Core Hippo pathway components act as a brake on Yap and Taz in the development and maintenance of the biliary network

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MS TITLE: Core Hippo pathway components act as a brake on Yap/Taz in the development and maintenance of the biliary network

AUTHORS: Zachary J. Brandt, Ashley E. Echert, Jonathan R. Bostrom, Paula N. North, and Brian A. Link

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and suggestions for improvements to your manuscript. In particular, they feel that although the genetic analyses are strong, you need to do some further characterisation of phenotypes to give confidence in the conclusions you draw from the experiments. Reviewer 1 makes a few additional requests, but I accept that these may be challenging to fully address if suitable tools /resources (such as a better reporter line) are not available within a reasonable time for revision. Assuming that you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions, please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Although the role of YAP signaling in the maintenance of biliary epithelial cells was recently reported in mice, its role in the extrahepatic biliary network and the gallbladder is not known, making this manuscript novel and significant.
Moreover, the finding that the morphology of the gallbladder and BECs is influenced by YAP signaling in hepatocytes is intriguing.

Comments for the author

This manuscript describes interesting defects in the gallbladder and hepatic biliary structure in sav1 mutants. Particularly, the finding that these defects were rescued by hepatocyte-specific expression of Sav1 is very intriguing. However, overall phenotypic analyses should be further improved to strongly support the conclusion of the manuscript. Furthermore, it should be investigated how the loss of Sav1 in hepatocytes results in the defects in the gallbladder and hepatic biliary structure. I have the following comments which may help to improve the current manuscript.

- It is not clear whether the defects in the gallbladder and hepatic biliary structure observed in sav1 mutants at 8 dpf are due to failed maintenance or impaired differentiation/maturation. To distinguish these two possibilities earlier stages, such as 5 dpf, should be analyzed. AnnexinA4 staining should be done at 4, 5 and 6 dpf in order to define when the morphology of biliary epithelial cells (BEC) become abnormal. In addition, the overall quality of the images showing the hepatic biliary structure can be improved with the Tg(Tp1:EGFP) or Tg(krt18:EGFP) line, which marks BECs in the liver.

- BODIPY C5 gallbladder fluorescence data in Fig. 1D show a severe defect in sav1 mutants: none of the mutant larvae (n = 12) exhibited normal gallbladder fluorescence. However, BODIPY C5 gallbladder fluorescence data in Fig. 3M show that majority of sav1 mutants (sav1-/-; yap1+/+) exhibited normal gallbladder fluorescence (5/8 larvae are normal). Due to this inconsistency, conclusions drawn from data in Fig. 3M are somewhat questionable.

- As PP2A inhibitor treatment reduced the level of ctgfa:d2GFP fluorescence in sav1 mutant livers to the level detected in wild-type livers, did the treatment also rescue the defects observed in sav1 mutants?

- Mechanism(s) by which induced Yap activity in hepatocytes due to the loss of Sav1 leads to the gallbladder and biliary defects should be investigated.

- Rescue data in Fig. 4 suggest that Yap activity is induced or enhanced in hepatocytes of sav1 mutant livers and that this induced Yap activity eventually impairs the gallbladder and biliary morphology in sav1 mutants. However, it is not shown that YAP activity (ctgfa:d2GFP expression) was indeed detected in hepatocytes of sav1 mutant livers.

- In mice, YAP activity is detected in a subset of BECs and is required for their maintenance (Pepe-Mooney BJ, 2019, Cell Stem Cell). To reveal this YAP activity in mouse BECs, the BAC-transgenic Cyr61:GFP line was used as a YAP reporter line. In contrast, in this manuscript, the ctgfa:d2GFP line was used as a YAP reporter line and revealed YAP activity in hepatic stellate cells, but not in BECs. This discrepancy between mice and zebrafish questions the zebrafish ctgfa:d2GFP line as a YAP reporter line for the liver. A better reliable YAP reporter line should be used to accurately define cell types exhibiting active YAP signaling in the liver.

- The authors concluded a cell-autonomous role of Hippo signaling in the extrahepatic biliary network, based on the observation that lfabp:GFP-2a-Sav1;sav1-/- adults eventually lost the gallbladder. However, this observation does not support such a cell-autonomous role. Instead, it suggests that Sav1 expression in non-hepatocytes is also required to maintain the gallbladder in sav1 mutant adults. Although these non-hepatocytes can be biliary cells in the gallbladder, the presented data do not show that the non-hepatocytes are indeed the biliary cells.

- Please point with arrows the presumptive AnnexinA4- gallbladder in the images of Fig. 2A-C.

- There is a typo in line 369: Fig. 6A should be Fig. 7A.

Reviewer 2

Advance summary and potential significance to field

Main findings. The authors investigate the role of Hippo signaling in liver development using the Zebrafish. They generated null mutants for Sav1, the ortholog of WW45, and found that Sav1 is required for normal morphology of intrahepatic bile ducts and for morphogenesis of the gallbladder. The function of Sav1 depended on its effector Yap (not Taz). Importantly, the gallbladder and intrahepatic duct anomalies in Sav1-/- mutants resulted from a non-cell autonomous activity of Sav1 in hepatocytes. The authors further provide genetic and pharmacological evidence that Sav1 functions in this context by activating Stk3, the ortholog of Mst1/2, through inhibition of phosphatase2A. The authors also show that Sav1 functions in part through mechanisms that are distinct from the Hippo-Yap pathway, and that rescue of Sav1 in a Sav1-/- mutants eventually leads to extrahepatic biliary carcinoma.

Strenghts. The work is original and, to the reviewer's knowledge, is the first to identify a role of Hippo signaling in extrahepatic biliary development. It is also the first to propose a cross-talk between hepatocytes and developing gallbladder, and to support the existence of a Hippo-Yap pathway-independent role for Stk3 in liver development . Most conclusions are well supported by the data, the paper is clearly written, and the figures are of very good quality. The unraveling of the mode of action of Sav1/Stk3/PP2A is very interesting.

Weaknesses. There is no attempt to unravel the mechanism by which hepatocytes control extrahepatic development, and an additional control experiment is required to fully support that Sav1 activity in hepatocytes impacts extrahepatic development. The phenotypic characterization of the abnormal biliary cells in Sav1-/- needs to be improved. The context in which cholangiocarcinoma develops (rescue of Sav1 in Sav1-/- background) is not clearly relevant to mechanisms of cholangiocarcinoma development in humans.

Comments for the author

MAJOR POINTS

#1. Fig. 1F-F'. Sav1-/- mutant liver show expanded staining of the biliary marker Annexin A4. Are the abnormal biliary cells in part fated to a hepatocyte phenotype; in other terms, do the biliary cells express some hepatocyte genes, like in many mouse or zebrafish mutants with biliary anomalies ? Do the hepatocytes express some biliary markers ? Can the authors address these questions by labeling the hepatic cells with hepatocyte and biliary markers, including other biliary markers than AnnexinA4 ?

#2. Results, lines 186-191. It is inconsistent to select AnnexinA4 as a biliary marker based on the fact that earlier work revealed that it labels hepatopancreatic progenitors, and then conclude in the present paper that AnnexinA4 is a marker of more mature biliary cells. This inconsistency undermines the conclusion that the gallbladder defects in Sav1-/- mutants results from dedifferentiation toward a progenitor-like state. In line with the reviewer's comment #1, can the authors address the possibility that the cells do not revert to a progenitor state but instead acquire in part the characteristics of hepatocytes ? Alternatively, can the authors strengthen their conclusion by using another progenitor marker ?

#3. The authors' experiments in which an lfabp/eGFP-2a/Sav1 transgene rescues the gallbladder phenotype in Sav1-/- mutants suggest for the first time that hepatocytes modulate gallbladder development. Can the authors eliminate the possibility that the lfabp/eGFP-2a/Sav1 transgene is transiently active in Sav1-/- gallbladder at an earlier stage of gallbladder development than 8dpf and would then be able to launch the process of gallbladder development ?

#4. How do the authors envisage that hepatocytes control extrahepatic biliary development ? Can this be discussed ? The current discussion only alludes to hepatocyte-intrahepatic biliary interactions.

MINOR POINTS

Abstract. Despite that the authors provide new data on intrahepatic duct development, the overall conclusions of the abstract (lines 23-25) only refer to extrahepatic ducts and gallbladder. The abstract should also make more clear what implication was found for understanding biliary carcinoma. The reviewer suggests to revise the abstract to make it more consistent with the paper.

Introduction. Lines 31-32. Stating that the biliary tract functions to transport bile acids is an oversimplification. The introduction should not recapitulate our knowledge on biliary physiology, but there is much more in bile than bile acids.

Introduction. Lines 36-37. The references associated to the statement that PFIC type 2 is studied in Zebrafish are not appropriate; the 3 references (Cofer, Lorent 2x) deal with biliary atresia and Alagille syndrome.

Introduction. Lines 48-49. It would be fair to mention the work by Spence et al. (Developmental Cell 17, 62–74, July 21, 2009) on the progenitors of extrahepatic ducts in mammals.

Results, lines 143-148. The authors state that reduced gallbaldder fluorescence does not result from "impaired gallbladder formation" since gallbladder formation is normal at 5 dpf. This is confusive, the initiation of gallbladder development is seemingly normal, but further development is severely affected at 8 dpf. Please rephrase.

line 292. typogarphic error: hepataocytes -> hepatocytes

Reviewer 3

Advance summary and potential significance to field

In this manuscript, Brandt et al. show the role of core Hippo pathway components in biliary duct and gallbladder maintenance in zebrafish. Generation of multiple mutant lines and epistasis analysis show that Yap1 and not Taz mediates Sav1 hepatobiliary functions, while Taz likely mediates additional roles required for survival. Transgenic expression of Sav1 in hepatocytes rescues all liver phenotypes, demonstrating non-cell autonomous functions between hepatocytes and biliary cells. Chemical PP2A inhibition and mutant analysis further indicate that showed that Sav1 is mediated partly by Stk3, and possible an additional kinase. Sav1 controls proliferation, as well as overall hepatobiliary differentiation, which in the case of the gall bladder appears convert into an unidentified tissue structure with suggested similarity to transformed cholangiocarcinoma-like tissue.
Overall, the text is well written and the data are of high quality and generally well presented. This study clearly establishes a specific requirement for Yap1 and not Taz downstream of Sav1-mediated Hippo pathway functions in intra- and extrahepatic ducts, required for maintaining hepatobiliary tissue structure, including gall bladder maintenance. Although the molecular details and interaction of the Hippo components have been elegantly dissected by genetic studies , the actual phenotype is only superficially analysed. There are a number of points that need to be addressed to further improve the presented work.

Comments for the author

- The biliary system in sav1 -/- is suggested to be immature, based on the dramatic changes to the IHD network and increased proliferation. Co-expression analysis of hepatoblast, hepatocyte and biliary duct genes should be performed to clarify the differentiation status of hepatocytes and biliary cells in sav1 mutants and whether this includes bipotential progenitor like characteristics.

- TEM at 8dpf supposedly supports a more immature state as indicated by a change of nuclear to cytoplasmic ratio in sav1 -/- biliary cells. The images are suggestive, however need more support by quantification and comparison to actual progenitor stages. Progenitor gene expression may be more straight-forward, see above point.

- Figure2A-C: To clarify which cell types proliferate in sav1 -/-, single focal planes of co-expression should be provided. Are mutant livers bigger than controls?
- Cell death was excluded as cause for the reduction and potential loss of sav1 -/- gall bladders starting after 5dpf and documented at 8 dpf, based on TUNEL staining at at 8dpf, when the defect is clearly detected.
Moreover, Fig2 suggests increase of proliferation in sav1-/- gall bladders. This contradictory point needs to be clarified. Additional time points for TUNEL should be examined to address whether size change could be due to cell death prior to 8dpf.
-Finally, how does the noticeably increased proliferation around the gallbladder relate to the observed defect.

- sav1 mutants suggest a specific requirement in the IHD maintenance and gallbladder survival. Which tissues and cell types express sav1 before and after 5 dpf? Moreover, to address possible cell type specificity, it should be determined whether hepatocytes are morphologically normal in sav1 -/-. Gene expression of functional hepatocyte genes and immunohistochemistry of canaliculi markers would provide insights into this point.

- The majority of phenotypes seems to manifest only after 5 dpf and later. How is this explained? To address this, it should be shown when the different pathway components are expressed during development and in which tissues. Are wild type maternal gene products present and rescue earlier defects? Or, could the late onset be related to changes in metabolic functions of the liver?

- Non cell autonomous rescue of the sav1 -/- IHD and gall bladder phenotypes by Sav1 over-expression in hepatocytes is very surprising. How is this explained mechanistically? In particular, the gall bladder is separated from hepatocytes, does this suggest secreted signals traveling long distance? What kind of signals?

-line 324-325: the cholangiocarcinoma-like tissue is suggested to be of biliary origin based solely on AnnexinA4 immunostaining. This statement needs to be adjusted or corroborated by lineage tracing.

Minor points:
- panels need better labeling of specific structures to make them accessible to non-experts. In particular larger overviews and H&E staninings.
- Line 174: what do ‘increased cell process diameters’ refer to? This needs to be clarified.
- Fig 3M and 8E– the results for gall bladder Bodipy FL C5 fluorescence in sav1 -/- gallbladders seem very different from those shown in Fig 1D. Please explain.
- Lines 211 – 214: the link between common progenitors and the changes within the hepatocyte population is unclear and needs elaboration.
- Title of figure4 implicates survival, although no cell death is reported, this needs to be clarified.

First revision

Author response to reviewers' comments

We thank all of the Reviewers and the Editor for their valuable insight and comments regarding this manuscript. In reflecting on the feed-back it was clear that our work would be enhanced by providing additional analysis of the phenotype of *sav1* mutants and addressing possible mechanisms underlying the non-cell autonomous signaling from hepatocytes to the biliary system. Below we respond to each critique and highlight the additions to our revised manuscript (bolded text). These revisions provided new Figures 2, 6, and 7, additional data to

Figures 5 and 9, as well as new supplemental data and edits to the manuscript text. Alterations to the revised manuscript are shown in red text. The new data include more detailed analysis of the timing of phenotype onset and progression, additional phenotyping of hepatocyte morphology and polarity, and characterization of hepatocyte-biliary junctions. We also provide discussion of possible mechanisms contributing to the non-cell autonomous signal from hepatocytes; however, we feel that to fully unravel the underlying mechanisms of this signaling is beyond the scope of the current study. We hope we have sufficiently addressed Reviewer questions and concerns regarding the publication of this work.

REVIEWER 1 ADVANCE SUMMARY AND POTENTIAL SIGNIFICANCE TO FIELD:

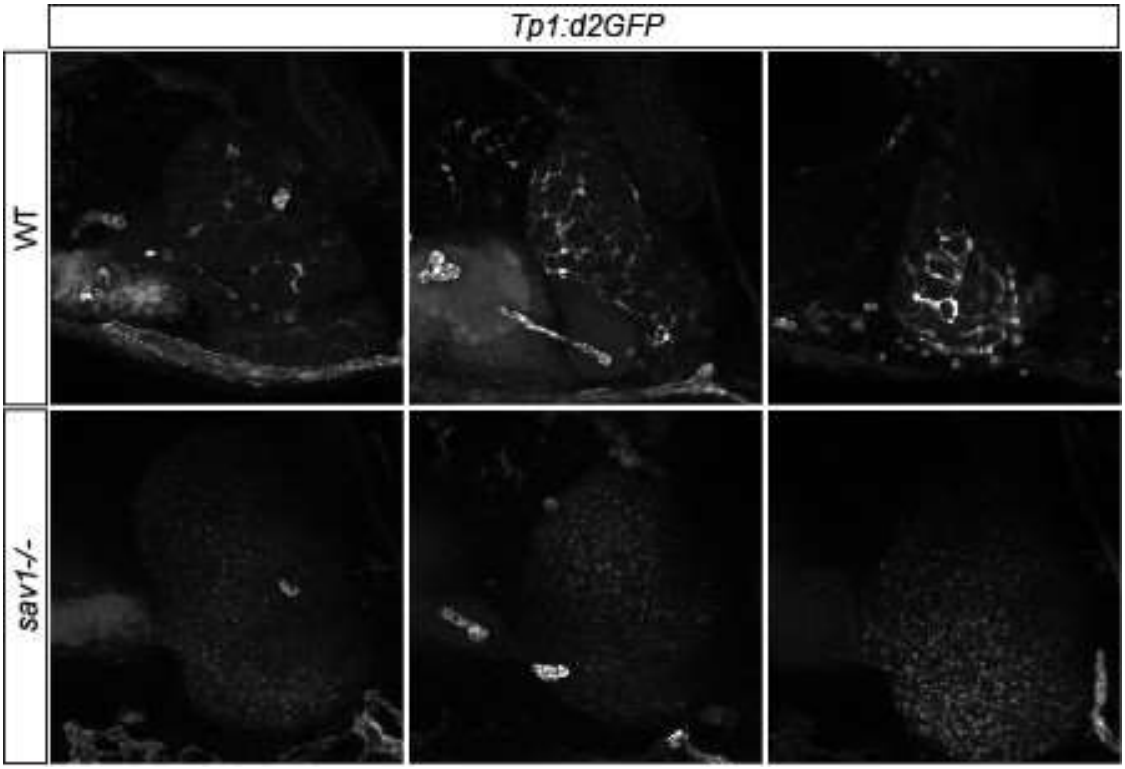
Although the role of YAP signaling in the maintenance of biliary epithelial cells was recently reported in mice, its role in the extrahepatic biliary network and the gallbladder is not known, making this manuscript novel and significant. Moreover, the finding that the morphology of the gallbladder and BECs is influenced by YAP signaling in hepatocytes is intriguing.

Reviewer 1 Comments for the Author:
This manuscript describes interesting defects in the gallbladder and hepatic biliary structure in sav1 mutants. Particularly, the finding that these defects were rescued by hepatocyte-specific expression of Sav1 is very intriguing. However, overall phenotypic analyses should be further improved to strongly support the conclusion of the manuscript. Furthermore, it should be investigated how the loss of Sav1 in hepatocytes results in the defects in the gallbladder and hepatic biliary structure. I have the following comments, which may help to improve the current manuscript.

- It is not clear whether the defects in the gallbladder and hepatic biliary structure observed in sav1 mutants at 8 dpf are due to failed maintenance or impaired differentiation/maturation. To distinguish these two possibilities, earlier stages, such as 5 dpf, should be analyzed. AnnexinA4 staining should be done at 4, 5 and 6 dpf in order to define when the morphology of biliary epithelial cells (BEC) become abnormal. In addition, the overall quality of the images showing the hepatic biliary structure can be improved with the Tg(Tp1:EGFP) or Tg(krt18:EGFP) line, which marks BECs in the liver.

In order to describe the timing of the phenotype more accurately, we included AnnexinA4 staining at 80hpf, 5dpf, 6dpf, 7dpf and categorized the severity of the biliary phenotype at each time point. In the initial manuscript we did not find differences at 5dpf based on gallbladder morphology viewed by brightfield microscopy and BODIPY C5 gallbladder fluorescence labeling. However, by carrying out the suggested earlier analyses using AnnexinA4, we were indeed able to see defects in *sav1* mutants as early at 5dpf. As previously described, though, when we examine later time points, we see an increase in the percentage of mutant larvae showing more severe phenotypes suggesting that the phenotypic spectrum at 8dpf does indeed represent the phenotype severity based on varying time of onset. However, our conclusion that initial gallbladder formation is unaffected in *sav1* mutants, is still supported by the lack of phenotype at 80hpf, shortly after the gallbladder is first identifiable. We have adjusted the manuscript to reflect this more detailed analysis. This additional data is shown in the new Figure 2.

To the point of utilizing Tg(*tp1:eGFP*) or Tg(*krt18:eGFP*), we have attempted live imaging with the *tp1:d2GFP* line. Initial results using this line suggested that *tp1:d2GFP* expression was lost in *sav1-/-* fish; however, WT siblings also showed what appeared to be somatic silencing of this transgene (see Reviewer Figure 1). With this potential silencing we did not feel confident in using this line to assess biliary structure.
Furthermore, Hippo signaling has been shown to interact with Notch signaling in several contexts, including the liver. Given that the *tp1* promoter is a Notch signaling reporter it would be interesting to use this or other Notch reporters in the future to assess Notch signaling in addition to biliary morphology, but interpretations as a biliary cell marker become complicated when Hippo signaling is manipulated. As for Tg(*krt18*:eGFP), we were unable to obtain this line.



Reviewer Figure 1. Top Row: Three examples of wild-type liver showing *tp1*:d2GFP fluorescence. Note the mosaic expression. Bottom Row: Three examples of *sav1*-/- livers showing *tp1*:d2GFP fluorescence.

- BODIPY C5 gallbladder fluorescence data in Fig. 1D show a severe defect in sav1 mutants: none of the mutant larvae (n = 12) exhibited normal gallbladder fluorescence. However, BODIPY C5 gallbladder fluorescence data in Fig. 3M show that majority of sav1 mutants (sav1-/-; yap1+/+) exhibited normal gallbladder fluorescence (5/8 larvae are normal). Due to this inconsistency, conclusions drawn from data in Fig. 3M are somewhat questionable.

The phenotypic onset in our mutants have a fair amount of variability, with some fish developing a phenotype as early as 5dpf while others initially appear normal at this stage. We find variability between individuals within a clutch, but also for the mean phenotype onset between clutches derived from different parents. The latter variability is likely due in part to genetic background differences introduced when combining multiple mutations. It appears that the *sav1*+/-; *yap1*+/- fish generated and in-crossed to obtain the data in Fig. 3M may have a background that causes a slightly less severe phenotype in the *sav1*-/- offspring as compared to the cohort used for Fig. 1D. Considering variability, we feel it is proper to restrict analysis to siblings in which phenotyping is first carried out and then the fish are genotyped. In accordance, for both datasets we followed this prescription and only compared mutant and wild-type siblings to control for background effects as much as possible. The goal of the experiment presented in Fig. 3M was to assess the consequences of *yap1* deletion on the *sav1* phenotype. We are confident in the conclusions drawn, particularly when considered together with the additional phenotype analysis including whole mount AnnexinA4/Edu staining and histopathology.

- As PP2A inhibitor treatment reduced the level of ctgfa:d2GFP fluorescence in sav1 mutant livers to the level detected in wild-type livers, did the treatment also rescue the defects observed in sav1 mutants?

To better characterize *sav1* mutants treated with PP2A inhibitor we repeated the experiment and assessed gallbladder morphology as well as reporter fluorescence. We found that in PBS treated controls 9/18 *sav1*-/- fish had lost normal gallbladder morphologyn (some showing no recognizable gallbladder), while in LB100 treated fish only 2/19 *sav1*-/- fish had such phenotypes. This analysis suggests that the PP2A inhibitor treatment did indeed partially rescue not only *ctgfa* expression, but also gallbladder differentiation and maintenance. We have added this additional assessment to the manuscript.

- Mechanism(s) by which induced Yap activity in hepatocytes due to the loss of Sav1 leads to the gallbladder and biliary defects should be investigated.

While we agree that these mechanisms should be further investigated, we feel that fully elucidating the non-autonomous mechanisms is beyond of the scope of the current study. However, we have outlined a working hypothesis in the Discussion.

- Rescue data in Fig. 4 suggest that Yap activity is induced or enhanced in hepatocytes of sav1 mutant livers and that this induced Yap activity eventually impairs the gallbladder and biliary morphology in sav1 mutants. However, it is not shown that YAP activity (ctgfa:d2GFP expression) was indeed detected in hepatocytes of sav1 mutant livers.

Supplemental Figure 3 shows AnnexinA4 staining alongside the *ctgfa*:d2GFP reporter, in both WT and sav1 mutant larvae. While we have not been able to immunostain for a unique hepatocyte marker we note that the *ctgfa*:d2GFP reporter labels both the phenotypically abnormal biliary epithelial cells as well as much of the surrounding tissue, including hepatocytes exhibiting increased Yap activity.

- In mice, YAP activity is detected in a subset of BECs and is required for their maintenance (Pepe-Mooney BJ, 2019, Cell Stem Cell). To reveal this YAP activity in mouse BECs, the BAC-transgenic Cyr61:GFP line was used as a YAP reporter line. In contrast, in this manuscript, the ctgfa:d2GFP line was used as a YAP reporter line and revealed YAP activity in hepatic stellate cells, but not in BECs. This discrepancy between mice and zebrafish questions the zebrafish ctgfa:d2GFP line as a YAP reporter line for the liver. A better, reliable YAP reporter line should be used to accurately define cell types exhibiting active YAP signaling in the liver.

While multiple reporter lines responsive to Yap transcriptional activity would certainly be interesting to evaluate, generating and then crossing such lines onto the *sav1* mutant background would be time prohibitive. We also note that while the *ctgfa*:d2GFP line reports high Yap activity in hepatic stellate cells, there is a subset of BECs co-labeled with AnnexinA4 that also show reporter activity. In addition, we see dramatic increases in *ctgfa*:d2GFP reporter activity in BECs of *sav1* mutants, suggesting that Yap activity is indeed significantly increased in these cells. The changes in expression of the *ctgfa*:d2GFP reporter with respect to the mutant gallbladder is also informative in the context of extrahepatic biliary phenotypes.

- The authors concluded a cell-autonomous role of Hippo signaling in the extrahepatic biliary network, based on the observation that lfabp:GFP-2a- Sav1;sav1-/- adults eventually lost the gallbladder. However, this observation does not support such a cell-autonomous role. Instead, it suggests that Sav1 expression in non-hepatocytes is also required to maintain the gallbladder in sav1 mutant adults. Although these non-hepatocytes can be biliary cells in the gallbladder, the presented data do not show that the non-hepatocytes are indeed the biliary cells.

Thank you for raising this issue, we acknowledge that our results do not prove a cell autonomous role for Hippo signaling in the extrahepatic biliary network. We have edited the manuscript to reflect this correction. We now suggest that there may be a cell autonomous role, but further experimentation is needed to clarify this point.

- Please point with arrows the presumptive AnnexinA4- gallbladder in the images of Fig. 2A-C.

We have added arrows indicating these structures.

- There is a typo in line 369: Fig. 6A should be Fig. 7A.

This typo has been corrected.

REVIEWER 2 ADVANCE SUMMARY AND POTENTIAL SIGNIFICANCE TO FIELD:

Main findings. The authors investigate the role of Hippo signaling in liver development using the Zebrafish. They generated null mutants for Sav1, the ortholog of WW45, and found that Sav1 is required for normal morphology of intrahepatic bile ducts and for morphogenesis of the gallbladder. The function of Sav1 depended on its effector Yap (not Taz). Importantly, the gallbladder and intrahepatic duct anomalies in Sav1-/- mutants resulted from a non-cell autonomous activity of Sav1 in hepatocytes. The authors further provide genetic and pharmacological evidence that Sav1 functions in this context by activating Stk3, the ortholog of Mst1/2, through inhibition of phosphatase2A. The authors also show that Sav1 functions in part through mechanisms that are distinct from the Hippo-Yap pathway, and that rescue of Sav1 in a Sav1-/- mutants eventually leads to extrahepatic biliary carcinoma.

Strengths. The work is original and, to the reviewer's knowledge, is the first to identify a role of Hippo signaling in extrahepatic biliary development. It is also the first to propose a cross-talk between hepatocytes and developing gallbladder, and to support the existence of a Hippo-Yap pathway-independent role for Stk3 in liver development. Most conclusions are well supported by the data, the paper is clearly written, and the figures are of very good quality. The unraveling of the mode of action of Sav1/Stk3/PP2A is very interesting.

Weaknesses. There is no attempt to unravel the mechanism by which hepatocytes control extrahepatic development, and an additional control experiment is required to fully support that Sav1 activity in hepatocytes impacts extrahepatic development. The phenotypic characterization of the abnormal biliary cells in Sav1-/- needs to be improved. The context in which cholangiocarcinoma develops (rescue of Sav1 in Sav1-/- background) is not clearly relevant to mechanisms of cholangiocarcinoma development in humans.

We concede that the context of cholangiocarcinoma development in a *sav1-/-* fish with WT *sav1* re-expressed in the hepatocyte population is unlikely directly translatable to human cholangiocarcinoma. However, this comment led us to wonder whether there may be clinical data supporting a role for Hippo signaling in extrahepatic cholangiocarcinoma. We addressed this using publicly available cBioportal human genomic data (www.cbioportal.org) and inserted the following text into the manuscript. We have also included a graphical summary of this data in Supplemental Figure 7, and a more detailed report of the data in Supplemental Table 1.

"To address this, we utilized genomic data from all liver and biliary tract cancers made available from cBioportal, a resource for cancer genomics. Using this database, we queried 15 studies and found that 151 of 1657 (~9%) patient samples had alterations in core Hippo pathway components. We queried all available samples for alterations in the following core Hippo pathway components: SAV1, STK3, STK4, LATS1, LATS2, MOB1A, MOB1B, YAP1, and WWTR1. Interestingly, while the number of samples available for extrahepatic biliary tract cancers is relatively small compared to liver cancers generally, we did find evidence of alteration in Hippo pathway components. While only 4 samples of perihilar cholangiocarcinoma were available, 2 of these had alterations in the pathway, with 1 containing a deep deletion in SAV1, and the other a mutation in STK3. 39 cases were identified as extrahepatic cholangiocarcinoma, and 2 of these samples had a Hippo pathway component altered with both being mutations in YAP1. Finally, 1 out of 134 cases of gallbladder carcinomas were altered, identifying a LATS1 amplification."

Certainly this sample size is too small to draw definitive conclusions; however, given our results and the knowledge that murine conditional *Alb:cre* knockouts of multiple Hippo pathway components can lead to both hepatocellular carcinoma and cholangiocarcinoma, it is reasonable to suggest that somatic Hippo pathway alterations in extrahepatic biliary cells could, and likely do, contribute to extrahepatic biliary tract carcinomas.

Reviewer 2 Comments for the Author:
MAJOR POINTS

#1. Fig. 1F-F'. Sav1-/- mutant liver show expanded staining of the biliary marker Annexin A4. Are the abnormal biliary cells in part fated to a hepatocyte phenotype; in other terms, do the biliary cells express some hepatocyte genes, like in many mouse or zebrafish mutants with biliary anomalies? Do the hepatocytes express some biliary markers? Can the authors address these questions by labeling the hepatic cells with hepatocyte and biliary markers, including other biliary markers than AnnexinA4?

Unfortunately, the commercial availability of hepatocyte markers for analysis in zebrafish is scarce. One commonly utilized antibody is the anti-goat HNF4a from Santa Cruz Biotechnolgy, which has since been discontinued. We were able to obtain a small aliquot of the legacy antibody from a colleague, but were unable to replicate the expected staining pattern reported in the literature, and thus have been unable to label and analyze hepatocytes by this marker. Another antibody used to mark hepatocytes in zebrafish, Bhmt, is not commercially available and we were unable to secure through requests.

#2. Results, lines 186-191. It is inconsistent to select AnnexinA4 as a biliary marker based on the fact that earlier work revealed that it labels hepatopancreatic progenitors, and then conclude in the present paper that AnnexinA4 is a marker of more mature biliary cells. This inconsistency undermines the conclusion that the gallbladder defects in Sav1-/- mutants results from dedifferentiation toward a progenitor-like state. In line with the reviewer's comment #1, can the authors address the possibility that the cells do not revert to a progenitor state but instead acquire in part the characteristics of hepatocytes? Alternatively, can the authors strengthen their conclusion by using another progenitor marker?

We attempted to use the progenitor marker Prox1 which has been reported to be present in hepatoblasts. However, using two independent antibodies against Prox1, both published as labeling hepatoblasts in early embryonic zebrafish, we have found that at 8 dpf Prox1 staining is expressed in both hepatocytes and intrahepatic biliary cells in WT larvae. In the context of the *sav1*-/- AnnexinA4 phenotype we did not see an increase in this progenitor marker, and in fact there was a slight loss of staining in the more severe mutants. In light of these new observations, we have altered our conclusion to no longer suggest a dedifferentiation to a more immature progenitor-like state, but rather a morphological change to a more dysplastic proliferative phenotype. We have included this result as Supplemental Fig. 2 in the revised manuscript. We also attempted to stain for another early marker of cholangiocytes (Sox9), which has been reported as one of the earliest markers of biliary differentiation. While this antibody has been published to work in the context of zebrafish retina, we were unable to see the predicted staining in our whole mount preparations of liver-gallbladder.

#3. The authors' experiments in which an lfabp/eGFP-2a/Sav1 transgene rescues the gallbladder phenotype in Sav1-/- mutants suggest for the first time that hepatocytes modulate gallbladder development. Can the authors eliminate the possibility that the lfabp/eGFP-2a/Sav1 transgene is transiently active in Sav1-/- gallbladder at an earlier stage of gallbladder development than 8dpf and would then be able to launch the process of gallbladder development?

To address this question we co-labeled transgenic *lfabp*:eGFP-2a-sav1 WT or *sav1* mutant fish with AnnexinA4 at 80hpf to mark the gallbladder and extrahepatic ducts. Staining revealed that the transgene is not transiently expressed at this early time point, when the gallbladder is first identifiable. We have added this observation to the manuscript, as additional data in Figure 5.

#4. How do the authors envisage that hepatocytes control extrahepatic biliary development? Can this be discussed? The current discussion only alludes to hepatocyte-intrahepatic biliary interactions.

We agree that our discussion should better highlight this interesting result and have altered the manuscript to better address this question. In particular we propose the hypothesis that bile acid transported from the hepatocytes to the extrahepatic ducts and gallbladder may carry important signals needed for continued maintenance.

MINOR POINTS

Abstract. Despite that the authors provide new data on intrahepatic duct development, the overall conclusions of the abstract (lines 23-25) only refer to extrahepatic ducts and gallbladder. The abstract should also make more clear what implication was found for understanding biliary carcinoma. The reviewer suggests to revise the abstract to make it more consistent with the paper.

We have revised our abstract to better summarize and reflect the content of the paper.

Introduction. Lines 31-32. Stating that the biliary tract functions to transport bile acids is an oversimplification. The introduction should not recapitulate our knowledge on biliary physiology, but there is much more in bile than bile acids.

Given one hypothesis for the non-cell autonomous effects we report, this is a particularly important point, and thus we have altered the text to more accurately discuss biliary tract and bile function.

Introduction. Lines 36-37. The references associated to the statement that PFIC type 2 is studied in Zebrafish are not appropriate; the 3 references (Cofer, Lorent 2x) deal with biliary atresia and Alagille syndrome.

There should have been 4 references here, with Ellis et. al. being the 4th reference associated with the PFIC type 2 statement. We have made sure this reference is included in the revised submission.

Introduction. Lines 48-49. It would be fair to mention the work by Spence et al. (Developmental Cell 17, 62–74, July 21, 2009) on the progenitors of extrahepatic ducts in mammals.

We thank the reviewer for pointing us to this paper we had missed and have revised our introduction to include it.

Results, lines 143-148. The authors state that reduced gallbladder fluorescence does not result from "impaired gallbladder formation" since gallbladder formation is normal at 5 dpf. This is confusive, the initiation of gallbladder development is seemingly normal, but further development is severely affected at 8 dpf. Please rephrase.

We have adjusted the text to address this issue. Our additional whole mount stains at multiple earlier time points should also help to clarify this point.

line 292. typographic error: hepataocytes -> hepatocytes

We've corrected this error.

REVIEWER 3 ADVANCE SUMMARY AND POTENTIAL SIGNIFICANCE TO FIELD:

In this manuscript, Brandt et al. show the role of core Hippo pathway components in biliary duct and gallbladder maintenance in zebrafish. Generation of multiple mutant lines and epistasis analysis show that Yap1 and not Taz mediates Sav1 hepatobiliary functions, while Taz likely mediates additional roles required for survival. Transgenic expression of Sav1 in hepatocytes rescues all liver phenotypes, demonstrating non-cell autonomous functions between hepatocytes and biliary cells. Chemical PP2A inhibition and mutant analysis, further indicate that showed that Sav1 is mediated partly by Stk3, and possible an additional kinase. Sav1 controls proliferation, as well as overall hepatobiliary differentiation, which in the case of the gall bladder appears convert into an unidentified tissue structure with suggested similarity to transformed cholangiocarcinoma-like tissue. Overall, the text is well written, and the data are of high quality and generally well presented. This study clearly establishes a specific requirement for Yap1 and not Taz downstream of Sav1-mediated Hippo pathway functions in intra- and extrahepatic ducts, required for maintaining hepatobiliary tissue structure, including gall bladder maintenance. Although the molecular details and interaction of the Hippo components have been elegantly dissected by

genetic studies, the actual phenotype is only superficially analyzed. There are a number of points that need to be addressed to further improve the presented work.

Reviewer 3 Comments for the Author:
- The biliary system in sav1 -/- is suggested to be immature, based on the dramatic changes to the IHD network and increased proliferation. Co-expression analysis of hepatoblast, hepatocyte and biliary duct genes should be performed to clarify the differentiation status of hepatocytes and biliary cells in sav1 mutants and whether this includes bipotential progenitor like characteristics.

This comments echoes Reviewer 2's points #1 and #2. Please see the response to those points.

- TEM at 8dpf supposedly supports a more immature state as indicated by a change of nuclear to cytoplasmic ratio in sav1 -/- biliary cells. The images are suggestive, however need more support by quantification and comparison to actual progenitor stages. Progenitor gene expression may be more straight-forward.

We have edited the manuscript to simply describe the morphology by TEM without suggesting they support a more progenitor state. See also our response to Reviewer 2 point #2.

- Figure2A-C: To clarify which cell types proliferate in sav1 -/-, single focal planes of co-expression should be provided. Are mutant livers bigger than controls?

We have added larger single focal plane panels of EdU and AnnexinA4 labeling to better show that both AnnexinA4+ and AnnexinA4- cells are proliferative, consistent with effects in both biliary cells and hepatocytes.

Mutant livers are indeed larger than controls. Text has been added to address this point.

- Cell death was excluded as cause for the reduction and potential loss of sav1 -/- gall bladders starting after 5dpf and documented at 8dpf, based on TUNEL staining at 8dpf, when the defect is clearly detected. Moreover, Fig2 suggests increase of proliferation in sav1-/- gall bladders. This contradictory point needs to be clarified. Additional time points for TUNEL should be examined to address whether size change could be due to cell death prior to 8dpf.

Through additional analysis, we conclude the gallbladder epithelial cells lose their normal epithelial identity and morphology, and instead become dysplastic and proliferative, which results in gallbladder reduction and eventual loss of normal markers. We have also included TUNEL staining at 5dpf, when the phenotype is first detectable, and show that there is no difference in this cell death marker at this time point as well.
We have added the 5dpf TUNEL analysis to Figure S1.

-Finally, how does the noticeably increased proliferation around the gallbladder relate to the observed defect.

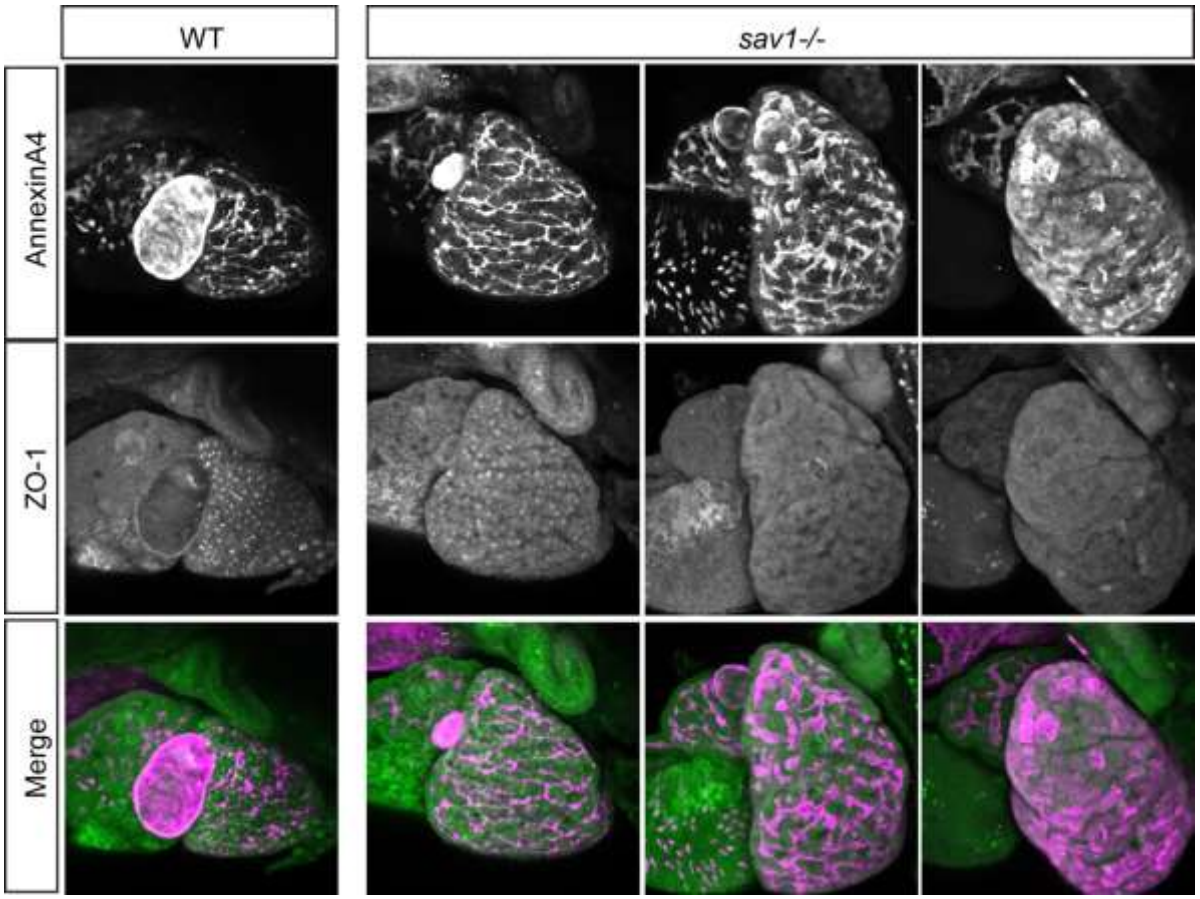
In line with the above comment we believe the normal gallbladder epithelium loses its normal identity and structure, and the increased proliferation consistent with increased Yap/Taz transcriptional activity results in dysplastic cells no longer recognizable as a gallbladder.

- sav1 mutants suggest a specific requirement in the IHD maintenance and gallbladder survival. Which tissues and cell types express sav1 before and after 5 dpf? Moreover, to address possible cell type specificity, it should be determined whether hepatocytes are morphologically normal in sav1 -/-. Gene expression of functional hepatocyte genes and immunohistochemistry of canaliculi markers would provide insights into this point.

We have included additional data to better address hepatocyte morphology. In particular we have added histology from Epon-embedded specimens and TEM data to address the hepatocyte morphology. We also carried out additional marker analysis for several cell junction and polarity proteins. Among these immunostains, aPKC marks the apical membrane of hepatocytes and thus the bile duct canaliculi. We note that these canaliculi are notably absent in *sav1*

mutants exhibiting abnormal biliary morphology, a finding that is corroborated by TEM analysis of canaliculi. aPKC staining is also increased around the majority of the hepatocyte borders, suggesting a possible expansion of the apical domain of hepatocytes and/or loss of proper polarity. Cdh2 or N- cadherin staining strongly marks the periphery of hepatocytes in WT fish but is proportionally lost in *sav1* mutants with respect to phenotype severity. We also see a marked increase in hepatocytes that lack peripheral Cdh2 and instead display an apparent collapse of the cadherin junctions. This data comprises the new Figure 7 in the revised manuscript.

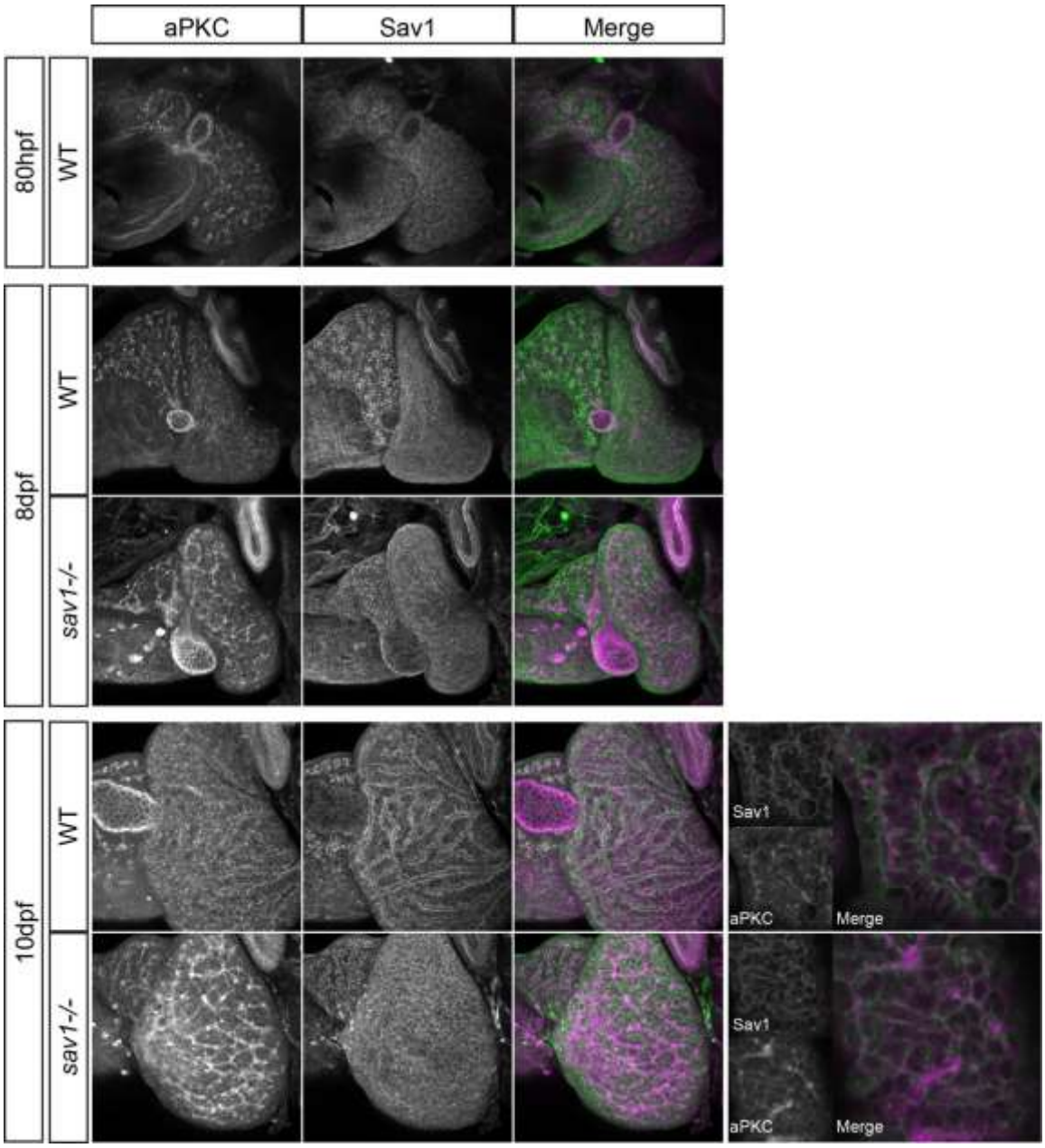
Finally, we attempted to stain with a ZO-1 antibody previously described to label bile canaliculi in zebrafish, presumably at the tight junctions present between hepatocytes and biliary cells. However, we were unable to see this staining pattern and instead noted peri-nuclear staining in WT larvae (Reviewer Figure 2). Interestingly this staining pattern is also lost as *sav1* mutant phenotypes increase in severity. Given the unexpected subcellular localization we have chosen not to include this in the manuscript. Cumulatively, however, these data illustrate morphological changes in hepatocytes that are concurrent with the changes in biliary morphology.



Reviewer Figure 2. Top Row: Wild-type and three examples of *sav1* mutant liver immunostained with AnnexinA4. Middle Row: Wild-type and three examples of *sav1* mutant liver immunostained with ZO-1. Bottom Row: Co-localization of Annexin (pink) and ZO-1 (green).

- The majority of phenotypes seems to manifest only after 5 dpf and later. How is this explained? To address this, it should be shown when the different pathway components are expressed during development and in which tissues. Are wild type maternal gene products present and rescue earlier defects? Or, could the late onset be related to changes in metabolic functions of the liver?

We now include 80hpf staining for the Hippo pathway effectors Yap and Taz. These stains show Taz to be enriched within the developing biliary system, with Yap staining low at this early time point. Yap staining is much more abundant at 8dpf, when we see robust phenotypic changes. This may suggest that the increase in Yap protein expression explains the phenotype, as *sav1*-/- mutants cannot properly mediate increased levels of Yap required at this time. This is consistent with the fact that additional deletion of *yap1* but not *taz* rescues the larval phenotypes seen in *sav1* mutants. We have included the new 80hpf data in Figure S5.
We have also performed whole mount staining with an antibody targeted against the C-terminal portion of human Sav1 protein. We see distinct and consistent staining patterns throughout the liver, pancreas and hepatopancreatic ductal system. However, this staining was not lost in *sav1*-/- fish at 80hpf or 8dpf. As the C-terminal portion of *sav1* contains the SARAH domain, which is shared with Stk3/4, as well as other proteins, the staining we observed may not be specific to *sav1* protein. Less likely, staining may represent Sav1 protein which is maternally provided. Because of the uncertainty of the Sav1 immunostaining, that data has not been included.



- Non cell autonomous rescue of the sav1 -/- IHD and gall bladder phenotypes by Sav1 over-expression in hepatocytes is very surprising. How is this explained mechanistically? In particular,

the gall bladder is separated from hepatocytes, does this suggest secreted signals traveling long distance? What kind of signals?

Elucidating the mechanisms behind this non-cell autonomous rescue is beyond the scope of this paper. We hypothesize that proper polarity, and secretion by hepatocytes is critical for maintaining the proper signaling in the biliary epithelial cells of the intrahepatic duct. Given the separation of the gall bladder from hepatocytes, a secreted signaling traveling long distance seems to be the most likely hypothesis. Given this hypothesis, a signal sent through the bile secretion seems most likely.

-line 324-325: the cholangiocarcinoma-like tissue is suggested to be of biliary origin based solely on AnnexinA4 immunostaining. This statement needs to be adjusted or corroborated by lineage tracing.

This statement was also made based on the fact that we are unable to identify gallbladder in sav1-/- rescued fish and the only obvious areas with this cholangiocarcinoma-like phenotype are always in the normal anatomical position of the gallbladder. We understand though that these observations do not prove that the cells are of biliary origin, and thus have adjusted our statement accordingly.

Minor points:
- panels need better labeling of specific structures to make them accessible to non-experts. In particular larger overviews and H&E staining

We have adjusted the labeling of figures accordingly and have also included cartoon depictions of large overviews when these data are first presented in order to help acclimate non-experts to the anatomy.

- Line 174: what do 'increased cell process diameters' refer to? This needs to be clarified.

We are referring more simply to the increased diameters of the intrahepatic biliary ducts and biliary cells as a whole and have adjusted this statement to clarify this.

- Fig 3M and 8E– the results for gall bladder Bodipy FL C5 fluorescence in sav1 -/- gallbladders seem very different from those shown in Fig 1D. Please explain.

See above response to Reviewer 1 on this same point.

- Lines 211 – 214: the link between common progenitors and the changes within the hepatocyte population is unclear and needs elaboration.

We have adjusted the text to further elaborate on this point.

- Title of figure4 implicates survival, although no cell death is reported, this needs to be clarified.

The survival is in reference to the survival of the larvae, not the cells. We have adjusted the title of this figure to clarify.

Second decision letter

MS ID#: DEVELOP/2019/184242

MS TITLE: Core Hippo pathway components act as a brake on Yap/Taz in the development and maintenance of the biliary network

AUTHORS: Zachary J. Brandt, Ashley E. Echert, Jonathan R. Bostrom, Paula N. North, and Brian A. Link

I hope that you are well and are coping OK in these extraordinary times.

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Most of the reviewer suggestions can be addressed without further experimentation although referee 3 did suggest a few further experiments. If you do not already have the requested data and your lab is currently closed, then I do understand that you will not be able to address these concerns in a timely fashion. In absence of further, new experiments, we would still be willing to publish a revised manuscript. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

- Although the role of YAP signaling in the maintenance of biliary epithelial cells was recently reported in mice, its role in the extrahepatic biliary network and the gallbladder is not known, making this manuscript novel and significant. Moreover, the finding that the morphology of the gallbladder and BECs is influenced by YAP signaling in hepatocytes is intriguing.

Comments for the author

This revised manuscript is much improved compared to the previous one. The authors have addressed well most of my comments, including the analysis of sav1 mutants at earlier stages and phenotype rescue by PP2A inhibitor. However, I still think that the mechanism by which induced Yap activity in sav1-/- hepatocytes results in biliary defects should be presented in the manuscript. The authors mentioned Notch signaling as a potential candidate for such a mechanism. Given the known roles of Notch signaling in biliary formation and morphogenesis, it is a good idea to test. If Notch signaling is reduced in sav1 mutants and if sav1 mutant phenotypes can be rescued by enhancing Notch signaling, even partially, then these new data will make the manuscript very solid for the journal of Development.

I have a technical suggestion:
Since the Tg(Tp1:d2GFP) line was mosaic or silenced, I suggest the Tg(Tp1:EGFP)um14 line, which is not silenced or mosaic. Several groups used this line to beautifully reveal biliary epithelial cells and intrahepatic biliary structure. The Tg(Tp1:VenusPEST)s940 line is also good.

Reviewer 2

Advance summary and potential significance to field

- Sav1, a regulator of Hippo signaling, is required for normal morphology of intrahepatic bile ducts and for morphogenesis of the gallbladder. Sav1 functions by activating Stk3, the ortholog of Mst1/2, through inhibition of phosphatase2A.
- The function of Sav1 depends on its effector Yap (not Taz).
- The gallbladder and intrahepatic duct anomalies in Sav1-/- mutants result from an uncharacterized non-cell autonomous activity of Sav1 in hepatocytes. This is the first evidence for a control of gallbladder development by developing hepatocytes.
- Rescue of Sav1 in a Sav1-/- mutants leads to extrahepatic biliary carcinoma.

Comments for the author

The authors have addressed my concerns in the revised version. They show more convincingly that the gallbladder defects in Sav1-/- mutants result from non-cell autonomous mechanisms. The lack of identification of these mechanisms is somewhat frustrating, but I agree that their characterization may require an excessive amount of work for the present paper.

The authors also corrected their interpretation of the phenotypic evolution of mutant biliary cells which are now more appropriately considered as dysplastic instead of progenitor-like. Consequently, my initial request to verify if biliary cells were in part fated to a hepatocyte phenotype has become less relevant. In that context, I can accept that the authors experienced difficulties with the use of hepatocyte markers, although other markers such a transferrin are available (e.g. : https://zfin.org/ZDB-FIG-090306-39).

Development of cholangiocarcinoma in rescued Sav1-/- mutants is still not clearly relevant to human cholangiocarcinoma. The fact that Hippo signaling is perturbed in human cholangiocarcinoma does not establish a convincing link with the authors' findings in the zebrafish. I suggest to delete the data on tumor development, or to keep it to a strict minimum.

Reviewer 3

Advance summary and potential significance to field

The authors have well addressed most of my comments apart from two points and a number of minor comments.
The studies provide genetic evidence supporting the role of Sav1 in the liver and in particular in the extrahepatic biliary system, including the gallbladder, which may provide entry points for new studies in homeostasis and cholangiocarcinoma of these important organs and ducts.

Comments for the author

A key point of this study is temporal requirement of Sav1 in the liver, e.g. the comparatively late onset of the tissue defects. Two points, which are both related to the onset of the phenotype, have not or only incompletely been addressed and require further clarification.
1 - The spatiotemporal expression of sav1 in the embryo and in particular the liver. Is it expressed before 5dpf? And which cell types express it from 5dpf onwards. Since the tested antibody didn't seem specific mRNA in situ hybridization should be used.
2 - Could maternal wild type sav1 gene products enable normal development of the early liver in sav1 -/- mutants? Could the same explain the late phenotype onset in the other mutants investigated in this study?
Related to this point, the description of the role of Sav1 is not entirely clear and/or consistently described throughout the manuscript. Does early liver development proceed independent of Sav1, and Sav1 specifically controls later maturation/differentiation and maintenance of tissue differentiation?

Introduction: consider adding recent and relevant work in the zebrafish extrahepatic biliary system by Thestrup et al. 2019 and Villasenor et al. 2020

Figure 3C-C'''', indicate area from which below inserts originate from.

Figure 3E+F: labelling of TEM panels. Due to the complexity of the structures, they are not very meaningful to readers without labelling.

Figure 4G: double labeling of biliary conduits difficult to see; use of alternative colours or adjustment of intensities.

Figure8 A-C: The suggestion that the GFP negative tissue could be cholangiocarcinoma-like tissue is interesting. Alternatively, could the lack of GFP expression be due to mosaic silencing of the transgene, which is commonly seen in adults in zebrafish?

Figure 8D-E': the magnified areas need to be indicated in the low magnification overviews. Moreover commonly low and high magnification of the same genotype are linked by same panel name, e.g. D and D' for low and high magnification of WT sections (currently D and E). The same could be applied to F-K'.

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Figure 11 A-D': outlining liver in sections, and/or show magnification of liver area.

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2 – Meyer et al. 2020, implicate the apical hepatocyte surface as mechano-sensor for bile acid load, including Yap activation. This is a very new study and seems very relevant to include in the discussion.

Discussion - Page 20, first paragraph: it states that " hepatic stellate cells are increased in number…", this needs to be toned down, since the identity has not been confirmed by cell type-specific expression.

Figure S1 title: change from 'abnormal biliary cell morphology', to 'biliary duct/conduit morphology', as the data do not provide single cell information.

Second revision

Author response to reviewers' comments

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We appreciate this suggestion and agree that utilization of the lines mentioned would be beneficial for future studies. However, we view a detailed investigation into the role of Notch signaling is beyond the scope of the current study.

Reviewer 2 Advance Summary and Potential Significance to Field:
- Sav1, a regulator of Hippo signaling, is required for normal morphology of intrahepatic bile ducts and for morphogenesis of the gallbladder. Sav1 functions by activating Stk3, the ortholog of Mst1/2, through inhibition of phosphatase2A.
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We are also eager to further investigate and identify the non-cell autonomous mechanism but appreciate the reviewer's acknowledgment of the time and work that will be required to properly do so.

The authors also corrected their interpretation of the phenotypic evolution of mutant biliary cells which are now more appropriately considered as dysplastic instead of progenitor-like. Consequently, my initial request to verify if biliary cells were in part fated to a hepatocyte phenotype has become less relevant. In that context, I can accept that the authors experienced difficulties with the use of hepatocyte markers, although other markers such a transferrin are available (e.g. : https://zfin.org/ZDB-FIG-090306-39.)

We thank the reviewer for the suggestion and agree that this and other *in situ* hybridization markers will be helpful for future work to circumvent the issues we faced with the availability and reproducibility of immunofluorescent antibody-based markers.

Development of cholangiocarcinoma in rescued Sav1-/- mutants is still not clearly relevant to human cholangiocarcinoma. The fact that Hippo signaling is perturbed in human cholangiocarcinoma does not establish a convincing link with the authors' findings in the zebrafish. I suggest to delete the data on tumor development, or to keep it to a strict minimum.

We have cut the paragraph on human cholangiocarcinoma and strictly limited the text on tumor development in humans.

Reviewer 3 Advance Summary and Potential Significance to Field:
The authors have well addressed most of my comments apart from two points and a number of minor comments.
The studies provide genetic evidence supporting the role of Sav1 in the liver and in particular in the extrahepatic biliary system, including the gallbladder, which may provide entry points for new studies in homeostasis and cholangiocarcinoma of these important organs and ducts.

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A key point of this study is temporal requirement of Sav1 in the liver, e.g. the comparatively late onset of the tissue defects. Two points, which are both related to the onset of the phenotype, have not or only incompletely been addressed and require further clarification.
1 - The spatiotemporal expression of sav1 in the embryo and in particular the liver. Is it expressed before 5dpf? And which cell types express it from 5dpf onwards. Since the tested antibody didn't seem specific, mRNA in situ hybridization should be used.
2 - Could maternal wild type sav1 gene products enable normal development of the early liver in sav1 -/- mutants?

Maternal contribution of WT sav1 gene products could potentially impact development and onset of phenotypes in our mutants. While *in situ* hybridization and spatiotemporal analysis of

***sav1* mRNA may provide insight into this possibility, ultimately Sav1 protein is what functionally matters, but we have been unable to identify an antibody specific for zebrafish Sav1 to address this possibility.**

Could the same explain the late phenotype onset in the other mutants investigated in this study?

We show that Stk3 protein is undetectable in *stk3-/-* mutants by Western Blot at 8dpf, which is a full 8 days earlier than we find a phenotype in stk3, suggesting that the presence of maternal gene products is not causing the late phenotype onset in this case. We provide data consistent with the idea that a redundant kinase may explain the delayed phenotype onset of *stk3* mutant fish.

Related to this point, the description of the role of Sav1 is not entirely clear and/or consistently described throughout the manuscript. Does early liver development proceed independent of Sav1, and Sav1 specifically controls later maturation/differentiation and maintenance of tissue differentiation?

Correct, our results suggest that early liver development does indeed proceed independent of Sav1. We have attempted to make this clear throughout the manuscript.

Introduction: consider adding recent and relevant work in the zebrafish extrahepatic biliary system by Thestrup et al. 2019 and Villasenor et al. 2020

We have included citations to these recent studies in revisions to the introduction.

Figure 3C-C’’’’, indicate area from which below inserts originate from.

Figure 3E+F: labelling of TEM panels. Due to the complexity of the structures, they are not very meaningful to readers without labelling.

Figure 3 has been revised accordingly.

Figure 4G: double labeling of biliary conduits difficult to see; use of alternative colours or adjustment of intensities.

We have adjusted the intensity of EdU labeling in Fig4 F’’’, G’’’, and H’’’ to make the Merge panels as clear as possible. Intensities were adjusted equally for each to preserve meaningful comparisons.

Figure8 A-C: The suggestion that the GFP negative tissue could be cholangiocarcinoma-like tissue is interesting. Alternatively, could the lack of GFP expression be due to mosaic silencing of the transgene, which is commonly seen in adults in zebrafish?

The loss of GFP is only ever observed in sav1 mutant fish, and is seen in all of these fish. None of their WT siblings display this phenomenon. We see no reason to think that a WT fish would be less susceptible to this potential silencing.

Figure 8D-E’: the magnified areas need to be indicated in the low magnification overviews. Moreover, commonly low and high magnification of the same genotype are linked by same panel name, e.g. D and D’ for low and high magnification of WT sections (currently D and E). The same could be applied to F-K'.

We have added outlines indicating the regions being shown in higher magnification panels.

Figure 9B: A protein ladder needs to be included in the panel showing the Stk3 western blot.

We have included the protein ladder for this blot.

Figure 11 A-D’: outlining liver in sections, and/or show magnification of liver area.

We have added outlines of liver tissue on these sections.

Discussion – page 17/18: Related to the suggested Sav1 function in hepatocytes, discussing the following two papers should be insightful:
1 - Pepe-Mooney and colleagues showed in their 2019 study that Yap function is dispensable in hepatocytes, as hepatocyte-specific Yap deletion causes no gross changes in morphology or blood chemistry.
2 – Meyer et al. 2020, implicate the apical hepatocyte surface as mechano-sensor for bile acid load, including Yap activation. This is a very new study and seems very relevant to include in the discussion.

We have added citations for these relevant papers to the Discussion.

Discussion - Page 20, first paragraph: it states that "hepatic stellate cells are increased in number…", this needs to be toned down, since the identity has not been confirmed by cell type-specific expression.

We have revised the text accordingly.

Figure S1 title: change from 'abnormal biliary cell morphology', to 'biliary duct/conduit morphology', as the data do not provide single cell information.

The title for Figure S1 has been revised accordingly.

Third decision letter

MS ID#: DEVELOP/2019/184242

MS TITLE: Core Hippo pathway components act as a brake on Yap/Taz in the development and maintenance of the biliary network

AUTHORS: Zachary J. Brandt, Ashley E. Echert, Jonathan R. Bostrom, Paula N. North, and Brian A. Link
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.