nature portfolio

Peer Review File

Cholangiocytes contribute to hepatocyte regeneration after partial liver injury during growth spurt in zebrafish

Corresponding Author: Professor Sumeet Singh

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Eski and co-authors designed two partial liver injury models in zebrafish and found that cholangiocyte-to-hepatocyte transdifferentiation is the primary mechanism of liver regeneration during rapid growth stages. They also found that mTORC1 regulates the plasticity of cholangiocytes. However, these findings are similar to a recently published paper, in which restricted hepatocyte ablation is sufficient to trigger local BEC aggregation and transdifferentiation (Ambrosio et al., Development 2024; doi:10.1242/dev.202217). Up to now, it is well known that cholangiocytes could transdifferentiate into hepatocytes in several liver injury models, and the roles of mTORC1 in cholangiocyte-to-hepatocyte transdifferentiation have been characterized in zebrafish and mice. So, the observation in this study that partial liver injuries, including partial hepatectomy and hepatocyte ablation, stimulate transdifferentiation of cholangiocytes has been identified and characterized, thus lacking novelty.

Major points
1. The manuscript describes cholangiocyte-to-hepatocyte transdifferentiation as the primary mechanism of liver regeneration during rapid growth stages. However, only a partial number of newly regenerated hepatocytes come from her2+ cells at 8 dpi after PHx in Fig.4F and G. According to the data, the H2B-mGL+ hepatocytes should be the primary cell sources for liver regeneration (Fig. 4D and F).

2. Fig.1E and G show that nearly all the newly regenerated hepatocytes (mTagBFP2+) were derived from non-hepatocytes. Meanwhile, less than half of hepatocytes marked by mCherry-NTR after 4-OHT treatment were ablated with Mtz incubation (Fig. 1F). What about the other half of hepatocytes during liver regeneration? Did the mGL+ hepatocyte proliferate, migrate or die during liver regeneration?

3. Most de novo regenerated hepatocytes are nls-mTagBFP2+ in Fig. 2F (non-hepatocyte-derived), but only partial newly regenerated hepatocytes are derived from her2+ cholangiocytes (Fig.4D-G). These data indicate other non-hepatocyte cell types besides cholangiocytes are also involved in hepatocyte regeneration. The authors need to address this important point.

4. The manuscript showed that the inhibition of Notch signalling led to the spontaneous emergence of hepatocytes. At the same time, the activities of mTORC1 in the larval cholangiocytes was stronger than those in adults. What is the relationship between Notch and mTORC1 in regulating cholangiocyte plasticity?

Minor points
1. It is essential to assess the ratios of non-hepatocyte-derived hepatocytes among all hepatocytes during liver regeneration.

2. Western blot is necessary to demonstrate the expression levels of pS6 in larval and adult zebrafish liver, as shown in Fig. 5D.

3. Could the inhibition of Notch signalling promote the conversion of cholangiocytes into hepatocytes in adult liver after PHx?

Reviewer #2

(Remarks to the Author)
The work by Eski investigates the regenerative response of the liver during a phase of rapid growth, as seen in young animals. Using zebrafish as a model, they employ two different ablation strategies, ablation of about 50% hepatocytes throughout the liver and PHx by surgical lobe resection. Based on genetic lineage tracing and slow histone turn-over tracing, they propose that new hepatocytes originate from cholangiocytes, rather than remaining hepatocytes or a mixed origin, as seen in adults or early larval zebrafish. Single cell transcriptomics are employed to corroborate cholangiocytes trans differentiation as source. Finally, these data further implicate enhanced mTORC1 signaling as mediator of this cholangiocyte plasticity during hightened growth phases, supported by subsequent inhibitor treatments.
Overall, the manuscript is well written and supported by imaging of excellent quality and appropriate quantifications. They also developed interesting new methodologies, including transgenic animals. The findings are overall novel and exciting, since little is known about mechanisms of postnatal/-embryonic regeneration. Therefore adding valuable insights to the hepatology field, and generally very interesting to the regeneration

1. De novo hepatocyte formation after partial hepatocyte ablation exclusively of cholangiocyte origin is an exciting proposal, and contrasts most liver regeneration in mammalian and zebrafish models, in which healthy hepatocytes contribute a substantial fraction of new cells by proliferation. To substantiate this intriguing interpretation and this key point of this work, complementary proliferation studies focusing on the different stages and cell types need to be performed, because current lineage tracing does for instance not reveal hepatocyte proliferation.

2. Here, it is notable that recombination is already initiated 5-6days before ablation, so that relatively large clones of NTR-mcherry form (see eg Fig 2A).
What is the recombination pattern and how does regeneration look like when recombination is induced shortly before NTR-ablation or PHx?

3. After PHx, which parts of the liver produce new hepatocytes? Surprisingly, this is not clearly described or shown. This is generally an important point, and specifically because after PHx in adult zebrafish new cells come either from a blastema or from compensatory growth and the reasons are not entirely clear yet (Oderberg and Goesling, 2021).
Schematic (Fig2D) indicates that the surgically removed part is replaced by epimorphic regeneration, in the sense that the missing tissue regrowth is exclusively formed by TagBFP2 cells. is this what is observed? The corresponding experimental results (Fig2F) are difficult to interpret, since it is unclear where in the image the resection site is located and which part of the liver is exactly shown.

It should be clearly addressed where in the liver, related to the resection site new hepatocytes from cholangiocyte transdifferentiation form. Analyzing and quantifying different regions in the same liver, e.g. left, right lobe and connecting part. Does the right lobe show a compensatory response contributing new hepatocytes to the regeneration of the liver? Like for the chemical NTR-model above, complementary proliferation studies are necessary to pinpoint the contributing of regional sources and cell types. Would that be expected given the ‘primed’ status of the cholangiocytes?

4. Further to the source of new cells following PHx, where is the resection side in Figure 4D and F? Where are the groups of mCherry cells located in the provided images in relation to it? Also please explain why there seem to be small focal clones of hepatocytes derived from cholangiocytes, rather than a more wider response throughout the remaining organ as one might expect when larval cholangiocytes show a generally higher plasticity? what is the spatial distribution of such clones?

5. PHx, the impaired growth response of the third lobe is interesting, can the authors exclude that they do not remove some of the ventral lobe progenitors? Similar right lobe PHx should be performed as appropriate control for this.

6. Also, please elaborate on the proposed morphogical response? is it possible that the anlage for the ventral lobe gets damaged during the left lobe surgery – representing the reason for smaller lobe?
Length measurement, even if only an approximation of regeneration, suggests that the left lobe recovers by an epimorphic mechanisms.

7. The authors make a strong point about unlimited food availability during the growth phase, as well as increased expression of components of mTORC1 signaling. Is this a real link? It should be tested whether limited feeding has an effect on the expression of mTORC1 signaling and the regeneration response.
Since in the late embryo/early larvae hepatocytes also contribute to regeneration, what makes their response different?
Finally, hepatocytes and cholangiocytes both express mTORC1 targets, what is the role in hepatocytes in this context? Do the inhibitor treatments give an indication?the authors should at least discuss possible mechanisms for different functions.

8. Given the use of the her2-driver line in this study, validation, including quantification, of cell type specificity should be provided (similar to figS8).
The same should be done for the her9-driver line. The images are excellent, though unclear which other cell types are marked and at which frequency.

9. Text and Figure S8: states that the her9+ lineage mainly contains cholangiocytes. Such a statement should be supported by appropriate quantification, which is essential for the conclusions that use this line.

Minor points:

1. Fig4D,F – please show consistently the larger magnifcations
2. For the cholangiocyte response, the authors use several times the term reprogramming, which is generally associated with the removal or remodeling of epigenetic marks, which they do not address. Hence consistent use of transdifferentiation seems more appropriate.
3. Please indicate what the data in Figure 1G, 2G and S7 E,G are normalized to.
4. Figure 4: mention of non-existing panel H in Figure legends.
5. Fig4D,F – indicate the resection site.
6. For a number of imaging data, it would be very helpful to see magnifications of the data, e.g. fig 2F or 5G,

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Again, I would repeat the key point of my original comments: The cholangiocyte-to-hepatocyte transdifferentiation has been intensively studied so that this manuscript is devoid of novelty.

Besides, the fact that this transdifferentiation only occurs in larvae, but not in adult zebrafish and mice after PHx, diminishes the significance of this finding.

Furthermore, some key tools used in this study are not optimal. For example, the her9 and her2 transgenes, because they label not only cholangiocytes but also other cell types such as satellite cells.

Therefore, I would not support publication of this paper at Nature Communications.

Reviewer #3

(Remarks to the Author)
The authors addressed several prior reviewers' comments and made additional important observations. These new data strengthen the paper and enhance its novelty. However, I have additional questions and comments:

1. Cholangiocyte Contribution to Hepatocyte Regeneration
The main conclusion of the paper is that cholangiocytes contribute to hepatocyte regeneration during growth spurts in zebrafish, even when a significant number of hepatocytes remain after injury. Cholangiocytes are heterogeneous based on their localization, morphology, and gene signatures. Which cholangiocyte population(s) specifically contribute to hepatocyte regeneration in the two injury models described in the paper? Furthermore, the current data do not provide sufficient evidence to exclude the possibility that hepatic progenitor cells, rather than differentiated cholangiocytes, contribute to this regeneration. Additional experiments should be conducted. Otherwise, the main conclusion from this paper is too strong of a statement.

2. Comparative Analysis of Partial Hepatectomy (PHx)
The authors show that partial hepatectomy (PHx) induces cholangiocyte transdifferentiation at 10 days post-fertilization (dpf), whereas previous studies reported that PHx at 5 dpf or in adult zebrafish does not induce cholangiocyte transdifferentiation. It is important to point out that different transgenic zebrafish models were used in these studies, which may explain the differing outcomes.

3. Control for PHx Experiments
What control was used in the PHx experiments? A sham control should be included to ensure that the surgical incision and removal of the skin do not influence ventral lobe growth independently of the hepatectomy.

4. Measurement of Liver Size
The exclusion of compensatory growth after PHx is primarily based on lobe length measurements (Figure 4D–H). However, liver area or volume measurements would be more appropriate to assess liver size accurately.

5. Identification of Her9-Lineage Cells
The authors concluded that 30% of her9-lineage cells are hepatic stellate cells. However, in Figure S8E, it is evident that all hand2:GFP+/Her9+ double-positive cells are located at the liver surface within the mesothelium. These cells are not hepatic stellate cells. The authors should revise this conclusion accordingly.

Additional comments regarding critique 1 (Cholangiocyte Contribution to Hepatocyte Regeneration):

To date, all published work on zebrafish liver regeneration involving cholangiocyte-to-hepatocyte transdifferentiation relies on Tp1-Cre or Tp1-CreERT2 to lineage trace existing cholangiocytes. However, Tp1, a Notch-responsive element, is not a cholangiocyte-specific promoter. It can also label hepatic progenitor cells. Additionally, the cholangiocyte markers commonly

used in zebrafish studies, such as 2F11, alcam, and epcam, are not exclusively expressed in differentiated cholangiocytes. This represents a significant limitation of these studies, which should be properly addressed.

Determining whether hepatic progenitors or differentiated cholangiocytes give rise to regenerating hepatocytes would require the development of additional tools and may be beyond the scope of this paper. However, I believe the single-cell RNA-seq data presented by the authors could provide valuable insights into this question. Within the identified "cholangiocyte" population, are there distinct subgroups? Specifically, are there more differentiated cholangiocyte subpopulations that express cholangiocyte-specific transporters and metabolic genes? Conversely, are there "cholangiocytes" that appear less mature and exhibit more progenitor-like properties?

Acknowledging the heterogeneity within Tp1+ populations and recognizing that different subsets may contribute differently to hepatocyte regeneration is a critical first step toward understanding the key cellular players in this process. Thus adding additional analysis of the scRNA-seq data will strengthen the paper.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The author's responses to reviewers' critiques have raised more questions as detailed below.

1. Newly generated data on liver regeneration after partial hepatectomy in juvenile mice.
The observation in Figure R2 neither supports nor refutes the authors' conclusion that cholangiocytes contribute to hepatocyte regeneration in juvenile mice following PHx. The percentage of CK19;HNF4α double-positive hepatocytes is not reported, nor is the number of animals analyzed. Moreover, in the absence of lineage tracing, the presence of these double-positive cells could also suggest that hepatocytes contribute to cholangiocyte regeneration after PHx.

2. The authors presented new scRNA-seq data demonstrating four cholangiocyte clusters in the control zebrafish liver: one enriched for krt4 (representing luminal ductal cells), two clusters comprising a mix of intrahepatic duct (IHD) and intermediate ductal cells, and a proliferative cholangiocyte cluster. Using pseudotemporal analysis, they proposed that IHD and intermediate ductal cells are the primary source of transdifferentiation.

However, the data in Supplemental Figure 6 raises more questions. In Fig. 6F, the "cholangiocytes rest" cluster (grey) appears to be more poised to transdifferentiation to hepatocytes than the other four cholangiocyte clusters, yet this cluster is not discussed in the text. The authors also refer to their previous scRNA-seq study of the adult zebrafish liver, in which IHD and intermediate ductal cells were also identified. Notably, PHx at the adult stage does not lead to cholangiocyte-to-hepatocyte transdifferentiation. Comparing the transcriptomes of IHD and intermediate ductal cells in juvenile versus adult livers would be crucial for uncovering the distinct mechanisms underlying liver regeneration at these two stages.

Cholangiocytes contribute to hepatocyte regeneration after partial liver injury during growth spurt in zebrafish.

Sema Elif Eski, Jiarui Mi, Macarena Pozo-Morales, Gabriel Garnik Hovhannisyan, Camille Perazzolo, Rita Manco, Imane Ez-Zammoury, Dev Barbhaya, Anne Lefort, Frédérick Libert, Federico Marini, Esteban N. Gurzov, Olov Andersson, Sumeet Pal Singh*

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We thank the reviewers for the encouraging comments. We have attempted to address all the issues outlined by the reviewers. We thank the reviewers for raising the concerns, which were all valid. Addressing the comments has, we think, strengthened the manuscript, and for this we appreciate the constructive feedback received from the reviewers.

Particularly, in the two main areas, the reviewer's feedback has been greatly beneficial. Firstly, in response to the comments, we have carried out a detailed, quantitative analysis of cellular proliferation in response to partial ablation and partial hepatectomy (PHx). In response to partial ablation, we observe a strong induction of cell-cycle in cholangiocytes. However, we do not observe any increase in proliferation in the spared hepatocytes, underscoring the specific response of cholangiocytes to the injury. In response to PHx, we observe an increase in hepatocyte proliferation. This, however, is

much smaller and shorter than the enhancement in cholangiocyte proliferation. Thus, in both injury models, cholangiocytes display a robust increase in cell-cycle.

Secondly, in response to the reviewer's comments, we have improved the presentation of our images. We have exchanged full liver images with zoom-in images, and we have opted to showcase a single confocal plane rather than maximum intensity projection images. This significantly increased the clarity of image presentation.

We thank the reviewers for their insightful feedback that helped improve the manuscript scientifically and visually.

For the revision, our response is in blue. To highlight specific image panels from the manuscript, we have reproduced them in the response letter. In this case, they are labeled with the numbers from the manuscript. Images specific to the rebuttal letter are labeled as Figure Rn.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Eski and co-authors designed two partial liver injury models in zebrafish and found that cholangiocyte-to-hepatocyte transdifferentiation is the primary mechanism of liver regeneration during rapid growth stages. They also found that mTORC1 regulates the plasticity of cholangiocytes. However, these findings are similar to a recently published paper, in which restricted hepatocyte ablation is sufficient to trigger local BEC aggregation and transdifferentiation (Ambrosio et al., Development 2024; doi:10.1242/dev.202217). Up to now, it is well known that cholangiocytes could transdifferentiate into hepatocytes in several liver injury models, and the roles of mTORC1 in cholangiocyte-to-hepatocyte transdifferentiation have been characterized in zebrafish and mice. So, the observation in this study that partial liver injuries, including partial hepatectomy and hepatocyte ablation, stimulate transdifferentiation of cholangiocytes has been identified and characterized, thus lacking novelty.

We would like to thank Reviewer #1 for their thorough evaluation and constructive comments on our manuscript. We appreciate the acknowledgment of our work in designing new liver injury models in zebrafish to explore the mechanisms of liver regeneration.

In response to the points regarding novelty, we cite Ambrosio et al. in the original manuscript and agree that their findings align well with our partial ablation model.

However, our work expands on these results in significant ways: 1. we show transcriptional convergence between *de novo* hepatocytes and spared hepatocytes using single-cell RNA sequencing, and 2. we employ lineage tracing with the Cre/Lox system to confirm cholangiocyte-to-hepatocyte transdifferentiation. Further, we develop and employ a partial hepatectomy (PHx) model to demonstrate cholangiocyte-to-hepatocyte conversion in an injury model where all cell populations of the liver are impacted. In contrast, Ambrosio et al. hypothesize that transdifferentiation is induced specifically by hepatocyte loss. Finally, the conversion of cholangiocytes into hepatocytes upon PHx is absent in mice (1), and, to our knowledge, no studies have reported this phenomenon in humans. **Thus, our study is the first to document cholangiocyte contributions to hepatocyte regeneration after PHx.**

Major points

1. The manuscript describes cholangiocyte-to-hepatocyte transdifferentiation as the primary mechanism of liver regeneration during rapid growth stages. However, only a partial number of newly regenerated hepatocytes come from her2+ cells at 8 dpi after PHx in Fig.4F and G. According to the data, the H2B-mGL+ hepatocytes should be the primary cell sources for liver regeneration (Fig. 4D and F).

The reviewer raises a valid point regarding the representation of our data. In the original manuscript, the her2 lineage tracing figures were shown as a single confocal plane, while the CellCousin experiment was represented with a maximum intensity projection image, which may have led to some confusion. We have now revised the CellCousin data to display a single confocal plane for consistency and clarity (Fig. 4C), which

clearly shows the restriction of *de novo* hepatocytes (mTagBFP+ cells) to a small region near the edge of the regenerate.

Additionally, we have quantified the labeling frequency for the *her2* line, finding that it labels 80.4% of cholangiocytes (Supplementary Fig. 7B). This means we are likely underestimating the extent of transdifferentiation based on her2-lineage tracing alone. However, by using a knock-in CreER line, we minimize potential non-specific expression that may arise from a promoter-driven Cre line. Therefore, despite the incomplete labeling, the her2 lineage tracing provides the strongest experimental evidence currently available for cholangiocyte-to-hepatocyte conversion. We believe that Cre/Lox-based lineage tracing is essential for confirming transdifferentiation, and this approach best supports our findings.

Furthermore, we would like to emphasize our findings from the cell-cycle analysis following partial hepatectomy (PHx). Using whole-mount EdU imaging, we found that EdU+ cells are confined to within 50 µm of the regenerate's edge (Fig. 3D-G; see also Supplementary Movie 1). This indicates the formation of a localized high-proliferation zone post-injury, suggesting a spatially focused regenerative response.

Within this proliferative zone, hepatocytes exhibit a significant increase in cell-cycle activity at 1 dpi, while cholangiocytes show a strong rise in cell-cycle activity at both 1 and 2 dpi. **Remarkably, 20 % of cholangiocytes within 50 µm of the regenerate edge**

enter S-phase at 1 dpi, highlighting a robust localized response. Beyond this zone, neither hepatocytes nor cholangiocytes display any increase in cell-cycle activity, confirming that the regenerative response is concentrated at the injury site. **Additionally, we observed that cholangiocytes maintain this proliferative activity longer and more intensely than hepatocytes, indicating a regionally focused role for cholangiocytes in liver regeneration.** These findings underscore the capacity of cholangiocytes to respond to PHx.

2. Fig.1E and G show that nearly all the newly regenerated hepatocytes (mTagBFP2+) were derived from non-hepatocytes. Meanwhile, less than half of hepatocytes marked by mCherry-NTR after 4-OHT treatment were ablated with Mtz incubation (Fig. 1F). What about the other half of hepatocytes during liver regeneration? Did the mGL+ hepatocyte proliferate, migrate or die during liver regeneration?

The reviewer raises an important question regarding whether the spared hepatocytes proliferate in response to partial ablation. Using whole-mount EdU imaging, we quantified cell proliferation across different stages post-injury (Fig. 2B). **Our findings reveal that spared hepatocyte proliferation does not significantly increase after partial ablation (Fig. 2C).** Instead, we observed a robust increase in cholangiocyte proliferation from 6 to 48 hours post-injury (Fig. 2C). Similarly, *de novo* hepatocytes, which are derived from cholangiocytes, display robust proliferation at 24- and 48-hours post-partial ablation. These results further support our interpretation that cholangiocytes, rather than spared hepatocytes, are the primary source of new hepatocytes in our regeneration model. We have added this data to the revised manuscript and updated

the Results sections to clarify this distinction.

Further, the spared mGL+ hepatocytes persist after partial ablation, as evidenced by their presence at 5 days post-partial ablation (dppa) in imaging (Fig. 1F) and in single-cell RNA sequencing data at 9 dppa (Fig. 5D-F).

3. Most de novo regenerated hepatocytes are nls-mTagBFP2+ in Fig. 2F (non-hepatocyte-derived), but only partial newly regenerated hepatocytes are derived from her2+ cholangiocytes (Fig.4D-G). These data indicate other non-hepatocyte cell types besides cholangiocytes are also involved in hepatocyte regeneration. The authors need to address this important point.

We thank the reviewer for raising this important point regarding the potential involvement of other non-hepatocyte cell types in hepatocyte regeneration. To address this, we performed an **unbiased pseudotemporal analysis using TSCAN** (1) to investigate the cellular trajectories during liver regeneration (Figure R1).

The pseudotemporal trajectory of cells during liver regeneration shows a continuous and unidirectional progression from cholangiocytes (blue) to hepatocytes (yellow). Importantly, no other cell types were found to exhibit a trajectory toward hepatocytes, as indicated by the absence of such transitions in the analysis. Cells in grey, including other non-hepatocyte and non-cholangiocyte populations, do not align with the pseudotemporal trajectory, supporting the conclusion that these cell types are not directly involved in the regeneration of hepatocytes. The *in silico* analysis demonstrates that non-cholangiocytes do not contribute significantly to hepatocyte regeneration.

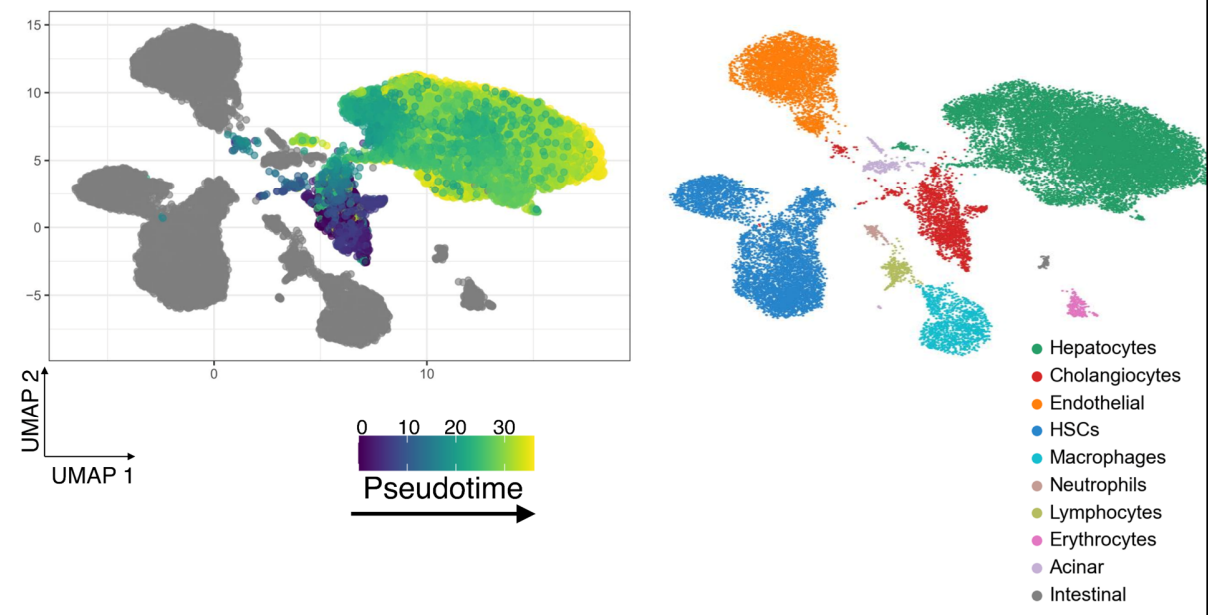

Figure R1: Unbiased pseudotemporal analysis identifies cholangiocytes as the source of hepatocytes. (Left) UMAP plot showing the pseudotemporal trajectory during liver regeneration in zebrafish. Cells are colored based on pseudotime, progressing from cholangiocytes (blue) to hepatocytes (yellow), highlighting a continuous transition from cholangiocytes to hepatocytes. No trajectory was identified for the cells in grey. **(Right)** UMAP plot with clusters annotated by cell type (same as Fig. 5B).

4. The manuscript showed that the inhibition of Notch signalling led to the spontaneous emergence of hepatocytes. At the same time, the activities of mTORC1 in the larval cholangiocytes was stronger than those in adults. What is the relationship between Notch and mTORC1 in regulating cholangiocyte plasticity?

This is an intriguing question. We propose that mTORC1 signaling does not influence Notch levels under homeostatic conditions. This is evidenced by the fact that in a growing liver, mTORC1 activity is high (Fig. 7D-F), yet no spontaneous transdifferentiation occurs. However, upon injury, we hypothesize that elevated mTORC1 levels enable cholangiocytes to effectively downregulate Notch signaling, promoting transdifferentiation. This distinction is especially clear when comparing 5 dpf and 10 dpf larval zebrafish. Previous studies, including our own, indicate that at 5 dpf, zebrafish are entering a fasting state (2–4), during which mTORC1 activity in cholangiocytes is low (Supplementary Fig. 10), and PHx does not induce cholangiocyte-to-hepatocyte transdifferentiation (5).

Based on this, below is a summary of the relationship between mTORC1 levels and transdifferentiation potential:

Stage	mTORC1 Levels	Cholangiocyte Transdifferentiation upon PHx
5 dpf	Low	Absent [From Zhang et al., *iScience*, 2021 (5)]
10 dpf	High	Present [This study]
Adult	Low	Absent [From Oderberg and Goessling, *JCI insight*, 2023 (6)]

Thus, it appears that the mTORC1 pathway primes cholangiocytes to alter their identity in response to injury. The precise molecular mechanisms underlying this process are currently unknown and may involve interactions between mTORC1 and injury-induced

signals. We are actively exploring this aspect as the basis for future studies.

Minor points

1. It is essential to assess the ratios of non-hepatocyte-derived hepatocytes among all hepatocytes during liver regeneration.

For PHx, we have now done this using FACS (Fig. 4E). This analysis showed that at 4 dpi, the de novo hepatocytes represent 18.0 ± 2.9 % of hepatocytes in PHx animals, as opposed to 1.2 ± 0.1 % of hepatocytes in control animals. Note that in PHx, we remove 17.3 % of hepatocytes (Fig. 3B).

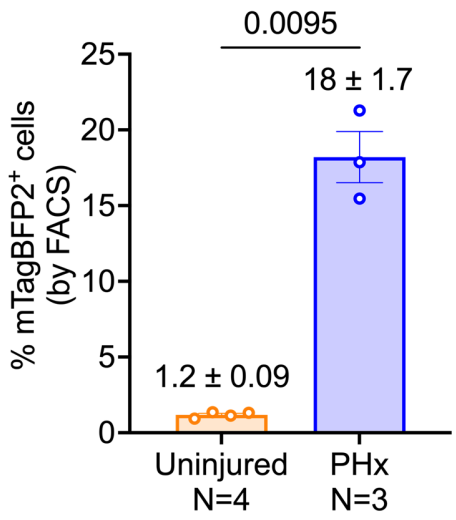

Figure 4E: Percentage of *de novo* hepatocytes after partial hepatectomy. Quantification of the percentage of mTagBFP2+ cells in uninjured (N=5) and PHx (N=3) liver tissue at 4 days post-injury (dpi) by FACS. Data are presented as mean ± SEM (Welch's t test).

For partial ablation, the ratios are presented in Fig. 1F. *De novo* hepatocytes represent 43.5 % of hepatocytes after ablation.

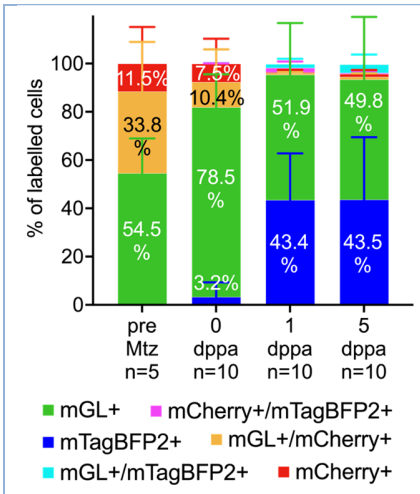

Figure 1F: Percentage of labeled cells after partial ablation. Mean ± SD of the percentage of hepatocyte labeling before (pre-Mtz, n=14) and after Mtz treatment (0,1 and 5 dppa, n=10 each).

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193 2. Western blot is necessary to demonstrate the expression levels of pS6 in larval and adult

194 zebrafish liver, as shown in Fig. 5D.

195 We have performed western blot and see a stronger signal for pS6 in larval liver as

196 compared to the adult liver.

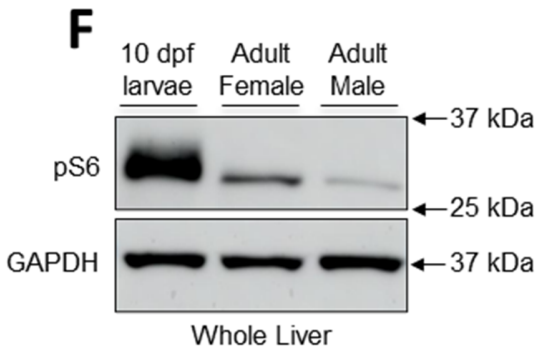

Figure 7F: Western blot analysis of protein expression from whole liver of 10 dpf larvae and female and male adults. The top panel shows the protein levels of pS6 and the bottom panel represents the loading control (GAPDH).

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199 3. Could the inhibition of Notch signalling promote the conversion of cholangiocytes into

200 hepatocytes in adult liver after PHx?

201 By using the exact same pharmacological protocol as utilized for larval zebrafish, the

adult zebrafish did not show any sign of transdifferentiation (Figure R2). We were unable to find a single example of overlap between cholangiocyte lineage and hepatocytes in adult zebrafish.

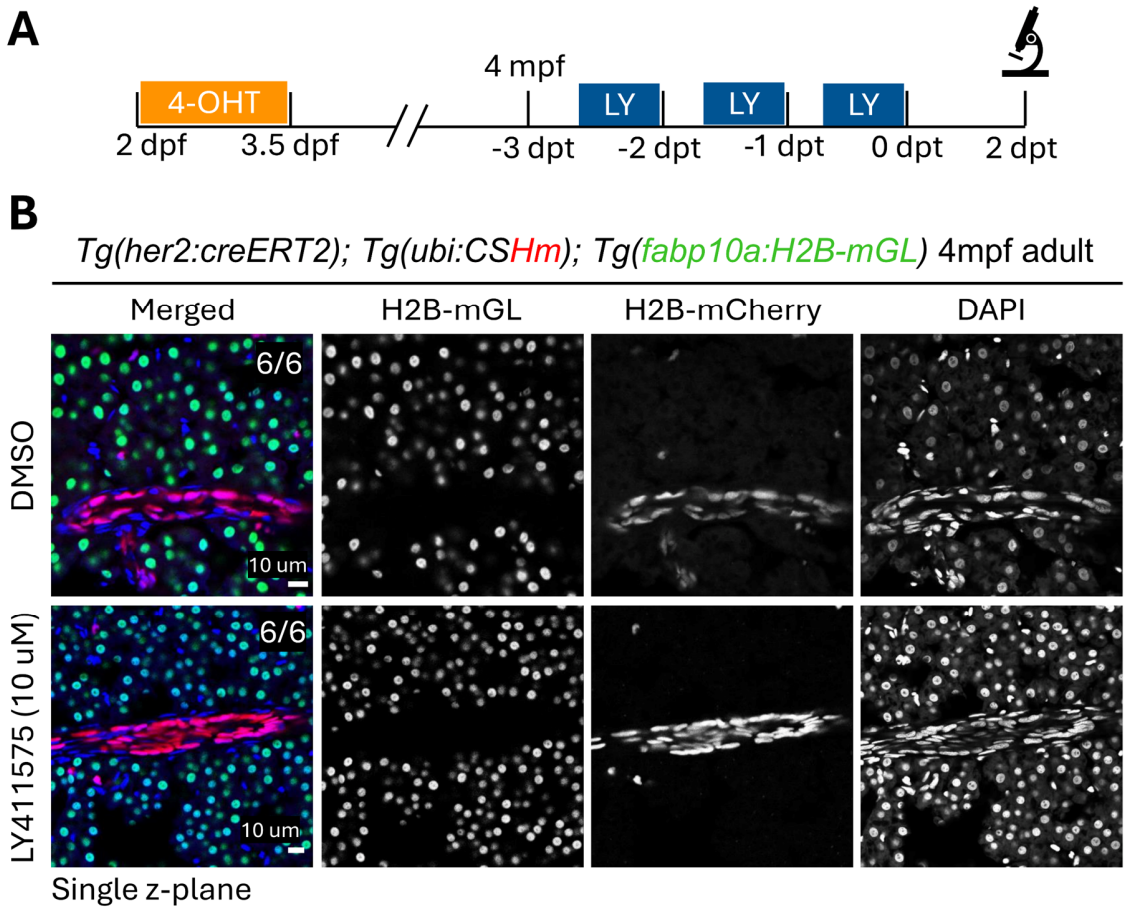

Figure R2: Inhibition of Notch signalling pathway in adult zebrafish does not induce spontaneous transdifferentiation. (A) Timeline for LY411575 treatment in adult zebrafish. 4-OHT was administered from 48 hpf to 78 hpf, and the her2 lineage was validated at the larval stage. Lineage tracing of her2 was performed at 4 mpf following treatment with 10 μM LY411575 for 16 hours over three consecutive nights in *Tg(her2:creERT2);Tg(ubi:CSHm);Tg(fabp10a:H2B-mGL)* transgenic zebrafish.

Samples were analyzed 2 days post-treatment (dpt). (B) Confocal images of adult liver treated with DMSO and 10 μM LY411575 for 3 days, showing no overlap or mutual expression between H2B-mGL and H2B-mCherry (n=6/6).

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Reviewer #2 (Remarks to the Author):

The work by Eski investigates the regenerative response of the liver during a phase of rapid growth, as seen in young animals. Using zebrafish as a model, they employ two different ablation strategies, ablation of about 50% hepatocytes throughout the liver and PHx by surgical lobe resection. Based on genetic lineage tracing and slow histone turn-over tracing, they propose that new hepatocytes originate from cholangiocytes, rather than remaining hepatocytes or a mixed origin, as seen in adults or early larval zebrafish. Single cell transcriptomics are employed to corroborate cholangiocytes trans differentiation as source. Finally, these data further implicate enhanced mTORC1 signaling as mediator of this cholangiocyte plasticity during hightened growth phases, supported by subsequent inhibitor treatments.

Overall, the manuscript is well written and supported by imaging of excellent quality and appropriate quantifications. They also developed interesting new methodologies, including transgenic animals. The findings are overall novel and exciting, since little is known about mechanisms of postnatal/-embryonic regeneration. Therefore adding valuable insights to the hepatology field, and generally very interesting to the regeneration

We thank Reviewer #2 for their positive appraisal of our study and for recognizing the novelty and relevance of our findings within the field of liver regeneration. We particularly acknowledge the constructive suggestions related to proliferative response to injuries, which prompted us to enhance our analysis of proliferative response, clarifying the contributions of cholangiocytes and hepatocytes to liver regeneration. We have also revised our imaging data presentation to clearly indicate resection sites.

230

231 1. De novo hepatocyte formation after partial hepatocyte ablation exclusively of cholangiocyte
232 origin is an exciting proposal and contrasts most liver regeneration in mammalian and zebrafish
233 models, in which healthy hepatocytes contribute a substantial fraction of new cells by
234 proliferation. To substantiate this intriguing interpretation and this key point of this work,
235 complementary proliferation studies focusing on the different stages and cell types need to be
236 performed, because current lineage tracing does for instance not reveal hepatocyte proliferation.
237

238 We thank the reviewer for highlighting this aspect of our study and for their suggestion
239 regarding complementary proliferation studies. In response, we conducted a
240 quantitative analysis of cell-cycle, using EdU incorporation, to assess cell proliferation
241 across different stages post-injury (Fig. 2B). **Our findings reveal that spared hepatocyte**
242 **proliferation does not significantly increase after partial ablation (Fig. 2C).** Instead, we
243 observed a robust increase in cholangiocyte proliferation from 6 to 48 hours post-injury
244 (Fig. 2C). Similarly, de novo hepatocytes, which are derived from cholangiocytes, display
245 robust proliferation at 24- and 48-hours post-partial ablation. These results further
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247 primary source of new hepatocytes in our regeneration model. We have added this data
248 to the revised manuscript and updated the Results sections to clarify this distinction.
249

250 2. Here, it is notable that recombination is already initiated 5-6days before ablation, so that
251 relatively large clones of NTR-mcherry form (see eg Fig 2A).
252 What is the recombination pattern and how does regeneration look like when recombination is

induced shortly before NTR-ablation or PHx?

Following 4-OHT treatment, we observe that the default blue fluorescence (nls-mTagBFP2) persists for approximately 3 days before reaching undetectable levels due to perdurance of the fluorescent protein, while mCherry-NTR fluorescence takes around 4 days to become robustly detectable (Supplementary Fig. 2C). To ensure clear lineage distinction, we wait 4.5 days from the end of 4-OHT treatment (5.5 days post-fertilization) to the induction of injury (10 days post-fertilization). This timing allows for sufficient transition between fluorescent markers and ensures that the labeled cells are effectively visualized at the onset of regeneration. We have added this clarification in the revised manuscript, which would be of interest to researchers who want to adapt CellCousin model to their organ system.

3. After PHx, which parts of the liver produce new hepatocytes? Surprisingly, this is not clearly described or shown. This is generally an important point, and specifically because after PHx in adult zebrafish new cells come either from a blastema or from compensatory growth and the reasons are not entirely clear yet (Oderberg and Goesling, 2021).
Schematic (Fig2D) indicates that the surgically removed part is replaced by epimorphic regeneration, in the sense that the missing tissue regrowth is exclusively formed by TagBFP2 cells. is this what is observed? The corresponding experimental results (Fig2F) are difficult to interpret, since it is unclear where in the image the resection site is located and which part of the liver is exactly shown.

It should be clearly addressed where in the liver, related to the resection site new hepatocytes

from cholangiocyte transdifferentiation form. Analyzing and quantifying different regions in the same liver, e.g. left, right lobe and connecting part. Does the right lobe show a compensatory response contributing new hepatocytes to the regeneration of the liver? Like for the chemical NTR-model above, complementary proliferation studies are necessary to pinpoint the contributing of regional sources and cell types. Would that be expected given the 'primed' status of the cholangiocytes?

This was an important point that we have now addressed. Using whole-mount EdU imaging, we observed that EdU+ cells are restricted to within **50 µm of the edge of the regenerate** (Fig. 3D-G; see also Supplementary Movie 1). This demonstrates that a localized region of high proliferation is established after injury, supporting a spatially focused response.

Within this proliferative zone, hepatocytes show a significant increase in cell-cycle activity at 1 dpi, whereas cholangiocytes display a robust increase in cell-cycle activity at both 1 and 2 dpi**. Notably, 20% of cholangiocytes within 50 µm of the regenerate edge are in S-phase at 1 dpi, indicating a strong localized response. Outside of this zone, neither hepatocytes nor cholangiocytes show any increase in cell-cycle activity, underscoring that the regenerative response is concentrated at the injury site.**
Additionally, we find that cholangiocytes sustain this proliferative response longer and more robustly than hepatocytes, indicating a regionally restricted contribution of cholangiocytes in liver regeneration. We refer to this area as the "wound border zone" in the Discussion section.

We have added these findings to the revised manuscript to clarify the spatial dynamics of hepatocyte and cholangiocyte contributions to regeneration post-PHx.

(In considering terminology, the corresponding author (SPS) was intrigued by the potential of labeling this region as a "blastema", due to previous experience in zebrafish fin regeneration. However, while the limb or fin blastema consists of multipotent cells, this may not apply to liver regeneration. Therefore, for accuracy, we have opted to describe this area as the "wound border zone," drawing on analogous terminology from heart regeneration studies (7).)

4. Further to the source of new cells following PHx, where is the resection side in Figure 4D and F? Where are the groups of mCherry cells located in the provided images in relation to it? Also please explain why there seem to be small focal clones of hepatocytes derived from cholangiocytes, rather than a more wider response throughout the remaining organ as one might expect when larval cholangiocytes show a generally higher plasticity? what is the spatial distribution of such clones?

We have now outlined the edge of the regenerate in the provided images for clarity. Our data indicate that cholangiocyte-derived hepatocytes are consistently found in close proximity to the regenerate edge, in line with our cell-cycle quantification. Regarding the reviewer's observation on clonality, we also noticed this focal clustering in all of our images, such as in Figures 4C and 7H. This pattern suggests that a small number of cholangiocytes may expand to drive regeneration.

Exploring the clonal dynamics of cholangiocytes post-PHx with a multi-color labeling strategy could provide valuable insights into whether a subset of cholangiocytes predominantly contribute to regeneration. However, clonal analysis of cholangiocyte contribution is beyond the scope of our current tools. We have noted this observation in the revised manuscript and hope to pursue it in future studies.

5. PHx, the impaired growth response of the third lobe is interesting, can the authors exclude that they do not remove some of the ventral lobe progenitors? Similar right lobe PHx should be performed as appropriate control for this.

We appreciate the reviewer's suggestion to perform partial hepatectomy (PHx) of the right lobe as a control for the impaired growth response of the ventral lobe. We attempted this experiment; however, it resulted in 80% lethality, requiring us to abort the experiment. We believe the high lethality may stem from the close proximity of the right lobe to the pancreas, which stores digestive enzymes, as well as to the gall bladder (Fig. R3). Injury to these adjacent structures likely impacts the animals' survival. In contrast, the left lobe is more isolated from other hepatopancreatic structures, allowing us to perform PHx safely in this region.

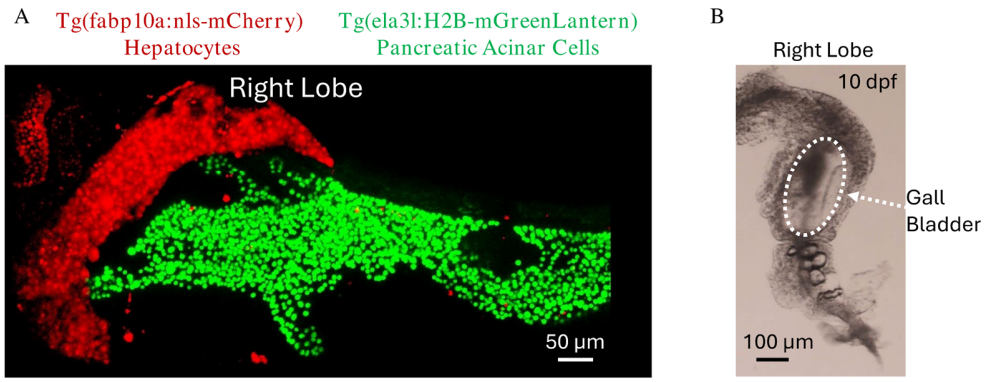

Figure R3: Proximity of the liver's right lobe to the pancreas and gall bladder. (A) Confocal image showing the proximity between the right liver lobe (red) and pancreatic acinar cells (green). (B) Bright-field image illustrating the right liver lobe positioned above the gall bladder at 10 dpf, highlighting their proximity.

6. Also, please elaborate on the proposed morphogical response? is it possible that the anlage for the ventral lobe gets damaged during the left lobe surgery – representing the reason for smaller lobe?

Length measurement, even if only an approximation of regeneration, suggests that the left lobe recovers by an epimorphic mechanisms.

We thank the reviewer for this thought-provoking question. Currently, due to the lack of a specific marker for the ventral lobe anlage in zebrafish, we are unable to delineate the exact mechanism underlying the observed morphological response. It is indeed possible that the anlage of the ventral lobe is directly impacted during left lobe PHx, which could explain the smaller size of the ventral lobe during regeneration. We have

added this consideration to the Results section of the revised manuscript (in bold): "Intriguingly, the ventral lobe remained shorter in the adults that underwent PHx as compared to controls (Supplementary Fig. 4G, H), suggesting that PHx has a long-term impact on the morphology of the liver in zebrafish. **However, since specific markers to label the ventral lobe anlage are currently unavailable in zebrafish, we are unable to track the precise origins or status of this anlage post-surgery. It is therefore possible that the ventral lobe anlage is directly impacted or even partially damaged during the left lobe PHx, which could account for its diminished growth.**"

7. The authors make a strong point about unlimited food availability during the growth phase, as well as increased expression of components of mTORC1 signaling. Is this a real link? It should be tested whether limited feeding has an effect on the expression of mTORC1 signaling and the regeneration response.

Since in the late embryo/early larvae hepatocytes also contribute to regeneration, what makes their response different?

Finally, hepatocytes and cholangiocytes both express mTORC1 targets, what is the role in hepatocytes in this context? Do the inhibitor treatments give an indication?the authors should at least discuss possible mechanisms for different functions.

To assess the relationship between feeding, mTORC1 signaling, we performed a 0.25x feeding regimen and observed a trend toward reduced pS6 levels, though this decrease was not statistically significant (Fig. R4). The rotifer feed has a high protein content (approximately 50% (8)), which likely sustains mTORC1 activity despite reduced feeding.

However, at 5 days post-fertilization (dpf), we and others have shown that the animals are entering a fasting state (2–4). At this stage, the pS6 levels are significantly low (Fig. R4). At 5 dpf, it has been shown that PHx at this stage does not induce transdifferentiation (5).

This suggests that mTORC1 activation promotes cholangiocyte plasticity.

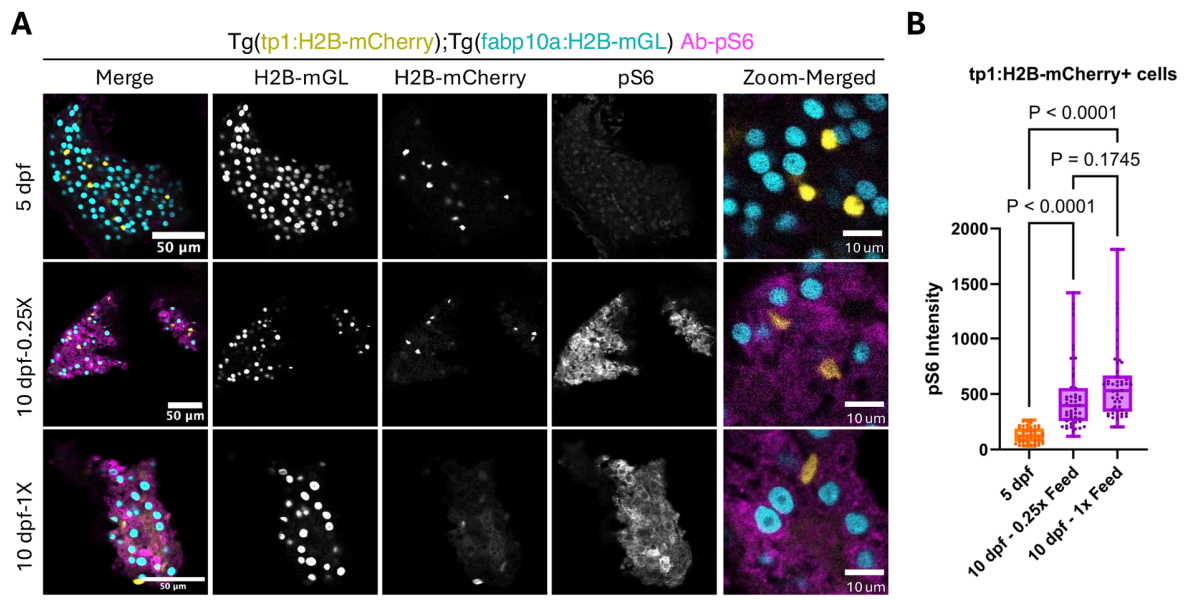

Figure R4: Effects of rotifer feeding on mTORC1 signaling activity. (A) Immunostaining for pS6 in cryosections from uninjured larvae at 5 dpf and 10 dpf fed with 1x and 0.25x rotifer. (B) Quantification of pS6 intensity in cholangiocytes from livers of 5 dpf, 1x and 0.25x fed 10 dpf zebrafish larvae.

The role of mTORC1 in hepatocytes likely revolves around their critical functions in protein processing and metabolism, given that mTORC1 activation is highly responsive

to protein uptake (9). Particularly, at 10 dpf, the animals are being fed with rotifers, a high protein diet (44 – 50 % protein content (8)). It would be of interest to raise zebrafish on diets varying in protein and lipid content and to evaluate mTORC1 levels and the cellular source of liver regeneration. This would be more informative rather than reducing the level of feed, as reduction in rotifer feed did not impact pS6 levels (Fig. R4). In the future, we can attempt to standardize alternative feeding regiment to investigate this question. We have added this point to the Discussion section of the manuscript.

8. Given the use of the her2-driver line in this study, validation, including quantification, of cell type specificity should be provided (similar to figS8).
The same should be done for the her9-driver line. The images are excellent, though unclear which other cell types are marked and at which frequency.

We have quantified the labeling frequency for both the her2 and her9 lines to validate cell type specificity. For the her2 line, we found that it labels 80.4% of cholangiocytes (Supplementary Fig. 7A-B). Within the her2-lineage cells, 93.7% were identified as cholangiocytes (Supplementary Fig. 7C)). Thus, the her2 lineage is predominantly specific to cholangiocytes in the liver.

For the her9 line, it labels 59.3% of cholangiocytes (Supplementary Fig. 8A-C). Within the her9 lineage, 60.2% are cholangiocytes (Supplementary Fig. 8D). Additionally, our single-cell RNA-Seq dataset reveals her9 expression in hepatic stellate cells (Fig. 5C). Using the *TgBAC(hand2:EGFP)* line, which specifically labels hepatic stellate cells, we identified that 30.0% of her9-lineage cells are stellate cells (Supplementary Fig. 8D, E).

It is important to note that our her2-lineage tracing demonstrates that cholangiocytes contribute to hepatocyte regeneration. While we do not propose *her9* as a specific cholangiocyte marker, as noted in the manuscript, it labels multiple cell types in the liver parenchyma. However, *her9* expression is completely excluding from hepatocytes. Thus, her9-lineage tracing indicates that non-hepatocytes can generate hepatocytes. We have added these quantifications to the revised manuscript to further clarify the cell type specificity and functionality of each driver line.

9. Text and Figure S8: states that the her9+ lineage mainly contains cholangiocytes. Such a statement should be supported by appropriate quantification, which is essential for the conclusions that use this line.

Please see the response to the previous question. In the text, we have removed the word “mainly” and added the appropriate quantifications.

Minor points:

1. Fig4D,F – please show consistently the larger magnifcations.

We have edited the figure to have larger magnifications.

2. For the cholangiocyte response, the authors use several times the term reprogramming, which is generally associated with the removal or remodeling of epigenetic marks, which they do not address. Hence consistent use of transdifferentiation seems more appropriate.

We have removed reprogramming from the manuscript. And used transdifferentiation

throughout the text.

3. Please indicate what the data in Figure 1G, 2G and S7 E,G are normalized to.

All data is normalized to volume. We mention this in the Methods Section and have also included this in the figures now.

4.Figure 4: mention of non-existing panel H in Figure legends.

Apologies for the error. We have corrected the figure legend.

5. Fig4D,F – indicate the resection site.

We added the edge of the regenerate with dotted line. It is difficult to identify the resection site as there aren't morphological landmarks to delineate the injury site. Thus, we can only identify the edge of the regenerating liver.

6. For a number of imaging data, it would be very helpful to see magnifications of the data, e.g. fig 2F or 5G,

Thank you for the suggestion. We have edited the figure panels in two ways:

1. Included high-magnification images
2. Edited the images from z-stack to a single plane for confocal images to increase clarity.

These edits dramatically improved the image presentation. We acknowledge the reviewer's feedback to improve the presentation.

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Cholangiocytes contribute to hepatocyte regeneration after partial liver injury during growth spurt in zebrafish.

Sema Elif Eski, Jiarui Mi, Macarena Pozo-Morales, Gabriel Garnik Hovhannisyan, Camille Perazzolo, Rita Manco, Imane Ez-Zammoury, Dev Barbhaya, Anne Lefort, Frédérick Libert, Federico Marini, Esteban N. Gurzov, Olov Andersson, Sumeet Pal Singh*

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We appreciate the constructive feedback provided by the reviewers. We have carefully considered the comments and have made substantial revisions to strengthen the manuscript. Below, we address each concern in detail, incorporating new data, clarifications, and modifications to improve the rigor and clarity of our study.

For the revision, our response is in blue. Figures specific to the rebuttal letter are labeled as Figure Rn.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Again, I would repeat the key point of my original comments: The cholangiocyte-to-hepatocyte transdifferentiation has been intensively studied so that this manuscript is devoid of novelty.

Besides, the fact that this transdifferentiation only occurs in larvae, but not in adult zebrafish and mice after PHx, diminishes the significance of this finding.

We acknowledge the reviewer's concerns regarding the novelty of our findings. While it is true that cholangiocyte-to-hepatocyte transdifferentiation has been studied in adult models, our work provides new insights into the context of liver regeneration during growth spurts. Unlike previously characterized adult systems, we demonstrate that this process occurs robustly in growing zebrafish larvae even when a significant number of hepatocytes remain after injury, challenging the prevailing model that transdifferentiation occurs only under conditions of severe hepatocyte loss. This highlights a previously underappreciated role of cholangiocyte plasticity in regeneration.

Furthermore, while transdifferentiation is absent in adult zebrafish and adult mice after partial hepatectomy, its presence in growing zebrafish larvae suggests that **developmental stage plays a key role in dictating cellular plasticity** .

Mouse Data (Redacted in Final Version)

Regarding the lack of evidence for this phenomenon in mice (CD1 background), we have conducted preliminary analyses in juvenile mice after PHx. First, we established a PHx model for juvenile mice (Fig. R1). Next, we performed partial hepatectomy, removing 50% of the liver in postnatal day 21 (P21) mice. We chose P21 mice because they are still in early postnatal development (1), not yet weaned, and their livers are actively maturing, with a higher percentage of proliferating hepatocytes compared to adult mice (2,3).

To assess potential cholangiocyte-to-hepatocyte conversion, we harvested the liver at postoperative day 2 and performed double immunohistochemistry for CK19 and HNF4α. We specifically looked for hepatocytes that co-expressed CK19 and HNF4α, which would indicate a transitional state between cholangiocytes and hepatocytes. Our analysis revealed the presence of CK19+/HNF4α+ hepatocytes within regenerating liver (Fig. R2A), indicating that a subset of hepatocytes may arise from cholangiocyte in juvenile mice following PHx. Notably, these cells appear despite the fact that native hepatocytes remain highly proliferative after PHx (Fig. R2B).

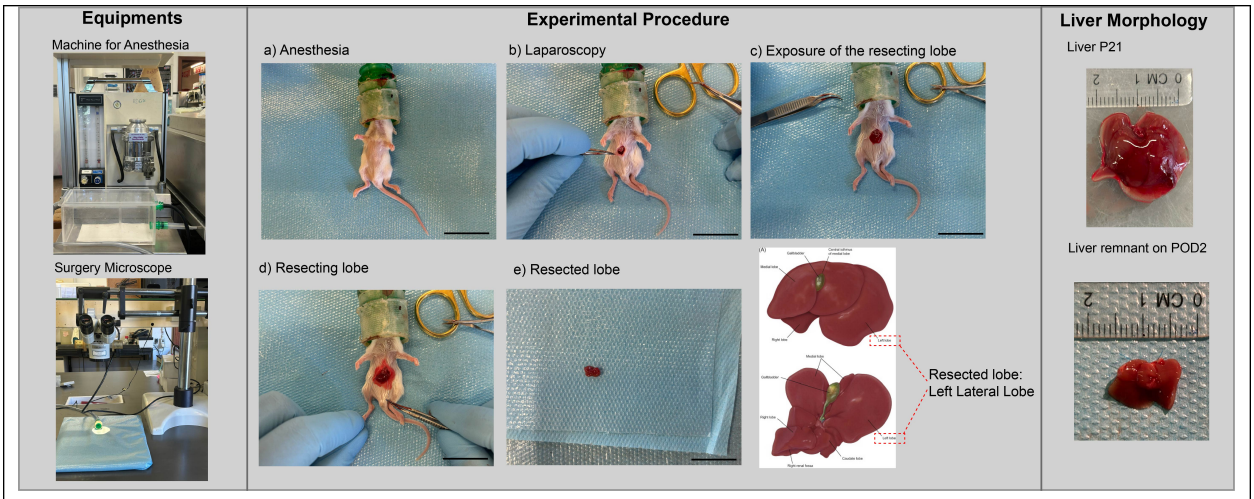

Figure R1. Schematic and representative images of the partial hepatectomy (PHx) procedure in juvenile mice.

(Left) Equipment setup for PHx, including an anesthesia machine (top) and a surgical microscope (bottom).

(Center) Stepwise surgical procedure:

(a) Induction of anesthesia and positioning of the juvenile mouse.

(b) Laparoscopy to access the abdominal cavity.

(c) Exposure of the left lateral liver lobe, which is targeted for resection.

(d) Surgical resection of the left lateral lobe.

(e) Isolated resected liver lobe post-PHx.

Schematic illustration of liver anatomy, indicating the location of the left lateral lobe, which is surgically removed during the procedure.

(Right) Liver morphology following PHx. P21 liver prior to surgery (top). Liver remnant on post-operative day 2 (POD2), illustrating the remaining liver tissue after partial resection (bottom).

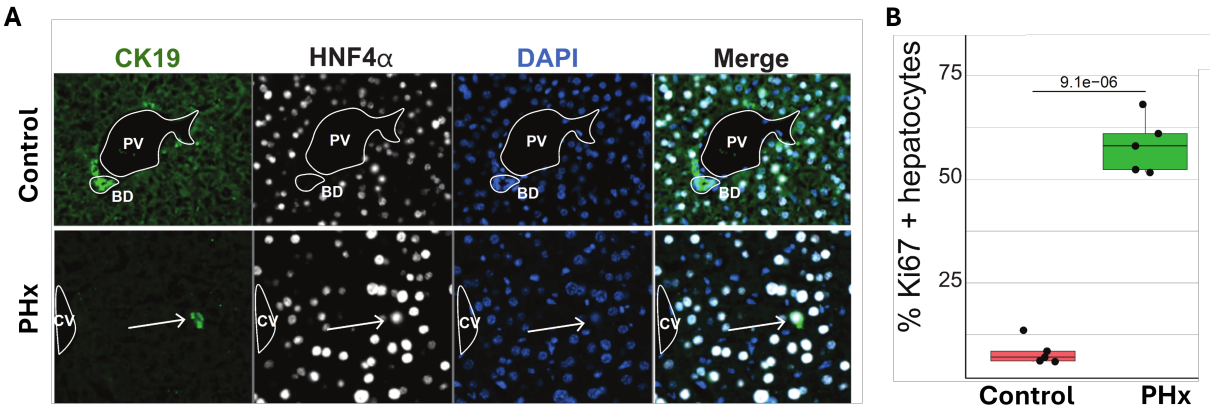

Figure R2: Cholangiocyte-to-hepatocyte contribution during liver regeneration in juvenile mice after PHx . (A) Representative immunohistochemistry images showing CK19 (green), HNF4α (white), and DAPI (blue) staining in liver sections from control P21 (CTL) and post-hepatectomy (PHx) mice at postoperative day 2 (POD2) following 50% (PH50) liver resection. White arrow indicates a CK19+/HNF4α+ double-positive hepatocyte. BD: bile duct; PV: portal vein; CV: central vein. **(B)** Quantification of Ki67+ proliferating hepatocytes in control and PHx mice, showing a significant increase in hepatocyte proliferation after PHx.

These findings, similar to our observations in zebrafish, **challenge the notion that cholangiocytes-to-hepatocytes transdifferentiation is strictly limited to adult individuals and occurs only when hepatocytes proliferation is impaired** . Instead, our data suggest that under specific conditions, such as postnatal liver development, cholangiocytes may contribute to hepatocyte regeneration even when hepatocyte-driven repair is still active. These findings support our hypothesis that **hepatic plasticity is enhanced during early postnatal growth** and that cholangiocytes may play a more

active role in liver regeneration when hepatocyte proliferation alone is insufficient. We acknowledge that definitive proof of transdifferentiation in mice requires lineage-tracing approaches, we are currently validating this observation using cholangiocyte-specific lineage tracing (osteopontin-iCreERT2 (4,5)) and single-nucleus RNA sequencing (snRNA-seq). These experiments aim to determine whether cholangiocytes directly contribute to hepatocyte regeneration in the postnatal liver. However, this investigation requires a comprehensive, standalone study and is beyond the scope of the current manuscript.

(End of Redaction)

Furthermore, some key tools used in this study are not optimal. For example, the her9 and her2 transgenes, because they label not only cholangiocytes but also other cell types such as satellite cells.

Regarding the specificity of our lineage-tracing tools, we would like to clarify that the *her2* transgene is specific to cholangiocytes (Fig. 5C) and does not label other liver cell types. Our validation experiments (Supplementary Fig. 7) show that **her2+ lineage cells predominantly mark cholangiocytes (93.7%)** , with minimal labeling of other cell types. This specificity strengthens our conclusions regarding the cholangiocyte origin of hepatocytes during regeneration.

Therefore, I would not support publication of this paper at Nature Communications.

Reviewer #3 (Remarks to the Author):

The authors addressed several prior reviewers' comments and made additional important observations. These new data strengthen the paper and enhance its novelty. However, I have additional questions and comments:

1. Cholangiocyte Contribution to Hepatocyte Regeneration

The main conclusion of the paper is that cholangiocytes contribute to hepatocyte regeneration during growth spurts in zebrafish, even when a significant number of hepatocytes remain after injury. Cholangiocytes are heterogeneous based on their localization, morphology, and gene signatures. Which cholangiocyte population(s) specifically contribute to hepatocyte regeneration in the two injury models described in the paper? Furthermore, the current data do not provide sufficient evidence to exclude the possibility that hepatic progenitor cells, rather than differentiated cholangiocytes, contribute to this regeneration. Additional experiments should be conducted. Otherwise, the main conclusion from this paper is too strong of a statement.

[The following Additional comment by the Reviewer was moved here by authors.]

Additional comments regarding critique 1 (Cholangiocyte Contribution to Hepatocyte Regeneration):

To date, all published work on zebrafish liver regeneration involving cholangiocyte-to-hepatocyte transdifferentiation relies on Tp1-Cre or Tp1-CreERT2 to lineage trace existing cholangiocytes. However, Tp1, a Notch-responsive element, is not a cholangiocyte-specific

promoter. It can also label hepatic progenitor cells. Additionally, the cholangiocyte markers commonly used in zebrafish studies, such as 2F11, alcam, and epcam, are not exclusively expressed in differentiated cholangiocytes. This represents a significant limitation of these studies, which should be properly addressed.

Determining whether hepatic progenitors or differentiated cholangiocytes give rise to regenerating hepatocytes would require the development of additional tools and may be beyond the scope of this paper. However, I believe the single-cell RNA-seq data presented by the authors could provide valuable insights into this question. Within the identified "cholangiocyte" population, are there distinct subgroups? Specifically, are there more differentiated cholangiocyte subpopulations that express cholangiocyte-specific transporters and metabolic genes? Conversely, are there "cholangiocytes" that appear less mature and exhibit more progenitor-like properties?

Acknowledging the heterogeneity within Tp1+ populations and recognizing that different subsets may contribute differently to hepatocyte regeneration is a critical first step toward understanding the key cellular players in this process. Thus adding additional analysis of the scRNA-seq data will strengthen the paper.

We appreciate the reviewer's insightful comments regarding the heterogeneity of the Tp1+ cholangiocyte population and the importance of further dissecting the cellular origins of transdifferentiation. Recent work from Jiarui Mi, Olov Andersson et al., currently posted on *bioRxiv* (6), has provided a refined classification of cholangiocyte

subpopulations in the zebrafish liver, identifying three distinct stages of maturity: Intrahepatic Duct (IHD) → Intermediate Duct → Luminal Duct. In this framework, IHD and intermediate duct cells are labeled by *her2*, while luminal ductal cells express *krt4*, representing the most mature cholangiocytes.

To further investigate this heterogeneity, we performed sub-clustering analysis on our control scRNA-seq dataset and identified four distinct cholangiocyte clusters: one enriched for *krt4* (luminal ductal cells), two clusters containing a mix of IHD and intermediate duct cells, and a proliferative cholangiocyte cluster (Supplementary Fig. 6A-E). To delineate the trajectory of cholangiocyte transdifferentiation, we conducted pseudotemporal analysis using Monocle, which revealed that IHD and intermediate duct cells are the primary source of transdifferentiation (Supplementary Fig. 6F-H).

Additionally, the results of the pseudotemporal analysis align well with our experimental findings using Cre/Lox-based lineage tracing with her2-CreER, demonstrating that IHD and intermediate duct cells contribute to hepatocyte regeneration upon injury, but not under homeostatic conditions. Importantly, these cells represent a bona fide cholangiocyte lineage and do not contribute to hepatocytes in the absence of injury.

In summary, our data suggests that transdifferentiation is driven by less mature cholangiocytes (IHD and intermediate duct cells). Future studies will be required to determine whether luminal cholangiocytes possess the capacity to alter their identity under specific conditions or if their fate is terminally differentiated. Thus, we have added this to the Discussion:

"Within the cholangiocyte population, our pseudotemporal analysis revealed that intrahepatic (IHD) and intermediate cholangiocytes serve as the primary source of cholangiocyte-to-hepatocyte transdifferentiation, while luminal cholangiocytes, represented by krt4 expression, do not appear to contribute (Supplementary Fig. 6). Future studies will be needed to determine whether luminal cholangiocytes retain the capacity to alter their identity under specific conditions or if they are terminally differentiated."

2. Comparative Analysis of Partial Hepatectomy (PHx)

The authors show that partial hepatectomy (PHx) induces cholangiocyte transdifferentiation at 10 days post-fertilization (dpf), whereas previous studies reported that PHx at 5 dpf or in adult zebrafish does not induce cholangiocyte transdifferentiation. It is important to point out that different transgenic zebrafish models were used in these studies, which may explain the differing outcomes.

We appreciate this point and have now explicitly addressed the differences in transgenic models used across studies in the Discussion Section:
"At 5 dpf, it has been shown that PHx does not induce cholangiocyte-to-hepatocyte transdifferentiation, similar to adult zebrafish. Notably, previous studies utilized *Tg(tp1:CreER)* for lineage tracing of cholangiocytes in 5 dpf larval and adult zebrafish, which may have influenced the observed absence of transdifferentiation."

3. Control for PHx Experiments

What control was used in the PHx experiments? A sham control should be included to ensure that the surgical incision and removal of the skin do not influence ventral lobe growth independently of the hepatectomy.

A sham control was performed to account for potential effects of the incision or skin removal. The data, presented in Supplementary Fig. 4I, demonstrate that the incision alone does not influence ventral lobe growth.

4. Measurement of Liver Size

The exclusion of compensatory growth after PHx is primarily based on lobe length measurements (Figure 4D–H). However, liver area or volume measurements would be more appropriate to assess liver size accurately.

We agree that liver volume measurements provide a more comprehensive assessment. While our imaging setup primarily allowed for 2D measurements, we have now performed additional volumetric quantifications at 11 dpi and updated the results in Supplementary Fig. 4J. The new quantifications demonstrate a significant reduction in liver volume after PHx as compared to uninjured or sham injured controls, further supporting our conclusions.

5. Identification of Her9-Lineage Cells

The authors concluded that 30% of her9-lineage cells are hepatic stellate cells. However, in Figure S8E, it is evident that all hand2:GFP+/Her9+ double-positive cells are located at the liver

surface within the mesothelium. These cells are not hepatic stellate cells. The authors should revise this conclusion accordingly.

We agree with the reviewer. *hand2+* cells include hepatic stellate cells, mesothelial cells, and perivascular cells. To ensure accuracy, we have revised the text and figure legend to describe the overlap between her9-lineage and *hand2+* cells without assigning a specific identity to these cells.

"Additionally, our single-cell RNA-Seq dataset revealed that *her9* is expressed in *hand2+* cells (Fig. 5C). Using the *TgBAC(hand2:EGFP)* line, which labels hepatic stellate cells, mesothelial cells and perivascular cells (7), we identified that 30.0% of her9-lineage cells were *hand2+* (Supplementary Fig. 9D, E)."

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Cholangiocytes contribute to hepatocyte regeneration after partial liver injury during growth spurt in zebrafish.

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We thank the reviewer for their continued engagement and thoughtful feedback. We have carefully considered each comment and have made additional revisions and clarifications to strengthen the manuscript. Below, we address the concerns in detail, incorporating new analyses and text modifications aimed at improving the rigor, clarity, and interpretability of our study.

For the revision, our response is in blue.

REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

The author's responses to reviewers' critiques have raised more questions as detailed below.

1. Newly generated data on liver regeneration after partial hepatectomy in juvenile mice.

The observation in Figure R2 neither supports nor refutes the authors' conclusion that cholangiocytes contribute to hepatocyte regeneration in juvenile mice following PHx. The percentage of CK19;HNF4α double-positive hepatocytes is not reported, nor is the number of animals analyzed. Moreover, in the absence of lineage tracing, the presence of these double-positive cells could also suggest that hepatocytes contribute to cholangiocyte regeneration after PHx.

We thank the reviewer for this important clarification. As noted in our previous revision, the mouse data were included **solely in response to Reviewer #1**, who had questioned whether the observed regenerative mechanism might be specific to zebrafish. We wish to emphasize again that **these mouse experiments are preliminary**, and we agree fully with the reviewer that lineage tracing is essential to draw any definitive conclusions.

We have only recently established the PHx procedure in postnatal mice and presented this early analysis to highlight a potential direction for future investigation. As the reviewer #3 rightly points out, the detection of CK19+/HNF4α+ cells without lineage tracing does not allow us to distinguish between cholangiocyte-to-hepatocyte or hepatocyte-to-cholangiocyte conversion. We are currently pursuing a dedicated study involving Opn:CreER-based lineage tracing and single-nucleus RNA sequencing to

resolve this question, but those experiments are outside the scope of the current manuscript.

Finally, these preliminary data are not part of the manuscript under consideration. However, we want to highlight that liver regeneration in the juvenile mouse remains an underexplored area, and we believe our new setup opens important avenues for comparative investigation.

2. The authors presented new scRNA-seq data demonstrating four cholangiocyte clusters in the control zebrafish liver: one enriched for krt4 (representing luminal ductal cells), two clusters comprising a mix of intrahepatic duct (IHD) and intermediate ductal cells, and a proliferative cholangiocyte cluster. Using pseudotemporal analysis, they proposed that IHD and intermediate ductal cells are the primary source of transdifferentiation.

However, the data in Supplemental Figure 6 raises more questions. In Fig. 6F, the "cholangiocytes rest" cluster (grey) appears to be more poised to transdifferentiation to hepatocytes than the other four cholangiocyte clusters, yet this cluster is not discussed in the text.

We thank the reviewer for pointing out the "cholangiocytes rest" and apologize for the lack of clarification. The "cholangiocytes rest" cluster corresponds to cholangiocytes derived from injured samples. Our subclustering analysis was specifically performed on control (uninjured) samples to identify distinct cholangiocyte states under uninjured conditions. These subclusters were then used to trace which populations in the uninjured liver might give rise to hepatocytes after injury.

However, to perform pseudotemporal trajectory analysis, the dataset must

contain a continuous sequence of states—from the starting population, through intermediate/transitional states (which arise only after injury), to the end point. Therefore, pseudotime inference was necessarily conducted using the entire scRNA-seq dataset, including both uninjured and injured samples.

Within this integrated dataset, the “cholangiocytes rest” cluster emerges from injured livers and represents a more heterogeneous population, which includes the transitional state. Our aim in the pseudotemporal analysis was to identify which uninjured subpopulation was most likely to initiate the transition toward hepatocyte identity. For this reason, we focused our analysis and interpretation on the uninjured cholangiocyte subclusters.

We have now updated the Methods and Figure Legend for Supplementary Figure 6 as follows to clarify this point. (**Edits in Bold**)

Methods

Subclustering and pseudotime trajectory analysis

For subclustering, cholangiocytes from uninjured (control) samples were subsetted from the dataset and processed following standard normalization procedures. Dimensionality reduction was performed by selecting the top 2,000 highly variable genes, followed by principal component analysis using the top 22 principal components. A neighborhood graph was computed with ‘n_neighbors=15’, and clustering was performed at a resolution of 0.4. Clusters enriched in acinar cell-specific markers (indicative of contamination) and those exhibiting high ribosomal and heat-shock gene expression (potentially damaged cells) were excluded from further analysis.

To reconstruct the differentiation trajectory, we used Monocle3 [76] **on the combined dataset of injured and uninjured livers, as pseudotemporal inference requires a continuum of cellular states that are not all present in control samples alone. Specifically, transitional or intermediate states appear only upon injury and were essential for trajectory learning.** Clustering was performed using the *cluster_cells()* function with a resolution parameter of 1e-4, and trajectory graph learning was conducted using the *learn_graph()* function with 'minimal_branch_len=10'. Cells were then ordered along the inferred trajectory using the *order_cells()* function with default parameters. The root node for pseudotime inference was manually defined within the IHD/intermediate cholangiocyte cluster from control samples to ensure biologically relevant reconstruction of the transdifferentiation process.

For visualization of subclusters within the cholangiocyte population, UMAP coordinates computed in Scanpy were used in place of the default Monocle3 layout to maintain consistency across all analyses. **The "cholangiocytes rest" cluster refers to the population of cholangiocytes derived from injured samples.**

Supplementary Figure 6 Legend

(F) UMAP visualization **of integrated single-cell data from both uninjured and injured livers, including cholangiocyte subclusters from controls (colored) and "cholangiocytes rest" (grey) from injured samples.** Hepatocytes are shown in green. (G) Pseudotime trajectory inferred using Monocle, showing progression from cholangiocytes to hepatocytes. **Analysis was performed on the full integrated dataset,**

including both uninjured and injured samples, to capture transitional states absent from control livers.

The authors also refer to their previous scRNA-seq study of the adult zebrafish liver, in which IHD and intermediate ductal cells were also identified. Notably, PHx at the adult stage does not lead to cholangiocyte-to-hepatocyte transdifferentiation. Comparing the transcriptomes of IHD and intermediate ductal cells in juvenile versus adult livers would be crucial for uncovering the distinct mechanisms underlying liver regeneration at these two stages.

We thank the reviewer for this insightful suggestion. In our original analysis, we had indeed performed a comparative transcriptomic analysis between cholangiocytes from 13 dpf larval and 13 mpf adult uninjured livers (Fig. 7A, B), which led to the identification of the mTORC1 pathway as being upregulated in larval cholangiocytes (Fig. 7C). This was validated by immunofluorescence and western blot analysis (Fig. 7D-F) Functionally, we showed that activation of this pathway in the uninjured context was sufficient to potentiate cholangiocyte-to-hepatocyte transdifferentiation after injury (Fig. 7G-I), highlighting a key regulator of regenerative plasticity.

However, as the reviewer correctly notes, our prior analysis was conducted on the entire cholangiocyte population, without distinguishing specific subtypes such as IHD/intermediate and luminal cholangiocytes.

To directly address this point, we have now performed a focused comparative analysis between the IHD/intermediate and luminal cholangiocyte clusters isolated from uninjured larval and adult zebrafish livers (Supplementary Fig. 11). This refined analysis identified 4415 genes significantly enriched in larval IHD/intermediate

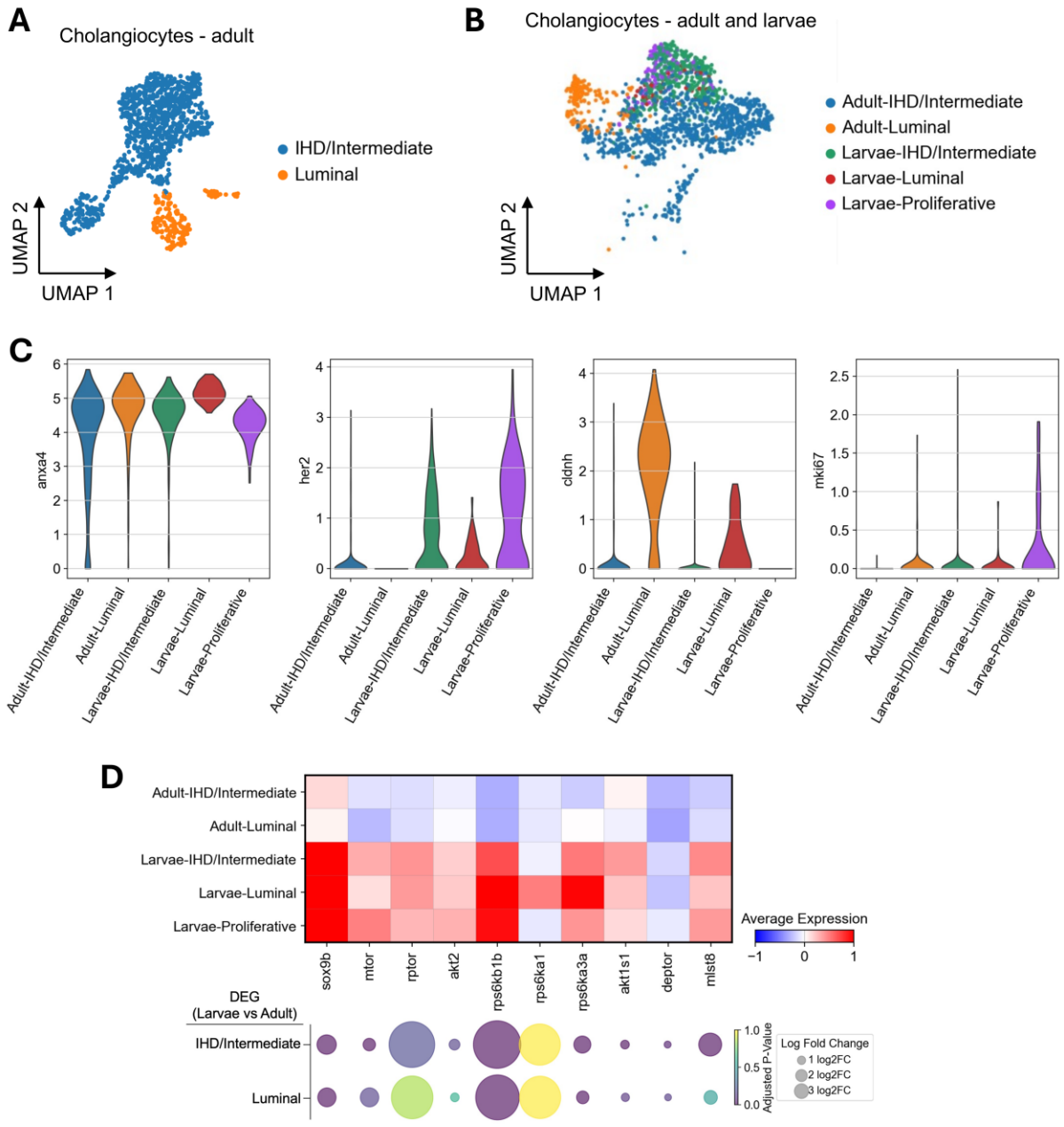
cholangiocytes (Supplementary Table 2), including multiple components of the mTORC1 signaling pathway (Supplementary Fig. 11D). Genes related to mTORC1 pathway were also upregulated in the luminal cholangiocytes from larval zebrafish (Supplementary Fig. 11D). These results validate and strengthen our original findings, now confirming that the differential activation of mTORC1 signaling is already present at the level of the IHD/intermediate cholangiocytes, which are potentially most relevant to transdifferentiation.

We have updated the Results section to reflect this new analysis and added the corresponding data to Supplementary Figure 11. The edits (in bold) are listed below:

Results (Page 17)

Furthermore, larval cholangiocytes exhibited upregulation of genes associated with the mTORC1 signalling pathway (Fig. 7C), which has been reported to be critical for the transdifferentiation of cholangiocytes [52–54]. **We also performed differential gene expression analysis of cholangiocyte subclusters (Supplementary Fig. 11A-C). Differentially expressed genes between larval and adult stage corresponding to the IHD/intermediate and luminal cholangiocytes are provided in Supplementary Table 2 and Supplementary Table 3, respectively. This analysis revealed that mTORC1 pathway activity-related genes are elevated in both IHD/intermediate and luminal cholangiocytes from larval livers compared to adults (Supplementary Fig. 11D-E).**

Supplementary Figure 11


A
Cholangiocytes - adult
IHD/Intermediate
Luminal
UMAP 2
UMAP 1
B
Cholangiocytes - adult and larvae
Adult-IHD/Intermediate
Adult-Luminal
Larvae-IHD/Intermediate
Larvae-Luminal
Larvae-Proliferative
UMAP 2
UMAP 1
C
anxa4
her2
cldnh
mki67
Adult-IHD/Intermediate
Adult-Luminal
Larvae-IHD/Intermediate
Larvae-Luminal
Larvae-Proliferative
D
Adult-IHD/Intermediate
Adult-Luminal
Larvae-IHD/Intermediate
Larvae-Luminal
Larvae-Proliferative
sox9b
mtor
rptor
akt2
rps6kb1b
rps6ka1
rps6ka3a
akt1s1
deptor
mlst8
Average Expression
DEG
(Larvae vs Adult)
IHD/Intermediate
Luminal
Adjusted P-Value
Log Fold Change
1 log2FC
2 log2FC
3 log2FC

Supplementary Figure 11: Comparative transcriptomic analysis of cholangiocyte subclusters from larval and adult zebrafish livers.

(A) UMAP visualization of cholangiocytes from uninjured 18 mpf adult zebrafish liver, showing subclustering into intrahepatic duct (IHD)/intermediate (blue) and luminal (orange) populations. **(B)** Integrated UMAP plot of cholangiocytes from 13 dpf larval and adult livers, color-coded by subcluster and developmental stage: Adult-IHD/Intermediate (blue), Adult-Luminal (orange), Larval-IHD/Intermediate (green), Larval-Luminal (red), and Larval-Proliferative (purple). **(C)** Violin plots showing expression of representative marker genes across cholangiocyte subclusters from larval and adult livers, including *anxa4* (general cholangiocyte), *her2* (IHD/intermediate), *cdh1* (luminal), and *mki67* (proliferative). **(D)** Top: Heatmap showing average expression of selected genes associated with mTORC1 signaling and cholangiocyte plasticity across each cholangiocyte subcluster. Bottom: Bubble plot showing differentially expressed genes (DEGs) related to the mTORC1 pathway between larval and adult cholangiocytes. Dot size represents the adjusted p-value, and color intensity reflects log2 fold change.

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