reactive protein (CRP), chitinase 3 like 1 (CHI3L1), serpin family A member 3 (SERPINA3) etc. We have now included excel sheets that show entire list of enriched gene ontology terms for each cell type, and the lists of genes that they correspond to in **Source Data**.

4. The adult livers show decreased accessibility around motifs for the TFs HNF4A, CEBPA and FOXA2 in SAH but not in AC. Why would the AC samples not show a decrease given that disease has progressed further? Similarly, the cell population differences are greater in SAH than AC.

As discussed under our response to Reviewer 1's comment 2, the effects of alcohol toxicity differ significantly between patients with SAH and those with AC. SAH patients requiring liver transplantation often drink heavily until their health deteriorates to the point of hospitalization for acute liver failure. They typically have stopped drinking for only a few weeks at the time of transplantation and urgently need a new liver to survive[4]. In contrast, AC patients experience gradual hepatic decompensation over years, and guidelines usually require them to demonstrate at least six months of sobriety before transplantation[5,6]. While both groups struggle with progressive hepatic dysfunction, the pace of decline is markedly different. SAH patients are "rapid progressors," experiencing swift deterioration, whereas AC patients are "slow progressors," facing a prolonged journey toward liver failure.

Another key difference between SAH and AC livers is the high degree of liver inflammation present in SAH in comparison to AC. The inflammatory cytokines produced in SAH livers reprogram the hepatocyte transcriptome to an immature cell state with reduced hepatic functions that are driven by rewiring of gene expression at the chromatin, transcriptional and post-transcriptional levels[1]. We find that this reprogramming includes the striking depletion of ESRP2, which collapses its downstream splicing program in SAH livers. Interestingly, ESRP2 deficiency in murine hepatocytes results in significant stunting of HNF4A- and CEBPA-driven gene programs[9], indicating that the absence of ESRP2 hinders hepatocytes from maintaining a mature gene expression profile.

Notably, ESRP2 is *much less downregulated* in AC compared to SAH livers **(Fig. 3d)**, supporting the hypothesis that loss of ESRP2 and other maturation factors in SAH promote adult to-fetal reprogramming, which parallel liver repopulation with quasi-progenitor hepatocytes. Conversely, we suspect that in the absence of ESRP2-depletion and/or hepatocyte reprogramming, the AC livers may instead get replaced by senescent hepatocytes over time that track with fibrosis and other liver dysfunctions[10].

5. If the TF activity is decreased according to the ATAC-seq, then the genomic loci for the TF should show decreased accessibility, but the opposite is seen. All 3 TFs seem to show increased accessibility in the SAH samples. The discrepancy needs to be addressed. Also, the data for HNF1A and B show that AC decreases accessibility but SAH does not (lettering needs to be consistent for these groups).

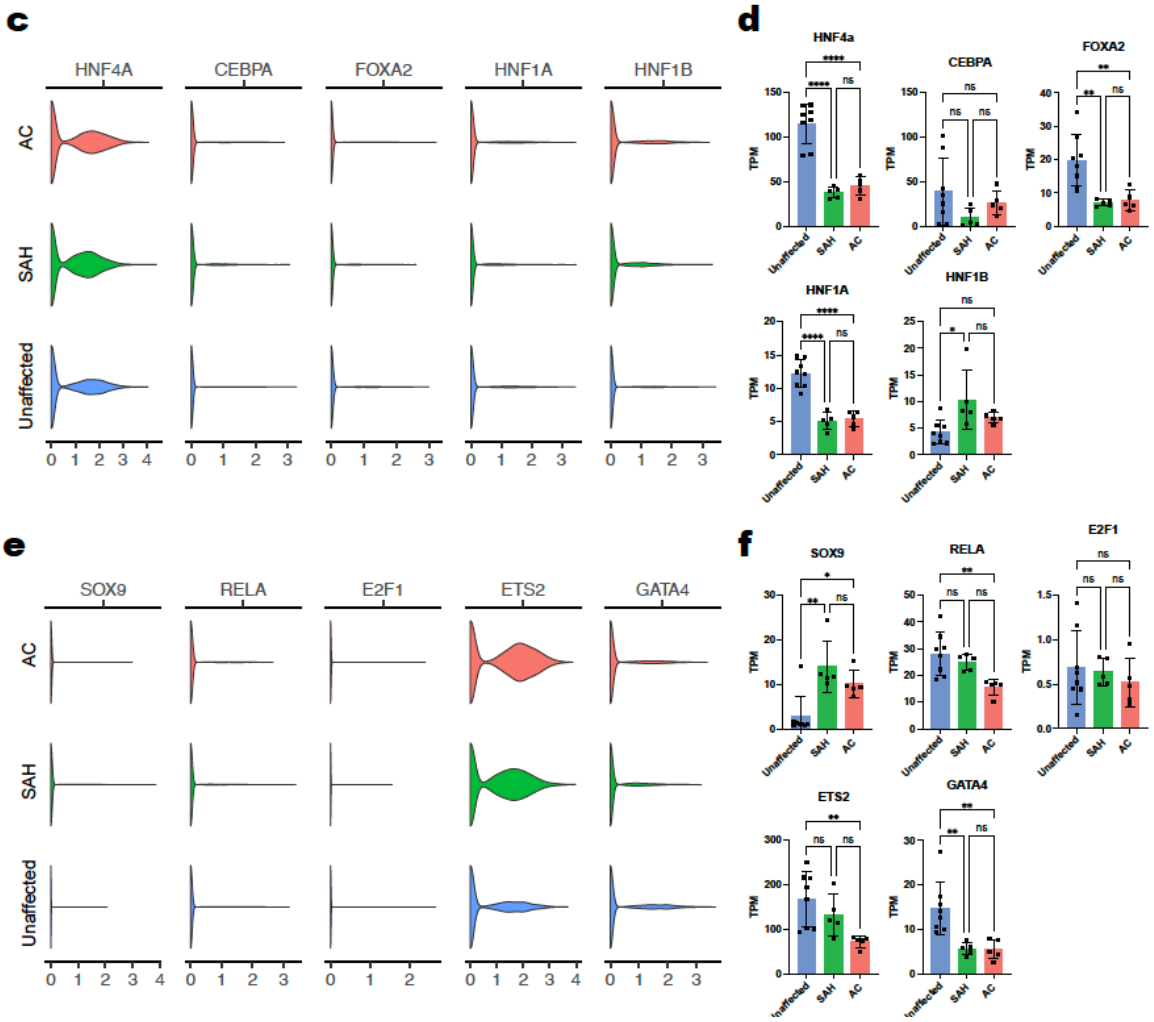
We thank the Reviewer for raising the concern about unexplained discrepancies observed here. We would like to clarify that while TF activity is reduced in SAH as evidenced from **Fig. 2A**, this might not necessarily imply a reduction in TF levels since the lack of activity might be explained by upstream signaling events. Secondly, as shown through analysis in **Fig. 3A**, for a large subset of genes changing in expression, genome accessibility can be largely unchanged, or sometimes changing in the opposite direction and therefore they might rely on post-transcriptional changes. Therefore, a myriad of possible molecular mechanisms could in effect explain reduced TF activity without a decrease in genome accessibility of the TF gene locus, itself.

Moving on to the precursor TFs, there is no accessibility around the motifs for SOX9, RELA or E2F1 in 2b and no changes in SAH or AC. The authors conclude that there is an incomplete transition to a progenitor state as there is no increase in SOX9, RELA or E2F1 "activity" despite increases in the proteins. This is based on negative results, i.e. not seeing a change in something. This is a difficult case to make as there could be many different explanations for a null result. Is there any change in genomic accessibility in the SOX9, RELA or E2F1 genes that would mirror the increase in expression? Expression values in Supp Fig 4 are also a concern as the snRNA-Seq data contradicts the bulk RNA-seq data, e.g. HNF4A, ETS2 etc.

The Reviewer is correct to point out that the absence of evidence isn't evidence of incomplete transition. To further explore the incomplete transition to a progenitor state, we compared gene expression profiles of Unaffected, SAH and AC hepatocytes to the published gene expression profiles of hepatocytes/hepatic progenitors through liver development[11]. We found that while there is reduced similarity in gene expression for SAH and AC samples to adult stage hepatocytes, the SAH hepatocytes show only a marginal increase in similarity to late fetal hepatocytes (FH2) compared to adult hepatocytes and almost none to early-stage fetal hepatocytes (FH1) **(see new Supplementary Fig. 4e)**. Furthermore, in case of E2F1, when we specifically compared

expression pattern of direct E2F1 gene targets[12] in SAH vs. Unaffected hepatocytes, only a handful of targets showed a significant change in expression, whereas the overall gene expression patterns were highly correlated, indicating limited increase in E2F1 activity within SAH hepatocytes **(see new Supplementary Fig. 4e).** This new information is added to the revised manuscript **(lines 146-154)**.

We appreciate the Reviewer's careful reading of the manuscript and pointing out the inconsistency in **Supplementary Fig. 4**. As shown below, in the first submission, we had provided gene expression estimates from both multiomics and bulk RNA-seq. However, as discussed in

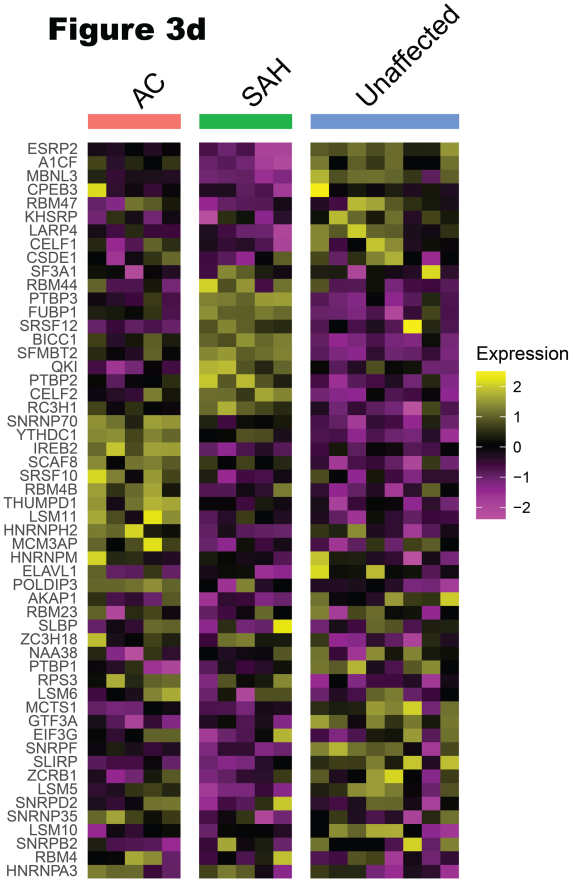


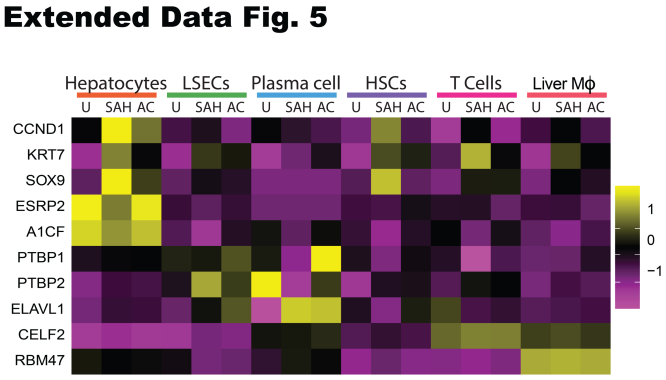
response to comment 5 from Reviewer 3, snRNA-seq is prone to loss of cytoplasmic and mitochondrial RNA information, decreased genes/UMI per cell detection, lack of transcript-wide coverage, and de-enrichment of fully spliced transcripts etc. We had included panels **d** and **f** in the original submission because snRNA-based expression data from most TFs presented in panel **c** and **e** were sparse limiting their reliability. The TPM values from bulk RNA-seq data also show that most of these TFs are very lowly expressed. To avoid any potential confusion, we have now removed **Supplementary Figure 4** panels **c** and **e** from the revised manuscript. We have kept only the panels showing expression of TFs based on their TPM values obtained from bulk RNA seq, which offers reliable estimation of gene expression especially for lowly expressed genes.

6. How did they pick the three loci in Fig. 3c? Heatmap in 3d is very low resolution as is the other heat map in Fig S5. Fig 3g is Venn diagram showing overlap of SAH and AC altered SEs. Does this figure really say that none of the splicing changes are in common? Where are the tables of splicing changes by rMATS? The authors verify selected SEs by RT-PCR. They need to state which exon or splice site is being described. The nomenclature used is the length of the exon which is not informative. Perhaps the exon number should be given.

The three loci shown in in **Fig. 3c** were hand-picked as examples to demonstrate *concordance* and *discordance* trends that we see between gene expression and accessibility.

Thank you for pointing out the issues with resolution of heatmaps. This is likely an upload/ download issue. We have included copies of heatmaps shown in **Fig. 3d and S5** below to ensure that the Reviewer can go through them. We will make sure that the final manuscript shows these heatmaps with high resolution and clarity.





We apologize for the confusion in **Fig. 3g**. In the original submission, **3g** showed the total number of alternative splicing events in SAH/AC and did not indicate the numbers of unique and common splicing events. Including all categories of alternative splicing, we identified a total of 893 events in SAH livers and 993 events in AC livers. We found that 260 of these events were shared between the two groups, which represent the intersection shown in **Fig. 3g**. We have now updated **Fig. 3g** to show the number of common as well as unique events and explain this in the main text **(lines 192-193)**.

We welcome the Reviewer's concern that exon names could be confusing and exon lengths might not uniquely describe an exon. However, in our experience, exon numbers can be even more

confusing, since exon numbers can change depending on which transcript of a gene one looks at. To ensure clarity, we have now included a supplementary table describing in detail the chromosome locations, size, and other details of each exon for which RT-PCRs (splicing assays) were performed **(see Source Data)**. We have also included supplementary tables listing significant alternative splicing events obtained from rMATS in SAH and AC livers **(see Source Data)**.

7. There is confusion in the manuscript regarding TCF4. The authors refer to TCF4 but in fact they mean TCF7L2 on chr 10. TCF4 is a separate gene on chromosome 18. The length of the TCF7L2 exon is 73 so this is an out of frame deletion. The authors need to explain what happens to this transcript isoform. The exon is at the C-terminus and causes a different C-terminus. As it is at the end, it might not cause NMD and the protein could be expressed. Fig. 5n makes it look like this exon is in frame. The SLK exon 13 is a known in-frame alternative exon.

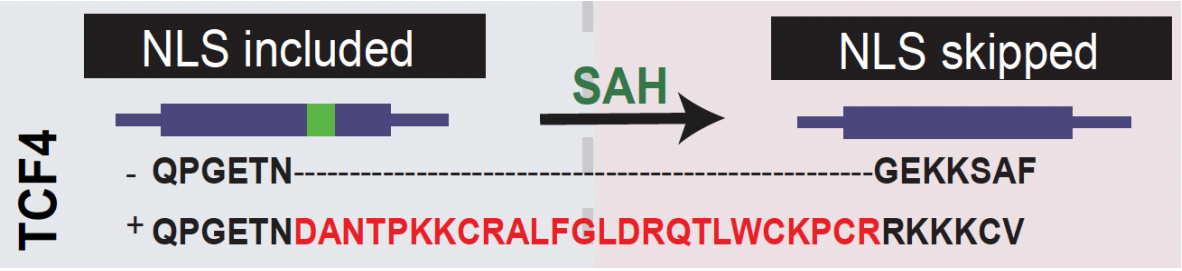
Thank you for this comment. As the Reviewer points out, TCF4 is the official symbol for 'transcription factor 4' (Gene ID: 6925). But 'TCF4' is also a widely used symbol/alias for TCF7L2 gene (known as 'T-cell-specific transcription factor 4' or 'transcription factor 7 like 2'), conflicting with the official symbol of transcription factor 4.

TCF4 and TCF7L2 gene pages on NCBI website do show a cautionary note highlighting this potential confusion. TCF7L2 was initially named (and was only referred to as) TCF4. It was renamed to TCF7L2 later, but most of the Wnt signaling community still refers to it as TCF4 for historical and other reasons, as pointed out in a review on gene symbol precision[13]. There are several recent examples for this trend in use of this nomenclature[14-19].

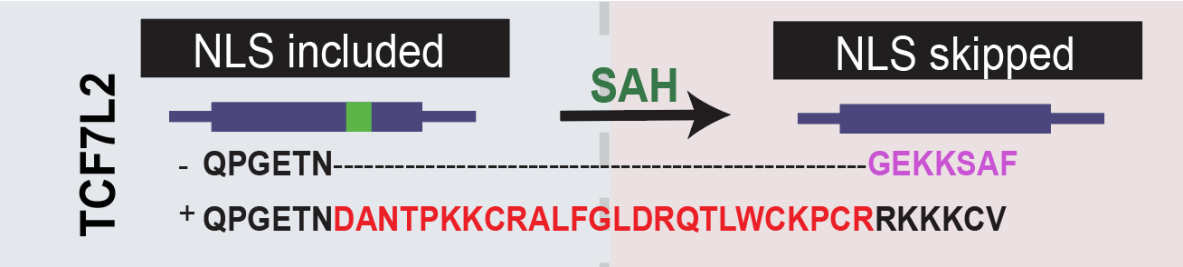
In our experience presenting this research, maintaining the nomenclature as TCF4 helped us convey the significance of this work better to Wnt researchers and hence chose to maintain the nomenclature in our initial submission. But as the Reviewer pointed out, we agree it is essential to use accepted conventions in nomenclature. Hence, we now refer to the gene as TCF4 throughout the manuscript considering it as a widely used alias, while also adding TCF7L2 in brackets wherever appropriate. We have added text description to clarify this in the manuscript **(lines 243-245)**.

We believe that these changes will promote accuracy without compromising visibility and ease of understanding among the broader scientific community.

As the reviewer pointed out the alternatively spliced exon in TCF7L2 that we refer to in this manuscript is 73 bp long, and when excluded it disrupts the reading frame of the downstream exons. **Fig. 5n** in the original submission, we did show this (Sequence of the peptide encoded by downstream exon changed from RKKKCV to GEKKSAF), but we understand that this aspect was not emphasized.



We have now highlighted the change in downstream exon in violet, and we have added text (**Fig. 5n legend)** to elaborate how exclusion of this exon changes the C-terminus of TCF7L2.



Briefly, the inclusion of 73-bp exon (exon 12) in *TCF7L2* transcript (as seen in Unaffected livers) produces an isoform that encodes in the +2 frame from downstream exon (exon 13, the last exon), expressing a 139 amino-acid long peptide at the c-terminus. But exclusion of this 73-bp exon leads to a change in the frame, producing a shorter c-terminus with only 26 amino-acids from the downstream exon. This is because of an earlier stop codon in the +1 frame on the downstream exon (exon 13). Note that the length of peptide expressed from exon 13 (139 aa or 26 aa) is contingent also upon inclusion of other exons early in the transcript. For instance, ENST00000536810 excludes the 73-bp exon but still expresses 139 amino acid peptides from the downstream exon. *TCF7L2* has several alternative exons leading to many possible transcripts. Hence, considering the lack of full-length transcript information in bulk short-read RNA-seq data, we are hesitant to draw conclusions on how the downstream exon is read.

Further, an important detail to note here is that this downstream exon (exon 13) is in fact the last exon. Both exclusion and inclusion isoforms encode stop codons on this exon (+1 as well as +2 frame) and there are no splice junctions downstream of this. Hence, we predict that these isoforms should not be subjected to NMD and are therefore annotated as protein coding.

8. No details are given for the *Slk* ASO. It seems to be very efficient unlike the *TCF4* ASO. In the ASO experiments it looks like the overall expression of SLK and TCF4 is decreased. Should be quantified. *TCF4* ASO has very little effect on target genes in 5m. Is this because the change in splicing is so weak? Are the changes significant? Can't tell from the heat map.

We apologize for not reporting the ASO sequence targeting the 93 bp exon of *SLK*. In the revised manuscript, we have included the ASO sequence and treatment protocol for the ASO in cell lines.

Thank you for noticing that the overall expression levels of SLK and TCF4 appear to have decreased in **Fig. 5l**. After reviewing the image files in **Fig. 5l**, we realized that this might be due to issues with scaling the intensity or how Adobe Illustrator processes image files in CMYK format rather than RGB format. To address this, we replaced the original images with the same images, which were scaled equally and prepared in the RGB format. As recommended by the Reviewer, we quantified the total nuclear as well as cytoplasmic SLK and TCF4 signal intensities of the cells, normalizing these values over the cellular area. Our analysis did not show any significant changes in the cumulative raw intensities. These quantifications are included in the revised manuscript as **Supplementary Figure 7a, 7b**. Furthermore, the **Supplementary Figure 7c, 7d** present five representative nuclei from different regions, all scaled equally. The corresponding quantification data shown in **Supplementary Figure 7e, 7f** illustrate that the sub-cellular distribution of SLK and TCF4 proteins is not related to their expression levels but is instead associated with the switch in splicing upon ASO treatment.

As noted by the Reviewer, representing the qRT-PCR data for known TCF4-target genes following GSK-3β inhibitor (CHIR99021) and *Tcf4*_73 ASO treatments as a heatmap may be confusing. We have replaced the heatmap with improved color scheme and added the corresponding bar plots in **Supplementary Fig. 7g** that display the relative fold change of TCF4 target genes after various treatments. We conducted a two-way ANOVA with uncorrected Fisher's LSD test and now present p-value statistics only for comparisons that showed significant differences with a p-value ≤ 0.05. Additionally, we included another control group involving the combination of CHIR99021 and control ASO treatment, which makes the comparisons easier to interpret. We also repeated the PCR-based splice assay of the *Tcf4*_73 skipping event using more cDNA and fewer PCR cycles to more accurately represent the changes in PSI values. The splice gel and quantifications in the figure have been updated accordingly. The ASO-mediated splicing redirection of *Tcf4* seems less robust when compared to *Slk*, however, when we tested the effectiveness of the *Tcf4* ASO at concentrations above 10 μM, we saw significant cell death. This was not the case with control ASO, suggesting increased skipping of *Tcf4*_73 exon is cytotoxic.

Authors describe the TCF4 exon as containing an ER retention signal. The speckled staining does not look like ER, so is this really an ER signal?

In **Fig. 4e**, the gene names were provided as representative examples for broad categories such as binding, catalytic activity, localization, PTM, and structure. These did not have a one-to-one correlation with the exon ontology subcategories (such as nucleotide binding site, protein binding, etc.) shown on the heat map on the right-hand side. Specifically, we did not intend to report an ER retention signal in TCF7L2, and we understand how its position adjacent to the ER signal in the heatmap could lead to this interpretation.

We have now edited **Fig. 4e** to reduce this potential confusion. In the revised figure, the list of genes is moved away from the ontology subcategories to the left-side of broad category labels. We apologize for this confusion and thank the Reviewer for pointing this out.

9. Fig. 6a and b need better description of the tracks. Are the green and blue tracks RNA-seq counts? And the top track eCLIP counts?

Thank you, we have now added the following description to the legend for **Fig. 6a, and 6b**. "The green and blue tracks visualize RNA-seq coverages from WT C57BL/6J and ESRP2 KO mouse hepatocytes on the UCSC genome browser respectively. eCLIP tags for ESRP2 are shown in black in the panel above RNA-seq data to demonstrate ESRP2 binding to the upstream introns."

10. Fig. 7 is not described adequately. What are we looking at in 7A?

We made several edits in the manuscript to describe **Fig. 7a** adequately. To improve clarity, we have now labeled distinct sections/plots of **Fig. 7a** (from 1 to 4) and explain what they correspond to in the figure legend as follows:

"NicheNet based cell interaction analysis summarizing (from left to right) **1)** Key ligands that regulate hepatocyte identities in SAH livers, **2)** Expression levels of ligands in different NPC cell types (Ligand producing "senders") in SAH livers, **3)** Log fold changes (LFC) in expression levels in SAH livers compared to Unaffected livers, and **4)** NicheNet-based prediction of a ligand's potential to regulate key hepatocyte target genes."

We have also provided more detailed description of the analysis and have referenced the sections of **Fig. 7a** individually to make the correlations from the results section easier.

Reviewer #3 (Remarks to the Author):

The current study performed bulk and single-nucleus RNA- plus ATAC-seq in healthy liver and livers with end-stage alcohol-related liver disease (ALD). The authors found profound changes in RNA binding protein (RBP) expression, that may cause misregulation RNA splicing. The authors propose that changes in stromal cell populations enrich failing ALD livers, which suppresses ESRP2-driven epithelial splicing program and replaces functional parenchyma with quasi-progenitor-like cells lacking liver-specific functions.

We thank the Reviewer for positively evaluating our work and for providing insightful comments.

1. The main weakness of the study is that ALD samples come from explant livers, from both advanced cirrhosis and alcohol-related hepatitis (AH) that have not respond to medical therapy. These livers typically show cirrhosis and marked liver failure with a massive ductular reaction and poor hepatocyte regeneration. Whether the results apply to earlier forms of ALD and compensated cirrhosis is unclear. While I understand that explant tissue allows the collection of larger liver tissue, this is a clear limitation of the study. The authors should perform some feasible studies in tissue other than explants to confirm their hypothesis.

We acknowledge the Reviewer's concern regarding the limitation of our study, as it is based on explanted liver tissues from patients with end-stage liver disease. Confirmation of our hypothesis using liver biopsy samples from patients who have not developed liver failure would indeed strengthen our findings. However, invasive liver biopsies are typically performed only in select cases where the diagnosis remains unclear or the disease exhibits unexpected progression, making such samples rarely available for research purposes.

We studied explanted human livers for two reasons: **1)** current clinical practice guidelines do not support diagnostic liver biopsies in humans with alcohol-related liver disease at any stage, and **2)** current animal models of alcohol-related liver disease (ALD) model conserved features of early-stage steatotic liver diseases (i.e., hepatocyte steatosis and mild lipotoxicity, liver inflammation and early fibrogenesis) but do not exhibit the full spectrum of progressive histopathology or hepatic decompensation that occurs in humans with advanced ALD. As our objective was to define mechanisms that drive hepatic decompensation, we focused on the human liver explants to delineate mechanisms responsible for hepatic decompensation as these liver explants are maximally dysfunctional. We used the mouse models as surrogates for earlier stages of ALD in humans. By integrating findings from livers with early (mouse model) and very advanced (human liver explants) ALD, we demonstrate that robust findings in late-stage disease emerge in milder form at earlier stages. Importantly, using the mouse models, we also show that the trajectory of ALD can be modified in early-stage disease by manipulating ESRP2 expression. Further, by complementing studies in mice and human livers with work in cultured cells, we identify biologically plausible ESRP2-sensitive mechanisms that regulate regenerative responses to alcohol-induced injury by showing that changing ESRP2 expression changes splicing events that control the cellular localization/function of ESRP2 targets that regulate Wnt signaling (an acknowledged mediator of hepatocyte metabolic function and proliferative capacity). Together, these results support our hypothesis that TGF-β-mediated signaling, ESRP2 suppression, and

splicing dysregulation are fundamental to ALD progression. Finally, it is important to note that since we first reported that hepatic accumulation of immature liver epithelial cells correlates with clinical predictors of mortality in human SAH in 2008[20], cumulating evidence from other groups supports our initial hypothesis that progressive replacement of mature liver parenchyma with immature hepatocytes drives liver dysfunction in ALD[6,20,21]. The current manuscript advances the field by demonstrating the importance of post-transcriptional mechanisms (e.g., particularly changes in multiple RBPs that lead to global changes in RNA splicing) in regulating these state transitions in liver cells. We have revised the discussion to clarify these issues **(lines 382-399)**.

Nonetheless, we are proactively reaching out to multiple liver centers and are working towards obtaining biopsy samples from patients with early-stage liver disease to address this limitation in future studies.

2. In fact, the animal model used in this study (i.e. control Lieber-DeCarli diet ad libitum for 5 days and then access for 20 days to the ethanol diet plus two doses of high ethanol content by gavage) does not cause liver failure, jaundice or advanced fibrosis, all features present in human samples. Although it is known that ethanol per se in unable to cause jaundice and cirrhosis in mice, the authors should confirm these results in an alternative model of more advanced liver disease.

Researchers in the ALD field are challenged by the dearth of small animal models that model clinically significant ALD in humans. Currently, the most *commonly used* models to investigate ALD pathogenesis are some sort of chronic moderate EtOH exposure (e.g, Lieber-DeCarli model in which EtOH is admixed in a nutritionally-replete liquid diet) versus varying numbers of acute, massive EtOH exposure binges to chow-fed mice (e.g., Bin Gao model) versus combination models of chronic EtOH exposure + intermittent acute ETOH challenges with or without other stressors (e.g., high fat diets, lipopolysaccharide, carbon tetrachloride)[21]**.** Despite the diversity of these approaches, none of them mimic the histopathology or clinical features that accompany alcohol-induced liver failure (i.e., the pathologic features observed in either the SAH or the AC human liver explants).

The model we selected (Lieber DeCarli + two EtOH binges) has become one of the standard models in the field as it reliably causes hepatic steatosis, lipotoxicity, liver inflammation and mild fibrogenesis. Importantly, we found that suppression of ESRP2 in this model of mild ALD significantly exacerbated liver damage - a finding that supports a role for reduced ESRP2 expression (and resultant effects on RNA splicing) in the pathogenesis of ALD progression. A strength of the model we used is that it recapitulates the drinking patterns of humans who are at risk for SAH or AC (i.e., bouts of heavy drinking interspersed upon daily 'social' drinking). We agree that alternative models, such as combined alcohol and CCl4 or high fat diet administration, could be studied but they are more contrived and thus, of uncertain relevance to human ALD. Also, we had previously collaborated with Dr Tsukamoto to study ESRP2 expression in his chronic EtOH/high fat diet continuous intragastric infusion mouse model of ALD. Although not widely used, that model does induce severe ALD that more closely resembles the histopathology of human ALD and we reported that ESRP2 is significantly down regulated in this mouse model of more advanced ALD[1].

Human ALD encompasses a complex interplay of genetic and epigenetic predispositions, alcohol metabolism, immune responses, and gut-derived endotoxins. None of the available mouse models captures the full spectrum of these processes. We have highlighted this point in the

revised discussion section **(lines 400-403)**.

3. Some of the conclusions of the study are too strong and should be tempered. For example, the statement that "altered immune milieu in ALD promotes a quasi-progenitor state of hepatocytes" is not fully proven by the data. Association of biological findings that could be related does not necessarily mean causality. The authors should temper their conclusions throughout the manuscript.

As suggested by the Reviewer, we have toned down the language in the revised manuscript.

1. We replaced the statement "altered immune milieu in ALD *promotes* a quasi-progenitor state of hepatocytes" with "altered immune milieu in ALD *associates with* a quasi-progenitor state of hepatocytes" **(line 70)**. Similarly, in the discussion section, the statement "Dysregulation of these processes *trap* hepatocytes in an intermediate state" is now changed to "Dysregulation of these processes *might trap* hepatocytes in an intermediate state" **(line 414)**
2. To delve deeper into the incomplete transition to a productive progenitor-like state, we compared gene expression profiles from Unaffected, SAH, and AC hepatocytes against those of hepatocytes and hepatic progenitors from published datasets during human liver development **(see new Supplementary Fig. 4e)**. Our new analysis revealed that while both SAH and AC samples display diminished similarity in gene expression to adult hepatocytes, SAH hepatocytes exhibit only a modest increase in similarity to late fetal hepatocytes compared to their adult counterparts, and barely any resemblance to early fetal hepatocytes. Furthermore, when we focused on the expression of direct E2F1 gene targets in SAH versus Unaffected hepatocytes, we uncovered only a handful of genes that showed significant changes in expression **(see new Supplementary Fig f)**. We have added this information to the revised manuscript **(lines 146-154)**.
3. We tempered the conclusions for **figures 5h and 5n** by replacing the statement "these data *indicate*" with the statement "these data *are consistent with the hypothesis*" in the revised manuscript **(line 266)**.
4. We acknowledged the limitations of AML12 cells in terms of their altered insulin responsiveness, limited capability to model gluconeogenesis, and their inability to replicate the complex cellular interactions present in a whole liver **(lines 359-367)**.

4. The role of inflammation (eg, PMNs) in ALD differs from early forms to severe AH. This should be acknowledged in the manuscript.

We agree with the Reviewer's observation that the inflammatory profile evolves across the spectrum of ALD. Our study highlighted the shift from Kupffer cell-mediated early inflammation to the predominance of monocyte-derived macrophages in SAH. This shift underscores the evolving immune landscape as the disease progresses from early form to advanced stages. Our findings demonstrated elevated levels of key inflammatory cytokines, including IL1-β, TNF-α, IFN-γ, and TGF-β, in SAH livers, which play pivotal roles in driving hepatocyte injury, inflammation, and fibrosis. These cytokines, along with the accumulation of PMNs in SAH, reflect the heightened inflammatory milieu characteristic of advanced disease. We have incorporated this perspective on neutrophil infiltration and its contribution to the pathophysiology of severe ALD in the revised manuscript **(lines 123-130)**.

5. The authors should discuss the potential limitations of using snRNAseq instead of scRNAseq, including the lack of mitochondrial DNA, etc.

In the revised manuscript, we have now added the limitations of using single nuclear RNA-seq approach compared to scRNA-seq and other RNA-seq approaches. We have highlighted issues such as loss of cytoplasmic and mitochondrial RNA information, decreased genes/UMI per cell detection, lack of transcript-wide coverage, de-enrichment of fully spliced transcripts and potential cell-type biases **(lines 76-84)**.

6. The term "regeneration failure" is not commonly used and it can be confused with "liver failure". I propose "impairs liver regeneration" or similar.

Thank you for the suggestion. We have changed "regeneration failure" to "impaired regeneration" in both the title and the body of the revised manuscript.

References

1 Hyun, J. *et al.* Epithelial splicing regulatory protein 2-mediated alternative splicing reprograms hepatocytes in severe alcoholic hepatitis. *J Clin Invest* **130**, 2129-2145 (2020). https://doi.org/10.1172/JCI132691

2 Yadav, D. K., Zhang, Q., Bai, X., Li, E. & Liang, T. Liver Transplantation for Alcohol-Related Liver Disease (ARLD): An Update on Controversies and Considerations. *Can J Gastroenterol Hepatol* **2020**, 8862152 (2020). https://doi.org/10.1155/2020/8862152

3 Mathurin, P. *et al.* Corticosteroids improve short-term survival in patients with severe alcoholic hepatitis: meta-analysis of individual patient data. *Gut* **60**, 255-260 (2011). https://doi.org/10.1136/gut.2010.224097

4 Lucey, M. R., Mathurin, P. & Morgan, T. R. Alcoholic hepatitis. *N Engl J Med* **360**, 2758-2769 (2009). https://doi.org/10.1056/NEJMra0805786

5 European Association for the Study of the, L. EASL Clinical Practice Guidelines: Management of alcohol-related liver disease. *J Hepatol* **69**, 154-181 (2018). https://doi.org/10.1016/j.jhep.2018.03.018

6 Louvet, A. & Mathurin, P. Alcoholic liver disease: mechanisms of injury and targeted treatment. *Nat Rev Gastroenterol Hepatol* **12**, 231-242 (2015). https://doi.org/10.1038/nrgastro.2015.35

7 Argemi, J. *et al.* Defective HNF4alpha-dependent gene expression as a driver of hepatocellular failure in alcoholic hepatitis. *Nat Commun* **10**, 3126 (2019). https://doi.org/10.1038/s41467-019-11004-3

8 Bhate, A. *et al.* ESRP2 controls an adult splicing programme in hepatocytes to support postnatal liver maturation. *Nat Commun* **6**, 8768 (2015). https://doi.org/10.1038/ncomms9768

9 Bangru, S. *et al.* ESRP2-microRNA-122 axis promotes the postnatal onset of liver polyploidization and maturation. *Genes Dev* **39**, 325-347 (2025). https://doi.org/10.1101/gad.352129.124

10 Aravinthan, A. *et al.* Hepatocyte expression of the senescence marker p21 is linked to fibrosis and an adverse liver-related outcome in alcohol-related liver disease. *PLoS One* **8**, e72904 (2013). https://doi.org/10.1371/journal.pone.0072904

11 Wesley, B. T. *et al.* Single-cell atlas of human liver development reveals pathways directing hepatic cell fates. *Nat Cell Biol* **24**, 1487-1498 (2022). https://doi.org/10.1038/s41556-022-00989-7

12 Denechaud, P. D. *et al.* E2F1 mediates sustained lipogenesis and contributes to hepatic steatosis. *J Clin Invest* **126**, 137-150 (2016). https://doi.org/10.1172/JCI81542

13 Bennani-Baiti, B. & Bennani-Baiti, I. M. Gene symbol precision. *Gene* **491**, 103-109 (2012). https://doi.org/10.1016/j.gene.2011.09.035

14 Kadosh, E. *et al.* The gut microbiome switches mutant p53 from tumour-suppressive to oncogenic. *Nature* **586**, 133-138 (2020). https://doi.org/10.1038/s41586-020-2541-0

15 Escribano-Nunez, A. *et al.* IGF1 drives Wnt-induced joint damage and is a potential therapeutic target for osteoarthritis. *Nat Commun* **15**, 9170 (2024). https://doi.org/10.1038/s41467-024-53604-8

16 Zou, Y. *et al.* N6-methyladenosine regulated FGFR4 attenuates ferroptotic cell death in recalcitrant HER2-positive breast cancer. *Nat Commun* **13**, 2672 (2022). https://doi.org/10.1038/s41467-022-30217-7

17 Jang, J. H. *et al.* Kindlin-1 promotes gastric cancer cell motility through the Wnt/beta-catenin signaling pathway. *Sci Rep* **15**, 2481 (2025). https://doi.org/10.1038/s41598-025-86220-7

18 Zheng, J. H. *et al.* A CLIC1 network coordinates matrix stiffness and the Warburg effect to promote tumor growth in pancreatic cancer. *Cell Rep* **43**, 114633 (2024). https://doi.org/10.1016/j.celrep.2024.114633

19 Li, Y. *et al.* BMP suppresses Wnt signaling via the Bcl11b-regulated NuRD complex to maintain intestinal stem cells. *EMBO J* **43**, 6032-6051 (2024). https://doi.org/10.1038/s44318-024-00276-1

20 Jung, Y. *et al.* Accumulation of hedgehog-responsive progenitors parallels alcoholic liver disease severity in mice and humans. *Gastroenterology* **134**, 1532-1543 (2008). https://doi.org/10.1053/j.gastro.2008.02.022

21 Hong, X. *et al.* Alcohol-related liver disease (ALD): current perspectives on pathogenesis, therapeutic strategies, and animal models. *Front Pharmacol* **15**, 1432480 (2024). https://doi.org/10.3389/fphar.2024.1432480