Peer Review File

Manuscript Title: Opposing Roles of Hepatic Stellate Cell Subpopulations in Hepatocarcinogenesis

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

Filliol et al. dissect the contributions of hepatic stellate cells to the development and progression of hepatocellular carcinoma (HCC). They use multiple murine models to manipulate HSC activation state and HSC abundance during hepatocarcinogenesis and correlate their findings to human clinical samples. With this rigorous approach, the authors present compelling evidence for both cancer-promoting and cancer-protective functions for HSCs in HCC. While previous studies have explored the contribution of HSCs to HCC, their results have been inconsistent. The present study leverages newly developed tools such as single cell RNA sequencing to develop a more detailed and nuanced understanding of the roles for HSCs in liver cancer. In particular, two molecules already known to be important in HCC, HGF and Col1a1, are identified as critical mediators of HSCs' divergent roles. This work helps clarify the contributions made by HSCs at different stages of HCC and may help to explain why previous studies reached contradictory conclusions.

Major points:

1. It would be helpful when comparing results across the several models used, to know what impact the genetic strategies (HSCΔYAP, HSCΔLHX2, etc) have on the ratio of the identified HSC subtypes: myHSC and cyHSC
2. The authors suggest that HSCs in the premalignant liver contribute to hepatocarcinogenesis of HCC but focus on HSC depletion at the end of the experimental window. It may be informative to establish the impact of HSC depletion earlier, prior to tumor development, in order to show that the effect is primarily in the PME and avoid the possibility that ablation of HSCs itself is exerting an anti-tumor effect.
3. In figure 2 C-D RNA-seq analysis shows greater changes to the gene expression of non-tumor tissue than tumor upon HSC-specific deletion of YAP, but it is hard to know if this signature is driven by hepatocytes or primary effects of YAP KO on HSCs – especially because the majority of HSCs are found in non-tumor tissue. Single nuclear sequencing or immunostaining for key altered genes would help to clarify which changes are specific to hepatocytes in the PME.
4. Figure 2E please clarify why ALT is 10x higher in aSMA-TKneg mice than in YAPfl/fl mice subjected to the same CCl4 regimen. Conversely, there are approximately 10x more TUNNEL+ hepatocytes per field in YAPfl/fl than in aSMA-TKneg mice. Please explain such large differences in control mice subjected to the same injury model.
5. The findings rely heavily on a DEN-CCl4, model of HCC. Evidence that the pathogenesis and gene expression profile of this model corresponds to human NASH-HCC is required to establish the relevance of these findings to human disease.

6. The data suggest that a subpopulation of hepatic stellate cells that are enriched in HGF contribute to the anti-carcinogenic activity of the 'cytokine producing HSC' population. The relative contribution of stellate cells versus endothelial cells in producing HGF needs to be quantified and some assessment of HGF protein in these cells and/or livers would strengthen the conclusion that HGF accounts for the anti-neoplastic effect. More importantly, since HGF mimetics are in development for liver disease, it would be extremely relevant to examine whether HGF administration, either transgenically or exogenously, rescues the cancer phenotype. Also, it would be important to show evidence of changes in HGF signaling, ideally by looking at the impact on phosphorylation of c-met, or an impact on pathways of HGF signaling based on transcriptomics.
7. Related to the above, the genetic deletion of Hgf seem to lead to increased fibrous Collagen staining (Extended Figure 14) which could serve as an alternative mechanistic explanation for why these mice demonstrate increased HCC burden (i.e. by promoting Col1a1 production and not by affecting hepatocyte turnover). This possibility needs to be addressed.
8. The findings are at odds with a recent paper in NATURE (Pfister et al, doi: 10.1038/s41586-021-03362-0) that clearly indicates evidence of impaired immune surveillance in NASH-HCC, and an earlier paper linking NASH to reduced CD4 lymphocytes (doi: 10.1038/nature16969), yet no impact was seen in the current study on any immune cell subsets. How are these findings from the current study reconciled with these other studies, and is this related to differences in the models used (see comment above)?
9. The contribution of collagens (DOI: 10.1111/liv.12465), tissue stiffness (DOI: 10.1152/ajpgi.00412.2010, DOI: 10.1002/hep.24108), and Taz (DOI: 10.1016/j.cmet.2020.03.010) have all been strongly speculated based on cell culture data or directly shown in vivo to drive liver disease in various publications. Similarly, the role of HGF on hepatocyte survival (reviewed in DOI: 10.1002/hep.28988), and the contribution of various forms of hepatocyte death to HCC has also been extensively published (e.g. DOI: 10.1038/s41586-018-0519-y). Since liver disease almost always precedes liver cancer and is the strongest predictor of HCC, therefore removing known drivers of liver disease and demonstrating a decrease in HCC formation is nice but not very surprising or novel. Given the trove of single cell transcriptomics data that have been generated in both mice and patients, the authors are well positioned to identify truly novel drivers and pathways affecting liver disease and HCC.
10. The authors have recently published two elegant papers (refs 52 & 53) that advance similar concepts regarding the role of type I collagen, and the presence of different HSC subsets in liver cancer (cholangiocarcinoma). How similar are the transcriptomic features of the HSC subsets described in the current paper to the 'myCAFs' and 'iCAFs' in reference 52? The classification of HSCs into cyHSC (which is essentially quiescent HSCs) and myHSC (which represent activated HSCs) has been established for decades (reviewed in DOI: 10.1038/nrgastro.2017.38). Unfortunately powerful, cutting-edge single cell technology has not been leveraged to identify new HSC subsets. Using a similar stellate cell enrichment strategy to enrich for HSCs, a recent publication has done a better job at identifying novel HSC subpopulations in liver fibrosis progression and regression (DOI: 10.1002/hep.31743).
11. Are the two major HSC subsets, 'myHSCs' and 'cyHSCs' derived from one another or are they programmed to these divergent fates? In other words, how are these different subtypes generated?
12. The authors state that HSC-specific deletion of Col1a1 was associated with reduced hepatocyte proliferation (line 276-277), but changes to ki-67+ hepatocytes did not reach significance in figure 4D (p=0.06). Reduced ki67 mRNA is not sufficient evidence of reduced hepatocyte proliferation. Please

revise the text to reflect this.

Minor points:

1. Fig 4 F western blot is of poor quality and hard to interpret.
2. Fig 2A labels on bar graph are out of alignment
3. Figure 4E, legend does not include a label for the data represented by open circles. Please add this.
4. Similar analyses (tumor burden, hepatocyte proliferation and hepatocyte apoptosis) are presented across multiple models. A summary figure or table of results would be helpful.
5. Figure 2G, 3F: From CellphoneDB analysis of scRNAseq data, it is quite clear that HSCs but not hepatocytes are a hub of cell-cell communication in the liver, which is in line with what has reported previously in a mouse NASH model (DOI: 10.1016/j.molcel.2019.07.028). Within all the cell types that HSCs communicate with, hepatocytes do not stand out as a main partner, the number of interactions between HSCs and hepatocytes is less than HSCs with endothelial, portal fibroblasts, cholangiocytes, macrophages, Kupffer cells, and DC. Focusing on hepatocyte as a hub of cell-cell communication in the liver seems somewhat misleading and not supported by the data.

Referee #2 (Remarks to the Author):

In the manuscript termed „An Impaired Balance of Tumour-Suppressing and Tumour-Promoting Hepatic 2 Stellate Cell Populations in Liver Fibrosis Contributes to Hepatocarcinogenesis" by Filliol and colleagues the authors dissect the role of hepatic stellate cells and factors expressed by the latter (e.g. HGF) in liver cancer in mouse models and human liver cancer specimen.

The study involves state of the art technologies and several different human specimen to characterize tumor associated HSCs – identifying tumor promoting (myHSC) and tumor suppressing (cyHSC) HSCs, which the authors can well define by single cell technologies and precise mouse models targeting HSCs (e.g. Lrat-Cre trasngenic).

This newly identified signature was also transferred into the real world – analyzing HCC risk of patients and the dysbalance of myHSC and CyHSC. Overall, this is a very interesting and innovative manuscript, - with some surprising findings (e.g. the existence of tumor suppressive HSCs; role of HGF) that is to the most degree also well controlled.

However, there are several additional questions that need to be addressed by the authors, and experiments need to be executed to clarify some unclear issues.

Generally, the manuscript would also profit from including one or the other spontaneous liver

cancer model (without the use of genetic drivers) – as the models used are in part artificial and might not reflect the natural activation of HSCs (when we think about transcriptional programs (e.g. like thorugh CCL4); or addition of tumor drivers –making a rather artificial genetic environment). This does not mean that this referee is against the models applied – on the contrary – but some of the findings need to be assured in spontaneous models. How do the authors know that there might not be some differences to the models used in this paper?

Another important point addresses the question whether HSCs interact early on with pre-neoplastic lesions in HCC (and the very interesting interaction HSCs-Hepatoctytes) and how the difference between HCC and ICC can be explained = linked to transdifferentiation programs?

Figures 1A-D: (also extended data figure 2): It should be demonstrated in more detail to what extent HSC activation and deactivation was achieved. It is not really clear whether Col1a1 indeed is the only good measure – what was the quality of the transcriptional programs (of activation and deactivation)? Also it is not clear whether the genetic activation renders additional "defects" in HSCs that contribute or affect the phenotype.
Are tumors different in their character (genetic, histology, immune cells)?

Figures 1E-H: Also here it is not clear – incidence of tumorigenesis is still 100% in both groups. What is altered in these tumors apart from size and number reduction? How is the TME altered in this context? Do the tumors look different?
Figures I-J (and Extended Data 2): The NASH model – needs to be better characterized (histology etc.) – and is less convincing as it does not include NASH-induced HCC but rather uses a genetic trick – to be combined with a NASH diet to end up in liver cancer.
Here the authors should indeed use a spontaneous model – as all the models used in Figure 1 do not display the spontaneous development of liver cancer but rather represent a "highly artificial" (still useful) set up of genetic environment.
So it is not quite clear how this would affect tumorigenesis in an spontaneous model. How sure are the authors at what time point exactly HSCs have been depleted – in correlation to the liver cancer development state?

Figure 2: Figure A is very convincing – again leaving however the question open – how does this look like in chronic inflammation induced liver cancer or spontaneous tumors? The difference between ICC and HCC is very convincing and clear-cut. At what time point does this "localization" occur? It would be very important, and interesting in the context of this paper to investigate whether HSCs maybe interact closely whit the tumor / pre-neoplastic lesions.
Figures 2E- and F: It is not clear where the tumor tissue is in relation to the performed stainings.
Potentially the N=1 analysis in Figure 2G – which I personally like – could be repeated to N=3?

Figure 3: The single cell analysis described are very nice and convincing. It is also very clear and nice that it is possible to change the plasticity of cyHSCs and myHSCs. Again this referee would advocate to investigate these 1. In at least one sponteanous liver cancer model – and maybe also characterize HSCs at the start of liver cancer and further on.
Figure 3J and K: Hgf and Col1a1 expression look very convincing. Is this to be found in other models of fibrosis similarly (induced or spontenaous). What about the human situation – in correlation with

the degree of fibrosis (e.g. HGF expression)? HGF expression in human livers?
Figure 3L: The RNA Scope analysis is overall very convincing. Can this be reproduced in other liver cancer models?

Figure 4C: Does the macroscopy in Figure 4C reflect a representative image (does not appear based on the quantifications). A more representative image should be shown.
Figure 4D: TUNEL Staining appear to be unspecific in some part – a double stain would clarify this.

Figure 5: Data on HGF are very convincing. Could the authors correlate this with a decrease of measurable HGF in serum? How does this HGF expression correlate in human liver and serum?
Is it also in the human situation that HGF is expressed by HSCs?
When is the HGF expression important – before (to protect damage) or during oncogenesis? Here experiments with blocking antibodies would be useful.
How does the lack of HGF also affect the immune cell environment of the liver contributing to HCC? Again here a spontaneous model of carcinogenesis would be very helpful to see whether this is reproducible.
Overall, the manuscript is well written and clear - as is the discussion.

Referee #3 (Remarks to the Author):

This manuscript is grounded in the controversy concerning the role of senescent hepatic stellate cells (HSCs) in the outcomes of liver injury. As you beautifully summarize in the introduction, some published data indicate that senescent HSCs promote liver cancer, while other data indicate the opposite effect. Your paper is a tour-de-force of mouse models and packed with data that are novel and likely to be highly utilized by other researchers. However, in my view, there are some significant limitations/weaknesses.

1 - None of the work that your present directly resolves the one fundamental research question that you posed in the manuscript's Introduction, i.e., do senescent hepatic stellate cells (HSCs) increase hepatocarcinogenesis or decrease hepatocarcinogenesis?

2 - Your results do indicate that at least a subpopulation of fibrogenic-activated/myofibroblastic HSCs are pro-carcinogenic and suggest that perhaps a subset of the remaining HSC population suppresses carcinogenesis. However, none of the data are informative regarding whether either the pro- or the anti-carcinogenic subpopulation is enriched with senescent cells (as indicated by beta-gal staining/expression of p16, p21, p27, p53, etc...). Assuming that you remain interested in resolving the controversy about the significance of HSC senescence (which is an important question), additional experiments/analyses are needed to address this question.

3 - Also, most of the data are descriptive, rather than mechanistic. For example,
a- although the data clearly show that the pro- and anti-carcinogenic HSC subpopulations exhibit differential expression of genes that encode various secreted proteins, no clear mechanism(s) is/are delineated to demonstrate how any of these factors either promote or antagonize hepato-

carcinogenesis. HGF is suggested to be a critical anti-carcinogenic factor because it protects cultured hepatocytes from experimentally-induced apoptosis. This is not surprising but falls short of explaining the net effects of the anti-carcinogenic HSC subpopulation in vivo given other evidence indicating that expression of multiple pro-inflammatory mediators is enriched in those cells (and inflammation is generally thought to promote cancer).
b - no experiments were done to define which of the various secreted gene products of the pro-carcinogenic HSC subpopulation promotes liver cancer (or to delineate how that factor enhances cancer growth).

4 - The identities of the large numbers of HSCs that exhibit neither the pro- or anti-carcinogenic HSC signature in all of the various mouse models has not been interrogated.
a - It would be helpful to know if signatures of these cells align with signatures that other groups have identified in various HSC subpopulations in healthy or fibrotic livers (According to those other two reports, there are at least 5 different HSC subpopulations in injured livers).
b - Heat map data in your Extended Materials suggest that both the more- and less-carcinogenic HSC populations share expression of many classical myofibroblast markers. This raises the possibility that HSCs are undergoing state transitions, an issue that could be examined with RNA velocity and/or other transition-type analyses. Another approach might be to assess if the anti-carcinogenic HSC subpopulation is enriched with "reverted" myofibroblastic HSCs that you and other groups have written about in previous publications.

5 - The idea of using HSC signatures generated in the mouse models to interrogate the pathogenesis of liver cancer in humans is appealing. Based on the mouse data, it would seem that the pro-carcinogenic HSC population should increase and the anti-carcinogenic population should decrease in cirrhotic versus non-cirrhotic liver. However, this hypothesis cannot be tested with the UMAP data presented because data sets from the two normal livers and two cirrhotic human livers were pooled. What are the results when you segregate the normal from the cirrhotic data sets?

6 - Finally, in my view, the experiments about Yap/Taz in hepatocytes distract from the HSC focus of the work. Further, some of the conclusions about the relative significance of Yap/Taz in the carcinogenic process are also difficult to justify based on the data presented. For example,
a - the text seems to suggest that Yap activation is important for the fibrogenic/pro-carcinogenic phenotype (the role of Taz is not addressed in HSCs) but that Taz is a more important procarcinogenic factor than Yap in hepatocytes. However, the models used to compare the two factors were not equivalent (the one showing the significance of Taz was more extreme than the one that was used to assess Yap) and there is abundant literature showing that activating Yap in hepatocytes promotes liver cancer. b - the rationale for these experiments that introduced constitutively active Yap/Taz mutants into hepatocytes is not explained since the primary assumption being investigated is whether or not HSCs regulate pro (or anti) carcinogenic signaling in hepatocytes.

Author Rebuttals to Initial Comments:

We would like to thank the reviewers and editors for their helpful comments, which have helped us to further improve our manuscript. We have spent the last 14 months carefully addressing all comments and have integrated a significant amount of additional data into the manuscript – including several spontaneous HCC models and a novel collagen-mediated tumor-promoting pathway via its receptor DDR1.

Referees' comments:

REFEREE #1 (REMARKS TO THE AUTHOR):

Filliol et al. dissect the contributions of hepatic stellate cells to the development and progression of hepatocellular carcinoma (HCC). They use multiple murine models to manipulate HSC activation state and HSC abundance during hepatocarcinogenesis and correlate their findings to human clinical samples. With this rigorous approach, the authors present compelling evidence for both cancer-promoting and cancer-protective functions for HSCs in HCC. While previous studies have explored the contribution of HSCs to HCC, their results have been inconsistent. The present study leverages newly developed tools such as single cell RNA sequencing to develop a more detailed and nuanced understanding of the roles for HSCs in liver cancer. In particular, two molecules already known to be important in HCC, HGF and Col1a1, are identified as critical mediators of HSCs' divergent roles. This work helps clarify the contributions made by HSCs at different stages of HCC and may help to explain why previous studies reached contradictory conclusions.

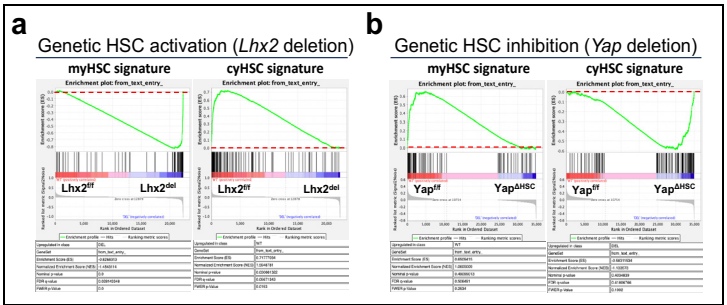
We would like to thank the reviewer for these positive comments, and have added experimental data to support the presented conclusions and further increase scientific rigor.

MAJOR POINTS:

1. It would be helpful when comparing results across the several models used, to know what impact the genetic strategies (HSCΔYAP, HSCΔLHX2, etc) have on the ratio of the identified HSC subtypes: myHSC and cyHSC.

We have followed up on this point and confirm that the genetic strategies alter the ratio between myHSC and cyHSC:

- In the Lhx2 deletion model, we observe a significant enrichment of the myHSC signature, accompanied by a decrease in the cyHSC signature, as determined by gene set enrichment analysis in isolated HSC. These data have been added to the manuscript (**Extended Data Figure 13a** – see right). The observed increase in the myHSC and decrease in the cyHSC is consistent with the observed increases HSC activation (***Figure 1A*** and ***Extended Data Fig.3d*** in the manuscript file).
- In the YAP deletion model, we observe a strong decrease of the myHSC signature, accompanied by an increase in the cyHSC signature, as determined by gene set enrichment analysis in isolated HSC. (**Extended Data Figure 13b**) and the observed changes are consistent with our finding that YAP deletion in HSCs decreases HSC activation (***Figure 1c*** and ***Extended Data Fig.3f*** in the manuscript file).

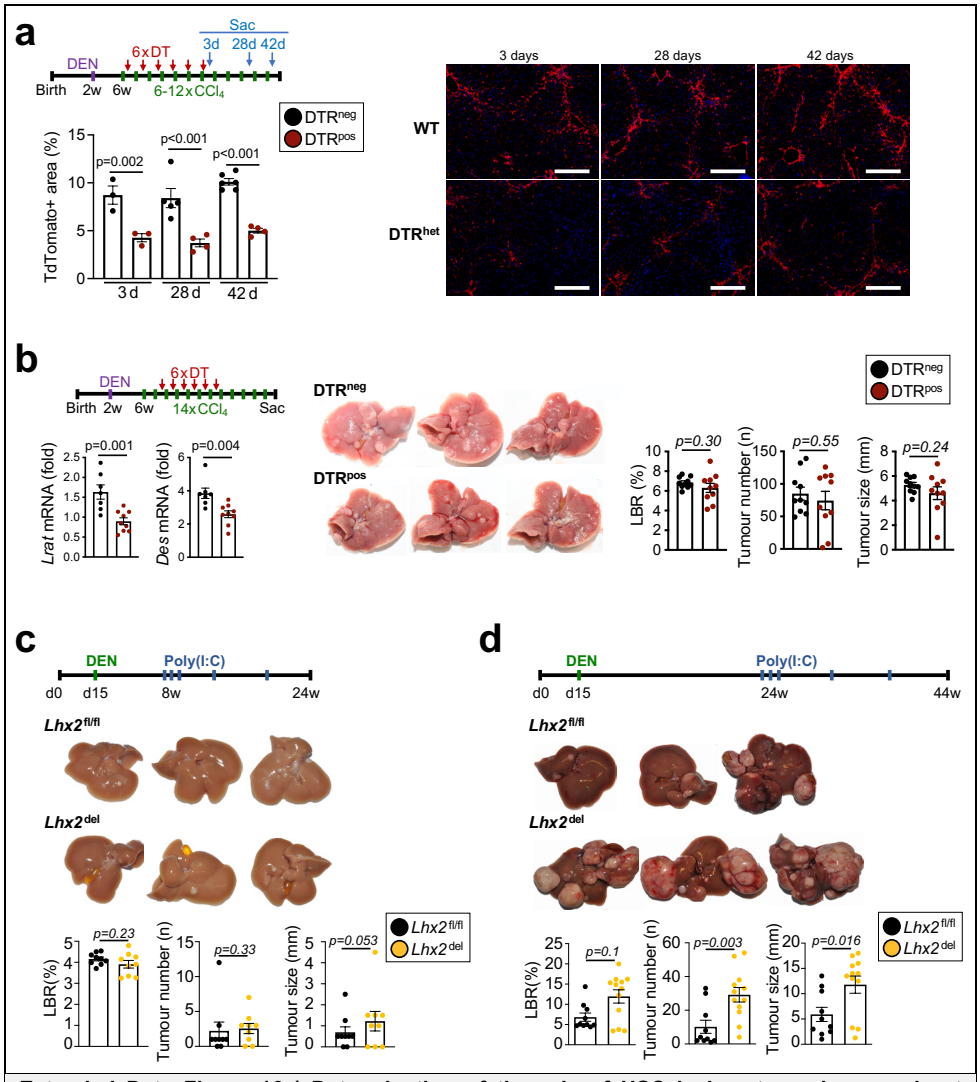


Extended Data Figure 13 | Genetic HSC activation and genetic HSC inhibition result in alterations mHSC and cyHSC signature enrichment. a-b, analysis of RNA-seq data from isolated HSC revealed that genetic HSC activation via Mx1Cre-mediated *Lhx2* deletion (n=4 *Lhx2* $^{fl/fl}$, n=4 *Lhx2*$^{\Delta HSC}$) resulted in enrichment of the myHSC signature and decreased cyHSC enrichment (a), whereas genetic HSC inhibition via LratCre-mediated Yap1 deletion (n=5 *Yap1*$^{fl/fl}$, n=4 *Yap1*$^{\Delta HSC}$) exerted the opposite effect (b).

2. The authors suggest that HSCs in the premalignant liver contribute to hepatocarcinogenesis of HCC but focus on HSC depletion at the end of the experimental window. It may be informative to establish the impact of HSC depletion earlier, prior to tumor development, in order to show that the effect is primarily in the PME and avoid the possibility that ablation of HSCs itself is exerting an anti-tumor effect.

We agree and have carefully considered the best approaches to address the extremely important points. We now exclude the "*possibility that ablation of HSCs itself is exerting an anti-tumor effect"* and our additional data also clearly show that there is no effect of HSC ablation on large established tumors. Our additional data, explained detail below, are consistent with the hypothesis that the bulk of tumor-promoting effects, mediated by *Col1a1*-expressing myHSC; occurs at later stages, when these cells most abundant and intensely interact with premalignant/transformed hepatocytes/early stage lesions; whereas tumor-suppressive effects, mediated by *Hgf*-expressing cyHSC, appear to be early, when these HSC subtypes are most abundant and protect hepatocytes from injury. Effects insides the tumor compartment in the setting of profound fibrosis, mediated by DDR1, will be discussed later.

- We have further analyzed the functional contributions of HSC to hepatocarcinogenesis at different stages using LratCre x DTR depletion. In the original submission, we genetically depleted HSC only in the second half of our hepatocarcinogenesis protocol as it is not possible to selectively deplete HSC in early stages. The early depletion (in the first half of DEN+CCl_4-mediated hepatocarcinogenesis), now added to manuscript, indeed shows longer-term depletion of HSC without major repopulation. As such, we observed reduced TdTom fluorescence 6 weeks after the last DT injection and reduced HSC markers *Lrat* and *Des* at sacrifice (**Extended Data Fig.16a-b**), meaning that this early DT treatment leads to reduced HSC throughout our hepatocarcinogenesis protocol, i.e. effectively achieving early AND late HSC depletion. Unexpectedly, but consistent with our hypothesis on HSC subpopulations with opposing functions in the regulation of hepatocarcinogenesis, we saw no decrease in tumors in the early depletion model (**Extended Data Figure 16b**). The lacking effects of this long-term HSC depletion method on HCC growth, in conjunction with the strong tumor-suppressive effects of late depletion (see ***Figure 1e,f,i,j, Extended Data Figure 3g-h*** in the manuscript) strongly suggests that effects of early HSC depletion oppose those of late depletion. We now also include data from the *Lhx2* deletion model, in which we made similar observations: Early activation of HSC via Mx1Cre-mediated Lhx2 deletion in the first half of our hepatocarcinogenesis protocol does not increase tumors, whereas activation of HSC via *Lhx2* deletion in the second half strongly increased tumors (**Extended Data Figure 16c-d**). Of note, there were either no or only very few and small macroscopic tumors when we started depleting, either via LratCre x DTR or αSMA-TK, in the second half of our hepatocarcinogenesis protocol (**Figure for Reviewers 1**). Together, these investigations at different time points revealed consistently effects of HSC at later stages in hepatocarcinogenesis, suggesting that effects are most likely on premalignant/transformed hepatocytes and/or early tumor lesions.



Extended Data Figure 16 | Determination of the role of HSC in hepatocarcinogenesis at different time points. a, the long-term effects of LratCre x DTR-mediated HSC depletion in the first half of DEN+CCl_4-induced hepatocarcinogenesis was tested by sacrificing mice 3 days (n=3 mice per group), 28 days (n=4-5 mice per group) and 42 days (n=4-6 mice per group) after the last DT injection and determining LratCre-induced TdTom expression. **b**, LratCre^{pos} DTR^{pos} mice with HSC depletion in the first half of DEN+CCl_4-induced hepatocarcinogenesis retained significant HSC depletion at time of sacrifice, as determined by qPCR for HSC markers *Lrat* and *Des* (n=7-9 mice per group), but did not show significant changes in liver body weight ratio (LBR), tumour number and tumour size compared to LratCre^{pos} DTR^{neg} littermates (n=10 mice per group). **c-d**, Effects of poly I:C-induced *Lhx2* deletion at early time points (**c**, n=9 mice per group) and late time points (**d**, n=10 Mx1Cre^{neg} *Lhx2* $^{fl/fl}$ and 12 Mx1Cre^{pos} *Lhx2* ^{del}) on DEN-induced HCC, as shown by representative images, liver body ratio (LBR), tumour number and tumour size.

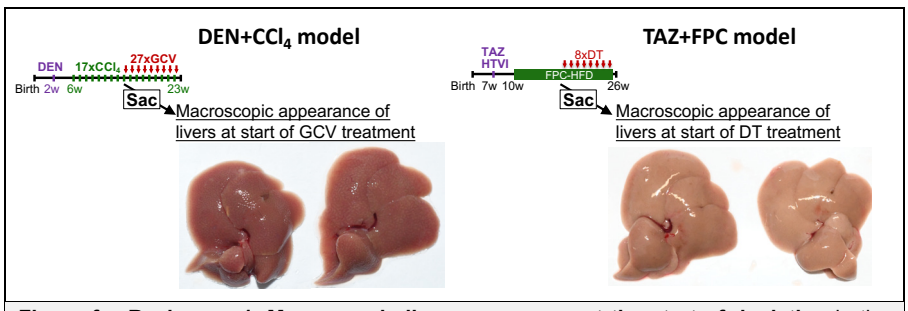
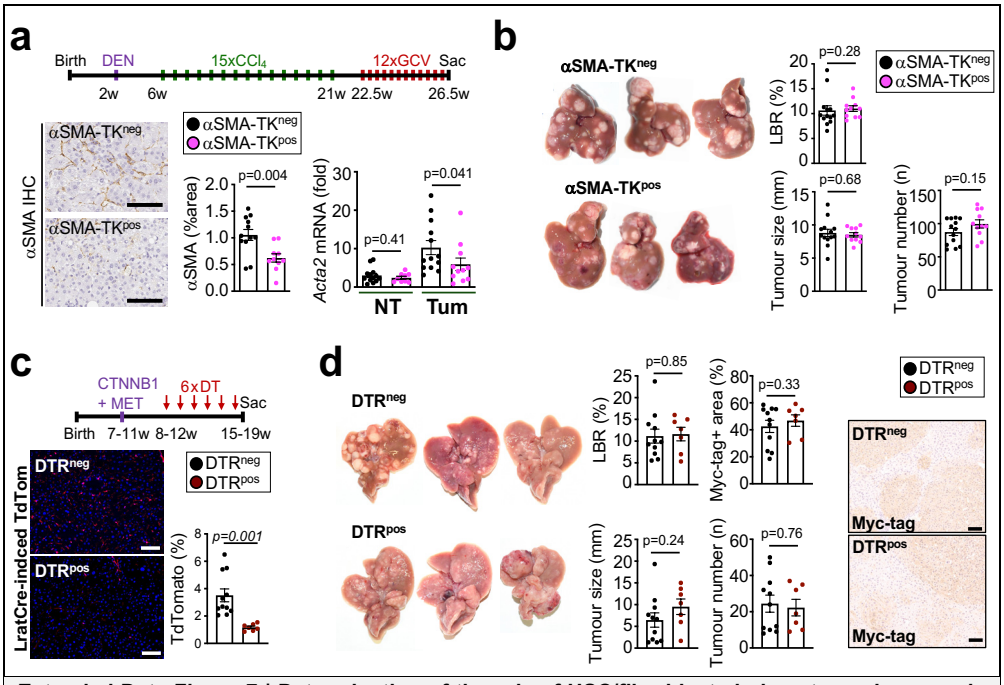


Figure for Reviewers 1. Macroscopic liver appearance at the start of depletion in the DEN+CCl_4 αSMA-TK TAZ-FPC (left panel) and LratCre x iDTR models (right panel).

- To further address the key question whether HSC/αSMA+ myofibroblasts

act mainly in the PME on premalignant/transformed hepatocytes/early tumor lesions or on large established tumor, we developed a depletion model in mice with established tumors, which allowed us to test the role of αSMA+ cells within tumors in a decisive manner. For this, we chose αSMA-TK mice and started depletion 10 days after the last CCl_4 injection, when activated αSMA+ myofibroblasts are primarily in the tumor (as opposed to the LratCre x DTR model, αSMA-TK mice do not deplete quiescent HSC). Our data in a large number of mice showed efficient depletion in tumors without effects in non-tumor areas (**Extended Data Fig.7a**). Importantly, there we found no reduction of tumor growth (**Extended Data Fig.7b**). These data clearly show that HSC/αSMA+ myofibroblasts do not play a role in promoting the growth of large tumors and are consistent with our data showing low numbers of HSC/αSMA+ myofibroblasts compared to the PME (see *Figure 2* in the manuscript). In summary, our data support our hypothesis that HSC/αSMA+ myofibroblasts promote the development of HCC through interactions with premalignant hepatocytes or in very early stages of tumor growth (when tumor cells are still in contact with HSC/αSMA+ myofibroblasts as opposed to large tumors, where HSC/αSMA+ myofibroblast numbers are low and interactions limited).



Extended Data Figure 7 | Determination of the role of HSC/fibroblasts in hepatocarcinogenesis in established tumors and in a non-fibrotic HCC model. a-b, ganciclovir injections into DEN+CCl_4-treated αSMA-TK^{pos} (n=11 mice) and αSMA-TK^{neg} (n=13 mice) littermates at late time points, when large tumours were established and CCl_4 injections had ceased for 1.5 weeks, resulted in a strong reduction of αSMA+ cells in tumour (Tum) but not in non-tumour (NT) areas (**a**), and did not affect tumour progression, as determined by liver body weight ratio (LBR), tumour number and tumour size (**b**). **c-d**, HSC depletion via LratCre x DTR in a non-fibrotic HCC model, induced by hydrodynamic tail vein injection and sleeping beauty-mediated expression MET + CTNNB1-Myc-tag, was highly efficient, as determined by TdTom fluorescence (**c**) but did not affect liver-body weight ratio, tumour size, tumour number or tumour area assessed by Myc-tag staining (n=7-11 per group) (**d**).

- Importantly, to exclude the "*possibility that ablation of HSCs itself is exerting an anti-tumor effect",* we investigated the development of HCC in the non-fibrotic CTNNB1+MET model. Despite efficient depletion of HSC via LratCre, we did not observe effects on tumor number, size or the liver body weight ratio (**Extended Data Figure 7c-d**).

In summary, our data clearly show no effect of activated HSC in established tumors. Our data strongly suggest that myHSC promote HCC by enhancing proliferation and survival of non-transformed hepatocytes (promoting regeneration) and transformed hepatocytes (promoting tumor growth) in later disease stages, when they are more abundant; whereas cyHSC protect from HCC by preventing hepatocyte injury predominantly in earlier disease stages, where they are most abundant. We have changed the manuscript to incorporate these updates.

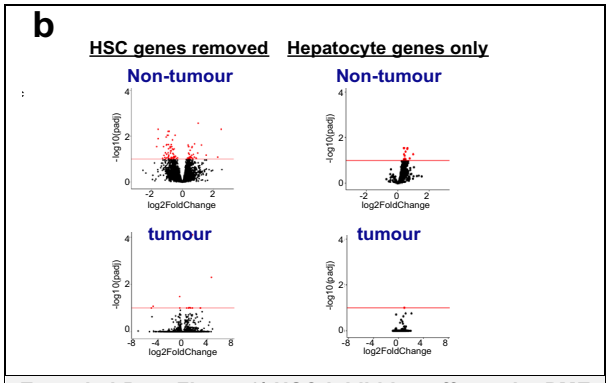
3. In figure 2 C-D RNA-seq analysis shows greater changes to the gene expression of non-tumor tissue than tumor upon HSC-specific deletion of YAP, but it is hard to know if this signature is driven by hepatocytes or primary effects of YAP KO on HSCs – especially because the majority of HSCs are found in non-tumor tissue. Single nuclear sequencing or immunostaining for key altered genes would help to clarify which changes are specific to hepatocytes in the PME.

We agree that the higher abundance of HSC in the non-tumor tissue is a potential confounder and have addressed this by two approaches in our data from mice with HSC-selective YAP deletion:

- As first approach, we filtered our bulk RNA-seq data focusing on genes that are strongly enriched in hepatocytes (using our scRNA-seq dataset to identify genes highly enriched in hepatocytes) Applying this filter to our RNAseq data from YAP$^{f/f}$ and YAP$^{\Delta HSC}$ mice, we still observe much higher changes in the non-tumor than tumor areas (**Extended Data Figure 6b**).

- As a second approach, we removed HSC-enriched genes (again using our scRNA-seq data) from our bulk RNA-seq data. Applying this filter to our RNAseq data from *Yap* $^{fl/fl}$ and *Yap* $^{\Delta HSC}$ mice, we still observe much higher changes in the non-tumor than tumor areas (**Extended Data Figure 6b**).

In summary, through these complementary approaches we have removed the potential bias from our analysis mentioned by the reviewer. In conjunction with abundant data confirming effects on hepatocytes (e.g. hepatocyte proliferation and cell death, *Fig.2E-F* in the manuscript file; and lacking effects of αSMA+ myofibroblasts depletion on tumors (see Extended Data Fig.7A-B shown above), we are confident that the main effects of HSC, revealed by our depletion and inhibition strategies in the DEN+14xCCl4 model, are indeed outside large tumors and on transformed hepatocytes or small tumors in the hepatic PME (please see also our extensive response to point 2).



Extended Data Figure 6| HSC inhibition affects the PME and reduces genes and pathways with relevance to tumourigenesis and fibrosis. b, comparison of bulk RNA-seq expression between YAP$^{fl/fl}$ and YAP$^{\Delta HSC}$ tumour and non-tumour areas in DEN+CCl$_4$-induced HCC (n=5/group; related to Main figure 2C) displaying data as volcano plot after removal of HSC genes, identified by scRNA-seq analysis, or filtering on hepatocyte genes, identified by scRNA-seq analysis.

4. Figure 2E please clarify why ALT is 10x higher in aSMA-TK^{neg} mice than in YAP$^{fl/fl}$ mice subjected to the same CCl$_4$ regimen. Conversely, there are approximately 10x more TUNEL+ hepatocytes per field in YAP$^{fl/fl}$ than in aSMA-TK^{neg} mice. Please explain such large differences in control mice subjected to the same injury model.

We agree but can explain this and also would like to confirm that all ALT measurements were performed by the same Columbia University facility using the same instrument (at over $10/sample rather than doing this in our lab as most commercial kits have a low bandwidth) and that the data within each experiment do not suffer from any bias:

- Differences between control mice during same injury model can be explained by different storage times: Some ALT measurements were done on samples stored after several years (this project initiated in 2011) and in others directly after blood collection. It is well established transaminase activity decreases over time, even in the freezer (Cuccherini et al, Lab Clin Med. 1983; 102(3):370-6)
- The subgroups for each set of mice were stored similarly, meaning that the comparisons are valid and not biased, even though there may have been a decrease in activity over time.
- In regard to the TUNEL+ hepatocytes, some experiments were quantified by different investigators, meaning the threshold of positive nuclei can vary between investigators. The αSMA-TK experiment (**Fig.2e**) was now analyzed by the same investigator (first author Filliol). While still a little bit lower than the YAP deletion model, the TUNEL+ hepatocytes are now in the same range.

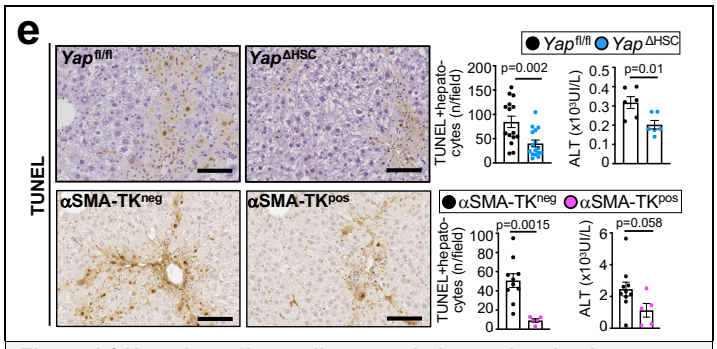


Figure 2 | Hepatic stellate cell accumulation and activation occurs predominantly in the non-tumour environment. e, cell death (e) and proliferation (Ff were determined by TUNEL assay and serum ALT measurement and Ki67 IHC, respectively, in non-tumour tissue from YAP$^{fl/fl}$ and YAP$^{\Delta HSC}$ littermates (n=15-16/group for TUNEL and n=10-11/group for Ki67; n=6/group for ALT, measured after six CCl$_4$ injections); as well as in the fibrotic liver induced by 6xCCl$_4$ from αSMA-TK^{neg} and αSMA-TK^{pos} littermates (TUNEL and ALT [n=5-9/group]).

5. The findings rely heavily on a DEN-CCl$_4$, model of HCC. Evidence that the pathogenesis and gene expression profile of this model corresponds to human NASH-HCC is required to establish the relevance of these findings to human disease.

While we agree that the DEN-CCl$_4$ model is the most common model in our study, we would like to point out that we have added more models to the manuscript and now confirmed the role of HSC in additional models. In addition to DEN-based toxic fibrosis (DEN+CCl4) and NASH-fibrosis (DEN+CDAA), our manuscript now incorporates **4 DEN-independent HCC models, including 3 models of NASH-HCC:** Cholestatic HCC (*Mdr2^{KO}*) and 3 models of NASH-HCC (TAZ+FPC; NICD+CDAA; and long-term CDAA diet). In addition, we have also used the CTNNB1+MET as model to exclude a role of activated HSC in a non-fibrotic model. In total, our study now employs 7 different HCC models (5 non-DEN models).

- In response to the reviewer's criticism as well as the request of Reviewer #2 to a model of spontaneous HCC, we added the ***Mdr2^{KO}* model as 3rd HCC model** to the already included DEN- CCl$_4$ model of chronic injury-associated

HCC and TAZ+FPC diet model of NASH-associated HCC. In the *Mdr2*^{KO} model, we confirmed reduced HCC development in mice with genetic HSC inhibition using PDGFRβ knockout in HSC via LratCre (**Figure 1g-h** - this approach works well in the *Mdr2*^{KO} model whereas the depletion of HSC or αSMA+ myofibroblasts in the *Mdr2*^{KO} HCC model is not sufficiently efficient over the 15-month period of this highly fibrotic model). Similar to human HCC and the other mouse models, we also observed more fibrosis in non-tumor areas than in tumor areas in *Mdr2*^{KO} mice (***Extended Figure 5b*** in the manuscript).

- In response to the reviewer's point that models mimicking human NASH-HCC are important, we have added two models of fibrotic NASH-HCC to investigate the role of HSC by our depletion approach, **DEN + HF-CDAA** and **NICD + HF-CDAA**. LratCre x DTR-mediated HSC depletion was highly efficient in the DEN+HF-CDAA model and results in a highly significant decrease in the liver body ratio, tumor number and tumor size (**Extended data Fig.4c-d**). In the NICD+HF-CDAA model, in which NICD expression is Cre-dependent (and thus not amenable to LratCre-mediated HSC depletion), we depleted αSMA+ myofibroblasts using αSMA-TK, and also observed efficient depletion and reduction of all tumor parameters (**Extended Data Fig.4e-f**).
- Furthermore, we have confirmed a second key finding of our study, the role of myHSC-expressed type I collagen, in **two spontaneous HCC models** with high levels of fibrosis: A model of cholestatic HCC induced by *Mdr2*^{KO} and a model of NASH-HCC, induced by long-term HF-CDAA diet. Deletion of *Col1a1* led, similar to the DEN+CCl_4 model (***Fig.4B-C*** and ***Extended Data Fig.19d-f*** in the manuscript files), led to a significant reduction of HCC development in this model of cholestatic and spontaneously arising HCC (**Extended Data Fig19a-b**) as did the HF-CDAA model (**Extended Data Fig.19g-h**) without affecting liver triglyceride or cholesterol levels. (**Extended Data Fig.19i**).

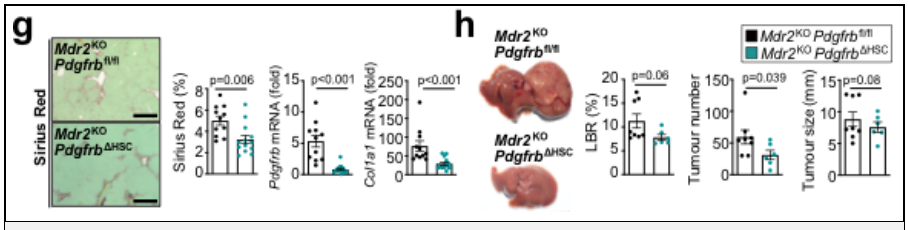
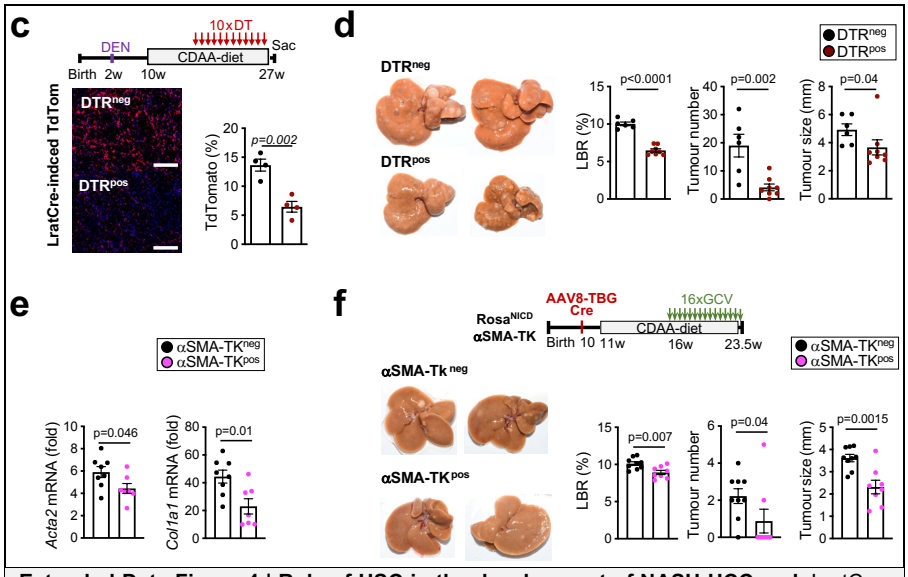
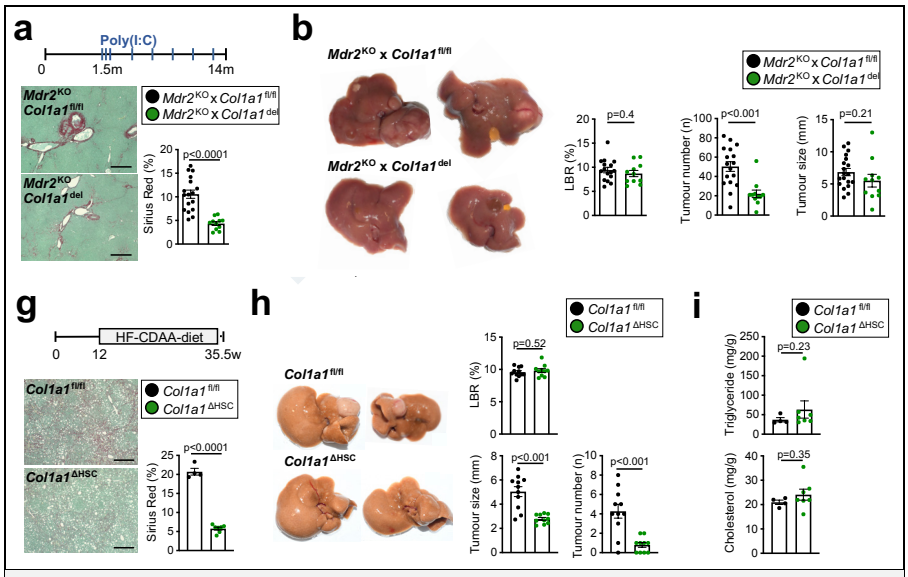


Figure 1 | Promotion of HCC development by hepatic stellate cells. g, Liver fibrosis and deletion of *Pdgfrb* were determined by Sirius red staining and qPCR for *Col1a1* and *Pdgfrb* in 4 months-old *Mdr2*^{KO} *Pdgfrb*$^{\Delta HSC}$ and *Mdr2*^{KO} *Pdgfrb*$^{fl/fl}$ female mice (n=11-13/group). **h**, Tumour development was determined in 15 months-old *Mdr2*^{KO} *Pdgfrb*$^{\Delta HSC}$ and *Mdr2*^{KO} *Pdgfrb*$^{fl/fl}$ female mice as described above (n=6-8/group).



Extended Data Figure 4 | Role of HSC in the development of NASH-HCC. c-d, LratCre-positive DTR^{pos} (n=8) or DTR^{neg} (n=6) mice were subjected to DEN+CDAA-HFD induced hepatocarcinogenesis, revealing efficient HSC depletion in non-tumour tissue (n=4 per group) (c) as well as reduced tumour development, as determined by liver body ratio, tumour number and tumour size in DTR^{pos} mice (d). **e-f,** αSMA-TK^{pos} mice (n=8 mice) or αSMA-TK^{neg} mice (n=9 mice) were subjected to NICD+CDAA-HFD induced hepatocarcinogenesis, revealing efficient HSC depletion (n=7-8 mice per group) (e) as well as reduced tumour development, as determined by liver body ratio, tumour number and tumour size in αSMA-TK^{pos} mice (f).



Extended Data Figure 19| Deletion of *Col1a1* reduces HCC development in DEN+CCl_4-, NASH diet- and *Mdr2* ^{KO}-induced HCC. a-b, Mx1Cre-mediated *Col1a1* deletion in *Mdr2* ^{KO} female mice (*Mdr2* ^{KO} *Col1a1* ^{del}, n=11) efficiently reduced liver fibrosis (**a**) and HCC development, as determined by liver body weight ratio, tumour number and tumour size (**b**), compared to Mx1Cre^{neg} *Mdr2*^{KO} littermates (*Mdr2* ^{KO} *Col1a1* $^{fl/fl}$, n=18). **g-i**, *Col1a1* $^{\Delta HSC}$ mice (n=10) displayed strong reductions of liver fibrosis (**g**) and HCC development, as determined by liver body weight ratio, tumour number and tumour size (**h**), but not in liver triglycerides and cholesterol content (**i**) compared to *Col1a1* $^{fl/fl}$ control mice (n=11 mice) in a model of NASH-associated HCC induced by 8 months of HF-CDAA diet.

- We do claim that the DEN+CCl_4 model represents human NASH-HCC as implied in the reviewer's comment. We therefore use it as one of several models in our study. DEN-based model are the most widely used and best established HCC models (107 PubMed-listed papers using the DEN+CCl_4 model, including Lujambio et al, *Cell*;153(2):449-60; Dapito et al, Cancer Cell 2012;21(4):504-16, Duran et al, *Cancer Cell* 2016;30(4):595-609; 1953 PubMed-listed papers using DEN-HCC models in general). To date, there is not a single mouse model that perfectly encompasses all aspects of human HCC. We therefore have confirmed key findings in above-listed models.
- While we have confirmed key aspects in multiple models and employed complementary approaches to increase rigor (e.g. two different methods of HSC/myofibroblast depletion + confirmation by genetic activation and inactivation), it is not possible to confirm every single experiment in our study in multiple HCC models. Models like *Mdr2*^{KO} require generation of triple transgenic mice and controls expressing the *Mdr2* allele, LratCre and the floxed allele (i.e. 12-15 months to generate mice and >1 year to expand the colony and investigate HCC development). We have a **very large number (a total of 16) of genetic approaches (see Extended Data Table 3)** in which we conditionally knocked out genes via Cre-Lox for the study of HCC (1. HSC depletion by LratCre x DTR; 2. HSC depletion by αSMA-TK; 3. HSC inhibition by either LratCre x *Yap*$^{fl/fl}$ or LratCre x *Pdgfrb*$^{fl/fl}$; 4. *Hgf* deletion in HSC via LratCre x *Hgf*$^{fl/fl}$; 5. *Col1a1* deletion in HSC via LratCre x *Col1a1*$^{fl/fl}$ or Mx1Cre x *Col1a1*$^{fl/fl}$; 6. *Has2* deletion in HSC via LratCre x *Has2*$^{fl/fl}$; 7. *Lhx2* deletion in HSC via LratCre x *Lhx2*$^{fl/fl}$ or Mx1Cre x *Lhx2*$^{fl/fl}$. 8. p53 deletion in HSC via LratCre x *Trp53*$^{fl/fl}$ 9. NFκB p65 deletion in HSC via LratCre x *Rela*$^{fl/fl}$ 10. DDR1 deletion in hepatocytes via AAV8-TBG-Cre x *Ddr1*$^{fl/fl}$; 11. ITGB1 deletion in hepatocytes via AAV8-TBG-Cre x *Itgb1*$^{fl/fl}$; 12. TAZ deletion in hepatocytes via AAV8-TBG-Cre x *Wwtr1*$^{fl/fl}$; 13. YAP deletion in hepatocytes via AAV8-TBG-Cre x *Yap*$^{fl/fl;}$ plus additional models not shown in much detail such as 14. HSC-selective deletion of *Hhip* via LratCre; 15. HSC-selective deletion of *Wntless* via LratCre (data not included in the manuscript for reasons of space– but can be added) and 16. HSC-selective deletion of *Cxcl12* via LratCre. The project was started in 2011, and might take another 2-3 years if we would confirm everything in all models.

6. The data suggest that a subpopulation of hepatic stellate cells that are enriched in HGF contribute to the anti-carcinogenic activity of the 'cytokine producing HSC' population. The relative contribution of stellate cells versus endothelial cells in producing HGF needs to be quantified and some assessment of HGF protein in these cells and/or livers would strengthen the conclusion that HGF accounts for the anti-neoplastic effect

We have addressed this point by multiple approaches including sc- and sn-RNA-seq analysis in mice and patients and measurement of HGF protein levels as well as review of published literature on this topic.

- Using scRNAseq we compared *Hgf* mRNA expression between HSC and endothelial cells in mouse liver. In normal liver, CCl_4-treated liver and HF-CDAA-treated liver, *Hgf* mRNA expression was significantly ($p<0.0001$) higher in the HSC population than in endothelial cells (**Figure for Reviewers 2**)

  Furthermore, we determined *HGF* mRNA expression between HSC and endothelial cells in human livers by snRNA-seq and found that *HGF* mRNA expression was significantly ($p<0.0001$) higher in the HSC population than in endothelial cells (**Figure for Reviewers 2**).

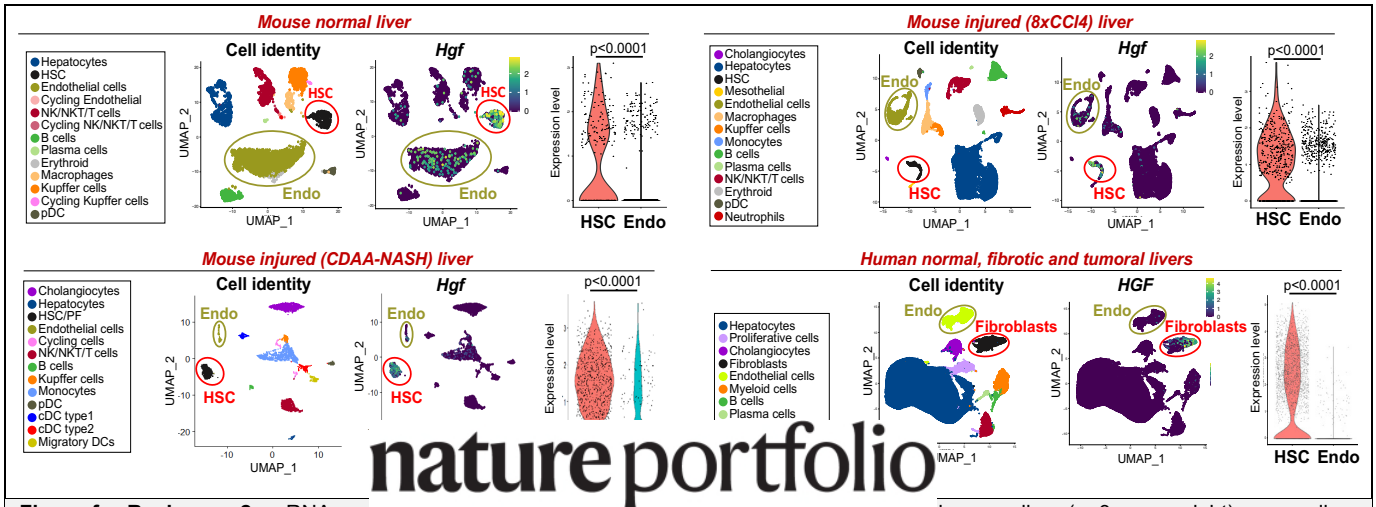


Figure for Reviewers 2. scRNA-analysis of normal mouse liver (n=1, upper left), 8xCCl4-treated mouse liver (n=3, upper right), mouse liver treated with HF-CDAA diet for 8 months (n=1, lower left) as well as snRNA-seq of human normal (n=4), cirrhotic (n=4) and HCC tumor (n=2) liver (all lower right). Shown are UMAPs highlighting the population sand *Hgf* expression as well as violin plots comparing *Hgf* expression between HSC and endothelial cells.

- Our quantification by qPCR and ELISA shows effective deletion of *Hgf* mRNA in whole liver extracts by HSC deleter

nature portfolio

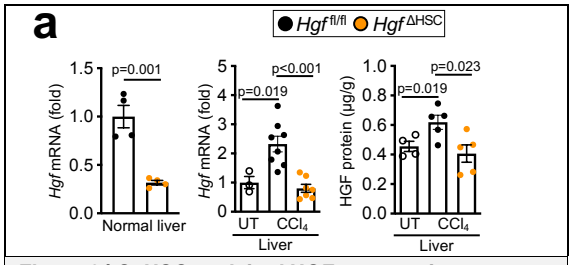


Figure 6 l CyHSC-enriched HGF protects hepatocytes from death and reduces HCC development. a, significant reduction of *Hgf* mRNA in normal and CCl_4 liver as well as of HGF protein in n=4-7 liver in LratCre^{pos} *Hgf* $^{fl/fl}$ mice (*Hgf* $^{\Delta HSC}$) compared to *Hgf* $^{fl/fl}$ mice (n=4-8/group).

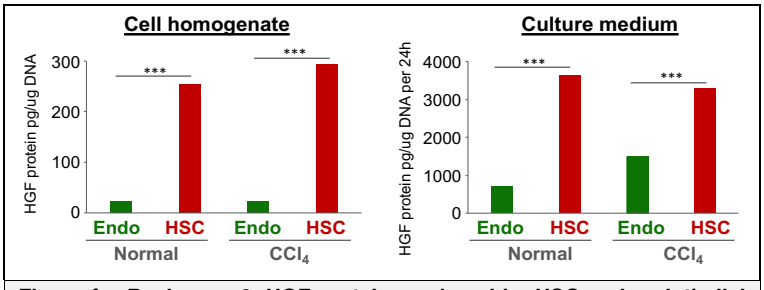


Figure for Reviewers 3. HGF protein produced by HSC and endothelial cells (Maher JJ, *J Clin Invest* 1993). HGF protein was measured by ELISA in endothelial cells or HSC in different conditions and normalized to DNA content as described in the original publication.

LratCre^{pos} in *Hgf*$^{\Delta HSC}$ mice, in normal liver (68%, p=0.0012, **Fig.6a**) and injured liver (65%, p=0.0009, **Fig.6a**). This ≈70% deletion by LratCre is consistent with our scRNA-seq analysis showing some *Hgf* expression in endothelial cells (see **Figure for Reviewers 2** on the previous page). The somewhat higher deletion efficacy in injured liver is consistent with the known up-regulation of HGF in LSEC during injury (Maher et al, J Clin Invest 1993;91(5):2244-2252 – see **Figure for Reviewers 3**). Moreover, we confirmed as requested by the reviewer at the protein level and saw a significant reduction of HGF protein in livers from *Hgf*$^{\Delta HSC}$ mice compared to *Hgf*$^{fl/fl}$ mice (**Fig.6a**). *In toto*, the deletion data are consistent with our scRNA-seq data and our data also show that the deletion of HGF in the HSC compartment exerts major biological effects (***Figure 6*** and ***Extended Data 28, 29*** in the manuscript files).

- The protein expression of HGF in HSC has been already extensively studied and compared to endothelial cells in normal liver and after CCl_4 treatment by Jackie Maher (*J Clin Invest 1993*;91(5):2244-52). As apparent from **Figure for Reviewers 3** (we converted the original table to a bar graph), their HSC ("lipocytes" in the original paper) produced several fold higher levels of HGF protein, although these differences become smaller following CCl_4.

More importantly, since HGF mimetics are in development for liver disease, it would be extremely relevant to examine whether HGF administration, either transgenically or exogenously, rescues the cancer phenotype.

Previous published studies have already shown protection from HCC development by HGF overexpression (Santoni-Rugiu et al, Proc Natl Acad Sci U S A, 1996;93:9577-8), making this point less relevant. Nonetheless, we want to follow up on the reviewer's suggestion.

With the HGF half-life being only 3 minutes, our two strategies were to use HGF mimetics and hepatic HGF overexpression. We unfortunately could not obtain HGF mimetics from Angion and in house synthesis did not yield mimetics that activated MET in mouse liver or in AML12 hepatocytes (**Figure for Reviewers 4A-B**). Instead, we attempted to overexpress HGF via AAV8-CMV-Cre. While there was an increase of serum HGF early on, the effects on liver injury were short lasting and we only observed a trend towards decreased tumor size (p=0.07) (**Figure for Reviewers 4C-D**). Most likely, this is due to loss of expression after repeated CCl_4 injections. Due to the high cost of this approach (>$5,000 for the AAV8-CMV-HGF and AAV8 Null for this cohort), we could not further pursue or optimize this approach (possibly, more injections might have improved this).

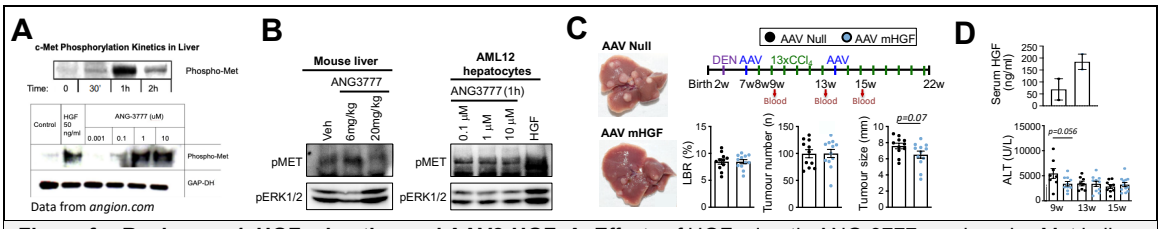


Figure for Reviewers 4. HGF mimetics and AAV8-HGF. **A.** Effects of HGF mimetic ANG-3777 on phospho-Met in liver and in vitro. **B**. In house synthesized ANG3777 did not increase phospho-MET in mouse liver or AML12 hepatocytes. **C-D.** Overexpression of HGF via injection of AAV8-TBG-Cre (twice) showed only a trend towards decreased tumor size (C). Serum HGF was increase by HGF, and liver injury was decreased at early but not late time points (D).

Also, it would be important to show evidence of changes in HGF signaling, ideally by looking at the impact on phosphorylation of c-met, or an impact on pathways of HGF signaling based on transcriptomics.

Unfortunately, we could not detect phosphorylation of Met in our models despite a strong signal in our positive control. This is due to insufficient sensitivity of the western blot. We think that this is largely related to the short half-life of HGF of just a few minutes – which is a completely different setting than our positive control, in which we injected an HGF bolus and extracted a few minutes later (**Figure for Reviewers 5**). As our biological readouts show clear and expected differences that largely mimic the Met knockout mouse, we consider this is a technical/sensitivity problem - there is no other established receptor for HGF. Unfortunately, there is no way of specifically determining

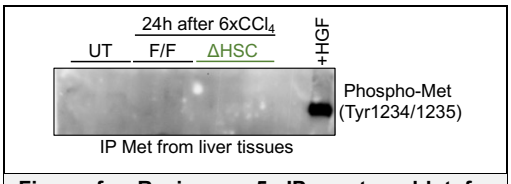
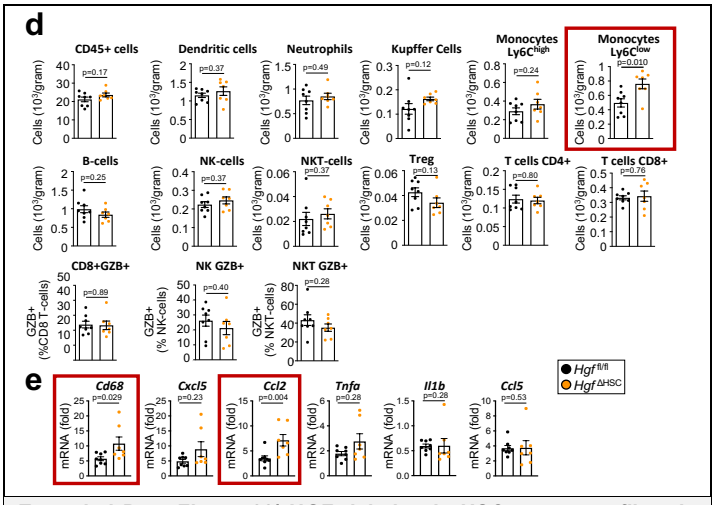


Figure for Reviewers 5. IP western blot for phospho-Met shows strong signal in HGF-treated liver but no signal in CCl_4-treated liver.

HGF signaling via transcriptomics as the pathway is largely activating prosurvival/proliferative kinase cascades that are shared with other growth factor signaling cascades.

7. Related to the above, the genetic deletion of Hgf seem to lead to increased fibrous Collagen staining (Extended Figure 14) which could serve as an alternative mechanistic explanation for why these mice demonstrate increased HCC burden (i.e. by promoting Col1a1 production and not by affecting hepatocyte turnover). This possibility needs to be addressed.



Extended Data Figure 28| HGF deletion in HSC promotes fibrosis and inflammation in CCl_4-induced liver injury. d, flow cytometric analysis of CD45+ cells as well as myeloid and lymphocyte populations in livers from *Hgf* $^{\Delta HSC}$ compared to *Hgf* $^{f/f}$ mice (n=7-8 mice per group) treated with injections of 6xCCl_4. GZB, granzyme B. **e**, qPCR for inflammatory genes from non-tumor tissue of *Hgf* $^{\Delta HSC}$ and and *Hgf* $^{f/f}$ littermates after treatment with 6xCCl_4 injections (n=7 mice per group).

- We completely agree with the reviewer that an increase of Col1a1 in *Hgf*$^{\Delta HSC}$ mice, based on the decrease of HCC observed in *Col1a1*$^{\Delta HSC}$ mice, is likely an important mechanism of increased HCC formation in these mice, highlighting the dichotomous functions of HGF and COL1A1 and the key role of their balance in hepatocarcinogenesis. This is consistent with the central finding of our manuscript that Col1a1 is a key driver of hepatocarcinogenesis (***Figure 4b-d***, ***Extended Figure 19a-i*** in the manuscript). **<u>However, increased injury in *Hgf*$^{\Delta HSC}$ mice represent the driving mechanism that precedes and triggers the increase inflammation and fibrosis</u>** (Friedman, *Gastroenterology* 2008; 134: 1655–1669; Koyama and Brenner, *J Clin Invest*. 2017;127:55-64) and not *vice versa*. Moreover, based on a large body of literature showing a key role for compensatory hepatocyte proliferation (e.g. Maeda et al, *Cell* 2005;12:977-90; Sakurai et al, *Cancer Cell* 2008;14:156-65) and inflammation (e.g. Haybeck et al, *Cancer Cell* 2009;16:295-308; Endig et al, *Cancer Cell* 2016;30:308-323; reviewed in Yang et al, *Semin Liver Dis*. 2019;39:26-42) in hepatocarcinogenesis, we cannot state that Col1a1 is the only factor responsible for increased HCC in *Hgf*$^{\Delta HSC}$ mice: Both compensatory hepatocyte proliferation (***Fig.6c*** in the manuscript files) and inflammation (**Extended Data Figure 28e**) are clearly increased. In addition to the increase in liver injury, evidenced by ALT and TUNEL analysis and by the protection from Fas-induced hepatocyte injury by HGF *in vitro* (***Fig.6c-d*** in the manuscript files), our immune cell analysis in *Hgf*$^{\Delta HSC}$ mice shows increase of classical Ly6C^{low} macrophages (**Extended Data Fig.28d**), which primarily serve to phagocytose, e.g. necrotic debris (Sprangers et al, J Immunol Res. 2016;: 1475435; Guillams et al, Immunity 201; 49:595-613). Finally, our data in the low dose DEN+CCl_4 model, in which we found decreased HCC in HSC/fibroblast-depleted mice and an increase in HCC in *Hgf*$^{\Delta HSC}$ but no difference in *Col1a1*^{del} mice (***Figure 1e-f, Extended Data Fig.3g-h, 4a-f and 18a-b and Figure 6b*** in the manuscript) support the idea that additional mediators than collagen are produced by HSC to promote HCC development. **<u>In response to above comment, we have changed our manuscript and included Col1a1 upregulation as likely mechanisms, but also point out compensatory hepatocyte proliferation and inflammation as additional mechanism.</u>**
- Knocking out *Col1a*1 in *Hgf*-deleted mice would be the only way to formally demonstrate the role of *Col1a1* specifically in this model (beyond our demonstration that HSC-derived Col1a1 has a key role in non-HGF-deleted mice in the DEN+CCl_4, *Mdr2*^{KO} and HF-CDAA models). We in fact have started breeding mice for this, but these experiments would stretch into 2023 (we have obtained first *Col1a1*$^{fl/fl}$ *Hgf*$^{fl/fl}$ homozygous mice, but need to expand the currently 3 homo mice into a larger breeding colony and then perform the DEN+CCl_4 injections; this model is also using Mx1Cre rather than LratCre to speed up breeding as LratCre can only be transmitted through females, making the generation of double floxed mice challenging). Based on the established role of other factors (compensatory proliferation and inflammation) and other Col1a1-independent ECM mediators (e.g. Has2/HA – see ***Extended Data Fig.26a-d*** in the manuscript files), we expect a significant but not complete reduction of HCC formation. As our revised manuscript already demonstrates the role of Col1a1 in three different models, adding data from this experiment, albeit elegant, would not change the message of our manuscript and cause a major delay.

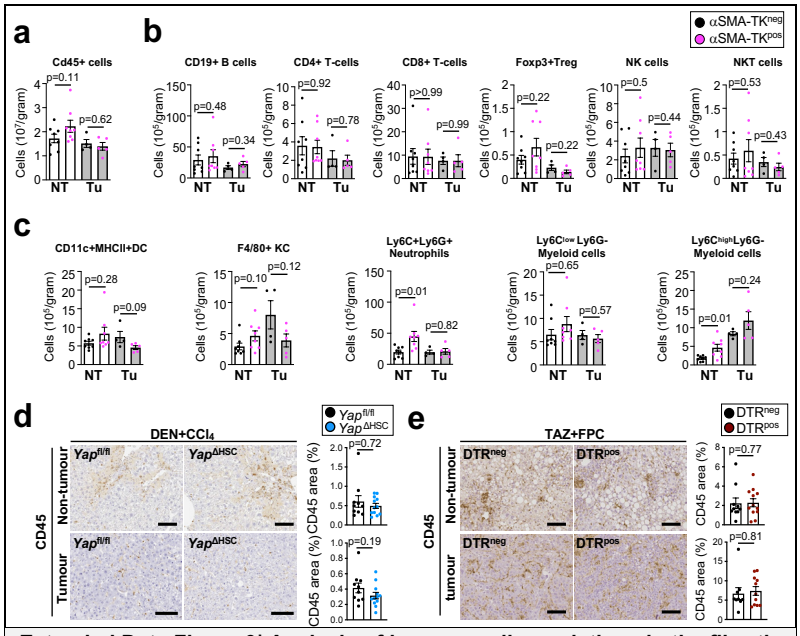
8. The findings are at odds with a recent paper in NATURE (Pfister et al, doi: 10.1038/s41586-021-03362-0) that clearly indicates evidence of impaired immune surveillance in NASH-HCC, and an earlier paper linking NASH to reduced CD4 lymphocytes (doi: 10.1038/nature16969), yet no impact was seen in the current study on any immune cell subsets. How are these findings from the current study reconciled with these other studies, and is this related to differences in the models used (see comment above)?

We completely agree that immune surveillance and its failure exert a key role in HCC and NASH-HCC, as shown in the above mentioned elegant studies by Pfister et al, (*Nature* 2021;592 :450–456) and Ma et al (*Nature* 2016; 531:253–257) as

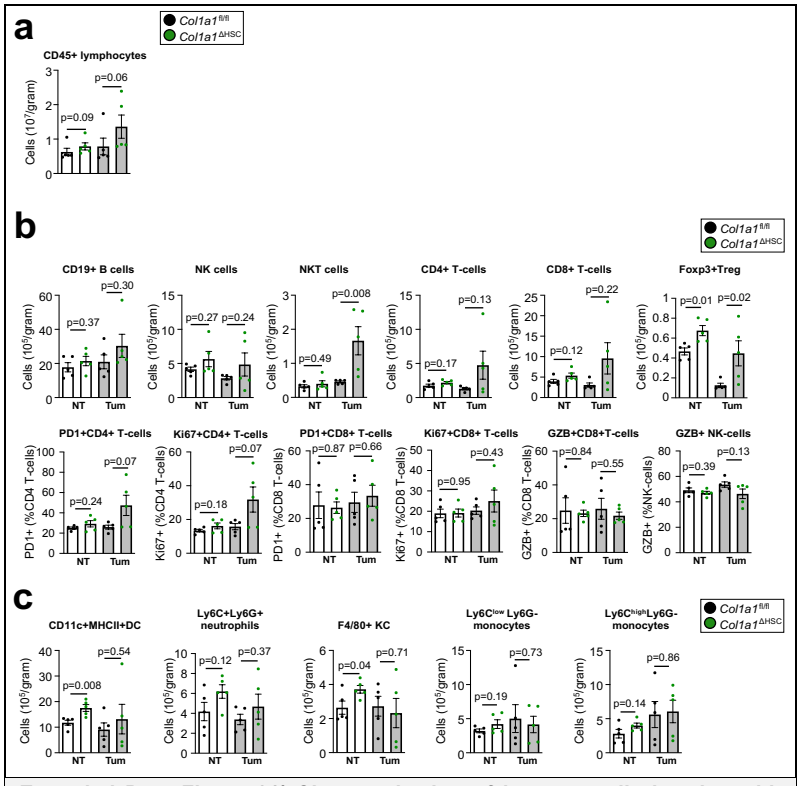
well as a large body of studies on the key role of immunosurveillance and its failure in HCC (e.g. Kang et al, *Nature* 2011; 201;479:547-51), further highlighted by the clinical success of immune checkpoint inhibitor therapy in HCC (e.g. IMbrave 150, Finn et al, *N Engl J Med* 2020; 382:1894-1905).

<u>We would like to clarify that our study does not question in any way the key role of immune surveillance and its failure in NASH.</u> In response to the reviewer's comments, we would like to explain this in further detail and also highlight additional experiments we have performed in this area to specifically address the role of HSC in this context:

- We do not think that a decrease in HCC seen in mice with modulation of HSC and HSC-expressed mediators has to be accompanied by alterations of immunosurveillance as the investigated pathways, e.g. HGF-Met, Col1-stiffness-TAZ and Col1–DDR1, have a key role in HSC-hepatocyte/tumor cell communication, shown by HSC- and hepatocyte-specific knockouts. Immune surveillance and its failure occur likely independent and in parallel to these pathways.
- As pointed out by the reviewer, we already performed an extensive characterization of immune cell subsets in myofibroblast-depleted mice in our original submission (**Extended data Figure 9a-c**). While we did not see differences in the non-tumor tissue of αSMA-TK+ depleted mice, there were some differences such as a significant increase of Ly6C+Ly6G+ neutrophils and Ly6C^{high} Ly6G^{neg} monocytes, most likely as response to the depletion of myofibroblasts, which is mediated by αSMA-TK-induced fibroblast apoptosis and typically requires removal of these dead cells by neutrophils and macrophages. Furthermore, we did not observe differences in CD45+ lymphocytes in the non-tumour and tumour compartments between *Yap*$^{fl/fl}$ and *Yap*$^{\Delta HSC}$ mice in the DEN+CCl$_4$ model or between DTR^{pos} HSC-depleted and DTR^{neg} control mice in the TAZ+FPC model (**Extended Data Fig.9d-e**).
- To follow up on the comment from the reviewer, we further tested the hypothesis that strong deposition of ECM could present a barrier for immune cell infiltration, in particular in the profound fibrosis models. Assessment of immune cell infiltration in the profound fibrosis DEN+CCl$_4$ model in *Col1a1*$^{\Delta HSC}$ mice indeed revealed differences in immune cell infiltration albeit modest, in comparison to Col1a1$^{fl/fl}$ control mice: There was a trend toward increased CD45+ cells (**Extended Data Fig.21a**), significant increases in Treg and a trend toward more CD8+ T cells (**Extended Data Fig.21b**), as well as increased dendritic cells and Kupffer cells (**Extended Data Fig.21c**) in *Col1a1*$^{\Delta HSC}$ mice compared to their *Col1a1*$^{fl/fl}$ littermates. The overall increase in immune cell infiltration (accompanied by increased infiltration of Treg, which typically goes hand in hand with increased infiltration of CD8+ T cells) is likely to contribute to increased immune surveillance and decreased tumor development in *Col1a1*$^{\Delta HSC}$ mice compared to their *Col1a1*$^{fl/fl}$



Extended Data Figure 9| Analysis of immune cell populations in the fibrotic liver or in HCC from mice with genetic HSC depletion or inhibition. a-c, FACS analysis of total CD45+ leukocytes (**a**), lymphocytes (**b**) and myeloid cells (**c**) in the PME and TME after αSMA+ cell depletion during HCC development induced by DEN+CCl$_4$ (n=8 mice/group for non-tumour and n=4-5 mice/group for HCC; related to experiments in Extended Data Fig.3g-h) shows increased neutrophil and Ly6C^{high} macrophage infiltration into non-tumour areas in aSMA-TK^{pos} mice; NT: non-tumour; Tu: Tumour. **d-e**, CD45 IHC staining of the non-tumour and tumour tissue in DEN+CCl$_4$ treated *Yap*$^{fl/fl}$ and *Yap* $^{\Delta HSC}$ mice (n=10-12 mice per group) (**d**), and in HSC-depleted DTR^{pos} mice or their DTR^{neg} littermates during HCC development induced by TAZ+FPC (n=9-12 mice per group) (**e**).



Extended Data Figure 21| Characterization of immune cells in mice with *Col1a1* deletion. a-c, determination of CD45+ lymphocytes (**a**), lymphocyte subpopulations (**b**) and myeloid subpopulations (**c**) by FACS in tumour (Tum) and non-tumour (NT) areas of LratCre^{neg} *Col1a1* $^{fl/fl}$ mice (n=5) and LratCre^{pos} *Col1a1* $^{fl/fl}$ mice (*Col1a1* $^{\Delta HSC}$, n=5).

littermates. We have included this data and interpretation in the manuscript and are also following up on the potential role of type I collagen, possibly together with other ECM components, as an immune cell barrier.

***In toto*, our data show a few but mostly moderate alterations in *Col1a1*$^{\Delta HSC}$ mice and little effect on immune cells in the αSMA-TK depletion model. These suggest that the majority of HSC effects are not mediated through tumor immunity, which is consistent with our discovery of several pathways that link HSC-secreted mediated mediators to receptors and pathways in hepatocytes (HGF-MET; Col I–stiffness-TAZ; Col I-DDR1). Finally, our findings on the role of HSC in HCC are independent of and do not contrast the progredient failure of anti-tumor immunity, which have been convincingly described by others in recent years.**

9. The contribution of collagens (DOI: 10.1111/liv.12465), tissue stiffness (DOI: 10.1152/ajpgi.00412.2010, DOI: 10.1002/hep.24108), and Taz (DOI: 10.1016/j.cmet.2020.03.010) have all been strongly speculated based on cell culture data or directly shown in vivo to drive liver disease in various publications. Similarly, the role of HGF on hepatocyte survival (reviewed in DOI: 10.1002/hep.28988), and the contribution of various forms of hepatocyte death to HCC has also been extensively published (e.g. DOI: 10.1038/s41586-018-0519-y). Since liver disease almost always precedes liver cancer and is the strongest predictor of HCC, therefore removing known drivers of liver disease and demonstrating a decrease in HCC formation is nice but not very surprising or novel. Given the trove of single cell transcriptomics data that have been generated in both mice and patients, the authors are well positioned to identify truly novel drivers and pathways affecting liver disease and HCC.

While we do not agree with the statement that the results are not surprising or novel as e.g. type I collagen is not a known driver of liver disease, but a consequence – we have taken these comments as motivation to further increase scientific novelty and included novel data on the DDR1 pathway as important driver of hepatocarcinogenesis.

Determining the role of HSC in hepatocarcinogenesis without asking the unanswered question about the role of type I collagen, the most abundant ECM component in the injured liver and key regulator of wound healing and mechanosignaling, in this process is not possible. Importantly, our studies are the **first to knockout type I collagen, and shown its role in HCC. Moreover, these studies clearly show that *Col1a1* deletion exerts little effects on CLD development but a big impact on HCC development. Hence, the Col I part of our study on mechanisms by which HSC regulate HCC development is not simply "*removing known drivers of liver disease and demonstrating a decrease in HCC formation"***. Based on published studies, our results are unexpected as the role of type I collagen and/or HGF in **HCC is opposite from other liver tumors (e.g. CCA and liver metastasis) as well as pancreatic adenocarcinoma (PDAC)**. Moreover, the only study investigating the role of type I collagen in HCC development, recently published by the Brenner group (Baglieri et al. Am J Pathol. 2021;191(9):1564-15), shows very different data: In that study, mice with non-degradable collagen, which have increased fibrosis, develop less HCC (in our study, less collagen results in less HCC. Our study and the accompanying manuscript by the Karin group now explain this, as collagen R/R-mediated alteration of the DDR1 pathway appear to override tumor-promoting effects of Col I. Finally, it is nearly impossible to investigate the complex TME-tumor interactions reliably by *in vitro* approaches. Cell culture artifacts represent a major problem for *in vitro* approaches used to date, e.g. our i*n vivo* studies clearly show that stiffness is not required for HSC activation in contrast to the above cited *in vitro* study (DOI: 10.1152/ajpgi.00412.2010). Importantly, we have also added abundant data on DDR1 and YAP-TAZ to further increase relevance and novelty.

- **The impact of collagen deletion on CLD or HCC has not been shown to date *in vivo*** (the cited paper DOI: 10.1111/liv.12465 is a review). Our study is the first to knock out one of the main collagens and assess impact on HCC development, showing an important role in CLD and hepatocarcinogenesis. The only studies on collagen *in vivo* have been using mice with non-degradable type I R/R collagen, published during our revision (Baglieri et al. Am J Pathol. 2021;191(9):1564-1579), which are opposite from ours, but explained in our manuscript and the accompanying manuscript by the Karin group as R/R collagen exerts specific effects on the collagen receptor DDR1. The only other studies that investigated the effects of decreased type I collagen expression on chronic liver disease - **but not HCC** - are by the group of Schuppan, using siRNA-mediated *Col1a1* silencing (Jiménez Calvente et al. Hepatology 2015;62:1285-97; Molokanova et al, Matrix Biol 2018;66:34-49). However, different from our studies, Col1a1 siRNA, which is delivered in large amounts to non-HSC populations in the liver, reduced HSC activation and inflammation. Our clean knockout studies using two different Cre- transgenic mice (MxCre to avoid potential developmental issues; LratCre to provide selective deletion in HSC in the liver) avoid potential non-specific effects and clearly show no role for Col1a1 in HSC activation, but nonetheless a strong effect on HCC development. Thus, the main part of our study on HSC mediators and pathways (i.e. the Col1a1 and DDR1 sections) does not simply "removing known drivers of liver disease and demonstrating a decrease of HCC", but instead show a pathway that promotes HCC without a major role in disease progression (it is even likely that there is some degree of protection by type I collagen that comes at the cost of higher cancer risk – currently under investigation).

- **Our results are novel and not predictable:**
  - **The role of Col1a1 in HCC in our study is opposite from its role in many other tumors and studies: There is increased tumor growth after Col1a1 deletion in desmoplastic liver metastasis and PDAC** (Bhattacharjee et al, *J Clin Invest*. 2021;131(11):e146987; Chen et al, *Cancer Cell* 2021;39(4):548-565); they are also different from intrahepatic cholangiocarcinoma (**no effect of Col1a1 deletion on tumor growth** – Affo et al, *Cancer Cell.* 2021;39(6):866-882).
  - We agree with the reviewer that for HSC-derived HGF, its main effect is on liver disease development and that effects on HCC development are secondary to this and a logical consequence. However, it needs to be pointed out **that HGF can also promote HCC growth** (MET inhibitors have been evaluated as HCC therapy in a large range of clinical studies). Also, **the role of HGF in HCC is opposite to studies in cholangiocarcinoma and liver metastasis, where it promotes tumor growth** (Affo et al, *Cancer Cell* 2021 and Bhattacharjee et al, *J Clin Invest* 2021). While we agree that our data on HSC-derived HGF are consistent with previous studies on MET in HCC (largely established by the Thorgeirsson group) and the known cytoprotective role of HGF, the net effect on cancer and the existence of an HGF-expressing protective HSC population are novel.
  - The manuscript **DOI: 10.1152/ajpgi.00412.2010** (Olsen et al, *Am J Physiol Gastrointest Liver Physiol*. 2011;301(1):G110-8.) does not investigate cancer, but only the role of stiffness in HSC activation *in vitro*. Not only are the topics/hypotheses different from our current study (Olsen et al investigate the hypothesis that HSC require a stiff environment to activate; we investigate the hypothesis that the stiff environment induced by HSC-secreted collagen affects hepatocarcinogenesis), but our *in vivo* data are also different from the Olsen et al in *vitro* data, in that we show the same degree of HSC activation in livers with less collagens and lower stiffness (**Extended Data Fig.18b-c** in the manuscript, while the Olsen et al paper shows less in vitro activation in the setting of lower stiffness).

- **The manuscript mentioned by the reviewer** (DOI: 10.1002/hep.24108; Schrader et al, *Hepatology* 2011;53(4):1192-205.) makes important contributions to better understand the role of stiffness in HCC biology. However, our study is completely different in that the Schrader et al paper is completely *in vitro* and that it does not investigate the role of HSC and ECM but uses a tunable *in vitro* stiffness system. By no means do we claim that we are the first to propose a role for stiffness in cancer or HCC. As mentioned above, there are several examples in which the effects of **collagen are the opposite, i.e. increased tumor growth, despite reduced stiffness, e.g. in PDAC (Chen et al, *Cancer Cell* 2021) and our own paper in desmoplastic liver metastasis (Bhattacharjee et al, *J Clin Invest*. 2021).** Hence, *in vitro* studies cannot address how HSC and fibrosis affect HCC and whether stiffness is a relevant to this or not.

Our revision now includes DDR1 as additional novel HCC-promoting pathway:

- Based on the reviewer's comments, we have focused our revision on identifying receptors that mediate effects of collagen and confirming that the effects of collagen are indeed mediated via hepatocytes. By western blot, scRNA-seq and tissue microarray analysis, we found that collagen receptor discoidin domain receptor 1 (DDR1) is upregulated in the fibrotic liver and in tumors in multiple models (**Figure 5a-c**). DDR1 was expressed in both primary hepatocytes and different HCC lines. Interestingly, we found that TGFβ – a master cytokine in fibrosis that

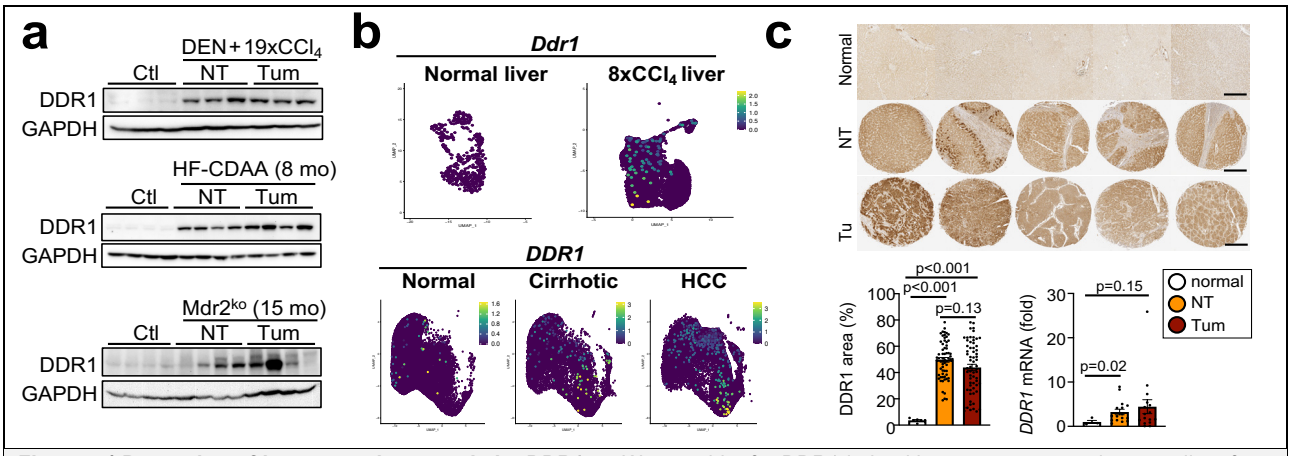


Figure 5 | Promotion of hepatocarcinogenesis by DDR1. a, Western blot for DDR1 in healthy, non-tumour and tumour liver from different mouse models of HCC. **b**, UMAPs of scRNA-seq data showing hepatocyte *Ddr1* expression from normal (n=1) and 8xCCl_4 fibrotic mouse liver (n=3) and of snRNA-seq from patients showing *DDR1* expression in the hepatocyte or tumor compartment from healthy patients (n=4), non-tumor (n=2) and cirrhotic (n=2) or HCC tumour (n=2) liver tissues. **c**, *DDR1* mRNA expression (n=4 healthy controls, n=16 non-tumor and tumor areas from HCC patients) and IHC and quantification in healthy human control livers (n=6), and in TMA containing non-tumour and tumour areas from patients (n=64 each).

triggers the activation of HSC – is an extremely potent inducer of DDR1 in both primary hepatocytes and HCC cell lines (**Figure 5d**). Thus, TGFβ upregulates both the DDR1 ligand, type I collagen, in HSC as well as its receptor on hepatocytes, **thereby activating this fibrosis- and HCC-relevant pathway on both ends.**

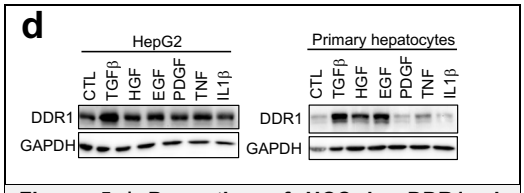


Figure 5 | Promotion of HCC by DDR1. d. determination of DDR1 expression in HepG2 and primary mouse hepatocytes by western blot after treatment with the indicated cytokines for 24h.

- To further test this signaling cascade in hepatocarcinogenesis, we generated mice with hepatocyte-specific deletion of DDR1 via AAV8-TBG-Cre, which led to a significant reduction of DDR1 expression (*Extended Data Fig.24b* in the manuscript). Of note, mice with hepatocyte-specific *Ddr1* deletion displayed a significantly reduction of HCC development (**Figure 5e**) similar to the deletion of Col1a1 our study. Our data in Col1a1-deleted mice showed a significant reduction of proliferation not only in non-tumor but also in tumor areas (*Figure 4d* in the manuscript files). Accordingly, observed significant reduction of proliferation in tumors from DDR1$^{\Delta Hep}$ mice (**Figure 5f**) fits with our finding in the Col1a1 deleted mice. Importantly, while the Col1-DDR1 pathway appears to be responsible for the Col I-mediated tumor proliferation, the Col1-stiffness-TAZ pathway is responsible for hepatocyte proliferation in the premalignant fibrotic liver (*Figure 4i* in the manuscript). Thus, these two pathways appear to jointly promote HCC growth but to operate independently and in two different compartments. This is the first study to determine the role of DDR1 in an endogenously arising model of HCC by conditional knockout. Together, our study shows that HSC-expressed Col1a1 and hepatocyte expressed TAZ and HCC-expressed DDR1 connect HSC activation and fibrosis to proliferation- and tumor-promoting signals in epithelial cells.

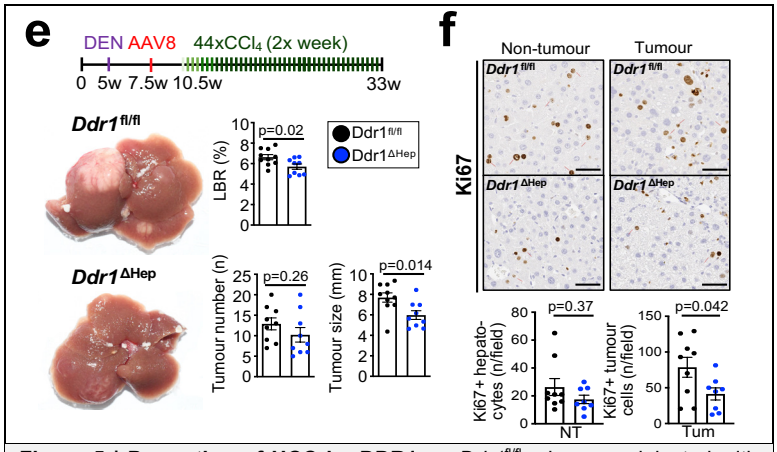
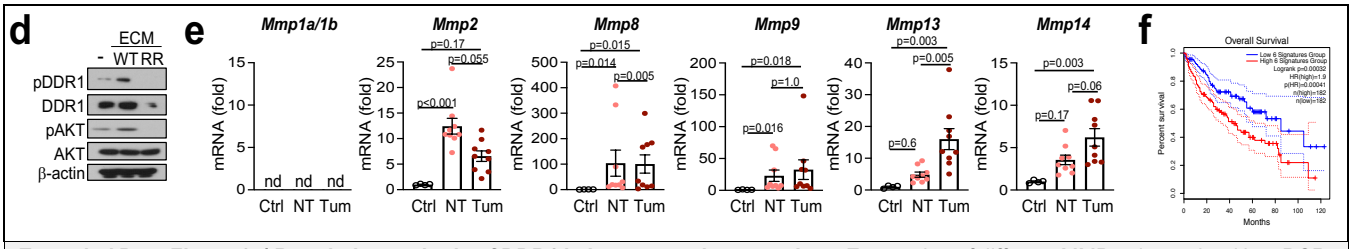


Figure 5 | Promotion of HCC by DDR1. e, *Ddr1*$^{fl/fl}$ mice were injected with AAV-TGB-empty (*Ddr1*$^{fl/fl}$, n=12) or AAV8-TBG-Cre (*Ddr1*$^{\Delta Hep}$, n=9) and HCC development was determined in the DEN+CCl$_4$ profound fibrosis HCC model. **f**, Proliferation was determined in the DEN+CCl$_4$ profound fibrosis HCC model by Ki67 IHC in the non-tumour and tumour compartments (n=7-8 per genotype).

- We also found that mutant MMP degradation-resistant collagen type 1 R/R fails to activate and even downregulates DDR1 (**Extended Data Fig.25d**), characterized in much further detail in the accompanying manuscript by Su et *al* from the Karin group. Along this line, we found that there was a strong increase in the



Extended Data Figure 25| Regulation and role of DDR1 in hepatocarcinogenesis. e, Expression of different MMPs, determined by qPCR, in the profound fibrosis DEN+CCl$_4$ model (n=4 normal liver controls, n=9 non-tumour and tumour samples). **f.** Huh7 cells were plated on plastic, WT ECM and type I collagen RR mutant ECM, followed by western blot for pDDR1, pAKT, DDR1, AKT and b-actin. **g,** effect of the expression of collagen degrading MMPs on survival of HCC patients in TCGA data, using a signature of six collagen degrading MMPs.

expression of collagen-cleaving MMPs (**Extended Data Fig.25e**) and a strong reduction of survival in HCC patients with high expression of a signature of six collagen-degrading MMPs (**Extended Data Fig.25f**).

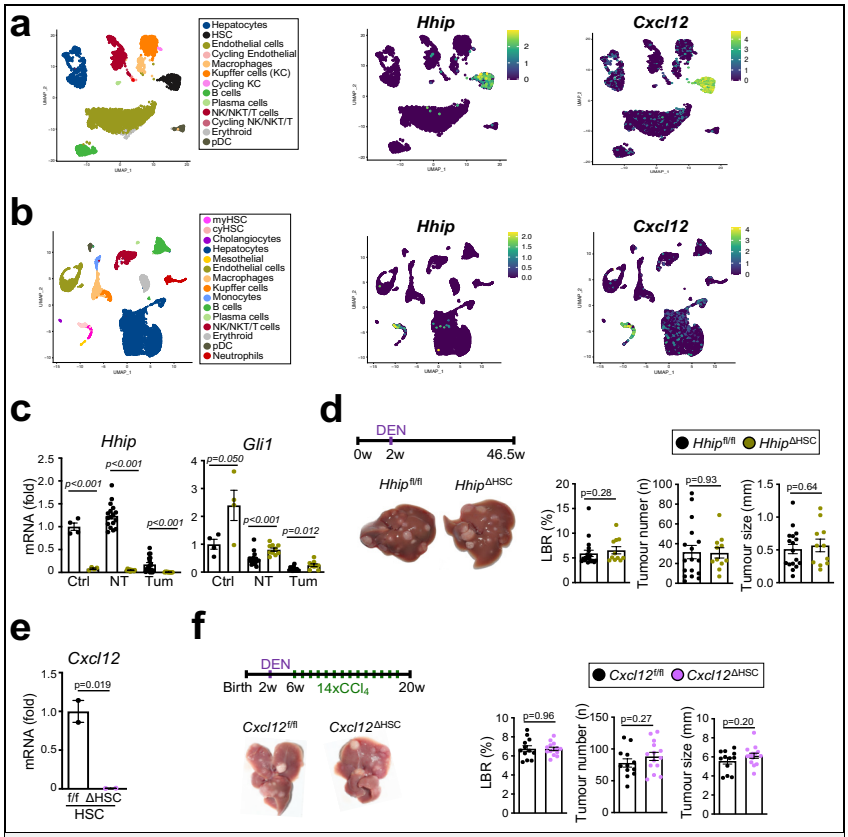
We have tested a large number of additional HSC- and fibrosis-related pathways in hepatocarcinogenesis (Itgb1, Wnt, Hh/Hedgehog, Cxcl12), many of these based on scRNA-seq expression data. In response to the reviewer's comments, we have included these data in our Extended Data Figures and/or as Figures for Reviewers. These data are not only evidence of our careful analysis of scRNA-seq data and consideration of many different mediators/pathways, but the negative data by above ko approaches further emphasize the important roles of HSC-expressed HGF, Col I and their receptors in regulating fibrosis-associated hepatocarcinogenesis.

- In response to the reviewer's comments, we have also included extensive additional data on other pathways including **HHIP/Hedgehog** (Hh) **and CXCL12**. scRNA-seq shows that *Hhip* and *Cxcl12* are strongly enriched in HSC from the normal mouse liver (**Extended Data Fig.27a**), and specifically in the cyHSC population (**Extended**

Data Fig.27b). HSC-selective deletion of *Hhip*, a negative regulator of the Hh pathway, by LratCre was highly efficient and resulted in an increase of Hh pathway activation as demonstrated by an increase of *Gli1* mRNA (**Extended Data Fig.27c**). However, despite efficient deletion and Hh pathway activation, we did not observe differences in DEN-induced hepatocarcinogenesis (**Extended Data Fig.27d**). Likewise, despite efficiently deletion of Cxcl12 by LratCre (**Extended Data Fig.27e)**, we did not observe differences in DEN+CCl_4-induced hepatocarcinogenesis (**Extended Data Fig.27f**).

- In scRNA-seq data, we found several Wnts, including Wnt4a, to be enriched in HSC (in particular in myHSC) but LratCre-mediated deletion of *Wntless*, which is required for secretion of all Wnts, did not change DEN+CCl_4-induced HCC development (**Figure for Reviewers 6**).

- We found only a minor tumor-promoting role of a second collagen receptor, integrin beta 1 (**Extended Data Figure 23**), with hepatocyte specific deletion of *Itgb1* via AAV8-TBG-Cre exerting significant effects on tumor size, but not on tumor number and liver body ratio. Previous studies using nanoparticle-mediated silencing with repeated (weekly) injections in the HCC model) have shown a role in liver regeneration (Speicher et al, *Nat Commun*. 2014;5:3862) and in the non-fibrotic MET+CTNNB1 HCC model (Bogorad et al, *Nat Commun*. 2014; 5: 3869). Thus, it appears that in fibrotic HCC, *Itgb1* only play a minor role, further highlighting the importance of epithelial DDR1 and TAZ.



Extended Data Figure 27| Expression and role of cyHSC-expressed HHIP and CXCL12 in hepatocarcinogenesis. a,b, UMAPs of *Hhip* and *Cxcl12* mRNA in normal mouse liver (**a**) and in CCl_4-treated liver (**b**). **c**, mice with LratCre-mediated conditional deletion of *Hhip* (*Hhip* $^{\Delta HSC}$) show efficient deletion in healthy control liver (n=4 mice), non-tumor tissue (n=10-17 mice per group) and tumor tissue (n=10-17 mice per group) as well as upregulation of Hedgehog target gene *Gli1* in comparison to *Hhip* $^{f/f}$ controls. **d**, *Hhip* $^{\Delta HSC}$ and *Hhip* $^{fl/fl}$ mice were subjected to DEN-induced hepatocarcinogenesis, followed by evaluation of the liver-body weight ratio (LBR), tumour size and tumour number. **e**, Deletion of *Cxcl12* was determined by qPCR in HSC isolated from mice with LratCre-mediated deletion (*Cxcl12* $^{\Delta HSC}$) in comparison to *Cxcl12* $^{fl/fl}$ HSC (n=2 mice per group). **f**, *Cxcl12* $^{\Delta HSC}$ and *Cxcl12* $^{fl/fl}$ mice (n=12-14 mice per group) were subjected to DEN+CCl_4 induced HCC followed by evaluation of the liver-body weight ratio, tumour size and tumour number.

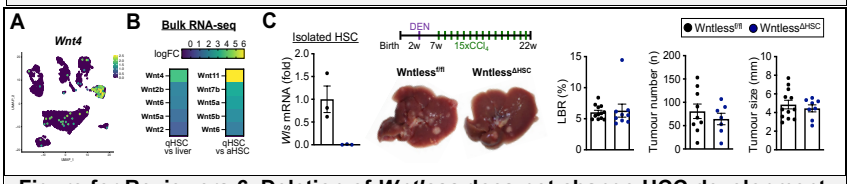
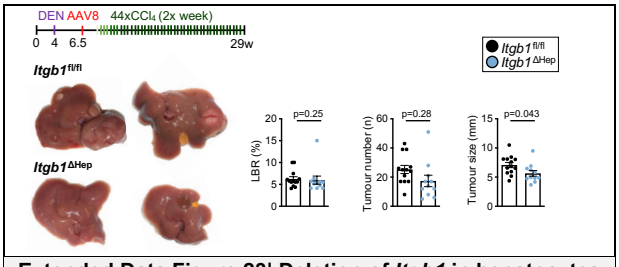


Figure for Reviewers 6. Deletion of *Wntless* does not change HCC development. A-B. Several Wnts are enriched in HSC (shown by scRNA-seq or bulk RNA-seq) or upregulated with HSC activation (shown by bulk RNA-seq). **C.** Deletion of *Wntless* (Wls), which is required for secretion of all Wnts, by LratCre was highly efficient, but did not alter DEN+CCl_4-induced HCC development. * p<0.05, **, p<0.01, ***, p<0.001

In summary, our data are novel, often unexpected and opposite from the roles of the identified pathways in other cancers. Addition data on DDR1 as well ITGB1, TAZ and YAP as well as cyHSC-enriched mediators HHIP and CXCL12 further highlight novelty, relevance and rigor of our studies.



Extended Data Figure 23| Deletion of *Itgb1* in hepatocytes exerts only minimal effects on DEN+CCl_4-induced hepatocarcinogenesis. *Itgb1* $^{fl/fl}$ mice were injected with either AAV-TGB-empty (*Itgb1* $^{fl/fl}$, n=13) or AAV8-TBG-Cre (*Itgb1* $^{\Delta Hep}$, n=11). Effects of hepatocyte-specific *Itgb1* deletion on HCC development were determined in the DEN+CCl_4 profound fibrosis HCC model by the liver-body weight ratio (LBR), tumour number and tumour size.

10. The authors have recently published two elegant papers (refs 52 & 53) that advance similar concepts regarding the role of type I collagen, and the presence of different HSC subsets in liver How similar are the transcriptomic features of the HSC subsets described in the current paper to the 'myCAFs' and 'iCAFs' in reference 52?

We appreciate this comment as indeed these functional classifications of cyHSC (equivalent of iCAF in non-tumor tissue) and myHSC (equivalent of iCAF in non-tumor tissue) are not vastly different. In the current paper, our goal was to understand the subpopulations in the non-tumor tissue from scratch rather than using our previous signatures, developed for CAF within tumors. Nonetheless, we compared these signatures and they do reveal similar results (**Figure for Reviewers 7A**), with the main difference that the newly developed cyHSC/myHSC signature provide a much stronger signal (as they have been developed specifically for this context). *Vice versa*, when applying the cyHSC and myHSC signatures to cholangiocarcinoma, we also see that this separates the CAF populations similar to the iCAF/myCAF signatures (**Figure for Reviewers 7B**). Based on above analyses, we consider the development and use of the cyHSC/myHSC signatures for the non-tumor context more appropriate.

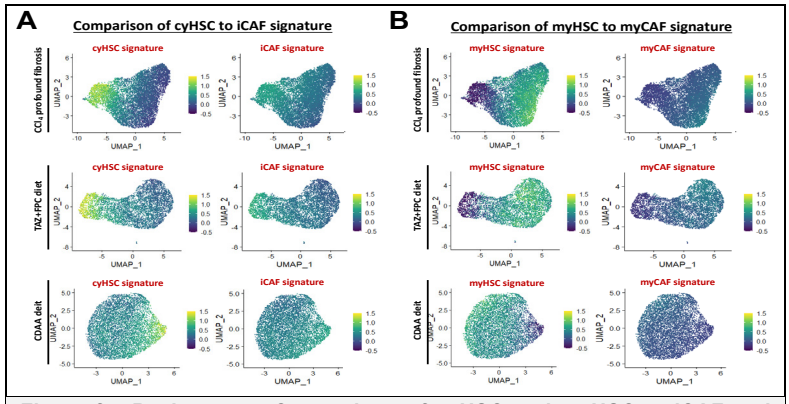


Figure for Reviewers 7. Comparison of cyHSC and myHSC to iCAF and myCAF signatures. A-B. iCAF (A) and myCAF (B) signatures from Affo et al. were applied to different HSC scRNA-seq datasets from our study and marked similar subpopulations as cyHSC and myHSC signatures, but less clearly.

The classification of HSCs into cyHSC (which is essentially quiescent HSCs) and myHSC (which represent activated HSCs) has been established for decades (reviewed in DOI: 10.1038/nrgastro.2017.38). Unfortunately powerful, cutting-edge single cell technology has not been leveraged to identify new HSC subsets. Using a similar stellate cell enrichment strategy to enrich for HSCs, a recent publication has done a better job at identifying novel HSC subpopulations in liver fibrosis progression and regression (DOI: 10.1002/hep.31743 – D. Brenner paper HSC in NASH).

- We agree with the reviewer that our concept is not different from quiescent and activated HSC, established long ago. **Our main goal was not to introduce a new classification, but to functionally analyze these cells, and based on this perform functional analyses by HSC-selective knockouts**. The more quiescent cells express high levels of cytokines – hence the terminology cyHSC instead of qHSC. One additional difference is that most of our comparisons are within injured livers, meaning that cyHSC are not completely quiescent.
- Our main goal was to identify HSC subpopulations and associated mediators with high functional relevance to HCC - rather than "novel subpopulations" - which we then tested in subsequent functional studies (knocking out myHSC mediators *Col1a1* and *Has2*; and cyHSC mediators *Hgf*, *Hhip* and *Cxcl12*). We do not think that novel HSC subpopulations/finer resolution/better functional classification via scRNA-seq analysis beyond what has already been published in the literature can be identified unless further in-depth analyses (e.g. concomitant scATAC-seq; concomitant high resolution spatial transcriptomics; or

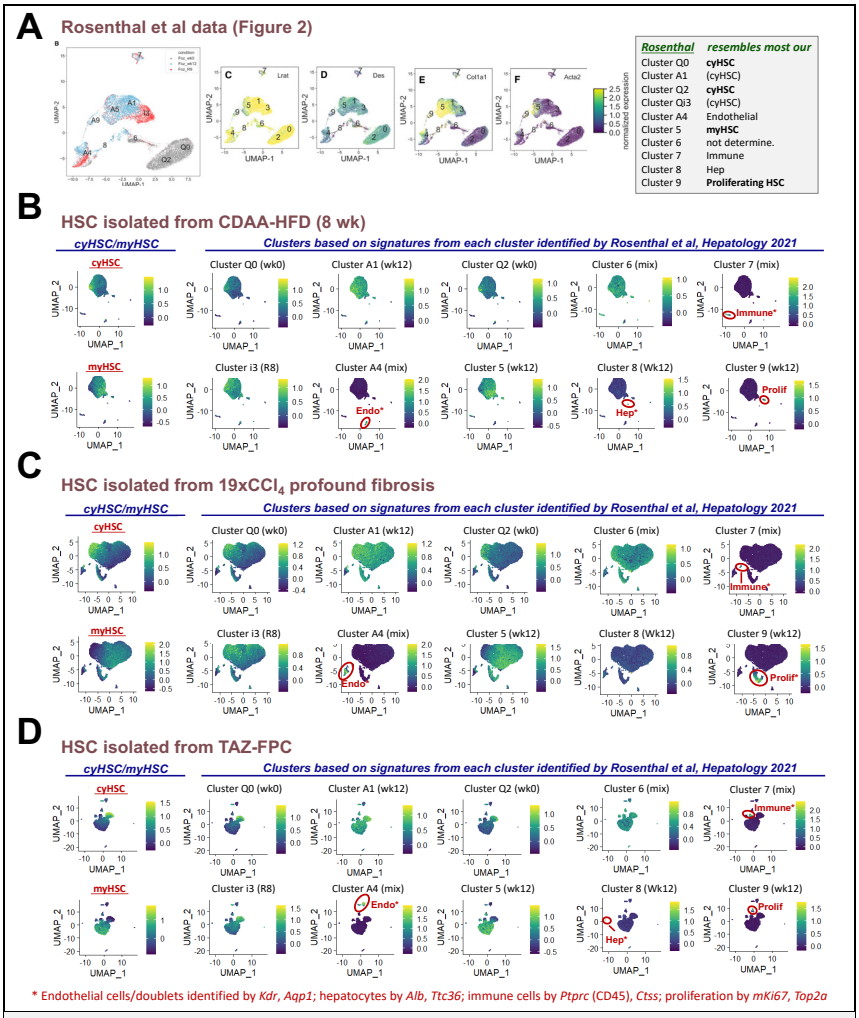


Figure for Reviewers 8. Comparison of the Rosenthal et al HSC clusters to our HSC clusters. A. Display of Rosenthal et al clusters and summary of comparison, showing which of our HSC clusters aligns with their clusters. **B-D.** Cluster-specific gene signatures for clusters 0-9 from Rosenthal et al were applied to HSC scRNA-seq data from the HF-CDAA (B), 19xCCl4 (C) and TAZ-FPC (D) models. Comparisons were made to data without removal of doublets or contaminating cells from our data, allowing to align some of the Rosenthal et al. clusters with these doublet/non-HSC clusters.

analysis of transcriptional regulators through reverse network engineering; both currently ongoing long-term projects in our lab) are applied.

- In response to the reviewer comments on the recent manuscript DOI: 10.1002/hep.31743 by the Brenner group (Rosenthal *et al*, Hepatology 2021;74(2):667-685), we have analyzed their clusters and compared them to our analyses (**Figure for Reviewers 8**), using a gene expression signatures for each cluster from their Supplementary Tables S3-S12. It should be noted that their paper not only used more conditions (resulting in more clusters), but also did not use genetic labeling strategies to sort HSC, which may result in additional clusters of non-HSC. A second paper by the Ravnskjaer group (Terkelsen et al, Hepatology. 2020;72(6):2119-2133) used approaches that are more similar to ours in that there is one continuous cluster of activated HSC, with the only differences that they also included quiescent HSC, which form a separate cluster (**Figure for Reviewers 9**). Overall, the results from the Brenner paper reveal expected finding, showing that the highly activated (A5 cluster) aligns with myHSC and the quiescent clusters (Q0,Q2) as well as the *Col1a1*^{low} and *Acta2*^{low} clusters (A1) with the cyHSC cluster (**Figure for Reviewers 8**). Notably, application of the A5 cluster (correlating to our myHSC cluster) covers a large proportion of our HSC. The proliferation cluster A9 also falls clearly into our clusters of proliferation HSC. The only notable difference is that cluster I3 (which is right adjacent to the A1 lowly activated HSC cluster in Rosenthal et al) largely labels cyHSC in our data, which is expected as (i) our model does not have a reversal phase and (ii) HSC after cessation of injury are expected to be lowly activated similar to the cyHSC status in our paper. Cluster 4, which was a mix of cells from different models and not clearly defined in the Rosenthal paper, marked a small cluster that was positive for endothelial markers *Kdr* and *Aqpr1* in our data (which may be contaminating LSEC or HSC-LSEC doublets). Clusters 7 signature marked a small cluster of cells in our that expressed immune cell markers *Ptprc* and *Ctss* in our (which may be contaminating immune cells or HSC-immune cell doublets). Cluster 8 marked a small cluster of contaminating hepatocytes, positive for *Alb* and *Ttc36*. For all analyses in our paper, doublets and contaminants (as well as proliferating cells) were removed (but are shown in the figure above to demonstrate contaminations).

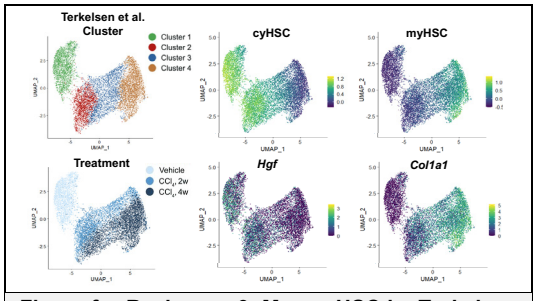


Figure for Reviewers 9. Mouse HSC by Terkelsen et al, Hepatology aligned with cyHSC and myHSC signatures. Shown are clusters of representing quiescent HSC (cluster 1) and HSC with different degrees of activation (Clusters 2-4), aligned with cyHSC/myHSC signatures, *Hgf* and *Col1a1*.

In summary, comparison of our data and analysis methods to the Rosenthal *et al* and Terkelsen *et al* papers shows (i) that our analyses are consistent with the clusters of Rosenthal et al (activated = myHSC; quiescent and *Col1a1*^{low} *Acta2*^{low} = cyHSC; proliferating = proliferating; (ii) that our data, like the one by Terkelsen et al (Figure for Reviewers 9), show less clusters due to the inclusion of fewer conditions and lower contamination due to use of LratCre labeling and doublet removal; and (iii) that our data had one large cluster of activated HSC with continuum of HSC activation similar to Terkelsen et al data. We believe that application of signatures from studies with multiple condition like Rosenthal et al (normal liver; fibrotic liver; reverted fibrosis liver) to our single condition progressive models does not provide a better resolution or better understanding of the underlying biology. Moreover, large clusters of HSC, with a continuous range from cyHSC to myHSC, seen in our scRNA-seq analysis, is similar to multiple other published studies (see Figure for Reviewers 10 and response to point 11 below).

11. Are the two major HSC subsets, 'myHSCs' and 'cyHSCs' derived from one another or are they programmed to these divergent fates? In other words, how are these different subtypes generated?

We used two approaches to investigate cyHSC and myHSC trajectories: (i) Analysis of already published scRNA-seq data with pseudotime and (ii) analysis of our own data. By both approaches, we found that myHSC are derived from cyHSC. Given that cyHSC represent the more quiescent and myHSC the more activated cell types, this is consistent with the well-established principle that activated HSC are derived from quiescent HSC.

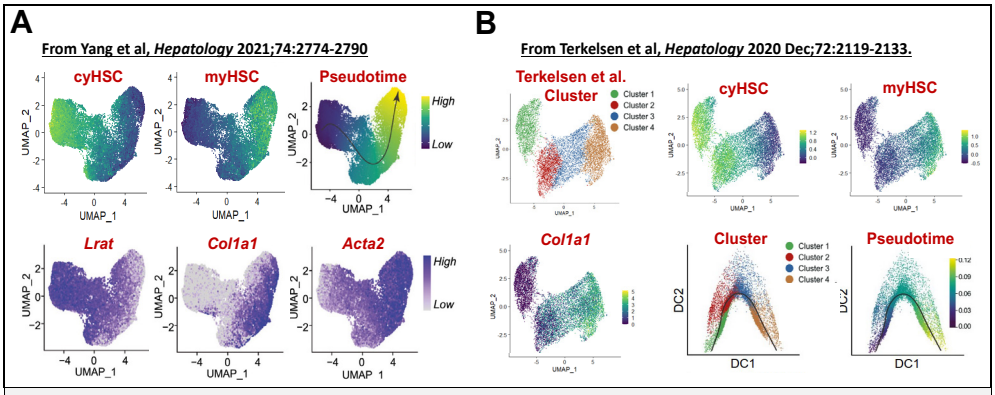
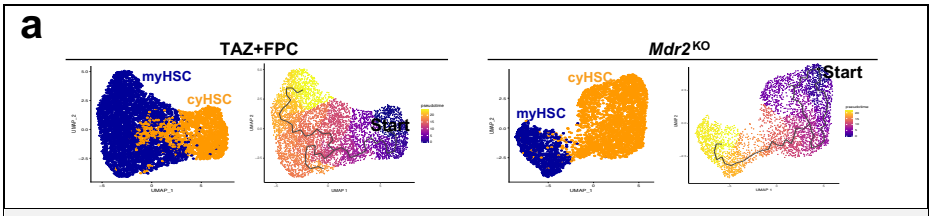


Figure for Reviewers 10. A-B. Applying our cyHSC and myHSC signatures to published pseudotime data from Yang et al (A) and Terkelsen (B) show trajectories from cyHSC to myHSC (see also Figure for Reviewers 9 for additional info on Terkelsen et al).

- For the first approach, we used data from Yang *et al* (*Hepatology* 2021; 74:2774-2790) and Terkelsen *et al* (Hepatology. 2020;72(6):2119-2133) and applied cyHSC and

myHSC signatures to their published pseudotime analysis as well displayed quiescence (*Lrat* mRNA) and activation (*Col1a1* and *Acta2* mRNA) marker genes. Not only did cyHSC and myHSC match quiescence and activation markers, respectively, but the pseudotime shows a trajectory from cyHSC to myHSC in both datasets (**Figure for Reviewers 10A-B**).



Extended Data Figure 14| Analysis of trajectories and mediators produced by myHSC and cyHSC populations by scRNA-seq. a, pseudotime analysis in HSC isolated from the TAZ+FPC (n=1) and *Mdr2* ^{KO} (n=1) models, juxtaposed to a UMAP showing cyHSC and myHSC.

- Similar findings were made in our own data in the TAZ+FPC and *Mdr2*^{KO} models, showing again trajectories from cyHSC to mHSC in our pseudotime analysis (**Extended Data Fig.14a**).

12. The authors state that HSC-specific deletion of Col1a1 was associated with reduced hepatocyte proliferation (line 276-277), but changes to ki-67+ hepatocytes did not reach significance in figure 4D (p=0.06). Reduced ki67 mRNA is not sufficient evidence of reduced hepatocyte proliferation. Please revise the text to reflect this.

We agree that this data was underpowered needed to be strengthened and have addressed this point in two ways:

- We increased the number of mice to n=13/group for the non-tumor area n=10-13 for the tumor areas (not all *Col1a1*-deleted mice had enough tumor tissue for our analyses). The data is now **highly significant at p=0.007 (Figure 4d)**.
- We have added a new cohort of mice with HSC-specific deletion via LratCre (the other set were mice with Mx1Cre-mediated deletion of *Col1a1*, which was our initial strategy as Col1a1 expression in largely HSC-specific). Similar to the Mx1Cre mice, we observed a significant (p=0.047) reduction of hepatocyte proliferation in the non-tumor area (***Extended Data Fig.19f*** in the manuscript files).
- We removed the *mKi67* mRNA data as this measures proliferation of all cells, opposed to the quantification of proliferation hepatocytes.

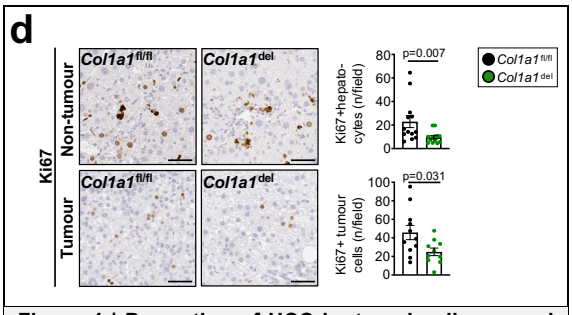


Figure 4 | Promotion of HCC by type I collagen and stiffness-associated accumulation of TAZ in hepatocytes. D, Ki67 staining as in non-tumour and tumour tissue (n=10-13 mice per group) show a reduction of hepatocyte proliferation and tumour cell proliferation in *Col1a1*^{del} mice compared to *Col1a1*$^{f/f}$ littermates.

MINOR POINTS:

1. Fig 4 F western blot is of poor quality and hard to interpret.

We have improved the western and our analysis shows significant differences and the results are further supported by our IHC analysis of hepatocyte-expressed TAZ in **Figure 4g**.

2. Fig 2A labels on bar graph are out of alignment

We have corrected this issue.

3. Figure 4E, legend does not include a label for the data represented by open circles. Please add this.

We have corrected this issue.

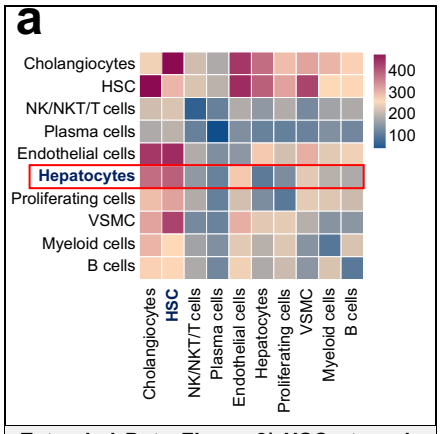
4. Similar analyses (tumor burden, hepatocyte proliferation and hepatocyte apoptosis) are presented across multiple models. A summary figure or table of results would be helpful.

As suggested, we have generated this table and also included it in the manuscript as **Extended Data Table 5**.

5. Figure 2G, 3F: From CellphoneDB analysis of scRNAseq data, it is quite clear that HSCs but not hepatocytes are a hub of cell-cell communication in the liver, which is in line with what has reported previously in a mouse NASH model (DOI: 10.1016/j.molcel.2019.07.028). Within all the cell types that HSCs communicate with, hepatocytes do not stand out as a main partner, the number of interactions between HSCs and hepatocytes is less than HSCs with endothelial, portal fibroblasts, cholangiocytes, macrophages, Kupffer cells, and DC. Focusing on hepatocyte as a hub of cell-cell communication in the liver seems somewhat misleading and not supported by the data.

- We completely agree with the comment that HSCs do represent a main hub of communication. We have included this in our manuscript also rephrased statements to emphasize that our specific focus in the context of hepatocarcinogenesis is on hepatocytes as they represent the source of origin for HCC (Mu et al, J Clin Invest 2015;125(10):3891-903) and HSC are their main interaction partner. We have included a statement in our manuscript: ".. HSC are a hub of cell-cell communication in the liver, as previously suggested by us and others (Xiong et al, *Mol Cell* 2019;75(3):644-660; Affo et al, *Cancer Cell.* 2021;39(6):866-882; Bhattacharjee et al, *J Clin Invest*. 2021;131(11):e146987)." Focusing on hepatocytes in the specific context of our HCC studies, we identified HSC as their main interaction partners (compared to all other hepatocyte-interacting populations)".

- We would like to point out that interactions of HSC with other cells could indirectly promote HCC. However, this would be more likely if non-HSC populations had more interaction with hepatocytes than HSC. Importantly, our focus revealed two HSC mediators, *Hgf* and *Col1a1*, which regulate HCC development through cognate receptors on hepatocytes (MET and DDR1, respectively), showing the relevance of direct HSC-hepatocyte interactions suggested by our CellPhoneDB analysis.
- Along the high relevance of hepatocytes as cells of origin for HCC, we would also like to point out that are various analyses in HSC-depleted or HSC-inactivated mice showed few differences in immune cells and endothelial cells (**Extended Data Fig.9** in the manuscript), but major differences in hepatocyte responses. Hence, focusing on interactions with hepatocytes is justifiable (and also lead to identification of several HSC-activated receptors/pathways that promote HCC in the second half of our study).
- We have additionally analyzed interaction in human single nucleus RNA-seq, showing that HSC are the cell type that hepatocytes interact the strongest with (**Extended Data Fig.8a**)



Extended Data Figure 8| HSC strongly interact and regulates hepatocytes proliferation. a, analysis of cell-cell interactions by CellPhoneDB in snRNA-seq data from cirrhotic liver patients (n=4) revealed HSC as a major cell type interacting with hepatocytes.

REFEREE #2 (REMARKS TO THE AUTHOR):

In the manuscript termed „An Impaired Balance of Tumour-Suppressing and Tumour-Promoting Hepatic 2 Stellate Cell Populations in Liver Fibrosis Contributes to Hepatocarcinogenesis" by Filliol and colleagues the authors dissect the role of hepatic stellate cells and factors expressed by the latter (e.g. HGF) in liver cancer in mouse models and human liver cancer specimen.

The study involves state of the art technologies and several different human specimen to characterize tumor associated HSCs – identifying tumor promoting (myHSC) and tumor suppressing (cyHSC) HSCs, which the authors can well define by single cell technologies and precise mouse models targeting HSCs (e.g. Lrat-Cre trasngenic).

This newly identified signature was also transferred into the real world – analyzing HCC risk of patients and the dysbalance of myHSC and CyHSC. Overall, this is a very interesting and innovative manuscript, - with some surprising findings (e.g. the existence of tumor suppressive HSCs; role of HGF) that is to the most degree also well controlled.

We would like to thank the reviewer for these positive comments.

However, there are several additional questions that need to be addressed by the authors, and experiments need to be executed to clarify some unclear issues.

We have worked diligently to address all points brought up by the reviewer,

Generally, the manuscript would also profit from including one or the other spontaneous liver cancer model (without the use of genetic drivers) – as the models used are in part artificial and might not reflect the natural activation of HSCs (when we think about transcriptional programs, e.g. like through CCL4); or addition of tumor drivers –making a rather artificial genetic environment). This does not mean that this referee is against the models applied – on the contrary – but some of the findings need to be assured in spontaneous models. How do the authors know that there might not be some differences to the models used in this paper?

We completely agree and have added the *Mdr2*^{KO} and HF-CDAA spontaneous HCC models to different parts of the manuscript. Both models have a strong degree of fibrosis and have common drivers of chronic liver disease (CLD) and HCC, i.e. non-alcoholic steatohepatitis (NASH) and cholestasis, thus making it highly relevant and well-suited to study the role of HSC and fibrosis in hepatocarcinogenesis. Of note, the *Mdr2* gene is the mouse orthologue of human *MDR3*, which is implicated in the development of chronic liver disease and liver cancer in patients (Trauner et al, *Semin Liver Dis* 2007;27:77-98). Unfortunately, due to COVID-related breeding constraints, we could not obtain sufficient *Hgf*$^{\Delta HSC}$ mice to study the role of HSC-derived HGF in additional spontaneous models.

- We have added the *Mdr2*^{KO} model to confirm the role of HSC in spontaneously arising HCC. For these studies, we used genetic inhibition model to assess the role of HSC as depletion via LratCre x DTR or αSMA-TK is technically difficult over such a long period. We in fact tested different model and found that HSC-selective LratCre-mediated deletion of *Pdgfrb* but not *Yap* (data not shown – but can be made available to the reviewers) resulted in efficient *Pdgfrb* deletion, inhibition of HSC activation and fibrosis as demonstrated by *Col1a1* qPCR and Sirius Red staining (**Figure 1 g**). Importantly, *Mdr2*^{KO} *Pdgfrb*$^{\Delta HSC}$ mice developed significantly less HCC than their *Mdr2*^{KO} *Pdgfrb*$^{fl/fl}$ littermates at 15 months (**Figure 1h**), which was also observed in a second cohort at 12 months (data not shown).

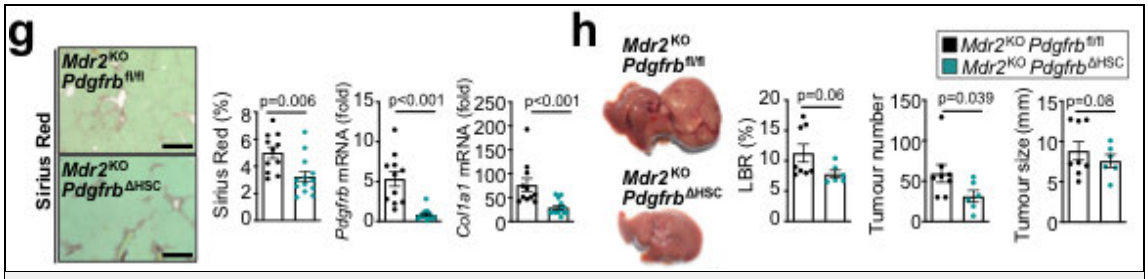
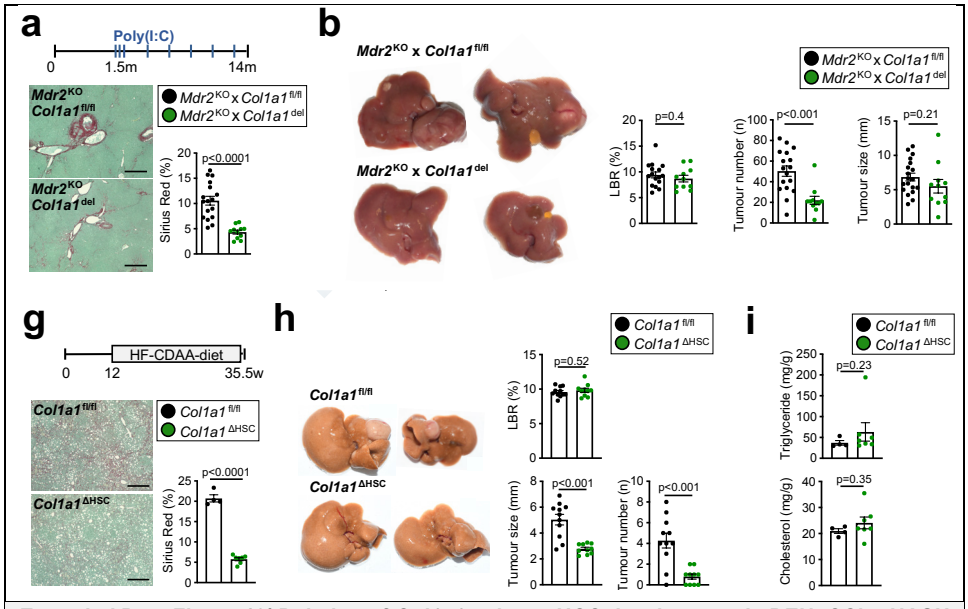


Figure 1 | Promotion of HCC development by hepatic stellate cells. g, Liver fibrosis and deletion of *Pdgfrb* were determined by Sirius red staining and qPCR for *Col1a1* and *Pdgfrb* in 4 month-old *Mdr2*^{KO} *Pdgfrb*$^{\Delta HSC}$ and *Mdr2*^{KO} *Pdgfrb*$^{fl/fl}$ female mice (n=11-13/group). **h**, Tumour development was determined in 15 month-old *Mdr2*^{KO} *Pdgfrb*$^{\Delta HSC}$ and *Mdr2*^{KO} *Pdgfrb*$^{fl/fl}$ female mice as described above (n=6-8/group).

- To further confirm the role of type I collagen in hepatocarcinogenesis, we employed the *Mdr2*^{KO} model of spontaneously arising HCC. Deletion of type I collagen was highly efficient as shown by Sirius Red staining.

Importantly, similar to the profound fibrosis DEN+CCl_4 model, we observed a highly significant reduction of the tumor number in *Col1a1*-deleted *Mdr2*^{KO} mice, (**Extended Data Figure 19a-b**). In the *Mdr2*^{KO} mice at this stage, we typically do not see a difference in liver body weight ratio as this is largely driven by the overall increased liver size rather than tumor mass (similar to NAFLD models).



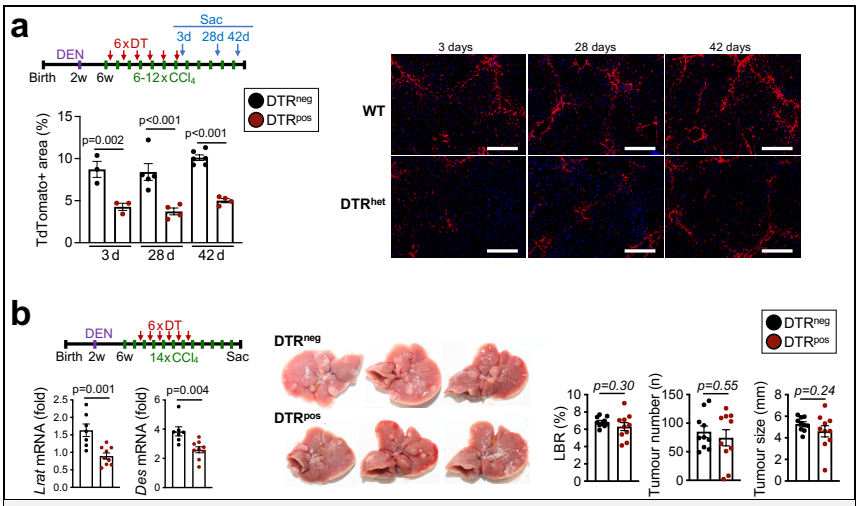
Extended Data Figure 19| Deletion of *Col1a1* reduces HCC development in DEN+CCl_4-, NASH diet- and *Mdr2* ^{KO}-induced HCC. a-b, Mx1Cre-mediated *Col1a1* deletion in *Mdr2* ^{KO} female mice (*Mdr2* ^{KO} *Col1a1* ^{del}, n=11) efficiently reduced liver fibrosis (**a**) and HCC development, as determined by liver body weight ratio, tumour number and tumour size (**b**), compared to Mx1Cre^{neg} *Mdr2* ^{KO} littermates (*Mdr2* ^{KO} *Col1a1* $^{fl/fl}$, n=18). **g-i**, *Col1a1* $^{\Delta HSC}$ mice (n=10) displayed strong reductions of liver fibrosis (**g**) and HCC development, as determined by liver body weight ratio, tumour number and tumour size (**h**), but not in liver triglycerides and cholesterol content (**i**) compared to *Col1a1* $^{fl/fl}$ control mice (n=11 mice) in a model of NASH-associated HCC induced by 8 months of HF-CDAA diet.

- We have additionally employed the HF-CDAA NASH-HCC model to confirm the role of type I collagen in spontaneously arising HCC. For this experiment, we used LratCre mice to specifically delete *Col1a1* in HSC (we also added the LratCre deletion approach for the DEN+CCl_4 model [see ***Extended Data Fig.19d-f*** in the manuscript files]). Deletion of type I collagen was highly efficient as shown by Sirius Red staining (**Extended Data Figure 19g**) Importantly, similar to the profound fibrosis DEN+CCl_4 model, we observed a reduction of HCC development in *Col1a1*-deleted mice in the HF-CDAA NASH-HCC model (**Extended Data Figure 19h**) and did not observe any effect on liver triglycerides or cholesterol levels (**Extended Data Figure 19i)**.

Another important point addresses the question whether HSCs interact early on with pre-neoplastic lesions in HCC (and the very interesting interaction HSCs-Hepatocytes) and how the difference between HCC and ICC can be explained = linked to transdifferentiation programs?

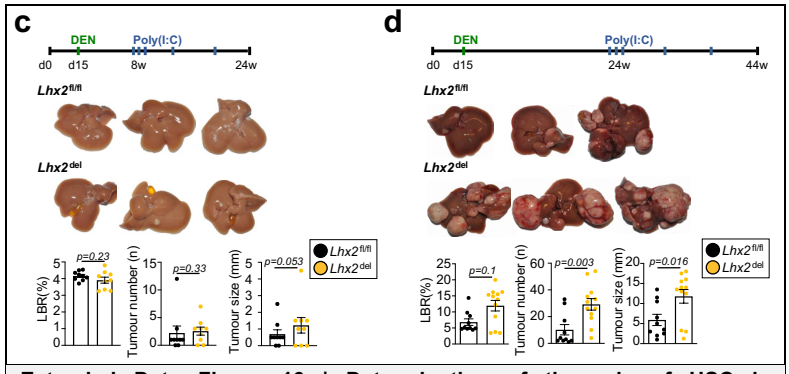
We have addressed these two important points by additional experiments and analyses, and now provide additional data on (i) functionally relevant interactions in different disease stages and (ii) on differences between HCC and ICC:

- We have further analyzed the functional contributions of HSC to hepatocarcinogenesis at different stages using LratCre x DTR depletion. In the original submission, we had genetically depleted HSC only in the second half of our hepatocarcinogenesis protocol as it is not possible to selectively deplete HSC only in early stages (without affecting also late stages). We now added early depletion experiments (in the first half DEN+CCl_4-mediated hepatocarcinogenesis), which indeed showed a strong depletion of HSC, observed 6 weeks after the last injection of DT and at sacrifice (**Extended Data Fig.16a-b**), meaning that this early DT treatment leads to reduced HSC throughout our hepatocarcinogenesis protocol, i.e. <u>early AND late HSC depletion</u>. Unexpectedly, but consistent with our hypothesis on HSC



Extended Data Figure 16 | Determination of the role of HSC in hepatocarcinogenesis at different time points. a, The long-term effects of LratCre x DTR-mediated HSC depletion in the first half of DEN+CCl_4-induced hepatocarcingenesis was tested by sacrificing mice 3 days (n=3 mice per group), 28 days (n=4-5 mice per group) and 42 days (n=4-6 mice per group) after the last DT injection and determining LratCre-induced TdTom expression. b, LratCre^{pos} DTR^{pos} mice with HSC depletion in the first half of DEN+CCl_4-induced hepatocarcinogenesis retained significant HSC depletion at time of sacrifice, as determined by qPCR for HSC markers *Lrat* and *Des* (n=7-9 mice per group), but did not show significant changes in liver-body weight ratio (LBR), tumour number and size compared to LratCre^{pos} DTR^{neg} littermates (n=10 mice per group).

subpopulations with opposing functions in the regulation of hepatocarcinogenesis, we saw no decrease in tumors in the early depletion model (**Extended Data Figure 16b**). The lacking effects of this long-term HSC depletion method on HCC growth, in conjunction with the strong tumor-suppressive effects of late depletion (see *Figure 1e,f,i,j* and Extended Data Fig 3g-h and 4c-f in the manuscript) strongly suggests that effects of early HSC depletion oppose those of late depletion.



Extended Data Figure 16 | Determination of the role of HSC in hepatocarcinogenesis at different time points. c-d, effects of poly I:C-induced *Lhx2* deletion at early time points (**c**, n=9 mice per group) and late time points (**d**, n=10 Mx1Cre^{neg} *Lhx2* $^{fl/fl}$ and 12 Mx1Cre^{pos} *Lhx2* ^{del}) on DEN-induced HCC.

- We made similar observations in our HSC activation model, which is amenable to genetic HSC activation at different time points via timed injection of poly I:C. In this model, we observed that early activation of HSC via and poly I:C-mediated *Lhx2* deletion, i.e. in the first half of our hepatocarcinogenesis protocol, did not increase tumors. In contrast, *Lhx2* deletion-mediated HSC activation in the second half significantly and potently increased tumors (**Extended Data Figure 16c-d**). These data have been added to the manuscript.

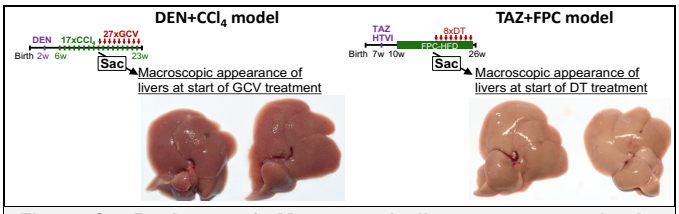
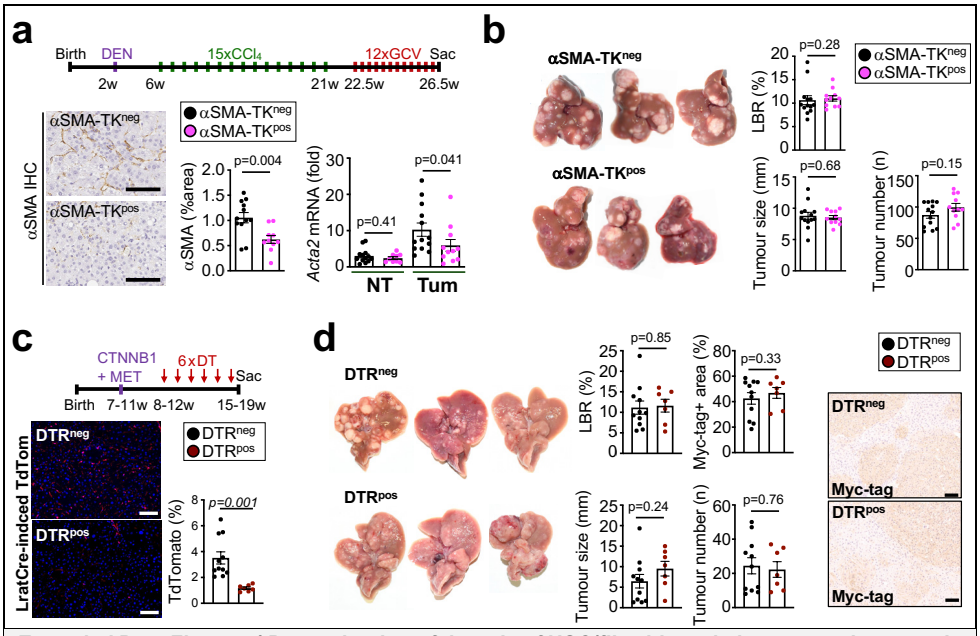


Figure for Reviewers 1. Macroscopic liver appearance in the DEN+CCl$_4$ model when αSMA-TK was started, or in the TAZ-FPC HCC model when LratCre x iDTR depletion was started.

- Of note, there were either no or only very few and small macroscopic tumors when we started depleting, either via LratCre x DTR or αSMA-TK, in the second half of our hepatocarcinogenesis protocol (**Figure for Reviewers 1**). Together with above studies that consistently revealed tumor promoting effects at later stages in hepatocarcinogenesis (but not on large established tumors – see below), these data suggest that effects are most likely on premalignant/transformed hepatocytes and/or early tumor lesions.

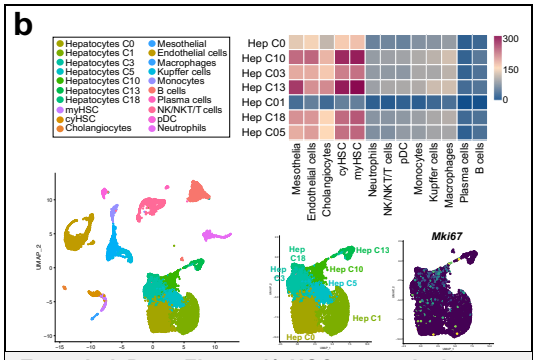
To further address the key question whether HSC/αSMA+ myofibroblasts act mainly in the PME on premalignant/transformed hepatocytes/early tumor lesions or on large established tumor, we developed a depletion model in mice with established tumors, which allowed us to test the role of αSMA+ cells within tumors. For this, we chose αSMA-TK mice and started depletion two weeks after the last CCl$_4$ injection, when activated αSMA+ myofibroblasts are primarily in the tumor (as opposed to the LratCre x DTR model, αSMA-TK mice do not deplete quiescent HSC). Our data in a large number of mice showed efficient depletion in tumors without effects in non-tumor areas (**Extended Data Fig.7a**). Importantly, we found no reduction of tumor growth – (**Extended Data Fig.7b**). These data clearly show that HSC/αSMA+ myofibroblasts do not play a role in promoting the growth of large tumors and are consistent with our data showing low numbers of HSC/αSMA+ myofibroblasts in tumors compared to the PME (see *Figure 2* in the manuscript). In summary, our data support our hypothesis that HSC/αSMA+ myofibroblasts promote the development of HCC through interactions with premalignant



Extended Data Figure 7 | Determination of the role of HSC/fibroblasts in hepatocarcinogenesis in established tumors and in a non-fibrotic HCC model. a-b, ganciclovir injections into DEN+CCl$_4$-treated αSMA-TK^{pos} (n=11 mice) and αSMA-TK^{neg} (n=13 mice) littermates at late time points, when large tumours were established and CCl$_4$ injections had ceased for 1.5 weeks, resulted in a strong reduction of αSMA+ cells in tumour (Tum) but not in non-tumour (NT) areas (**a**), and did not affect tumour progression, as determined by liver body weight ratio (LBR), tumour number and tumour size (**b**). **c-d**, HSC depletion via LratCre x DTR in a non-fibrotic HCC model, induced by hydrodynamic tail vein injection and sleeping beauty-mediated expression MET + CTNNB1-Myc-tag, was highly efficient, as determined by TdTom fluorescence (**c**) but did not affect liver-body weight ratio, tumour size, tumour number or tumour area assessed by Myc-tag staining (n=7-11 per group) (**d**).

hepatocytes or in very early stages of tumor growth (when tumor cells are still in contact with HSC/αSMA+ myofibroblasts as opposed to large tumors, where HSC/αSMA+ myofibroblast numbers are low and the interactions are limited).

- To further substantiate above findings, we performed scRNA-seq-based CellPhoneDB analysis looking at hepatocyte subpopulations. Consistent with our finding that manipulations of HSC and HSC-secreted mediators affect proliferation, we found that HSC most intensely interacted with the proliferating hepatocyte subcluster C10 as well as with a metabolically active subcluster, C13 (see **Extended Data Fig.8b** – data on pathway analysis of hepatocyte subclusters in ***Extended Data Fig.8c*** in the manuscript.)



Extended Data Figure 8| HSC strongly interact and regulates hepatocytes proliferation. a, analysis of cell-cell interactions by CellPhoneDB in snRNA-seq data from cirrhotic liver patients (n=4) revealed HSC as a major cell type interacting with hepatocytes. **b**, CellPhoneDB analysis showing cell-cell interactions between different hepatocytes clusters and liver cells populations in the mouse fibrotic liver induced by 8xCCl_4 (n=3) as well as UMAP visualization of the proliferation marker *Mki67*.

…….and how the difference between HCC and ICC can be explained = linked to transdifferentiation programs?

- In regard to the fascinating differences of fibroblasts within the TME of HCC and ICC, we have performed analyses that provide explanations but cannot address this topic in depth, i.e. through causation experiment with knockout or overexpression of below-identified genes as this would go far beyond the scope of our study. In response the reviewer's suggestion, we have tried to understand factor that differ between HCC and ICC and may explain why ICC generates a fibrogenic environment that promotes the activation and proliferation of HSC-derived myofibroblasts, whereas HCC only contains few activated myofibroblasts. Analysis of scRNA-seq data that contains both HCC and ICC patient samples reveals much higher expression of two most powerful drivers of fibroblasts activation and proliferation, TGFβ and PDGF, respectively. As shown in **Figure for Reviewers 11**, there is a <u>significantly higher expression of *TGFB1*, *TGFB2*</u> in tumor cells from ICC than HCC. We believe that this is important in establishing a niche that is either highly desmoplastic (ICC) or not (HCC).

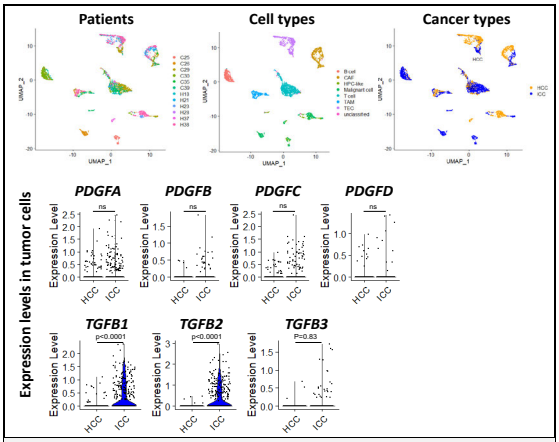


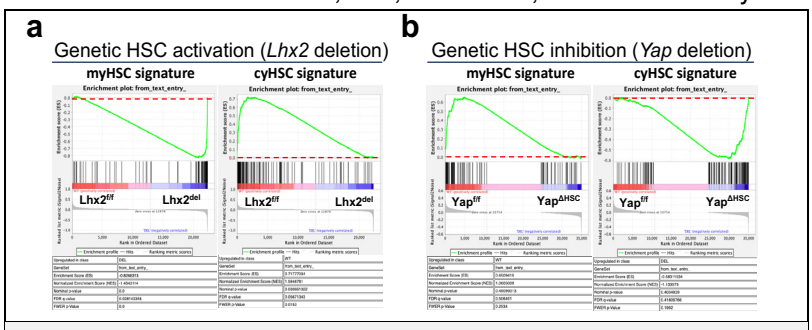
Figure for Reviewers 11. Increased expression of PDGF and TGFB isoforms in ICC compared to ICC. scRNAseq analysis show an increase of TGFB and PGFD isoform expression by ICC tumor cells compared to HCC tumor cells in human patients (published dataset from Ma *et al*, Cancer Cell, 2019; 36(4):418-430).

- With regard to fibroblast interactions in HCC and ICC, we see major functional differences in interaction partners and, related to that, also in functional consequences:
  - **In ICC, HGF expression is tumor-promoting (Affo et al, *Cancer Cell.* 2021;39(6):866-882)– whereas it is tumor suppressive in HCC as shown by our current study.** We believe that these opposing functions are linked to different interaction partners, with HGF-expressing CAF in ICC interacting with tumor cells and their main function being proliferation of tumor cells, resulting in ICC progression, but its dominant action in HCC being the protection of hepatocytes from injury.
  - **In ICC, Col1a1 does not promote tumor growth (Affo et al, *Cancer Cell.* 2021;39(6):866-882), whereas it promotes the growth of HCC in our current study.** Col1a1-expressing HSC are predominantly found in close proximity to hepatocytes, where they promote TAZ expression, AKT activation and proliferation of hepatocytes via stiffness. As biology is often complex, there is an additional tumor-promoting Col I-DDR1 pathway in the tumor compartment, as apparent by the reduced proliferation of tumor cells in *Col1a1*^{del} mice (***Figure 4d*** – in the manuscript) and *Ddr1* $^{\Delta Hep}$ mice ***Figure 5e-f*** – in the manuscript). In contrast to HCC, *Col1a1*-expressing HSC are almost exclusively within tumors in ICC. In ICC, Col I biology is also complex, with most likely tumor-promoting YAP/TAZ activation being offset by mechanical tumor restriction (as shown in our published data in desmoplastic liver metastasis - Bhattacharjee et al, *J Clin Invest.* 2021;131(11):e146987, where *Col1a1* deletion even increased tumor growth).

Figures 1A-D: (also extended data figure 2): It should be demonstrated in more detail to what extent HSC activation and deactivation was achieved. It is not really clear whether Col1a1 indeed is the only good measure – what was the quality of the transcriptional programs (of activation and deactivation)? Also it is not clear whether the genetic activation renders additional "defects" in HSCs that contribute or affect the phenotype. Are tumors different in their character (genetic, histology, immune cells)?

While *Col1a1*/type I collagen is directly linked to fibrosis and its clinical complications, we appreciate the feedback from the reviewer and have additionally evaluated myHSC/cyHSC signature activities to highlight transcription activation programs:

- Given that (i) the degree of fibrosis is the most relevant determinant of outcomes in chronic liver diseases including NASH (Sanyal et al, *New Engl J Med* 2021;385:1559-1569; Vilar-Gomez et al, *Gastroenterology* 2018;155:443-457; Hagström et al, *J Hepatol*. 2017;67:1265-1273; Angulo et al, *Gastroenterology* 2015;149:389-97), not only predicting death but also the development of HCC (Sanyal et al, *New Engl J Med* 2021;385:1559-1569; Simon et al, *Hepatology* 2021;74:2410-2423; Vilar-Gomez et al, *Gastroenterology* 2018;155:443-457) and that (ii) Col1a1 is the best established marker of fibrogenic activation in HSC, we consider the Sirius Red-positive area and Col1a1 as highly relevant histological and RNA parameters, respectively.
- To address the related question whether we see the activation and deactivation of HSC, we have performed additional analyses: (i) We analyzed cyHSC and myHSC signatures in isolated HSC from the *Lhx2*^{del} HSC activation and the *Yap*$^{\Delta HSC}$ HSC inhibition models. Our analysis shows a decrease of cyHSC and an increase of myHSC signature activity in HSC from the *Lhx2*^{del} HSC activation model; and, *vice versa*, an increase of cyHSC and a decrease of myHSC signature acvity in HSC from the *Yap*$^{\Delta HSC}$ HSC inhibition model (**Extended Data Fig.13a-b**).
- We have carefully investigated the HSC activation model via *Lhx2* and show that the majority of significantly genes in HSC isolated from *Lhx2*^{del} mice are similar genes significantly altered in HSC isolated from CCl_4- and BDL-treated mice as shown in a Venn Diagram (**Figure for Reviewers 12A**). Moreover, the top up- and downregulated genes in the *Lhx2*^{del} HSC show a similar up and downregulation with *Disp2* being the only exception (**Figure for Reviewers 12B**).



Extended Data Figure 13 | Genetic HSC activation and genetic HSC inhibition result in alterations mHSC and cyHSC signature enrichment. a-b, analysis of RNA-seq data from isolated HSC revealed that genetic HSC activation via Mx1Cre-mediated *Lhx2* deletion (n=4 *Lhx2* $^{fl/fl}$, n=4 *Lhx2*$^{\Delta HSC}$) resulted in enrichment of the myHSC signature and decreased cyHSC enrichment (a), whereas genetic HSC inhibition via LratCre-mediated Yap1 deletion (n=5 *Yap1*$^{fl/fl}$, n=4 *Yap1*$^{\Delta HSC}$) exerted the opposite effect (b).

In summary, above data show that there are no apparent unphysiological "defects" that confound our studies on hepatocarcinogenesis in these models. Moreover, above-discussed HSC activation and inhibition are complemented by a vast array of additional approaches confirming the overall tumor-promoting role of HSC – i.e. two methods of HSC/fibroblast depletion via LratCre x DTR and αSMA-TK as well as the deletion of tumor-promoting genes such as *Col1a1* in HSC by two genetic approaches (Mx1Cre and LratCre) and *Has2* deletion.

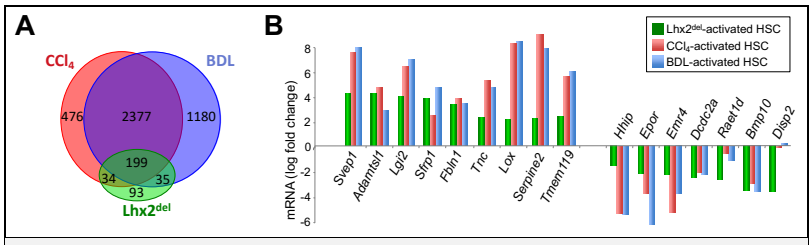


Figure for Reviewers 12 | Genetic HSC activation and genetic HSC inhibition result in alterations mHSC and cyHSC signature enrichment. A-B. Analysis of RNA-seq data from isolated HSC revealed that genetic HSC activation via Mx1Cre-mediated Lhx2 deletion (n=4 *Lhx2* $^{fl/fl}$, n=4 *Lhx2*$^{\Delta HSC}$) resulted in enrichment of the myHSC signature and decreased cyHSC enrichment (A), whereas genetic HSC inhibition via LratCre-mediated Yap1 deletion (n=5 *Yap*$^{fl/fl}$, n=4 *Yap*$^{\Delta HSC}$).

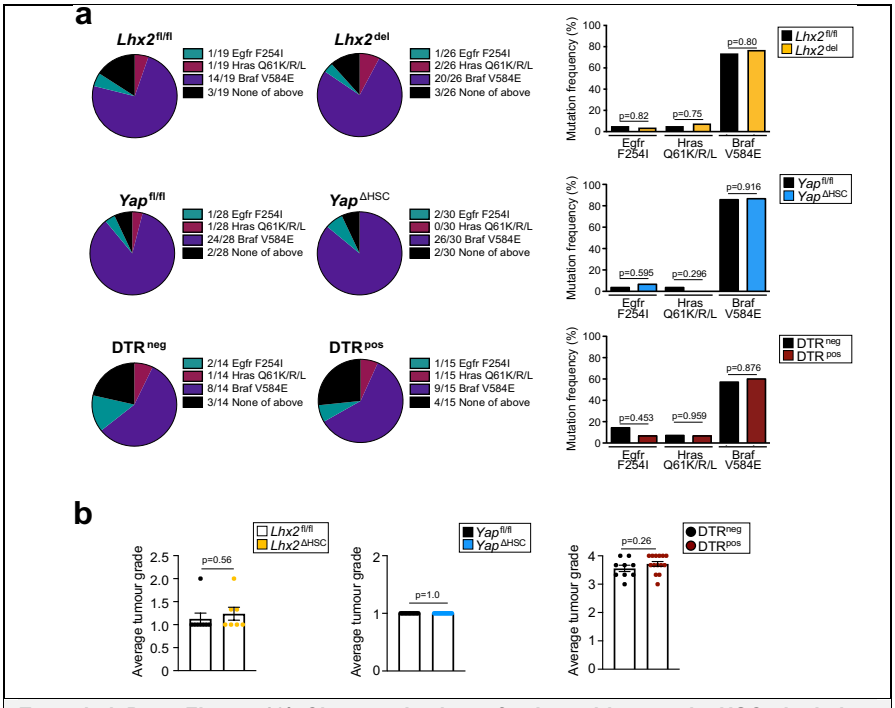
Are tumors different in their character (genetic, histology, immune cells)? Figures 1E-H: Also here it is not clear – incidence of tumorigenesis is still 100% in both groups. altered in these tumors apart from size and number reduction? How is the TME altered in this context? Do the tumors look different?

It is expected that the tumor incidence is 100% as DEN mouse models typically have 100% incidence, even when DEN is used in the absence of CCl_4 or HF-CDAA diet as non-fibrotic model. Hence, the anticipated alterations in such models are not tumor incidence, but tumor size and number. Overall, the findings are consistent with our hypothesis that HSC and fibrosis do not actively trigger cancer development but can accelerate the development of HCC by providing more or less favorable conditions.

In response to these important comments, we have further characterized the tumors by (i) determination of hotspot mutations; (ii) expert pathological evaluation and grading; as well as (iii) gene expression and (iv) determination of immune cells by FACS in select models:

- Based on previous studies, we evaluated known and common hotspot mutations in *Hras*, *Braf* and *Egfr*, in the DEN and DEN+CCl_4 models established by the group of Duncan Odom (Connor et al, J Hepatol. 2018 Oct;69(4):840-850) and confirmed in additional studies by Hao Zhu's group (Chung et al, Cancer Prev Res. 2020;13(11):911-922). We did not observe significant differences in the frequency of these mutations in tumors from the *Lhx2*^{del} HSC activation, the *Yap*$^{\Delta HSC}$ HSC inhibition and the LratCre x DTR HSC depletion models (**Extended Data Fig.10a**)

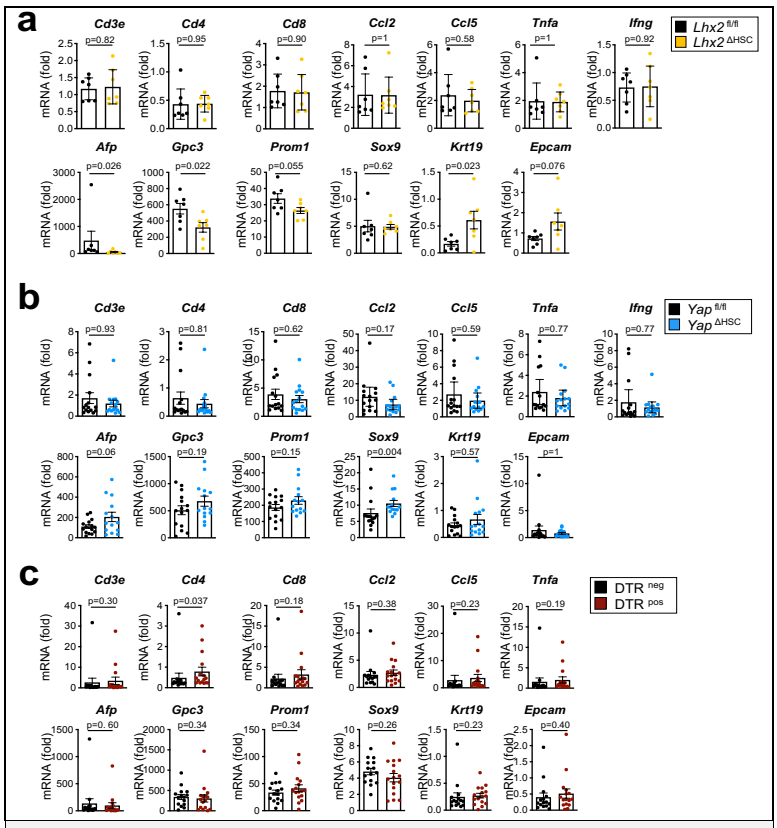
- The tumors were also evaluated by a board-certified pathologist with more than 25 years professional experience (co-author Helen Remotti). There were no differences in tumor grade between the *Lhx2*^{del} HSC activation, the *Yap*$^{\Delta HSC}$ HSC inhibition and the LratCre x iDTR HSC depletion models (**Extended Data Fig.10b**), nor any histological differences or abnormalities in mice with HSC activation, inhibition or depletion (data not shown).



Extended Data Figure 10| Characterization of mice with genetic HSC depletion, activation of inhibition. a, mutations detected by PCR and sequencing for Hras Q61K/R/L, Braf V584E and Egfr F254I in *Lhx2*$^{fl/fl}$ vs *Lhx2*$^{\Delta HSC}$ /*Lhx2* ^{del}; *Yap*$^{fl/fl}$ vs *Yap*$^{\Delta HSC}$ and DTR^{neg} vs DTR^{pos} mice treated with DEN or DEN+CCl_4. **b**, tumour grading in *Lhx2*$^{fl/fl}$ and *Lhx2*$^{\Delta HSC}$ mice subjected to the DEN model (n=7-8 mice per group – average of 3 tumours/mouse); in *Yap* $^{fl/fl}$ and *Yap* $^{\Delta HSC}$ mice subjected to DEN+CCl_4 (n=14-16 mice per group – average of 3 tumours/mouse) and DTR^{neg} and DTR^{pos} mice subjected to the TAZ+FPC HCC model (n=9-14 mice per group – average of 3 tumours/mouse).

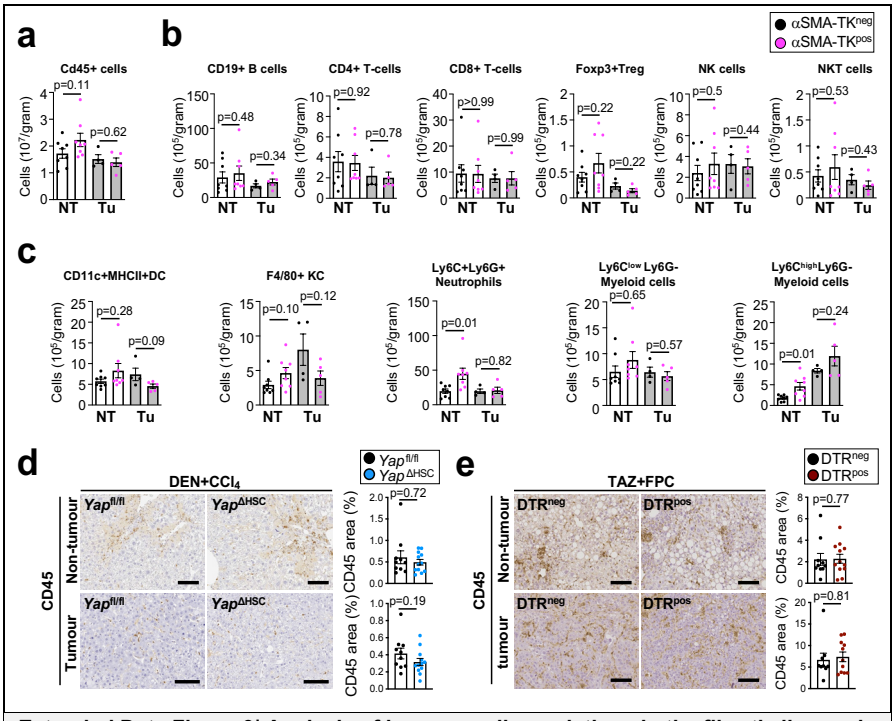
- We additionally evaluated gene expression profiles. In addition to our RNA-seq analysis comparing tumor from *Yap*$^{\Delta HSC}$ and *Yap*$^{fl/fl}$ mice, which showed almost no significant differences in gene expression inside tumors in contrast to strong differences outside tumors (see *Figure 2C* in the manuscript), we also assessed by qPCR the expression of HCC-associated marker genes associated with tumor aggressiveness such as *Afp*, *Gpc3*, *Sox9*, *Epcam*, *Prom1* and *Krt19*, markers of relevant immune populations including *Cd3e*, *Cd4*, *Cd8*, and inflammation-related genes *Ccl2*, *Ccl5* and *Tnfα* in the Lhx2^{del} HSC activation, the YAP$^{\Delta HSC}$ HSC inhibition and the Lrat x DTR HSC depletion models (**Extended Data Fig.10a-c**). While there were a few genes with significant differences, overall did not show consistent differences.

- In addition to above qPCR analyses on inflammation, we characterized immune cell subsets by FACS and also added IHC staining. Overall, there were no differences in overall CD45+, lymphocyte populations and the majority of myeloid populations in both the non-tumor and tumor tissue of αSMA-TK+ depleted mice, there were a few and largely expected differences in the non-tumor tissue (**Extended Data Fig.9a-c)**, which is enriched in fibroblasts (as shown in *Figure 2* in our manuscript). These differences



Extended Data Figure 11| Extended Data Figure 9| Characterization of inflammation, immune cell and tumor markers in mice with genetic HSC depletion, activation of inhibition. a-c, characterization of inflammation, immune cells and tumors by qPCR for T cell markers, inflammatory genes and tumor-relevant marker genes in tumors from *Lhx2*$^{\Delta HSC}$ mice with HSC activation in comparison to mice to *Lhx2*$^{f/f}$ littermates (n=7 mice/group) (**a**); *Yap*$^{\Delta HSC}$ mice with HSC inhibition compared to *Yap*$^{fl/fl}$ mice (n=14-15 mice/group) (**b**); DTR ^{pos} mice with HSC depletion compared to DTR ^{neg} mice (n=15-16 mice/group) (**c**).

included significant increases of Ly6C+Ly6G+ neutrophils and Ly6C^{high} Ly6C^{neg} monocytes, most likely as response to the depletion of myofibroblasts, which is mediated by αSMA-TK-induced fibroblast apoptosis and typically requires removal of these dead cells by neutrophils and macrophages. Furthermore, we did not observe differences in CD45+ lymphocytes between YAP$^{fl/fl}$ and YAP^{ΔHSC} mice in the DEN+CCl$_4$ model or between DTR^{pos} HSC-depleted and DTR^{neg} control mice in the TAZ+FPC model as shown by immunohistochemistry (**Extended Data Fig.9d-e**).

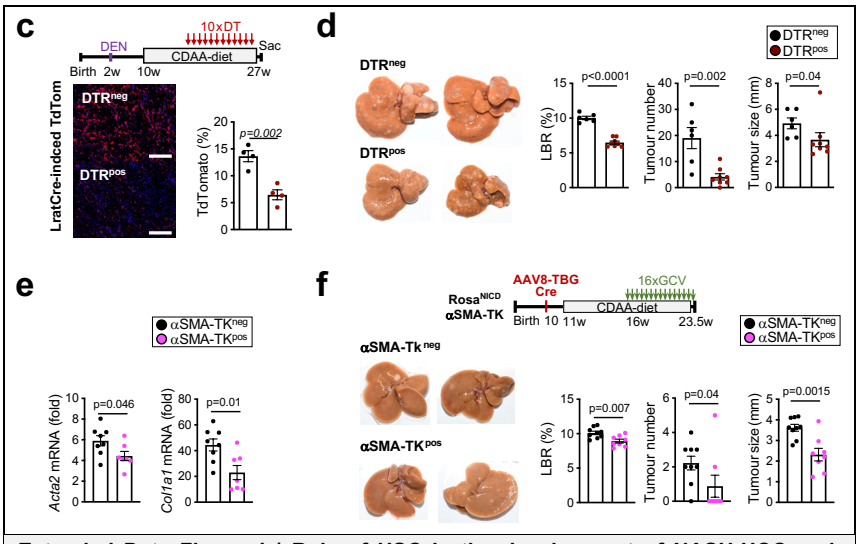


Extended Data Figure 9| Analysis of immune cell populations in the fibrotic liver or in HCC from mice with genetic HSC depletion or inhibition. a-c, FACS analysis of total CD45+ leukocytes (**a**), lymphocytes (**b**) and myeloid cells (**c**) in the PME and TME after αSMA+ cell depletion during HCC development induced by DEN+CCl$_4$ (n=8 mice/group for non-tumour and n=4-5 mice/group for HCC; related to experiments in Extended Data Fig.3g-h) shows increased neutrophil and Ly6C^{high} macrophage infiltration into non-tumour areas in aSMA-TK^{pos} mice; NT: non-tumour; Tu: Tumour. **d-e**, CD45 IHC staining of the non-tumour and tumour tissue in DEN+CCl$_4$ treated *Yap*$^{fl/fl}$ and *Yap* ^{ΔHSC} mice (n=10-12 mice per group) (**d**), and in HSC-depleted DTR^{pos} mice or their DTR^{neg} littermates during HCC development induced by TAZ+FPC (n=9-12 mice per group) (**e**).

In summary, our extensive and multi-modal analysis, including mutational hotspot analysis, expert pathological tumor evaluation and grading, RNA-seq, qPCR analysis of tumor-, immune- and inflammation-related genes as well as FACS and immunohistochemistry revealed no appreciable effects of genetic HSC activation, inhibition and depletion on tumors, consistent with our finding that most HSC/fibroblast are outside of tumors and that the majority of their effects are in this compartment and that their role in hepatocarcinogenesis is the creation of an environment in which tumors can thrive.

Figures I-J (and Extended Data 2): The NASH model – needs to be better characterized (histology etc.) – and is less convincing as it does not include NASH-induced HCC but rather uses a genetic trick – to be combined with a NASH diet to end up in liver cancer.

In response to the reviewer's comments, we have included additional NASH models in different parts of the paper – now a total of 4 NASH-HCC models: 1. TAZ+FPC (see *Figure 1i-j* in the manuscript); 2. DEN + HF-CDAA, 3. NICD + HF-CDAA (**Extended Data Fig.4c-f**); and 4. A long-term HF-CDAA (**Extended Data Fig.19g-i** on the next page), and also further characterized the TAZ+FPC model. In addition to the histopathological evaluation of this model we have also included qPCR analysis for HCC markers including *Afp* and *Gpc3*, which are also strongly increased compared to control liver. The long-term HF-CDAA diet has not been used for genetic depletion but for deletion of Col1a1 (as long-term depletion can be technically difficult). While there is no perfect model of NASH and NASH-HCC, the employed models are all highly fibrotic, complement each



Extended Data Figure 4 | Role of HSC in the development of NASH-HCC. c-d, LratCre-positive DTR^{pos} (n=8) or DTR^{neg} (n=6) mice were subjected to DEN+CDAA-HFD induced hepatocarcinogenesis, revealing efficient HSC depletion in non-tumour tissue (n=4 per group) (c) as well as reduced tumour development, as determined by liver body ratio, tumour number and tumour size in DTR^{pos} mice (d). **e-f,** αSMA-TK^{pos} mice (n=8 mice) or αSMA-TK^{neg} mice (n=9 mice) were subjected to NICD+CDAA-HFD induced hepatocarcinogenesis, revealing efficient HSC depletion (n=7-8 mice per group) (e) as well as reduced tumour development, as determined by liver body ratio, tumour number and tumour size in αSMA-TK^{pos} mice (f).

other and consistently show a similar role for HSC in hepatocarcinogenesis, which is also similar to the other non-NASH HCC models in our study. Our histopathological characterization of the TAZ-FPC model showed that it induces very high-grade tumors, which is distinct from other NASH-HCC models and show loss of the typical reticulin staining of hepatocyte plates (**Figure for Reviewers 13**). The TAZ-FPC is also best-suited for genetic ablation due to its shorter duration (as is the NICD-HF-CDAA). In the NICD+HF-CDAA HCC model (which is Cre-induced and thus not amenable to LratCre-mediated HSC depletion), we confirm the role of HSC in NASH-HCC by a second depletion method, mediated by αSMA-TK. The common feature of all employed models is a high degree of fibrosis.

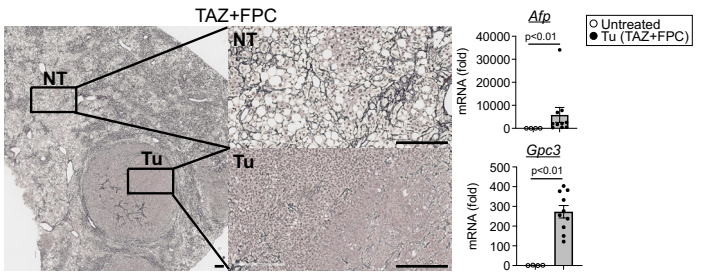


Figure for Reviewers 13. Characterization of the TAZ-FPC HCC model, showing reticulin staining with loss of staining/patterning in tumors (Tu) but not in non-tumor tissue (NT). Also, HCC markers *Afp* and *Gpc* were strongly increased compared to untreated control liver.

Here the authors should indeed use a spontaneous model – as all the models used in Figure 1 do not display the spontaneous development of liver cancer but rather represent a "highly artificial" (still useful) set up of genetic environment. So it is not quite clear how this would affect tumorigenesis in a spontaneous model.

In response to the reviewer's suggestions, we have added two well-established spontaneous HCC models, the *Mdr2*^{KO} model (relevant as patients with mutations in the human homologue *MDR3* develop chronic liver disease and liver cancer) and the long-term HF-CDAA model:

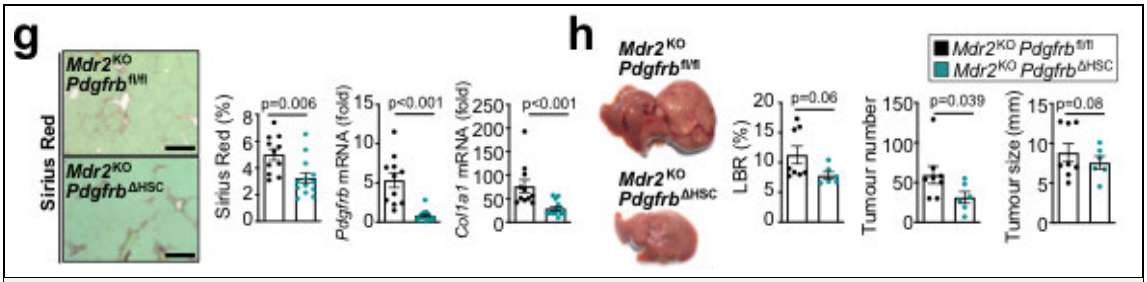
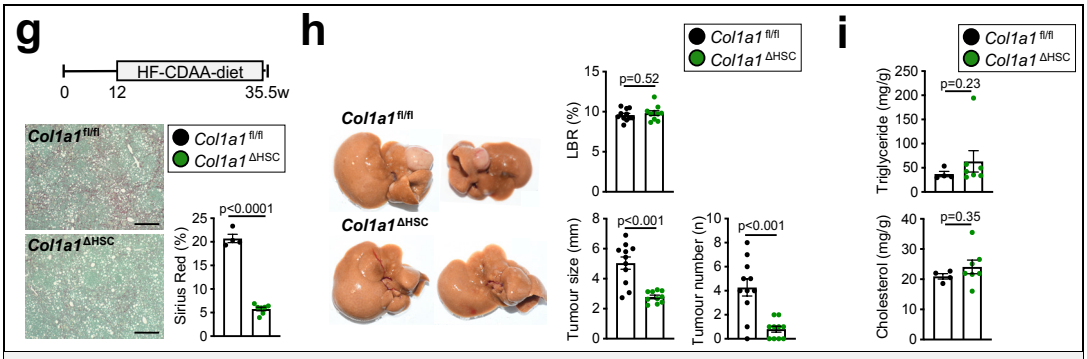


Figure 1 | Promotion of HCC development by hepatic stellate cells. g, Liver fibrosis and deletion of *Pdgfrb* were determined by Sirius red staining and qPCR for *Col1a1* and *Pdgfrb* in 4 months-old *Mdr2*^{KO} *Pdgfrb*$^{\Delta HSC}$ and *Mdr2*^{KO} *Pdgfrb*$^{fl/fl}$ female mice (n=11-13/group). **h**, Tumour development was determined in 15 month-old *Mdr2*^{KO} *Pdgfrb*$^{\Delta HSC}$ and *Mdr2*^{KO} *Pdgfrb*$^{fl/fl}$ female mice as described above (n=6-8/group).

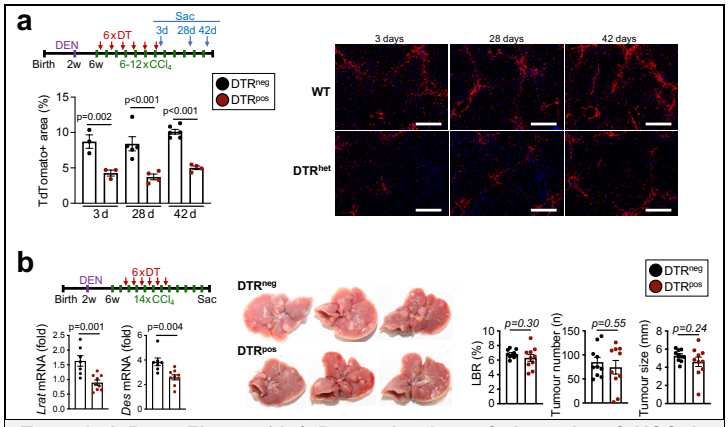
- We have used the *Mdr2*^{KO} model in conjunction with LratCre-mediated deletion of *Pdgfrb*, which efficiently reduced HSC activation/expansion and fibrosis in this model; as well as for the deletion of *Col1a1* by Mx1Cre in the second part of the manuscript. Our data in this model show a significant reduction of tumor number as well as a trend to reduced LBR and tumor size (**Figure 1g-h**), again supporting a tumor-promoting role of HSC shown by numerous other models and approaches in our manuscript. In addition, *Col1a1* deletion in the *Mdr2*^{KO} model showed a reduction of HCC development similar than in the LratCre *Pdgfrb*$^{\Delta HSC}$ mice and *Col1a1* deletion in the DEN+CCl_4 profound fibrosis model demonstrating the role of type I collagen in hepatocarcinogenesis (see ***Extended Data Fig.19a-b*** in the manuscript).
- The other spontaneous model added to our manuscript is the long-term HF-CDAA model. This model was used to demonstrate the role of type I collagen – due to its length it is not ideal for depletion studies, in our opinion, and we simply did also not have capacity for yet another model (**Figure 1** and related Extended Data Figures already show 5 different genetic approaches to activate, inhibit or deplete HSC in a total of 6 different HCC models) in this already very expansive project. Similar to our other approaches, LratCre-mediated deletion of *Col1a1* showed a highly significant effect on HCC promotion in the HF-CDAA model as shown (**Extended Data Fig.19 g-i**).



Extended Data Figure 19| Deletion of *Col1a1* reduces HCC development in DEN+CCl_4-, NASH diet- and *Mdr2*^{KO}-induced HCC. g-i, *Col1a1*$^{\Delta HSC}$ mice (n=10) displayed strong reductions of liver fibrosis (**g**) and HCC development, as determined by liver body weight ratio, tumour number and tumour size (**h**), but not in liver triglycerides and cholesterol content (**i**) compared to *Col1a1*$^{fl/fl}$ control mice (n=11 mice) in a model of NASH-associated HCC induced by 8 months of HF-CDAA diet.

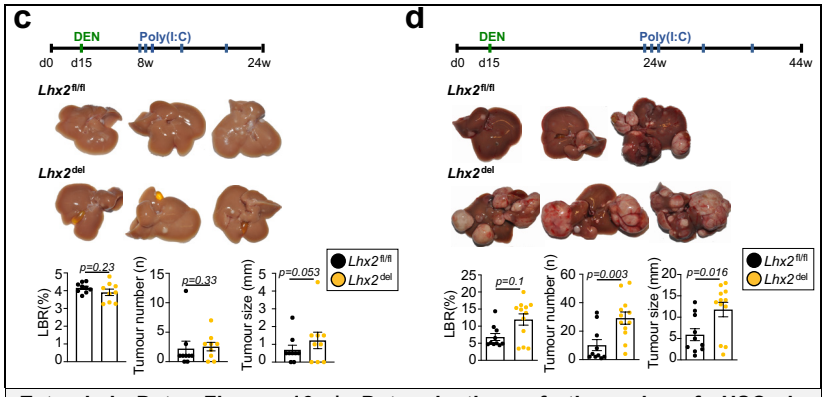
How sure are the authors at what time point exactly HSCs have been depleted – in correlation to the liver cancer development state?

Depletion is highly efficient already after a single injection and occurs within 1-2 days in both the DTR and αSMA-TK models (we did not include data on this as the efficient depletion via these methods is well established across cell types – but can add if needed). As already shown in response to a comment from the reviewer 1, we have now included a detailed analysis on the time frame in which HSC exert effects on hepatocarcinogenesis, which are not occurring in large established tumor and are consistently seen in later stages of hepatocarcinogenesis, coinciding with the time when tumors start to develop and a stronger dysbalance between cyHSC and myHSC at these advanced stages:



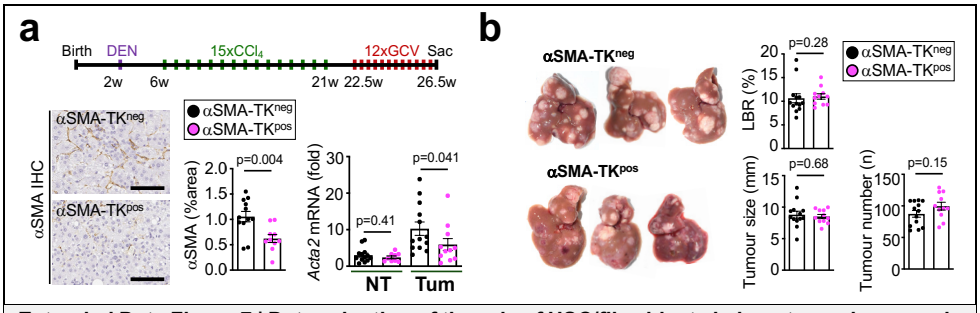
Extended Data Figure 16 | Determination of the role of HSC in hepatocarcinogenesis at different time points. a, the long-term effects of LratCre x DTR-mediated HSC depletion in the first half of DEN+CCl_4-induced hepatocarcinogenesis was tested by sacrificing mice 3 days (n=3 mice/group), 28 days (n=4-5 mice/group) and 42 days (n=4-6 mice/group) after the last DT injection and determining LratCre-induced TdTom expression. **b**, LratCre^{pos} DTR^{pos} mice with HSC depletion in the first half of DEN+CCl_4-induced hepatocarcinogenesis retained significant HSC depletion at time of sacrifice, as determined by qPCR for HSC markers *Lrat* and *Des* (n=7-9 mice/group), but did not show significant changes in liver body weight ratio (LBR), tumour number and tumour size compared to LratCre^{pos} DTR^{neg} littermates (n=10 mice/group).

- As requested by this and the other reviewers, we have investigated the effects of early HSC depletion via LratCre x DTR in the DEN+CCl_4 model. We demonstrate that depletion lasted for at least 6 weeks (**Extended Data Fig.16a**). Nonetheless, early HSC depletion only resulted in a trend towards reduced tumors (**Extended Data Fig.16b**) in contrast to the late depletion which led to a highly significant reduction of tumors (liver body weight ration; tumor number; tumor size).
- We additionally added data using *Lhx2* deletion-induced HSC activation, which could be achieved at early and late time points via Mx1Cre, as second approach. Again, we saw no appreciable differences in the liver body weight ratio, tumor number and tumor size when HSC were activated at early time points via *Lhx2* deletion (**Extended Data Fig.16c**), whereas there were strong effects when HSC activation was achieved via *Lhx2* deletion in the second half of our hepatocarcinogenesis protocol (**Extended Data Fig.16d**).



Extended Data Figure 16 | Determination of the role of HSC in hepatocarcinogenesis at different time points. c-d, Effects of poly I:C-induced *Lhx2* deletion at early time points (**c**, n=9 mice per group) and late time points (**d**, n=10 Mx1Cre^{neg} *Lhx2*$^{fl/fl}$ and 12 Mx1Cre^{pos} *Lhx2* ^{del}) on DEN-induced HCC, as shown by representative images, liver body ratio (LBR), tumour number and size.

- We also did not see an effect of fibroblast depletion within large established tumors. For this, depletion was started 10 days after the last CCl_4 injection in the DEN+CCl_4 protocol so that the majority of αSMA+ cells were within tumors. This led to efficient depletion in the tumor but not in the non-tumor compartment (**Extended Data Fig.7a**). Importantly, despite efficient depletion, we did not observe effect on tumor size, number and liver body weight ratio (**Extended Data Fig.7b)**, clearly showing that there is no major role for αSMA+ fibroblast in the tumor compartment of large established tumors.

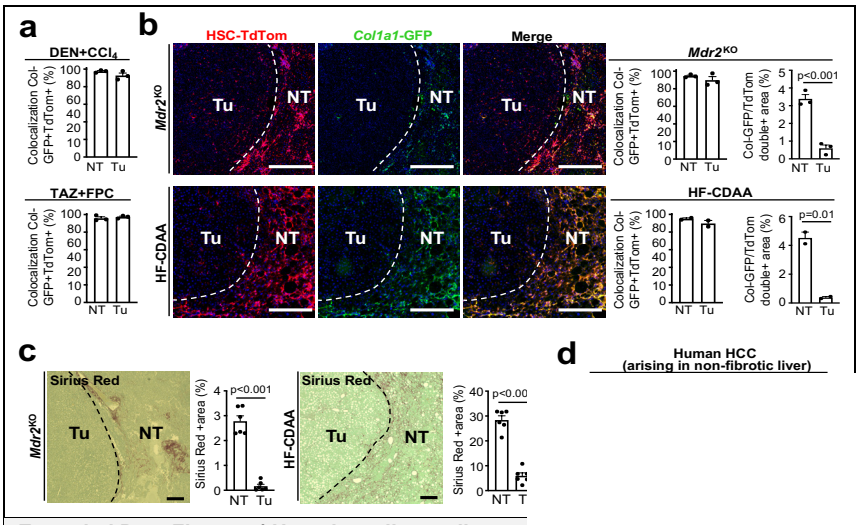


Extended Data Figure 7 | Determination of the role of HSC/fibroblasts in hepatocarcinogenesis in established tumors and in a non-fibrotic HCC model. a-b, ganciclovir injections into DEN+CCl_4-treated αSMA-TK^{pos} (n=11 mice) and αSMA-TK^{neg} (n=13 mice) littermates at late time points, when large tumours were established and CCl_4 injections had ceased for 1.5 weeks, resulted in a strong reduction of αSMA+ cells in tumour (Tum) but not in non-tumour (NT) areas (**a**), and did not affect tumour progression, as determined by liver-body weight ratio (LBR), tumour number and size (**b**).

These data demonstrate that HSC (and their depletion) affect HCC development in the premalignant liver at a time when tumors are developing or small tumors are present. Given the profound effects at late stages and insignificant effects at early stages, both for the HSC depletion and *Lhx2* deletion HSC activation model, we are confident to state that tumor-promoting effects of HSC occur at late stages.

Figure 2: Figure A is very convincing – again leaving however the question open – how does this look like in chronic inflammation induced liver cancer or spontaneous tumors? The difference between ICC and HCC is very convincing and clear-cut.

As suggested by the reviewer, we have investigated the patterns in two models of spontaneous/chronic inflammation-induced tumor, induced by *Mdr2*^{KO} or HF-CDAA (**Extended Data Fig.5a-b**). These findings not only match the predominant accumulation of HSC-derived LratCre-labeled fibroblasts and of Sirius Red+ fibrosis outside of tumors in the DEN+CCl_4, TAZ+FPC mouse models but also the human data (*Figure 2a-b* in the manuscript). We have also seen this pattern in additional model (e.g. TAK1-deletion, NICD1-HFD HCC model and others) but not included this in the manuscript as we do not have the LratCre TdTom labeling for those models.



Extended Data Figure 5 | Hepatic stellate cell accu in the non-tumour environment. a, co-localization of *Col1a1*-GFP+ and LratCre-induced TdTom was quantified in non-tumour (NT) and tumour (Tu) areas of DEN+CCl_4-induced (n=3) and TAZ-FPC-induced (n=3) HCC (related to Fig.2a). **b**, LratCre-TdTom+ HSC and *Col1a1*-GFP+ fibroblasts were visualized and co-localization of *Col1a1*-GFP+ and LratCre-induced TdTom as well as the *Col1a1*-GFP/TdTom double-positive area were quantified in 15 months-old *Mdr2* ^{KO}-induced (n=3 mice) and HF-CDAA-diet-induced (n=2 mice) HCC. **c**, the Sirius Red-positive area was visualized and quantified in 15 months-old *Mdr2*^{KO}-induced and HF-CDAA-diet-induced HCC (n=6/group).

In summary, the predominant localization of fibroblasts and fibrosis outside of tumors is in different mouse models of HCC, including spontaneous models, as well as in patients.

At what time point does this "localization" occur? It would be very important, and interesting in the context of this paper to investigate whether HSCs maybe interact closely whit the tumor / pre-neoplastic lesions.

HSC and hepatocytes are in close proximity and close interaction partners in the normal and injured liver – the concept of the" stellome" (Wake, K. Proc Jpn Acad Ser B Phys Biol Sci. 2006; 82(4):155-640) suggests connections of one HSC to multiple hepatocytes. Our findings suggest that the interaction between HSC and hepatocytes is lost with increasing tumor size as the interface decreases the larger the tumor becomes and that this already occurs in sub 1mm tumors - see example of a 0.5 mm tumor (**Figure for Reviewers 14**). At the same time, the functionally critical time for this interaction seems to occur in the second half of our hepatocarcinogenesis protocol, when tumors make the transition from microscopic to macroscopic. It is likely that this is a critical phase where premalignant hepatocytes and small tumors benefit most from this interaction and that large tumors not only have a strongly diminished interface with HSC (see our finding in **Figure 2** that there are very few HSC within tumors – and the interface to surrounding liver becomes smaller and long-distance for most tumor cells in larger tumors) but that also large established tumors may not need HSC support for proliferation and survival anymore.

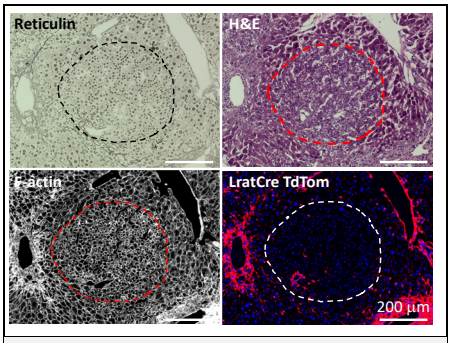


Figure for Reviewers 14. DEN+CCl_4-induced HCC with 0.5 mm tumor size shows only a small tumor-HSC interface.

Figures 2E and F: It is not clear where the tumor tissue is in relation to the performed stainings.

The picture is all non-tumor tissue as indicated in the figure legend. All non-tumor tissue in our study is tumor-distant. Our manuscript also incorporates determination of tumor tissue in multiple sections.

Potentially the N=1 analysis in Figure 2G – which I personally like – could be repeated to N=3?

As requested by the reviewer, we have increased the analysis to n=3. The results of this n=3 analysis was displayed in a single CellPhoneDB plot (**Figure 2g**). The findings are the same as before, showing (i) HSC as hub of cell-cell communication and (ii) that HSC represent the cell type, with which hepatocytes interact most intensely with.

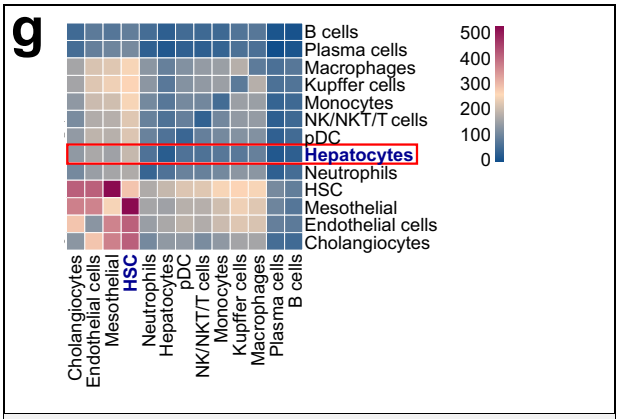
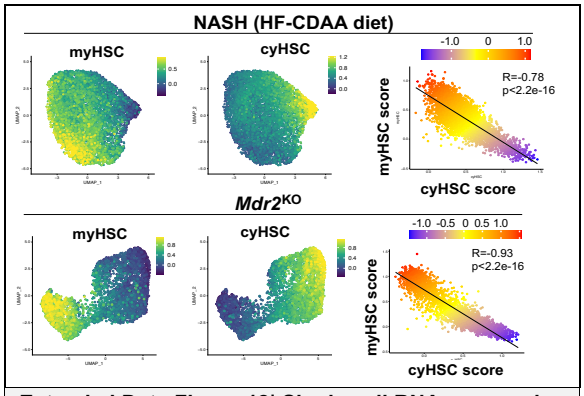


Figure 2 | Hepatic stellate cell accumulation and activation occurs predominantly in the non-tumour environment. g, analysis of cell-cell interactions by CellPhoneDB in scRNA-seq data from 8xCCl_4-injured liver (n=3) revealed HSC as a major cell type interacting with hepatocytes

Figure 3: The single cell analysis described are very nice and convincing. It is also very clear and nice that it is possible to change the plasticity of cyHSCs and myHSCs. Again this referee would advocate to investigate these in at least one spontaneous liver cancer model – and maybe also characterize HSCs at the start of liver cancer and further on.



Extended Data Figure 12| Single cell RNA-sequencing analysis of mouse hepatic stellate cells in fibrotic livers. a, UMAPs of the myHSC and cyHSC populations, each visualized by specific signatures as well as the correlation of cyHSC and myHSC signature score in HSC isolated from mice fed with HF-CDAA NASH diet for 12 weeks (n=1) or from 3 month old *Mdr2*^{KO} mice (n=1).

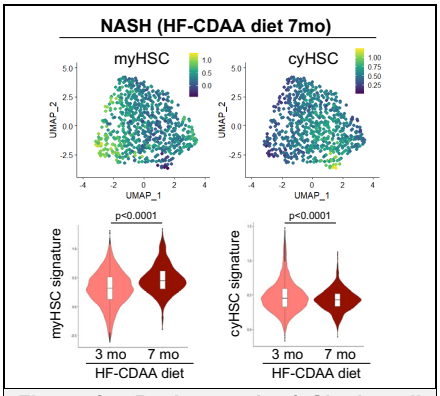
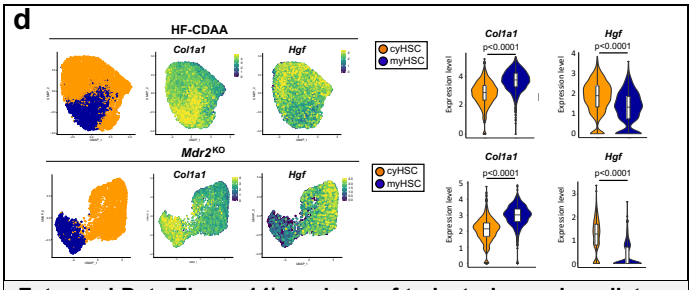


Figure for Reviewers 15. | Single cell RNA-sequencing showing myHSC and cyHSC in the HF-CDAA model at 7 months (at a time when tumors arise)**. Comparison of the 3 months and 7 months HF-CDAA shows higher myHSC and lower cyHSC signature at 7 months.**

As suggested by the reviewer, we added scRNA-seq and cyHSC/myHSC analyses from two spontaneous liver cancer models, the HF-CDAA diet (isolated at 3 months of diet) and from the *Mdr2*^{KO} model (**Extended Data Fig.12a**). Our data on myHSC and cyHSC in these two spontaneous models are similar to our other models as well as patients, show the presence of these subpopulations in all investigated models and support the overall hypotheses of our study. Furthermore, we also included single cell analysis in the HF-CDAA model at 7 months, i.e. at a time when tumors arise and see similar cyHSC and myHSC populations (**Figure for Reviewers 15** – not included in the manuscript as it looks almost identical to the already included 3 month HF-CDAA analysis).

Figure 3J and K: Hgf and Col1a1 expression look very convincing. Is this to be found in other models of fibrosis similarly (induced or spontaneous).

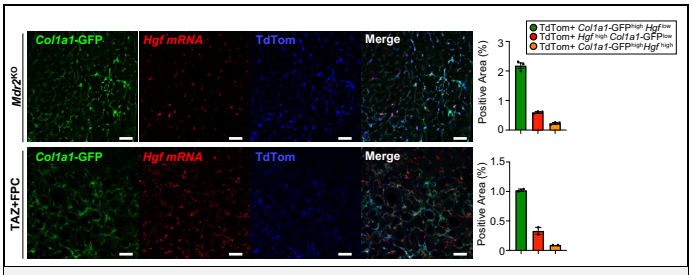
Thank you – we have added an scRNA-seq analysis of *Hgf* and *Col1a1* for the two spontaneous models, CDAA and *Mdr2*^{KO}-associated HCC (**Extended Data Figure 14d**), again showing enrichment of *Hgf* in cyHSC and *Col1a1* in myHSC populations.



Extended Data Figure 14| Analysis of trajectories and mediators produced by myHSC and cyHSC populations by scRNA-seq. d, *Col1a1* and *Hgf* mRNA expression within myHSC and cyHSC subpopulations in scRNA-seq of isolated HSC from a mouse fed with HF-CDAA NASH diet (n=1) or a 3 month old *Mdr2* ^{KO} mouse (n=1).

Figure 3L: The RNA Scope analysis is overall very convincing. Can this be reproduced in other liver cancer models?

As requested by the reviewer, we confirmed the RNAscope analysis in two additional models (*Mdr2*^{KO} and TAZ+FPC - **Extended Data Fig.15d**), making it a total of 3 models together with the previously already DEN+CCl$_4$ model (***Figure 3L*** in the manuscript). In all three models, we see distinctly located *Col1a1*-GFP^{high} Hgf$^{low/neg}$ LratCre-TdTom+ myHSC and *Hgf* ^{high} *Col1a1*-GFP$^{low/neg}$ LratCre-TdTom+ cyHSC.



Extended Data Figure 15| In situ analysis of myHSC and cyHSC populations in fibrotic mouse liver. *In situ* analysis of *Hgf* mRNA, visualized by RNAscope, *Col1a1*-GFP and LratCre-induced TdTom and subsequent quantification shows separate populations of *Col1a1*-GFP ^{high} *Hgf*^{low} myHSC, and Col1a1-GFP ^{low} *Hgf* ^{high} cyHSC in *Mdr2*^{KO} (n=3) and TAZ+FPC-treated livers (n=1).

Figure 4C: Does the macroscopy in Figure 4C reflect a representative image (does not appear based on the quantifications). A more representative image should be shown.

We have changed the macroscopic image in Figure 4C with a more representative one as suggested.

Figure 4D: TUNEL Staining appear to be unspecific in some part – a double stain would clarify this.

Higher magnification shows that TUNEL+ hepatocytes were clearly identified.

Figure 5: Data on HGF are very convincing. Could the authors correlate this with a decrease of measurable HGF in serum?

We show a decrease of HGF protein in the liver by ELISA (**Figure 6a**). Due to the short-half life and contribution of organs to serum levels, we consider this

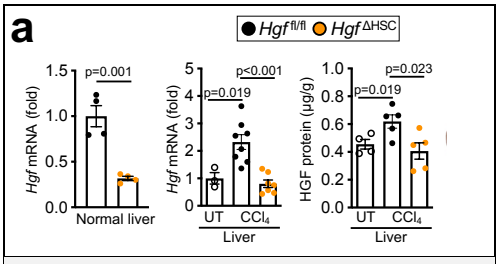


Figure 6 | CyHSC-enriched HGF protects hepatocytes from death and reduces HCC development. a, significant reduction of *Hgf* mRNA in normal and CCl$_4$ liver as well as of HGF protein in n=4-7 liver in LratCre^{pos} *Hgf* $^{fl/fl}$ mice (*Hgf* $^{\Delta HSC}$) compared to *Hgf* $^{fl/fl}$ mice (n=4-8/group).

more accurate to show decreased HGF in the most relevant compartment, where its biological effects take place (for this, one needs to consider HGF has a very short half-life of 3.8 minutes as shown by Appasamy et al, Lab Invest, 1993;68(3):270-6.).

How does this HGF expression correlate in human liver and serum?

Our manuscript already correlated hepatic HGF mRNA levels and risk to develop liver cancer development **(Fig.6g)**. Unfortunately, we do not have data to correlate serum levels with HCC development.

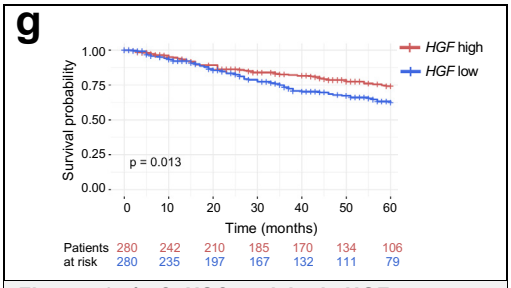


Figure 6 | CyHSC-enriched HGF protects hepatocytes from death and reduces HCC development. g, survival of HCC patients with low *HGF* (n=280) or high *HGF* (n=280) expression.

Is it also in the human situation that HGF is expressed by HSCs?

We in fact demonstrated the predominant HGF expression by HSC in the human situation by snRNA-seq (**Figure for Reviewers 2**, see also ***Extended Data Fig.17b*** in the manuscript).

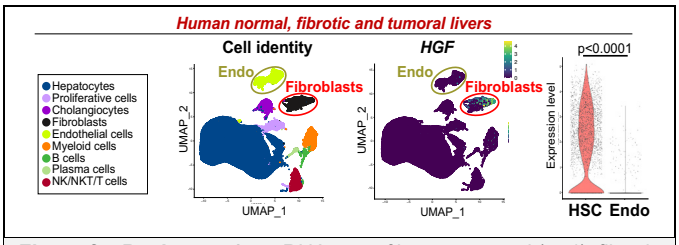


Figure for Reviewers 2. snRNA-seq of human normal (n=4), fibrotic (n=4) and HCC tumor (n=2) liver. Shown are UMAPs highlighting the populations and *Hgf* expression as well as violin plots comparing *Hgf* expression between HSC and endothelial cells.

When is the HGF expression important – before (to protect damage) or during oncogenesis? Here experiments with blocking antibodies would be useful.

We consider it most likely that HGF expression exerts key effects at early stages, based on its higher abundance at this time point as well as the lacking effects of early HSC depletion on HCC development (early depletion removes HSC at early and late time points – suggesting that there are HSC mediators that counter the tumor-promoting effects of HSC at later time points), suggesting protective effects of HSC at early time points. However, we were unfortunately not able to investigate this sufficiently as our HGF overexpression strategy only achieved temporary effects and did not sufficiently affect hepatocarcinogenesis (**Figure for Reviewers 4C-D**). We had intended to overexpress HGF early or late to specifically investigate this point). We also had considered to use HGF mimetics to address this point and also to capture the therapeutic aspect suggested by Reviewer 1, but unfortunately did not see effects of the HGF mimetic ANG3777 (synthesized at Columbia University – as we were not able to obtain it from Angion). We hope to address the question of the critical time point in future studies that focus on these therapeutic aspects – as adding further approaches and testing of blocking strategies would likely require additional 9-12 months and significantly delay our manuscript.

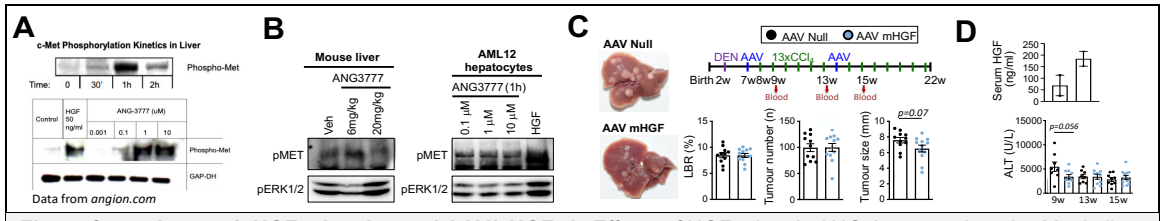
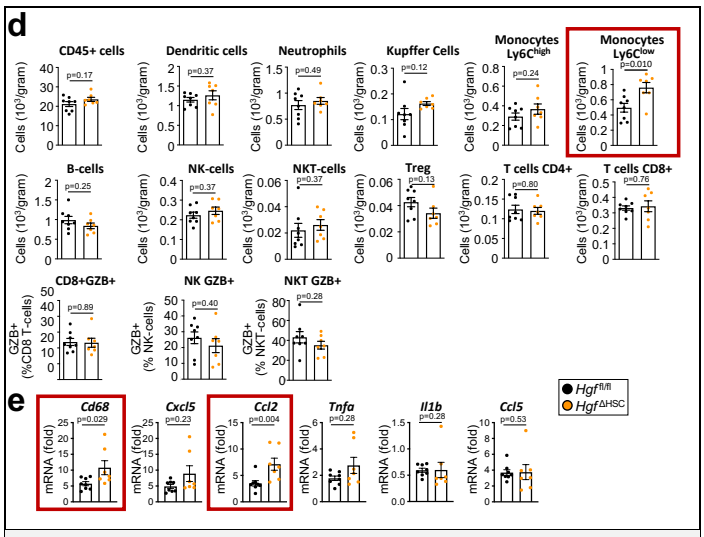


Figure for reviewers 4. HGF mimetics and AAV8-HGF. **A.** Effects of HGF mimetic ANG-3777 on phospho-Met in liver and in vitro. **B.** In house synthesized ANG3777 did not increase phospho-MET in mouse liver or AML12 hepatocytes. **C-D.** Overexpression of HGF via injection of AAV8-CMV-Cre (twice) showed only a trend towards decreased tumor size compared to mice that received AAV8-CMV-Null (C). Serum HGF was increase by HGF, and liver injury was decreased at early but not late time points (D).

How does the lack of HGF also affect the immune cell environment of the liver contributing to HCC? Again here a spontaneous model of carcinogenesis would be very helpful to see whether this is reproducible.

We have performed the analysis suggested by the reviewer. Careful analysis of immune subpopulations by FACS in *Hgf*$^{f/f}$ and *Hgf* $^{\Delta HSC}$ mice shows as main change an increase of classical Ly6C^{low} macrophages in *Hgf* $^{\Delta HSC}$ mice (**Extended Data Fig.28d**), which primarily serve to phagocytose, e.g. necrotic debris (Sprangers et al, J Immunol Res. 2016; 1475435; Guillams et al, Immunity 201; 49:595-613). qPCR analysis reflects these findings with an increase of *Cd68* and *Ccl2* mRNA in *Hgf* $^{\Delta HSC}$ mice compared to *Hgf* $^{\Delta HSC}$ mice (**Extended Data Fig.28e**). Altogether, this is consistent with our finding of increased liver injury in *Hgf*$^{\Delta HSC}$ mice.



Extended Data Figure 28| HGF deletion in HSC promotes fibrosis and inflammation in CCl$_4$-induced liver injury. d, flow cytometric analysis of CD45+ cells as well as myeloid and lymphocyte populations in livers from *Hgf* $^{\Delta HSC}$ compared to *Hgf* $^{f/f}$ mice (n=7-8 mice per group) treated with injections of 6xCCl$_4$. GZB, granzyme B. e, qPCR for inflammatory genes from non-tumor tissue of *Hgf* $^{\Delta HSC}$ and and *Hgf* $^{f/f}$ littermates after treatment with 6xCCl$_4$ injections (n=7 mice per group).

Overall, the manuscript is well written and clear - as is the discussion.

We would like to the thank the reviewer for this positive comment.

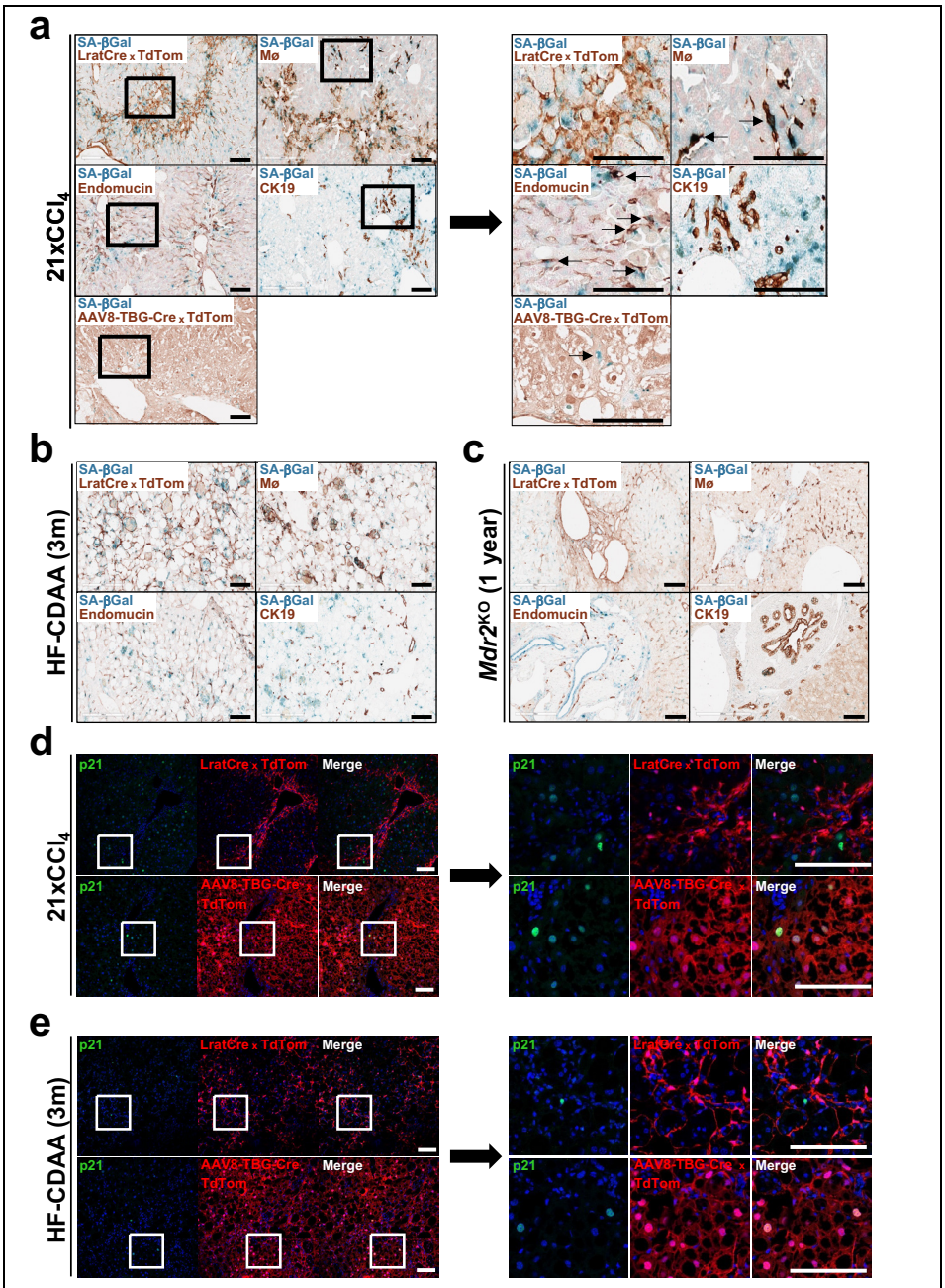
REFEREE #3 (REMARKS TO THE AUTHOR):

This manuscript is grounded in the controversy concerning the role of senescent hepatic stellate cells (HSCs) in the outcomes of liver injury. As you beautifully summarize in the introduction, some published data indicate that senescent HSCs promote liver cancer, while other data indicate the opposite effect. Your paper is a tour-de-force of mouse models and packed with data that are novel and likely to be highly utilized by other researchers. However, in my view, there are some significant limitations/weaknesses.

We would like to thank the reviewer for the positive comments.

1 - None of the work that your present directly resolves the one fundamental research question that you posed in the manuscript's Introduction, i.e., do senescent hepatic stellate cells (HSCs) increase hepatocarcinogenesis or decrease hepatocarcinogenesis?

We would like to take this opportunity to point out that the overarching goal of our studies was to understand the role of HSC and HSC-expressed mediators in the process of hepatocarcinogenesis rather than to specifically study HSC senescence in this context. While we did start out with HSC senescence based on reports from the literature, we neither saw a role for HSC-expressed p53 (***Extended Data Fig.1a-d*** in the manuscript) and *RelA* (***Extended Data Fig.1e-h*** in the manuscript) in hepatocarcinogenesis, nor did we see a high amount of HSC senescence, by senescence-associated beta-galactosidase staining (**Extended Data Fig.2a-c**), p21 IHC (**Extended Data Fig.2d-e**) as well as senescence signatures in scRNA-seq analysis in different models (**Figure for Reviewers 16** next page). Instead, we observed that the majority of b-gal staining in the endothelial cell and macrophage/Kupffer cell populations. These findings are consistent with a recent study by Omori et al (Cell Metabol 2020;32(5):814-828), which showed that HSC only represent 1% of p16^{high} cells in NASH livers fed CD-HFD for 6 months (**Figure for Reviewers 17** next page). As the main research question that we wanted to study was the role that HSC exert in hepatocarcinogenesis, almost all of our subsequent studies therefore focused on identifying pathways and mediators (such as *Hgf*, *Col1a1* and large range of additional mediators) without specific focus on their link to senescence. We have expressed this more clearly in the manuscript. Although senescence is not widespread in HSC and does not appear to contribute to hepatocarcinogenesis in the models we studied, we do not want to completely exclude a role and now discussed the



Extended Data Figure 2 | Determination of cellular senescence in murine models of fibrosis. **a-c,** representative images showing senescence in specific cell types by beta-galactosidase staining and co-staining for markers or lineage tracers of HSC (LratCre x TdTom), macrophages (anti-macrophage antibody), endothelial cells (endomucin antibody), cholangiocytes (CK19 antibody) and hepatocytes (AAV8-TGB-Cre x TdTom) in the CCl_4 (**a**), HF-CDAA diet (**b**) and *Mdr2* ^{KO} (**c**) mouse models of fibrosis (from n=1-3 mice per group). **d-e**, representative images showing senescence in specific cellular compartments by p21 IHC in combination with lineage markers for HSC (LratCre x TdTom) and hepatocytes (AAV8-TGB-Cre x TdTom) in the CCl_4 (**d**) and HF-CDAA diet (**e**) mouse models of fibrosis (from n=1 mouse per group).

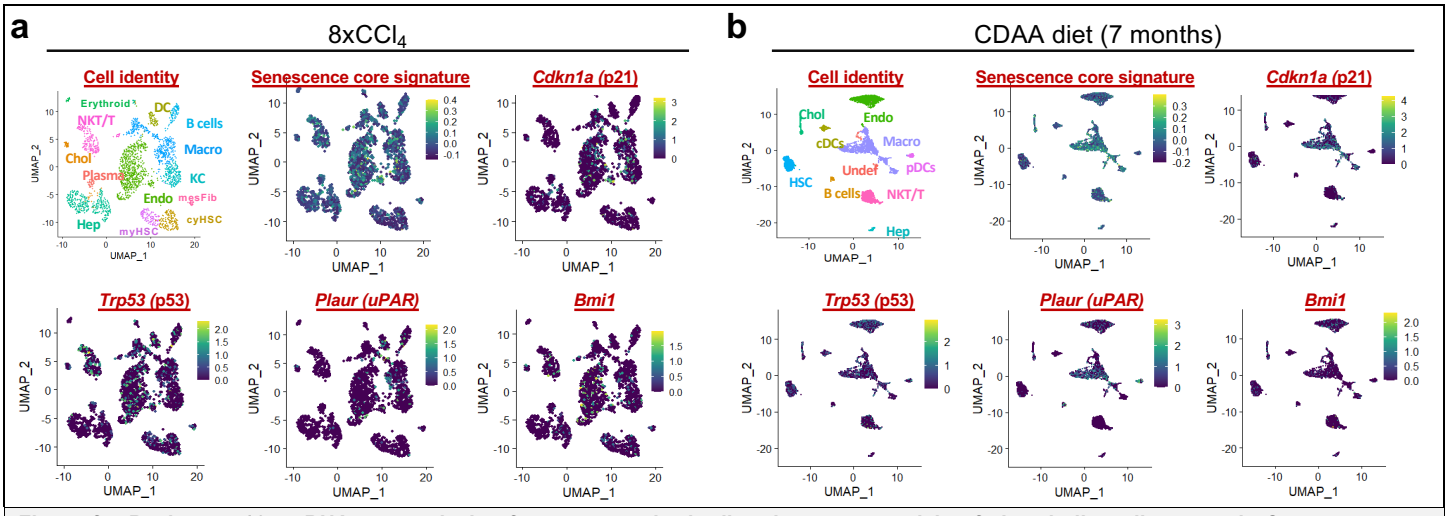


Figure for Reviewers 16. scRNA-seq analysis of senescence in the liver in mouse models of chronic liver disease. a-b. Senescence was analyzed in scRNA-seq data from CCl_4-treated (a) and 7 months HF-CDAA -fed mice (b) using a senescence core signature (xxx et al) and also displaying several single markers genes including *Cdkn1a*, *Trp53*, *Plaur* and *Bmi1*. Consistent with previously studies and our beta-gal staining, we found the high levels of senescence in macrophages/Kupffer cells (KC) and endothelial cells.

known link between the expression of type I collagen, which we identified as key HSC-secreted tumor-promoting mediator, and senescence and pointed out that numerous studies have shown that senescent fibroblasts/HSC produce lower amounts of type I collagen (e.g. Schnabl et al, Hepatology. 2003 Mar;37(3):653-64; Krizhanovsky et al, Cell 2008;134:657-67)

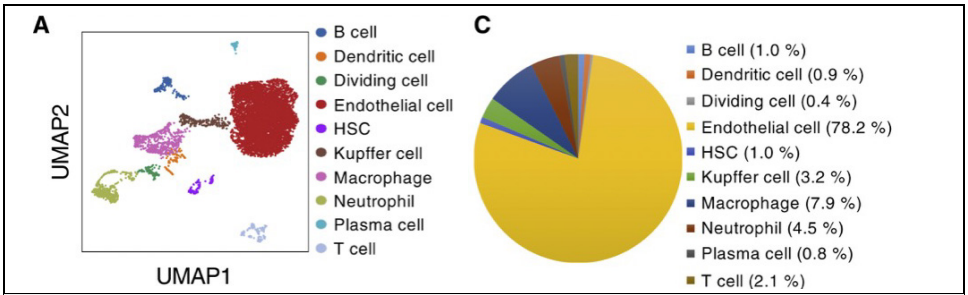


Figure for Reviewers 17. "Figure 4. Single-Cell Mapping of p16^{high} Cells in the Liver of a NASH Mouse (Omori et al, Cell Metabol 2020;32:814-828). A. UMAP for the cell-type identification of 7,129 single NPC transcriptomes from the liver of a p16-CreERT2-tdTomato mouse with NASH as in Figure 3A. **C.** The cell-type composition of Tom+ NPCs sorted from liver of a NASH mouse.

2 - Your results do indicate that at least a subpopulation of fibrogenic-activated/myofibroblastic HSCs are pro-carcinogenic and suggest that perhaps a subset of the remaining HSC population suppresses carcinogenesis. However, none of the data are informative regarding whether either the pro- or the anti-carcinogenic subpopulation is enriched with senescent cells (as indicated by beta-gal staining/expression of p16, p21, p27, p53, etc...). Assuming that you remain interested in resolving the controversy about the significance of HSC senescence (which is an important question), additional experiments/analyses are needed to address this question.

As alluded to above, our studies and those from other groups found only little HSC senescence in the liver and our functional studies also did not support an important role for HSC senescence in promoting hepatocarcinogenesis. To address the specific point whether the activated myHSC population, which we found to be tumor-promoting, has higher levels of senescence, we analyzed myHSC and cyHSC senescence in our single cell RNA-seq data (since this allows reliable identification of both the HSC status as well as senescence). We did not observe significant differences in senescence, both

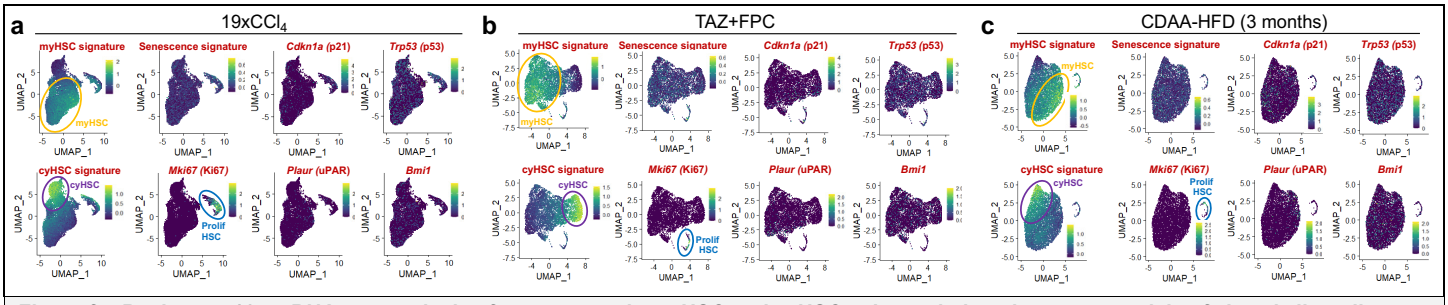


Figure for Reviewers 18. scRNA-seq analysis of senescence in myHSC and cyHSC subpopulations in mouse models of chronic liver disease. a-c, senescence was analyzed in scRNA-seq data from CCl_4-treated (**a**), TAZ+FPC (**b**) or HF-CDAA-treated mice (**b**) using the same senescence core signature as in Fig.for Reviewer 16 above and also displaying myHSC and cyHSC signature activities, proliferation marker *Mki67* as well as several single senescence markers genes including *Cdkn1a*, *Trp53*, *Plaur* and *Bmi1*

by a senescence signature as well as by multiple single markers between the two subpopulations (**Figure for Reviewers 18**).

3 - Also, most of the data are descriptive, rather than mechanistic. For example, a - although the data clearly show that the pro- and anti-carcinogenic HSC subpopulations exhibit differential expression of genes that encode various secreted proteins, no clear mechanism(s) is/are delineated to demonstrate how any of these factors either promote or antagonize hepato-carcinogenesis.

We have taken this comment - although we do not agree with the term "descriptive" as all our experiments include knockout of HSC mediators, and often receptor or downstream mediators in hepatocytes - as motivation to include more data and causation experiments on HSC mediators, pathways and receptors, now revealing a more refine picture of pro-tumor and anti-tumor effects of HSC (see our comments in the next paragraphs):

HGF is suggested to be a critical anti-carcinogenic factor because it protects cultured hepatocytes from experimentally-induced apoptosis. This is not surprising but falls short of explaining the net effects of the anti-carcinogenic HSC subpopulation in vivo given other evidence indicating that expression of multiple pro-inflammatory mediators is enriched in those cells (and inflammation is generally thought to promote cancer).

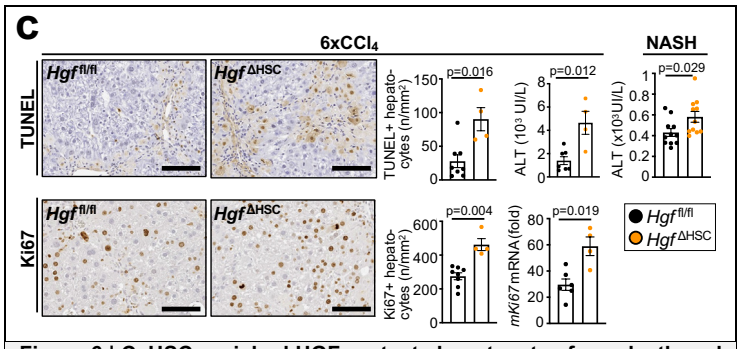
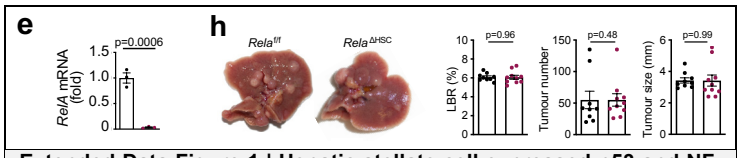
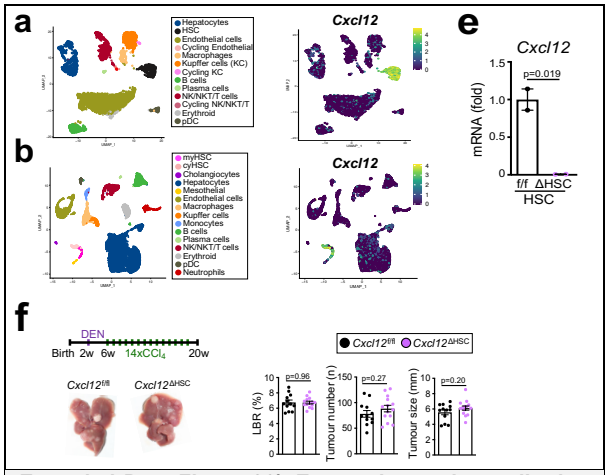


Figure 6 | CyHSC-enriched HGF protects hepatocytes from death and reduces HCC development. C. Hepatocyte death assessed by TUNEL staining and ALT measurement as well as hepatocyte Ki67 IHC and *mKi67* RNA were increased in $Hgf^{\Delta HSC}$ mice compared to $Hgf^{fl/fl}$ (n=4-8/group); $Hgf^{\Delta HSC}$ mice under CDAA-diet for 1 month display more liver injury assessed by ALT measurement compared to $Hgf^{fl/fl}$ (n=9/group).



Extended Data Figure 1 | Hepatic stellate cell-expressed p53 and NF-kB RelA do not modulate DEN+CCl4-induced HCC development. e. qPCR showing *Rela* mRNA in HSC isolated from FACS-sorted HSC from $Rela^{f/f}$ and $Rela^{\Delta HSC}$ mice (n=3 mice/group). **h,** HCC was induced in $Rela^{f/f}$ and $Rela^{\Delta HSC}$ mice (n=9-10 mice/group) by injection of DEN (i.p. 25 mg/kg at 2 weeks old) followed by 20 CCl_4 injections (1x/week). HCC burden is shown by representative pictures, liver-body ratio, tumour number and size.

- While agree that the *in vitro* hepatocyte data is not surprising, we would like to point out that our study clearly shows differences in hepatocyte death *in vivo* (**Figure 6c**), both by TUNEL staining and serum ALT. As such, hepatocyte death is considered the primary driving mechanism that triggers subsequent inflammation and fibrosis in patients with chronic liver disease and in many mouse models, including the $Hgf^{\Delta HSC}$ mice (Friedman, *Gastroenterology* 2008; 134: 1655–1669; Koyama and Brenner, *J Clin Invest*. 2017;127:55-64). In the long run, hepatocyte death triggers a number of tumor-promoting consequences (reviewed in Luedde, Kaplowitz and Schwabe, *Gastroenterology*. 2014;147:765-783 and Schwabe and Luedde, Nat Rev Gastroenterol Hepatol. 2018;15:738-752.), including compensatory hepatocyte proliferation (e.g. shown by Maeda et al, *Cell* 2005;12:977-90; Sakurai et al, *Cancer Cell* 2008;14:156-65), inflammation (e.g. shown by Haybeck et al, *Cancer Cell* 2009;16:295-308; Endig et al, *Cancer Cell* 2016;30:308-323; reviewed in Yang et al, *Semin Liver Dis*. 2019;39:26-42), and fibrosis/stiffness (shown in our current study). Our data clearly shows increased compensatory hepatocyte proliferation (**Fig.6c**) and inflammation (***Extended Data Figure 28e*** in the manuscript) in the $Hgf^{\Delta HSC}$ mice. We also would like to point out that studies with hepatocyte-specific deletion of Met have already consistently shown increased HCC (Takami et al, Cancer Res. 2007;67:9844-51), thus confirming the HGF-MET receptor-ligand pair as direct link between HSC-produced HGF and hepatocyte injury/protection. In response to this comment, we have **added *in vivo* experiments in our manuscript:**



Extended Data Figure 26|. Expression and contribution of Hhip and Cxcl12 in hepatocarcinogenesis. a-b, UMAPs of *Cxcl12* mRNA in normal (a) and in CCl_4-treated mouse liver (b)**. e,** *Cxcl12* deletion was determined by qPCR in HSC from mice with LratCre-mediated deletion ($Cxcl12^{\Delta HSC}$) in comparison to $Cxcl12^{fl/fl}$ HSC (n=2/group). **f,** $Cxcl12^{\Delta HSC}$ and $Cxcl12^{fl/fl}$ mice (n=12-14/group) were subjected to DEN+CCl_4-induced hepatocarcinogenesis, followed by evaluation of the liver-body weight ratio, tumour size and number.

- Although inflammation is considered a driver of carcinogenesis as pointed out by the reviewer and discussed above, we clearly observed that HSC-mediated inflammation does not make a major contribution:
  - Mice with HSC-selective deletion of NF-kB p65/*RelA* did not show any differences in hepatocarcinogenesis (**Extended Data Fig.1e,h**).

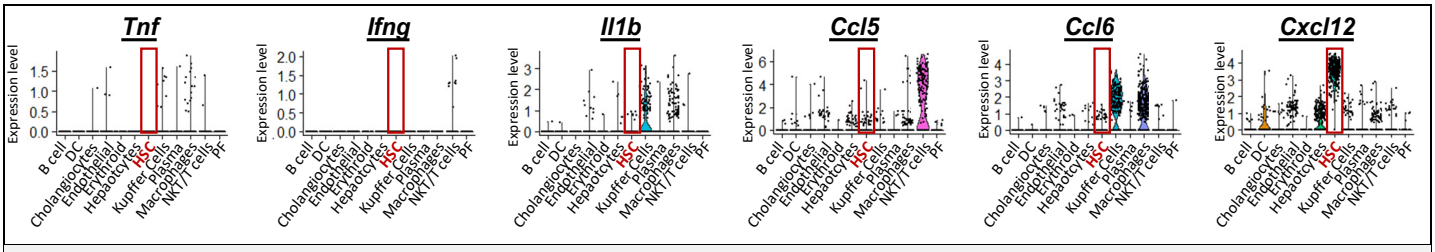
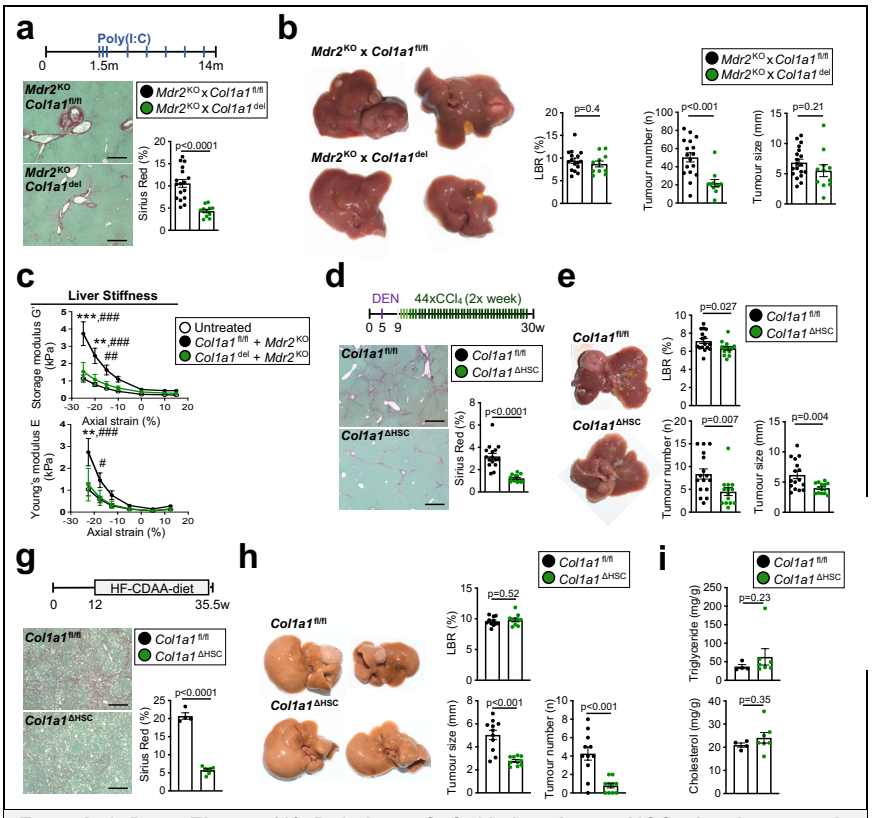


Figure for Reviewers 19. Expression of inflammatory mediators by HSC and other cell types. scRNA-seq data from CCl4-treated mouse liver shows predominant expression of inflammatory mediators by immune cells and not by HSC.

- We now added studies in mice with HSC-selective deletion of *Cxcl12* (*Cxcl*12$^{\Delta HSC}$ mice) which is strongly enriched in HSC and specifically in the cyHSC subpopulation, to the manuscript. *Cxcl12*$^{\Delta HSC}$ mice did not show alterations in hepatocarcinogenesis compared to their floxed littermates (**Extended Data Fig.26 a,b,e,f**).
- While cyHSC are enriched in growth factors and cytokines vs myHSC, the majority of inflammatory mediators and growth factors is much more highly expressed in non-HSC populations than in HSC (see **Figure for Reviewers 19**). HGF and also CXCL12 are notable exceptions in that are strongly enriched in HSC and here within the cyHSC subpopulation. Deletion of *Cxcl12* did not affect hepatocarcinogenesis and based on above data and our data in mice with HSC-selective deletion of NF-kB subunit RelA (**Extended Data Fig.1e,h** last page), we do not think that HSCs are a major instigators of inflammation and do not affect hepatocarcinogenesis via inflammation.

b - no experiments were done to define which of the various secreted gene products of the pro-carcinogenic HSC subpopulation promotes liver cancer (or to delineate how that factor enhances cancer growth).



Extended Data Figure 19| Deletion of *Col1a1* reduces HCC development in DEN+CCl$_4$-, NASH diet- and *Mdr2*^{KO}-induced HCC. a-b, Mx1Cre-mediated *Col1a1* deletion in *Mdr2*^{KO} female mice (*Mdr2*^{KO} *Col1a1* ^{del}, n=11) efficiently reduced liver fibrosis (**a**) and HCC development, as determined by liver body weight ratio, tumour number and tumour size (**b**), compared to Mx1Cre^{neg} *Mdr2*^{KO} littermates (*Mdr2*^{KO} *Col1a1* $^{fl/fl}$, n=18). **c**, *Col1a1* deletion in 22 week old *Mdr2*^{KO} male mice (n=2 mice) reduces stiffness assessed by rheometry compared to *Col1a1* $^{fl/fl}$ *Mdr2* ^{KO} (n=3 mice), livers from 8 week old untreated mice were used as control (n=3) **d-f**, HSC-selective ablation of *Col1a1* in LratCre^{pos} *Col1a1* $^{f/f}$ (*Col1a1* $^{\Delta HSC}$, n=14 mice) efficiently reduced liver fibrosis (**d**) and HCC development, as determined by liver body weight ratio, tumour number and tumour size (**e**) compared to Lrat^{neg} littermate controls (*Col1a1* $^{fl/fl}$, n=16 mice) in the DEN + CCl$_4$ profound fibrosis model. **g-i**, *Col1a1* $^{\Delta HSC}$ mice (n=10) displayed strong reductions of liver fibrosis (**g**) and HCC development, as determined by liver body weight ratio, tumour number and tumour size (**h**), but not in liver triglycerides and cholesterol content (**i**) compared to *Col1a1* $^{fl/fl}$ control mice (n=11 mice) in a model of NASH-associated HCC induced by 8 months of HF-CDAA diet. Data are shown as mean ± SEM, each data point represents one individual· scale bars: 200 µm (**a**,**c** and **f**), 50 µm (**e**).

Our previous data had clearly shown a role for type I collagen as myHSC-secreted tumor-promoting gene product. In response to above comment, we not only significantly expanded the section on type I collagen by adding two additional spontaneous models of HCC induced by HF-CDAA- and *Mdr2* ^{KO} as well as deletion via LratCre in the DEN+CCl$_4$ model, but also substantial novel data on downstream mechanisms, now revealing Col I–Stiffness–TAZ and Col I–DDR1 as two independent pathways that promote hepatocarcinogenesis in different compartments:

- We now show by 2 different genetic approaches (Mx1Cre and LratCre) in 3 different HCC models (DEN+CCl$_4$, *Mdr2*^{KO} and HF-CDAA-) a tumor-promoting role of myHSC

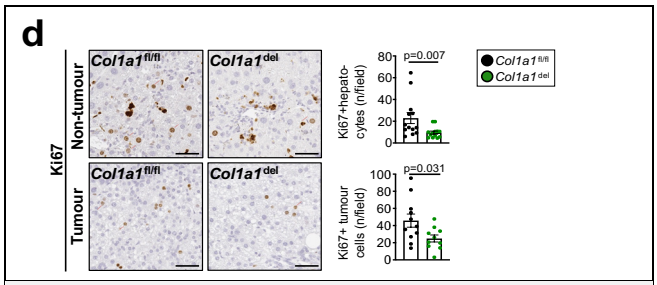


Figure 4 | Promotion of HCC by type I collagen and stiffness-associated accumulation of TAZ in hepatocytes. d, Ki67 staining as in non-tumour and tumour tissue (n=10-13 mice per group) show a reduction of hepatocyte proliferation and tumour cell proliferation in *Col1a1*^{del} mice compared to *Col1a1*$^{f/f}$ littermates.

secreted mediator *Col1a1* (**Extended Data Figure 19a-i** and *Figure 4b-d* in the manuscript). Mice with Col1a1 deletion display a reduction of hepatocyte proliferation in non-tumor liver as well as a reduction of tumor cell proliferation (**Figure 4d**).

Downstream of type I collagen, we now added further data showing that the stiffness-TAZ pathway is an important mediator of hepatocarcinogenesis induced in hepatocytes and distinct from the Col I-DDR1 pathway active in tumor cells (see below). We had already shown a reduction of TAZ expression by western blot analysis and hepatocyte TAZ expression by IHC (*Figure 4f-g* in the manuscript) in mice with Cola1a1 deletion. We now have added new data that shows reduced HCC development in mice with TAZ but not YAP hepatocyte-specific deletion (**Figure 4h** and *Extended Data Fig.22a,b* in the manuscript). Importantly hepatocyte proliferation in the non-tumor compartment was reduced in mice with hepatocyte-specific deletion of TAZ (**Figure 4i**).

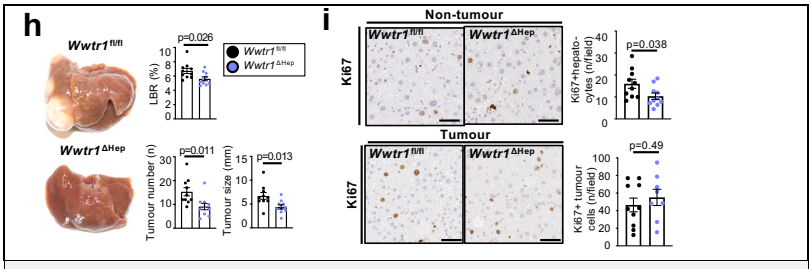


Figure 4 | Promotion of HCC by type I collagen and stiffness-associated accumulation of TAZ in hepatocytes. h, Mice with hepatocyte-specific TAZ deletion (*Wwtr1*$^{\Delta Hep}$, n=10) showed reduced HCC development compared to *Wwtr1*$^{fl/fl}$ controls (n=10) in the DEN+44xCCl$_4$ HCC model. **i,** Ki67 staining and quantification of Ki67+ hepatocytes in tumour and non-tumour sections of *Wwtr1*$^{\Delta Hep}$ and *Wwtr1*$^{fl/fl}$ mice (n=8-10 mice/group).

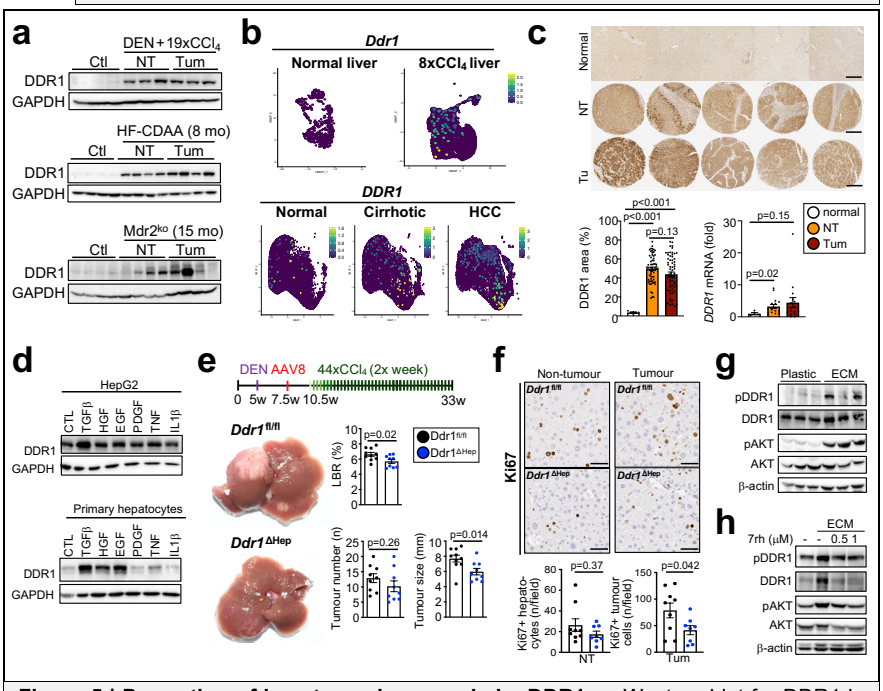
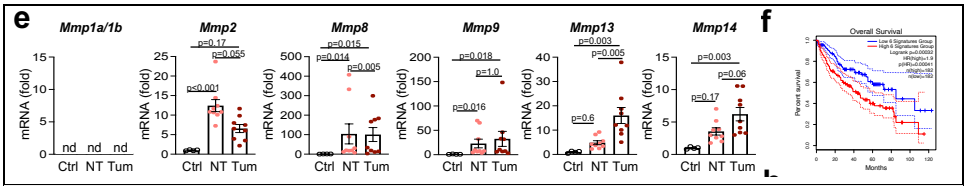


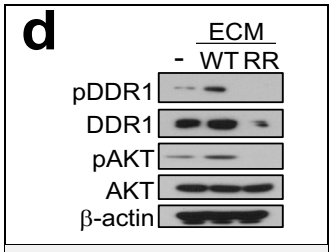
Figure 5 | Promotion of hepatocarcinogenesis by DDR1. a, Western blot for DDR1 in healthy, non-tumour and tumour liver from different mouse models of HCC. **b**, UMAPs of scRNA-seq data showing hepatocyte *Ddr1* expression from normal (n=1) and 8xCCl$_4$ fibrotic mouse liver (n=3) and of snRNA-seq from patients showing *DDR1* expression in the hepatocyte or tumor compartment from healthy patients (n=4), non-tumor (n=2) and cirrhotic (n=2) or HCC tumour (n=2) liver tissues. **c**, *DDR1* mRNA expression (n=4 healthy controls, n=16 non-tumor and tumor areas from HCC patients) and IHC and quantification in healthy human control livers (n=6), and in TMA containing non-tumour and tumour areas from patients (n=64 each). **d**, Western blot for DDR1 in HepG2 and primary mouse hepatocytes after treatment with the indicated cytokines for 24h. **e**, *Ddr1*$^{fl/fl}$ mice were injected with AAV-TGB-empty (*Ddr1*$^{fl/fl}$, n=12) or AAV8-TBG-Cre (*Ddr1*$^{\Delta Hep}$, n=9) and HCC development was determined in the DEN+CCl$_4$ profound fibrosis HCC model. **f**, Proliferation was determined in the DEN+CCl$_4$ profound fibrosis HCC model by Ki67 IHC in the non-tumour and tumour compartments (n=7-8 per genotype). **g**, western blot for phospho-DDR1 (pDDR1), pAKT, DDR1, AKT and b-actin in HepG2 cells were plated on plastic or ECM. **h-i**, western blot for pDDR1, pAKT, DDR1, AKT and b-actin in Huh7 cells plated on plastic, or ECM and treated with DDR1 inhibitor 7rh (**h**) or plated on plastic, WT ECM and type I collagen RR mutant ECM (**i**). **j**, impact of the expression of six collagen-degrading MMPs on survival of HCC patients in TCGA data. Data are shown as mean ± SEM, each datapoint (a, c-h) represents one individual. Scale bars: 500 μm (c), 50 μm (f).

- We added a completely new section to the manuscript in which we show upregulation of DDR1 in the non-tumour and tumour compartments, as well as a key role for DDR1 in the promotion of hepatocarcinogenesis within the tumor compartment. Our findings on the Col I-DDR1 signaling pathway which complements the above-described role of the stiffness-TAZ pathway in the non-tumor compartment of the liver: Specifically, we found that DDR1 expression is upregulated in the injured liver, both in mice and patients, as shown by western blotting, qPCR and tissue microarray (**Figure 5a-c**), and that this occurs within the hepatocyte compartment as well as in tumor cells. As such, TGFβ, the fibrogenic master regulator that triggers activation and collagen production by HSC was the strongest inducer of DDR1 in primary hepatocytes and tumor cells (**Figure 5d**). Thus, TGFβ not only acts as powerful inducer of ECM production but also upregulates receptor systems on epithelial cells for fine-tuning of epithelial responses to type I collagen.



Extended Data Figure 25| Regulation and role of DDR1 in hepatocarcinogenesis. e, e, expression of collagen-degrading MMPs, determined by qPCR, in the profound fibrosis DEN+CCl$_4$ model (n=4 normal liver controls [Ctrl], n=9 mice for non-tumour [NT] and tumour [Tum] samples). **f**, survival analysis in the TCGA dataset based on the expression of a collagen-degrading MMP signature (*MMP1*, *MMP2*, *MMP8*, *MMP9*, *MMP13* and *MMP14*).

Importantly, we demonstrated a tumor-promoting role of DDR1 by hepatocyte-specific deletion of DDR1, which strongly reduced the development of HCC (**Figure 5e**). While deletion of TAZ reduced proliferation in premalignant or early-stage lesions in the non-tumor compartment, deletion of DDR1 reduced proliferation in the tumor compartment (**Figure 5f**). Thus, Col I (which promotes proliferation in both compartments) acts through DDR1 in the tumor cells and through TAZ in hepatocytes.



Extended Data Figure 25| Regulation and role of DDR1 in hepatocarcinogenesis. d, Huh7 cells were plated on ECM from wild-type fibroblasts or from MMP-resistant Col I (RR) fibroblasts, followed by western blot for pDDR1, DDR1, pAKT, AKT and b-actin.

- In additional experiments, which are linked to a co-submitted manuscript by the Karin group (Su et al, we also showed that in tumor cells, DDR1 and its downstream pathway AKT are activated by Col I-containing ECM, which is inhibited by DDR1 inhibitor 7rh (**Figure 5 g,h**) but not by ECM which contains non-degradable type I collagen (**Extended Data Fig.25d**). Furthermore, we show that the expression of collagen-degrading MMPs is increased in non-tumor and especially in tumor areas (**Extended Data Fig.25e**), and that high expression of collagen-degrading MMPs as well as high expression of DDR1 confer a worsened prognosis for HCC patients (**Extended Data Figure 25f**). These data may also explain why in a recently published study non-degradable type I collagen, which increases type I collagen levels in the liver but, as we show (**Extended Data Fig.25d**), inhibits the activation of DDR1, was associated with reduced rather than increased HCC formation (Baglieri et al. Am J Pathol. 2021;191(9):1564-15).

- We also analyzed a large number of additional HSC-enriched secreted mediators including Wntless (required for the secretion of all Wnts – data not shown, but can be provided to reviewers), *Cxcl12* and hedgehog inhibitor *Hhip*. We did not observe effects of these mediators in hepatocarcinogenesis (***Extended Data Figure 27a-f*** in the manuscript), highlighting the relevance of the identified myHSC mediators *Col1a1* and *Has2* and cyHSC mediator *Hgf* in hepatocarcinogenesis.

4 - The identities of the large numbers of HSCs that exhibit neither the pro- or anti-carcinogenic HSC signature in all of the various mouse models has not been interrogated.

We would like to point out that the tumor-suppressing and tumor-promoting gene signatures and associated populations investigated in our studies represent gradients. While we have used thresholds to assign HSC as cyHSC or myHSC for some figures, the cells represent a continuum in all investigated scRNA-seq dataset, including those from other groups. Please see also our response to comments from reviewer #1, who had asked for comparisons to previously published studies and potentially more detailed subclustering. There are virtually no HSCs that do not express the cyHSC or myHSC signature and our statistical analysis show a strong inverse correlation between the two, i.e. cyHSC expressed low levels of myHSC signature and vice versa. We do not think that the cells between cyHSC and myHSC represent a distinct biological entity - as this is continuous gradient. Accordingly, our pseudotime analysis shows a clear trajectory from cyHSC to myHSC (***Extended Data Fig.14a*** in the manuscript).

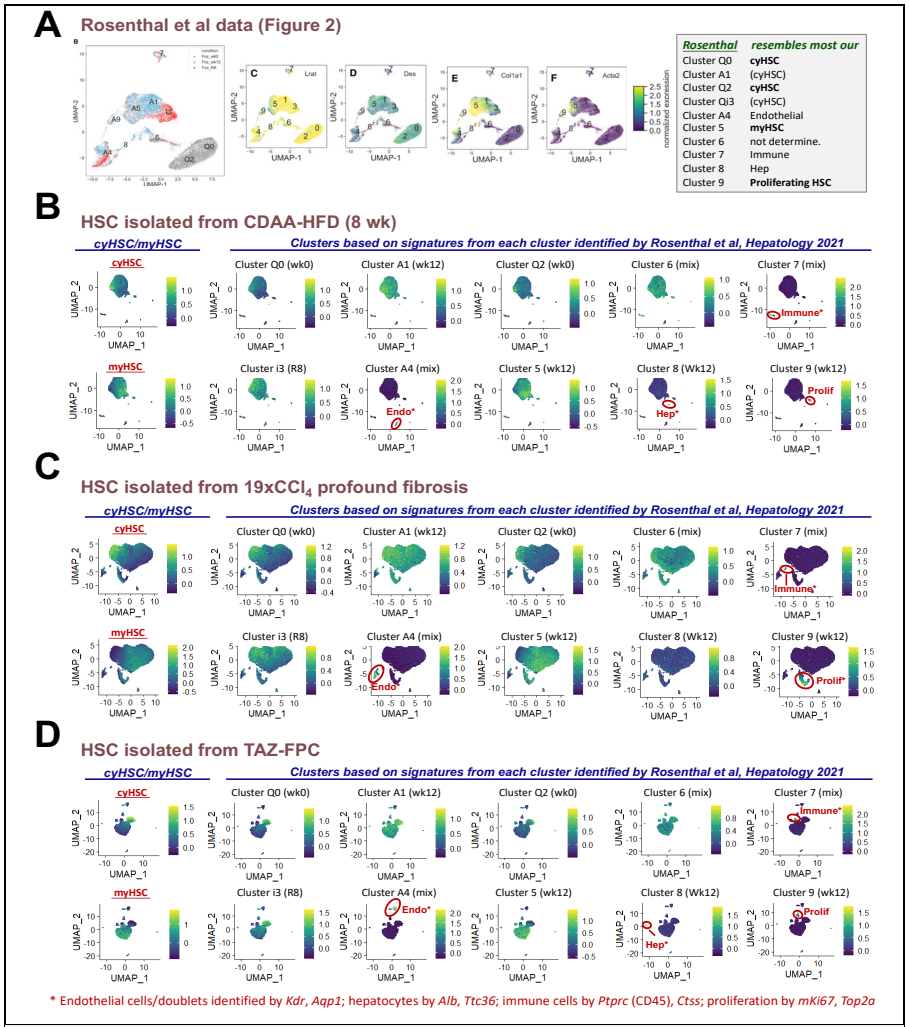


Figure for Reviewers 8. Comparison of the Rosenthal et al HSC clusters to our HSC clusters. A. Display of Rosenthal et al clusters and summary of comparison, showing which of our scRNA-seq HSC clusters aligns with their clusters. **B-D.** Cluster-specific gene signatures for clusters 0-9 from Rosenthal et al were applied to HSC scRNA-seq data from the HF-CDAA (B), 19xCCl4 (C) and TAZ-FPC (D) models. Comparisons were made to data without removal of doublets or contaminating cells from our data, allowing to align some of the Rosenthal et al. clusters with these doublet/non-HSC clusters.

a - It would be helpful to know if signatures of these cells align with signatures that other groups have identified in various HSC subpopulations in healthy or fibrotic livers (According to those other two reports, there are at least 5 different HSC subpopulations in injured livers).

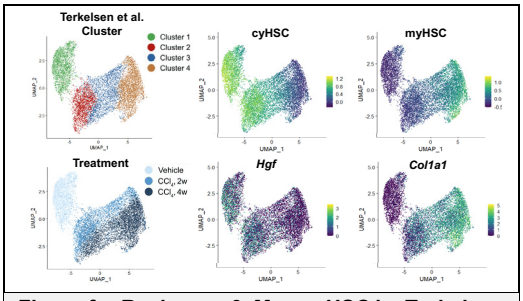


Figure for Reviewers 9. Mouse HSC by Terkelsen et al, Hepatology aligned with cyHSC and myHSC signatures. Shown are clusters of quiescent HSC (cluster 1) and HSC with different degrees of activation (Clusters 1-4), aligned with cyHSC/myHSC signatures, *Hgf* and *Col1a1*.

- Our main goal was to identify HSC subpopulations and associated mediators with high functional relevance to HCC - rather than identifying "novel subpopulations" - which we then tested in subsequent functional studies (knocking out myHSC mediators such as *Col1a1*; *Has2*; and cyHSC mediators *Hgf, Hhip* and *Cxcl12*). We also do not think that novel HSC subpopulations/finer resolution/better functional classification via scRNA-seq analysis beyond what has already been published in the literature can be identified unless further in-depth analyses (e.g. concomitant scATAC-seq; concomitant high resolution spatial transcriptomics; or analysis of transcriptional regulators through reverse network engineering; both currently ongoing long-term projects in our lab) are applied.

- In response to the reviewer comments on the recent manuscript DOI: 10.1002/hep.31743 by the Brenner group (Rosenthal *et al*, Hepatology 2021;74(2):667-685), we have analyzed their clusters and compared them to our analyses, using a gene expression signatures for each cluster from their Supplementary Tables S3-S12 (**Figure for Reviewers 8**). It should be noted that their paper not only used more conditions (resulting in more clusters), but may also have less pure HSC preps (not using LratCre and not removing contaminating non-HSC populations/doublets from scRNA-seq data). A second paper by the Ravnskjaer group (Terkelsen et al, Hepatology. 2020;72(6):2119-2133) used approaches that are more similar to ours in that there is one continuous cluster of activated HSC, with the only differences that they also included quiescent HSC, which form a separate cluster (**Figure for Reviewers 9**). Overall, the results from the Brenner paper reveal expected finding, showing that the highly activated (A5 cluster) aligns with myHSC and the quiescent clusters (Q0,Q2) as well as the *Col1a1*^{low} and *Acta2*^{low} clusters (A1) with the cyHSC cluster (**Figure for Reviewers 8**). Notably, application of the A5 cluster (correlating to our myHSC cluster) covers a large proportion of our HSC. The proliferation cluster A9 also falls clearly into our clusters of proliferation HSC. The only notable difference is that cluster i3 (which is right adjacent to the A1 lowly activated HSC cluster in Rosenthal et al) largely labels cyHSC in our data, which is expected as (i) our model does not have a reversal phase and (ii) HSC after cessation of injury are expected to be lowly activated similar to the cyHSC status in our paper. Cluster 4, which was a mix cells from different models and not clearly defined in the Rosenthal paper, marked a small cluster that was positive for endothelial markers *Kdr* and *Aqpr1* in our data (which may be contaminating LSEC or HSC-LSEC doublets). Clusters 7 signature marked a small cluster of cells in our that expressed immune cell marker in our (which may be contaminating immune cells or HSC-immune cell doublets). Cluster 8 marked a small cluster of contaminating hepatocytes, positive for *Alb* and *Ttc36*. For all analyses in our paper, doublets and contaminants (as well as proliferating cells) were removed (but are shown in the figure above).

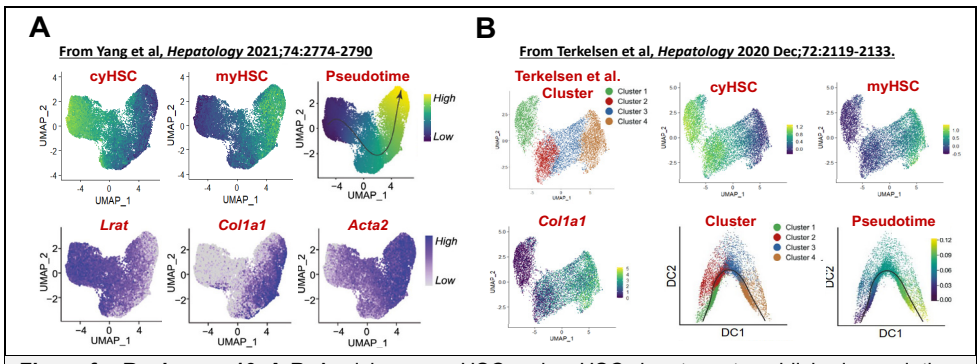


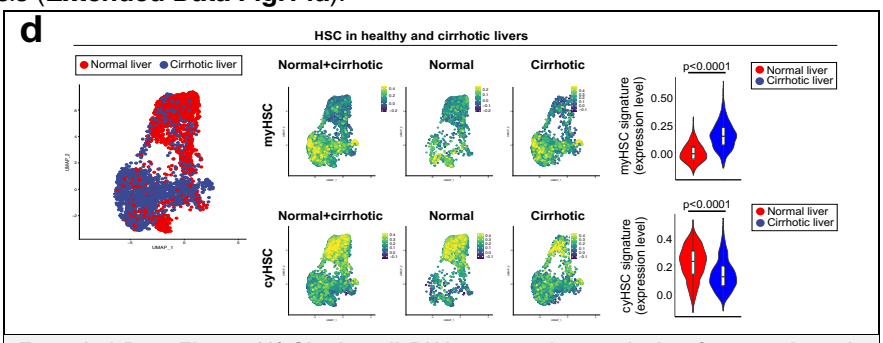
Figure for Reviewers 10. A-B. Applying our cyHSC and myHSC signatures to published pseudotime data from Yang et al (A) and Terkelsen (B) show trajectories from cyHSC to myHSC (see also Figure for Reviewers x for additional info on Terkelsen et al).

b - Heat map data in your Extended Materials suggest that both the more- and less-carcinogenic HSC populations share expression of many classical myofibroblast markers. This raises the possibility that HSCs are undergoing state transitions, an issue that could be examined with RNA velocity and/or other transition-type analyses. Another approach might be to assess if the anti-carcinogenic HSC subpopulation is enriched with "reverted" myofibroblastic HSCs that you and other groups have written about in previous publications.

We used two approaches to investigate cyHSC and myHSC trajectories: (i) Analysis of already published scRNA-seq data with pseudotime and (ii) analysis of our own data. By both approaches, we found that cyHSC are derived from myHSC. Given that cyHSC represent the more quiescent and myHSC the more activated cell types, this is consistent with the well-established principle that activated HSC are derived from quiescent HSC. For reverted HSC, we do not see a clear reverted HSC population as all employed models are progressive and do not include fibrosis resolution, please see also comments above and **Figure for Reviewers 6**.

- For the first approach, we used data from Yang *et al* (*Hepatology* 2021; 74:2774-2790) and Terkelsen *et al* (Hepatology. 2020;72(6):2119-2133) and applied cyHSC and myHSC signatures to their published pseudotime analysis as well displayed quiescence (*Lrat* mRNA) and activation (*Col1a1* and *Acta2* mRNA) marker genes. Not only did cyHSC and myHSC match quiescence and activation markers, respectively, but the pseudotime shows a trajectory from cyHSC to myHSC in both datasets (**Figure for Reviewers 8A-B**).
- Similar findings were made in our own data in the TAZ+FPC and Mdr2^{KO} models, showing again trajectories from cyHSC to mHSC in our pseudotime analysis (**Extended Data Fig.14a**).

5 - The idea of using HSC signatures generated in the mouse models to interrogate the pathogenesis of liver cancer in humans is appealing. Based on the mouse data, it would seem that the pro-carcinogenic HSC population should increase and the anti-carcinogenic population should decrease in cirrhotic versus non-cirrhotic liver. However, this hypothesis cannot be tested with the UMAP data presented because data sets from the two normal livers and two cirrhotic human livers were pooled. What are the results when you segregate the normal from the cirrhotic data sets?



Extended Data Figure 12| Single cell RNA-sequencing analysis of mouse hepatic stellate cells in fibrotic livers. d, Visualization and quantification of myHSC and cyHSC populations in HSC from healthy (n=4) and cirrhotic (n=4) human livers by snRNAseq.

We increased the number of samples to n=4 normal and n=4 cirrhotic and segregated as suggested. The data show a clear increase in the myHSC signature and decrease in the cyHSC signature in cirrhosis vs healthy (**Extended data Fig.12d).**

6 - Finally, in my view, the experiments about Yap/Taz in hepatocytes distract from the HSC focus of the work. Further, some of the conclusions about the relative significance of Yap/Taz in the carcinogenic process are also difficult to justify based on the data presented. For example,

a - the text seems to suggest that Yap activation is important for the fibrogenic/pro-carcinogenic phenotype (the role of Taz is not addressed in HSCs) but that Taz is a more important procarcinogenic factor than Yap in hepatocytes. However, the models used to compare the two factors were not equivalent (the one showing the significance of Taz was more extreme than the one that was used to assess Yap) and there is abundant literature showing that activating Yap in hepatocytes promotes liver cancer.

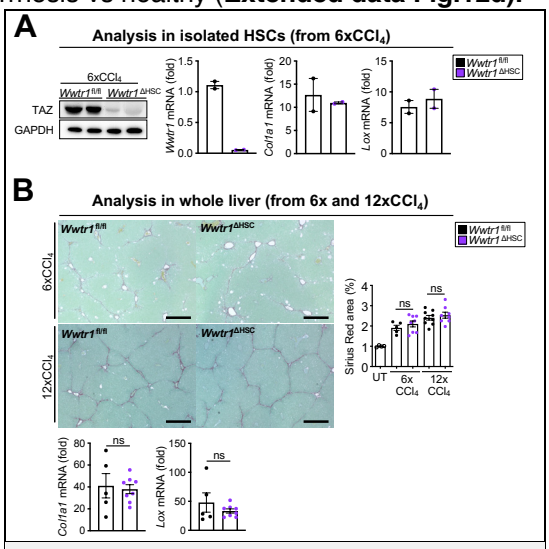


Figure for Reviewers 20. Effect of TAZ deletion of HSC activation and liver fibrosis. A. HSC were isolated from 6xCCl$_4$-treated LratCre^{pos} *Wwtr1*$^{\Delta HSC}$ or LratCre^{neg} *Wwtr1*$^{fl/fl}$ mice and expression of *Wwtr1* (encoding for TAZ), *Col1a1* and *Lox* were determined by qPCR (n=2 per group). **B.** *Wwtr1*$^{\Delta HSC}$ and *Wwtr1*$^{fl/fl}$ mice were subjected to 6xCCl$_4$ (n=5-8 per group) or 12xCCl$_4$ (n=8-9 per group) treatment and liver fibrosis was determined by Sirius red staining and analysis for fibrogenic genes *Col1a1* and *Lox*.

- We did investigate the role of TAZ in HSC but did not include this into the manuscript as LratCre-mediated TAZ deletion in HSC did not affect HSC activation. In this regard, we observed efficient deletion of TAZ but no effect on the expression of profibrogenic gene in HSC isolated from 6xCCl$_4$-treated mice (**Figure for Reviewers 20A**). Likewise, we did not observe effects on histological fibrosis, assessed by the Sirius Red-positive area, in 6xCCl$_4$ and 12xCCl$_4$-treated mice as well as no effect on fibrogenic gene expression in the liver (**Figure for Reviewers 20B**).
- To specifically address the reviewer's comment about the literature providing evidence for YAP promoting liver cancer and using the same conditions to evaluate the role of YAP and TAZ, we now include genetic deletion of either hepatocyte YAP or TAZ as main strategy to determine their role in HCC development. Deletion of TAZ but not YAP in hepatocytes strongly reduced DEN+CCl$_4$-induced HCC. (**Figure 4h** and ***Extended Data Fig.22a,b*** in the manuscript).

b - the rationale for these experiments that introduced constitutively active Yap/Taz mutants into hepatocytes is not explained since the primary assumption being investigated is whether or not HSCs regulate pro (or anti) carcinogenic signaling in hepatocytes.

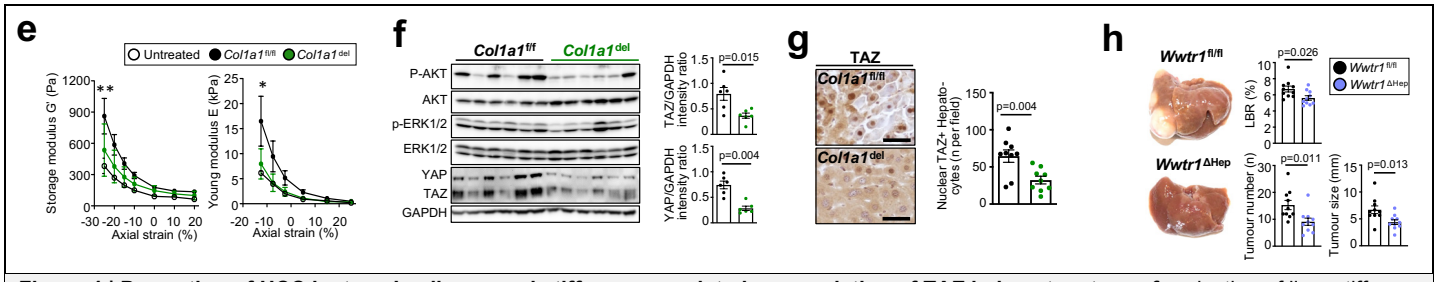


Figure 4 | Promotion of HCC by type I collagen and stiffness-associated accumulation of TAZ in hepatocytes. e-f, reduction of liver stiffness, determined by rheometry (control: n=1; DEN+19xCCl4: n=4/group) (e), and of phospho-AKT, phospho-ERK, YAP and TAZ in non-tumour liver from *Col1a1*^{del} mice and *Col1a1*$^{f/f}$ littermates (n=6/group) as determined by western blot at a time point preceding HCC development in the DEN+19xCCl_4 injections profound fibrosis model (**f**). **g**, TAZ nuclear translocation, revealed by IHC in non-tumour tissue of *Col1a1*^{del} mice and *Col1a1*$^{f/f}$ littermates (n=9 each) in the DEN+19xCCl_4 profound fibrosis model. **h**, mice with hepatocyte-specific TAZ deletion (*Wwtr1*$^{\Delta Hep}$, n=10) showed reduced HCC development compared to *Wwtr1*$^{fl/fl}$ controls (n=10) in the DEN+44xCCl_4 HCC model.

We have changed the experimental design. Rather than introducing constitutively active YAP and TAZ, we now delete YAP and TAZ selectively in hepatocytes – <u>to functionally study the effect of inhibiting the procarcinogenic signaling that may be upregulated by HSC</u>. This is based on our findings that liver stiffness and hepatocyte TAZ were increased in our profound fibrosis model, and that both were strongly reduced in mice with deleted Col1a1 (**Fig.4e-g**), which is consistent with the the known role of stiffness in the activation of the YAP/TAZ pathway. Thus, our hepatocyte TAZ deletion experiments, showing a significant reduction of HCC (**<u>Fig.4h)</u>**, now link HSC-produced type I collagen and stiffness to procarcinogenic hepatocyte mechanosignaling.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have extensively addressed the reviewer comments and the work represents a massive effort with a large amount of informative data.

Comments:

1. The effect of collagen 1 and DDR1 on immune cell infiltration aligns with a recent paper in Nature that should be cited (Sun et al, https://doi.org/10.1038/s41586-021-04057-2)

2. The findings still do not clearly demonstrate that HSCs have direct tumor promoting effects rather than the procedure of HSC depletion having a tumor promoting effect. In their new data, they removed all HSCs with early stage depletion and observed no HSC repopulation subsequently in their model. The complete removal of HSCs (Extended Fig 16b) did not seem to affect tumor formation at all (tumor number and tumor size are identical compared to non-depleted mice Fig 1f). The most straight forward explanation is that HSCs are not having any tumor modulatory effect and that the effect of late depletion may be due to the procedure itself, (e.g. HSC death leading to recruitment of anti-tumorigenic immune cells) and/or an effect of collagen I.

Referee #2 (Remarks to the Author):

The authors have now answered all my questions completely and have performed a tremendous amount of beautiful work. This manuscript is now very strong, innovative and will certainly have a big impact in the field.

Referee #3 (Remarks to the Author):

You have done an enormous amount of work to address substantive concerns raised by the three initial reviewers. As a result your revised manuscript includes a vast amount of information that will be of great interest to liver researchers.
In addition the rationale for the studies has been changed and no longer focuses on clarifying the role of hepatic stellate cell (HSC) senescence in hepatocarcinogenesis. Your rationale for that decision is based on new experimental evidence that HSC-specific deletion of either p53 or RelA do not change tumorigenesis in your models. However, it is not clear from the data you show whether either strategy actually reduced HSC senescence or suppressed the senescence-associated secretome. Further, you have not attempted to reconcile your findings with discrepant reports about this issue. That said, the lingering questions about the significance of HSC senescence are not the main reason for my hesitancy about your revised submission.

I struggled with your re-submission because your conclusions derive from multiple models of liver fibrosis that varied with regards to the type(s) hepatic stellate cell populations (HSC) that were manipulated, when during the disease trajectory the various HSC types were targeted, and when effects on HSC and liver phenotypes were assessed. Your data are vast and solid but, in my view, many of your conclusions are much more speculative.

My uncertainty stems from the fact that the complex mechanisms driving liver fibrosis and hepatocarcinogenesis are dynamic and the main targeting approach that you used in most of these experiments (Lrat-Cre-mediated deletion of different HSC genes) constitutively deletes floxed alleles from both cyHSC and myHSCs (because Lrat is expressed in both cell states and the two states appear to be inter-related). Hence, efforts to overcome this using Lrat-Cre driven induction of diptheria toxin receptor (DTR) expression do not resolve this concern because that approach will reduce both cy-HSC and my-HSC following diptheria toxin administration. The asma-TK model is a more specific approach to delete myHSC but heatmap data presented in your supplementary materials demonstrate that Yap-depleted HSC (which you argue are less myHSC-like and more cy-HSC-like) express asma (albeit at lower levels than wild type HSC).

Given this, I'm still not sure which HSC phenotype (cyHSC or myHSC) drove the observed biological changes in nontumor liver and/or the HCCs in the mouse models or the human livers. The revised submission does a better job explaining that these two HSC phenotypes are reciprocally regulated (i.e., cyHSC tend to decrease when myHSC increase and vice versa) and trajectory analysis suggests that they are different states of the same HSC subpopulation. You indicate that cyHSC transition to become myHSC, but what is the evidence that the approaches used can exclude the alternative (i.e., that myHSC can become cyHSC)?

In any case, your data do support the concept that certain nontumor/tumor phenotypes emerge when the myHSC state is predominant and that different liver phenotypes associate with cyHSC-predominant states. Thus, the data support your general "take home message" that pathology results when there is imbalance within the HSC population. This realization per se is not particularly novel, however. It has long been known that conditions that lead to predominant accumulation of myofibroblastic HSCs promote both liver fibrosis and hepatocellular carcinoma. Previous reviewers requested re-analysis of your initial data to incorporate findings from single cell/single nuclei RNA-seq data sets that have been reported by other groups in order to clarify the mechanisms driving state transitions within the HSC population. Such insight would be novel and very important. What novel information did you uncover regarding mechanisms that determine HSC state?

Previous reviewers also requested more mechanistic data to clarify how cyHSC or myHSC regulate hepatocarcinogenesis. Evidence is presented that Hgf (which is highly expressed in cyHSC) protects from HCC. You extrapolate from this definitive finding to conclude that loss of cyHSC promotes HCC by decreasing HGF which in turn, decreases hepatocyte viability and thereby, triggers regenerative responses that are pro-carcinogenic. However, you did not offer an explanation for this paradox (i.e., how loss of a key paracrine hepatocyte growth factor increased proliferation of remaining hepatocytes and triggered their malignant transformation) and data regarding how deleting Hgf in HSC impacted HSCs themselves and/or other cell types involved in liver repair/regeneration were not presented.

Conclusions regarding the role of col1a1 in mediating the pro-carcinogenic actions of myHSCs may also be somewhat premature. For example, it was difficult for me to follow the logic regarding col1a1, liver stiffness and Yap/Taz induction in hepatocytes as a driving paracrine force for myHSC-related HCC. Some of the text in that section of the manuscript (which indicates that col1a1 deletion in HSCs increases hepatocyte Taz) does not seem to match data that you show in the figures and discuss in other parts of the text (i.e., that cola1a deletion reduces liver stiffness and inhibits hepatocyte Taz). Perhaps this was a typo?

If/how cola1a deletion impacts Yap/Taz in HSC themselves is also confusing based on the info presented. Given that matrix stiffness regulates HSC state transitions and the mechanism is thought to involve Yap/Taz, wouldn't loss of col1a1 in HSC be predicted to deplete myHSC (and thus, relatively enrich for cyHSC)? However, you state that cola1a depletion in HSC had no effect on the HSC themselves despite decreasing matrix stiffness significantly. Also how do you explain the lack of effect on tumorigenesis when Yap is depleted in HSC given that Yap is a major inducer of the fibrogenic myHSC state and liver fibrosis increases liver stiffness and liver stiffness increases hepatocarcinogenesis? When Yap is depleted in HSC did cyHSC and/or hgf increase?

In my view, the most exciting aspect of your work is the identification of signatures for cyHSC and myHSC because this information has immediate translational implications for biomarker development, as well as the potential to unravel fundamental mechanisms that control HSC state transitions and thus, may be targeted therapeutically. I suggest deferring speculation about these mechanisms until more research has capitalized upon these discoveries.

Author Rebuttals to First Revision:

We would like to thank the reviewers for their positive comments. We have addressed each comment in below point-by-point response.

Reviewer #1

1. The effect of collagen 1 and DDR1 on immune cell infiltration aligns with a recent paper in Nature that should be cited (Sun et al, https://doi.org/10.1038/s41586-021-04057-2).

We completely agree and have cited this paper (reference 39 in the revised manuscript).

2. The findings still do not clearly demonstrate that HSCs have direct tumor promoting effects rather than the procedure of HSC depletion having a tumor promoting effect. In their new data, they removed all HSCs with early stage depletion and observed no HSC repopulation subsequently in their model. The complete removal of HSCs (Extended Fig 16b) did not seem to affect tumor formation at all (tumor number and tumor size are identical compared to non-depleted mice Fig 1f). The most straight forward explanation is that HSCs are not having any tumor modulatory effect and that the effect of late depletion may be due to the procedure itself, (e.g. HSC death leading to recruitment of anti-tumorigenic immune cells) and/or an effect of collagen I.

- Because of potential side effects and confounding effects of HSC manipulations, we have addressed the role of HSC by multiple approaches. Besides depletion, we have also inhibited HSC activation (by *Yap* and by *Pdgfrb* deletion) and activated HSC (by Lhx2 deletion) and consistently see an overall tumor-promoting effect of HSC. Hence, we politely disagree with the proposed interpretation of the reviewer as these multiple approaches consistently show an overall tumor-promoting effect of HSC.
- We are aware of potential side effects of depletion, such as immunomodulation (although our FACS data did not show this) and have further emphasized potential confounders in our manuscript to address this reviewer's concern.
- The effect of late depletion on collagen I, proposed by the reviewer, would be in support of tumor-promoting effects at late stages (as collagen I accumulates at these stages and promotes HCC).
- Unfortunately, we cannot selectively deplete HSC at early tumor stages as they fail to completely repopulate. We have therefore investigated the role of HSC at early and late stages also by Lhx2 deletion-mediated HSC activation and, consistent with our depletion data, see effects of HSC at late stages. We therefore think that the most straight forward explanation of these multiple approaches and in conjunction with the protective effects of HGF at early stages is that HSC are overall tumor-promoting, but that there are stage-specific effects, i.e. protection by HSC at early stages and tumor-promotion at later stages. The fact that the "complete removal of HSC" (i.e. removal at early and late stages) exerted no net effect would be a logical result of these distinct functions at different stages.
- Moreover, the proposed effect of HSC depletion via recruitment of anti-tumorigenic immune cells by HSC depletion should in fact have a strong impact on early stages as early-stage immune

surveillance of pre-malignant is highly relevant in DEN-induced HCC as shown by the Zender group (Kang et al, *Nature*. 2011 Nov 9;479:547-51.).

Reviewer #2

We would like to thank the reviewer for the positive comments.

Reviewer #3

You have done an enormous amount of work to address substantive concerns raised by the three initial reviewers. As a result your revised manuscript includes a vast amount of information that will be of great interest to liver researchers. In addition the rationale for the studies has been changed and no longer focuses on clarifying the role of hepatic stellate cell (HSC) senescence in hepatocarcinogenesis. Your rationale for that decision is based on new experimental evidence that HSC-specific deletion of either p53 or RelA do not change tumorigenesis in your models. However, it is not clear from the data you show whether either strategy actually reduced HSC senescence or suppressed the senescence-associated secretome. Further, you have not attempted to reconcile your findings with discrepant reports about this issue. That said, the lingering questions about the significance of HSC senescence are not the main reason for my hesitancy about your revised submission.

- We would like to thank the reviewer for the comments. In regards to HSC senescence, we did not further follow up on effects of p53 and *RelA* deletion within HSC as results were negative despite highly efficient deletion and as these are well-known regulators of senescence and SASP. Our manuscript tried to uncover the role of HSC in hepatocarcinogenesis rather than studying the role of HSC senescence – hence we did not focus too much on negative results. We could remove these data but believe that the p53 knockout data are valuable and we also demonstrated that senescence in HSC was less than in other cell types – detecting a decrease in the small amount of senescence we saw in vivo would be difficult.

I struggled with your re-submission because your conclusions derive from multiple models of liver fibrosis that varied with regards to the type(s) hepatic stellate cell populations (HSC) that were manipulated, when during the disease trajectory the various HSC types were targeted, and when effects on HSC and liver phenotypes were assessed. Your data are vast and solid but, in my view, many of your conclusions are much more speculative. Your conclusions derive from multiple models of liver fibrosis that varied with regards to the type(s) hepatic stellate cell populations (HSC) that were

manipulated, when during the disease trajectory the various HSC types were targeted, and when effects on HSC and liver phenotypes were assessed.

- We consider the use of multiple models and approaches (two depletion approaches, HSC activation, HSC inhibition, HSC-specific knockout of key mediators; DEN+CCl_4, NASH and $Mdr2^{ko}$ models) a strength as most results were consistent in these complementary approaches and/or in different models. We have pointed out limitations in our revision to address the reviewers' concerns (see below).

My uncertainty stems from the fact that the complex mechanisms driving liver fibrosis and hepatocarcinogenesis are dynamic and the main targeting approach that you used in most of these experiments (Lrat-Cre-mediated deletion of different HSC genes) constitutively deletes floxed alleles from both cyHSC and myHSCs (because Lrat is expressed in both cell states and the two states appear to be inter-related). Hence, efforts to overcome this using Lrat-Cre driven induction of diptheria toxin receptor (DTR) expression do not resolve this concern because that approach will reduce both cy-HSC and my-HSC following diptheria toxin administration. The asma-TK model is a more specific approach to delete myHSC but heatmap data presented in your supplementary materials demonstrate that Yap-depleted HSC (which you argue are less myHSC-like and more cy-HSC-like) express asma (albeit at lower levels than wild type HSC). Given this, I'm still not sure which HSC phenotype (cyHSC or myHSC) drove the observed biological changes in nontumor liver and/or the HCCs in the mouse models or the human livers. The revised submission does a better job explaining that these two HSC phenotypes are reciprocally regulated (i.e., cyHSC tend to decrease when myHSC increase and vice versa) and trajectory analysis suggests that they are different states of the same HSC subpopulation.

- We would like to thank the reviewer for the positive comments on the reciprocal regulation of cyHSC and myHSC. We agree that some of our in vivo approaches are not perfect as LratCre affects genes in both cyHSC and myHSC. However, via LratCre we target myHSC-enriched genes (e.g. *Col1a1* and *Has2*) and cyHSC-enriched genes (e.g. Hgf) to investigate the function of these genes (our manuscript does not claim that we investigate the functions in one subopoulation only). Likewise, YAP deletion in HSC does not wipe out all myHSC – it just reduces them. These limitations are expected as we are working in complex *in vivo* systems, that unfortunately do not always allow specific targeting of subpopulations or complete genetic inhibition. Our manuscript also demonstrates that YAP deletion reduced myHSC signature activity and increased cyHSC signature activity and that Lhx2 deletion achieved the opposite. In summary, a large number of complementary approaches through which we shift the balance of HSC of the different subpopulations and subpopulation-enriched mediators support our interpretation on the role of HSC, the shift of subpopulations, and the role of subpopulations-enriched mediators.
- To address the reviewer's concerns, we have added further sentences on limitations to the discussion that address specifically these points.
- To address above concerns, we also ensured to state that cyHSC-enriched and myHSC-enriched genes affect hepatocarcinogenesis rather than stating that cyHSC affect HCC (our aSMA-TK approach is largely targeting myHSC, hence this is experimentally proven) and also do not state anywhere that deletion of genes such as *Hgf* or *Col1a1* in specific subpopulations mediates effects on HCC.

You indicate that cyHSC transition to become myHSC, but what is the evidence that the approaches used can exclude the alternative (i.e., that myHSC can become cyHSC)?

- The overall starting point is quiescence=cyHSC as this is the state in which virtually all HSCs are in the normal liver and the overall tendency is a net increase in myHSC as pointed out by the reviewer below.
- Our own work and that of others in principle have shown the possibility of myHSC to cyHSC transition in regression states. We think that myHSC can become cyHSC in diseases where there is undulation (like in viral and autoimmune disease, where there are often flares followed by remission). While we strongly believe that in progressive models studied here the main direction is cyHSC to myHSC, we acknowledge now in the manuscript that the myHSC and cyHSC states, based on our work and that by the Kisseleva and Brenner groups, are may be more dynamic than our single time point scRNA-seq analysis without formal tracing shows, and that some myHSC may return to the cyHSC state in some diseases of models such as CCl_4 , especially with injections at longer intervals. These points have been added to the discussion.

In any case, your data do support the concept that certain nontumor/tumor phenotypes emerge when the myHSC state is predominant and that different liver phenotypes associate with cyHSC-predominant states. Thus, the data support your general "take home message" that pathology results when there is imbalance within the HSC population. This realization per se is not particularly novel, however. It has long been known that conditions that lead to predominant accumulation of myofibroblastic HSCs promote both liver fibrosis and hepatocellular carcinoma.

- We completely agree that accumulation of myHSC is known and we have also removed the statement from the discussion that promotion of HCC by an HSC subpopulation is unexpected.
- However, the novelty of our paper is the demonstration of their functional effects and the responsible mediators. To date, the role of collagen type 1 in HCC has not been demonstrated *in vivo*. Moreover, its functions in HCC are quite distinct and often even opposite from other liver cancers (ICC, metastasis) or PDAC.
- To date, there is not a single paper showing a tumor-restricting function of HSC in HCC, shown here.

Previous reviewers requested re-analysis of your initial data to incorporate findings from single cell/single nuclei RNA-seq data sets that have been reported by other groups in order to clarify the mechanisms driving state transitions within the HSC population. Such insight would be novel and very important. What novel information did you uncover regarding mechanisms that determine HSC state?

- Our analysis of scRNA-seq data (requested by the reviewers) focused on subpopulations rather than state transitions. Using multiple datasets, we confirmed the populations found here in other datasets and also applied their subpopulation markers to our datasets, without major changes in interpretation.

- We agree that state transitions are fascinating and highly relevant. We are indeed analysing the transitions from cyHSC/quiescent HSC to myHSC/activated HSC in sc/snRNA-seq data in large murine and human datasets with the goal to uncover gene regulatory networks in HSC and key drivers of state transitions. This is a long-term effort that requires adaption of current analysis tools (adaptation of ARACNe and VIPER developed by the Califano lab – analyses using standard tools such as SCENIC are not sufficient) in combination with siRNA and CRISPR in vitro and in vivo screens. We anticipate to complete this work, confirm key regulators and identify novel regulators of state transition in the next 2 years, and compare these to the regulation of fibroblast activation in other organs. Even if this work was already completed, it would be impossible to integrate a large number of additional and highly complex figures and methodologies into the current manuscript (and in the context of HCC, genetic inhibition of state transition regulators would also be required).

Previous reviewers also requested more mechanistic data to clarify how cyHSC or myHSC regulate hepatocarcinogenesis. Evidence is presented that Hgf (which is highly expressed in cyHSC) protects from HCC. You extrapolate from this definitive finding to conclude that loss of cyHSC promotes HCC by decreasing HGF which in turn, decreases hepatocyte viability and thereby, triggers regenerative responses that are pro-carcinogenic. However, you did not offer an explanation for this paradox (i.e., how loss of a key paracrine hepatocyte growth factor increased proliferation of remaining hepatocytes and triggered their malignant transformation) <u>and data regarding how deleting Hgf in HSC impacted HSCs themselves and/or other cell types involved in liver repair/regeneration were not presented.</u>

- We agree that this is an important point that was not completely explained in our manuscript. We therefore have included on the role of HGF in hepatocyte regeneration. Our data clearly show that deletion of HSC-derived HGF does not affect hepatocyte proliferation after PHx and liver regeneration. This is consistent with the literature (e.g. studies by the Michalopoulos group) showing the activation of multiple pathways and a high degree of redundancy between these pathways (e.g. inhibition of both HGF/MET and EGFR needed to block liver regeneration). Thus, our data support the interpretation that HGF is very important for cytoprotection but only one of multiple mediators in regeneration and thus not essential for this process. This is now stated in the updated manuscript.
- Besides analyzing effects of HGF deletion on hepatocytes, we had already included effects of HGF on immune cells (**Extended Data Figure 12g**) through an extensive FACS analysis and also effects on HSC activation/fibrosis (**Extended Data Figure 12e-f**).

Conclusions regarding the role of col1a1 in mediating the pro-carcinogenic actions of myHSCs may also be somewhat premature. For example, it was difficult for me to follow the logic regarding col1a1, liver stiffness and Yap/Taz induction in hepatocytes as a driving paracrine force for myHSC-related HCC. Some of the text in that section of the manuscript (which indicates that col1a1 deletion in HSCs increases hepatocyte Taz) does not seem to match data that you show in the figures and discuss in other parts of the text (i.e., that cola1a deletion reduces liver stiffness and inhibits hepatocyte Taz). Perhaps this was a typo?

- We apologize for this mistake - we had wrongly states “revealing increased nuclear translocation of TAZ” (the figure showed a significant decrease of TAZ). We have corrected this error.

If/how cola1a deletion impacts Yap/Taz in HSC themselves is also confusing based on the info presented. Given that matrix stiffness regulates HSC state transitions and the mechanism is thought to involve Yap/Taz, wouldn't loss of col1a1 in HSC be predicted to deplete myHSC (and thus, relatively enrich for cyHSC)? However, you state that cola1a depletion in HSC had no effect on the HSC themselves despite decreasing matrix stiffness significantly.

- Our previous data indeed showed no differences of HSC activation in mice with Col1a1 deletion, i.e. with decreased stiffness, in the DEN+CCl_4 model (**Extended Data Figure 9b**).
- To address this point further, we have investigated the effect of Col1a1 deletion on HSC activation in HF-CDAA-induces fibrosis and Mdr2KO-induced fibrosis as two additional models. In both models, we do not see decreases of HSC activation when Col1a1 is deleted (**Extended Data Figure 9c,d**). As discussion in our previous point-by-point response, all data on HSC activation to date is derived from *in vitro* studies and the role of stiffness on HSC activation *in vivo* has not yet been determined to date. Our data suggest that stiffness induced by type I collagen is not essential for HSC activation. An alternative interpretation would be that collagen receptors on HSC (e.g. DDR1 or DDR2) provide negative feedback instructing HSC to produce less collagen. We are following up on this point.
- In summary, our data clearly show that the effect of Col1a1 deletion on HCC is not merely due to a reduction in HSC activation/state transition – which is consistent with our finding that deletion of collagen receptor DDR1 or TAZ in hepatocytes reduces HCC.

Also how do you explain the lack of effect on tumorigenesis when Yap is depleted in HSC given that Yap is a major inducer of the fibrogenic myHSC state and liver fibrosis increases liver stiffness and liver stiffness increases hepatocarcinogenesis? When Yap is depleted in HSC did cyHSC and/or hgf increase?

- When YAP is deleted in HSC, we do see a significant reduction of HCC (**Figure 1c,d**) – not “a lack of tumorigenesis”.
- As discussed above, deletion of YAP in HSC increased cyHSC signature activity (**Extended Data Fig.6c**)

In my view, the most exciting aspect of your work is the identification of signatures for cyHSC and myHSC because this information has immediate translational implications for biomarker development, as well as the potential to unravel fundamental mechanisms that control HSC state transitions and thus, may be targeted therapeutically. I suggest deferring speculation about these mechanisms until more research has capitalized upon these discoveries.

We would like to thank the reviewer for these positive comments. As already discussed above, we have modified our manuscript to address these concerns and also added sentences describing limitations to the discussion section.