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Title: Reactive cholangiocytes differentiate into proliferative
hepatocytes with efficient DNA repair in mice with chronic liver injury

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Abstract: Background and Aim: Chronic liver diseases are characterized by expansion of the small immature cholangiocytes - a mechanism named ductular reaction (DR) - which have the capacity to differentiate in hepatocytes. We investigated the kinetics of DR differentiation into hepatocytes as well as several important features as functional maturity, clonal expansion and resistant to stress of the newly formed hepatocytes in mice with long-term liver damage.

Methods: We tracked cholangiocytes using osteopontin-iCreERT2 and hepatocytes with AAV8-TBG-Cre. Mice received carbon tetrachloride (CCl4) for >24weeks to induce chronic liver injury. Livers were collected for reporter proteins, cell proliferation and death, DNA damage, and nuclear ploidy analysis; hepatocytes were isolated for RNAseq.

Results: During liver injury we observed a transient DR and the differentiation of DR-cells into hepatocytes as clones that expanded to occupy 12% of the liver parenchyma by week 8. By lineage tracing, these new hepatocytes derived from cholangiocytes but not from native hepatocytes. They had all features of mature functional hepatocytes. In contrast to the exhausted native hepatocytes, these newly emerged hepatocytes had higher proliferation, less apoptosis, a lower proportion of highly polyploid nuclei and had better eliminated DNA damage.

Conclusions: In chronic liver injury, DR-cells differentiate into stress resistant hepatocytes that repopulate the liver. The process might account for the observed parenchymal reconstitution in livers of patients with advanced-stage hepatitis and can be a target for regenerative purpose.

Response to Reviewers:

Brussels, January 21, 2019

Dear Dr Sancho-Bru,
Dear Dr Lotersztajn,
Dear Dr Jalan,

First of all, we would like to thank you again for giving us the opportunity to address your and the reviewer's concerns regarding our study and to submit a revised version of the manuscript.

You had 2 main requests:

1. To discuss the specificity of the Cre expression in the OPN-CreER^{T2} transgenic mouse model we used,
2. To evaluate the senescence marker.

We carefully addressed these questions and we report below the results.

1. SPECIFICITY IN THE OPN-CreER^{T2} TRANSGENIC MOUSE MODEL and POTENTIAL EFFECTS OF LEAKY EXPRESSION OF Cre: We rigorously addressed the questions of specificity and potential leakiness of the transgenic mouse model used and now provide ample evidence that shows that:

- OPN is immunodetected only in cholangiocytes in the healthy liver;
- YFP is not found in untreated OPN-iCreER^{T2};Rosa26R^{YFP};
- In the healthy liver TAM drives Cre expression in cholangiocytes and not in any other cell type;
- Tamoxifen drives the expression of the reporter protein in cholangiocytes and not elsewhere.
- In the absence of tamoxifen, disease-induced OPN expression in hepatocytes and other liver cells does not drive transgene expression

The results of the experiments that allow these conclusions are discussed below.

Opn gene regulatory regions were selected to drive iCreER^{T2} expression¹. The Cre recombinase activity and the nuclear translocation, are modulated by a mutated ligand-binding domain of the estrogen receptor (ER), which is sensitive only to tamoxifen and not to the (endogenous) estrogens^{2,3}. Mice were crossed with the Rosa26R^{YFP} reporter mice or the Rosa26R^{mt/mG} reporter mice: in Rosa26R^{YFP} reporter mice a loxP-flanked stop cassette in the Rosa26R^{YFP} reporter locus is removed at a specific time-point and in a specific cell population by tamoxifen-inducible iCreER recombinase, thereby enabling

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expression of YFP. In the double fluorescent reporter mice Rosa26R^{mT/mG} all cells express membrane tdTomato fluorescence (mT) prior to Cre-mediated excision and membrane EGFP (mG) protein after excision, therefore cannot express simultaneously eGFP and tdTomato. So Cre recombinase will be activated and translocate into the nucleus to excise the stop cassette in front of the reporter gene allowing expression of fluorescence protein **if and only if** the two conditions of osteopontin promotor activation **and** presence of tamoxifen are met **at the same time**.

OPN is immunodetected only in cholangiocytes in the healthy liver. In the healthy adult mouse liver, OPN expression was found only in cholangiocytes whether lining the canals of Hering, the intralobular ductules and the interlobular bile ducts (BD)⁴. As we also reported elsewhere¹, OPN was only immunodetected in Ck19⁺/Sox9⁺ cholangiocytes but not in hepatocytes nor in any other liver cell type in the healthy liver (Figure 4A for the reviewers and ¹). This analysis is repeatedly performed in the control group of each experiment (cumulatively, more than 50 livers were examined over the years) with identical observations. Moreover, we sorted the various liver cell population from healthy OPN-CreER^{T2};Rosa26R^{mT/mG} mouse control liver (see M&M section in the manuscript) and found a high OPN gene expression in cholangiocytes, but it was not expressed in hepatocytes and barely detected in the other liver cell types (Figure 4B for the reviewers).

YFP is not found in untreated (no TAM-treated) OPN-iCreER^{T2};Rosa26R^{YFP}. In the liver of OPN-iCreER^{T2}; Rosa26R^{YFP} mice, OPN is strongly expressed in cholangiocytes but YFP was never detected whether in young or aging mice. This analysis is repeatedly performed in control group of each experiment and, cumulatively, more than 50 livers from mice of both genders were examined over the years. Specifically for this paper, the livers of 7 female and 5 male untreated (no TAM injection) OPN-iCreER^{T2};Rosa26R^{YFP} mice have been analyzed. Livers were harvested from the mice between 3-6 month of age. We found no YFP expression (Figure 5 for the reviewers and Suppl. Fig 1b in the manuscript).

In the healthy liver TAM drives Cre expression in cholangiocytes and not in any other cell type. We examined the livers of OPN-iCreER^{T2}; Rosa26R^{YFP} mice who did not receive TAM or 3 days after the last TAM injection and detected the Cre recombinase by immunostaining. As shown in Suppl. Fig. 1 in the manuscript, nuclear Cre recombinase was not detected in the livers of mice who did not receive TAM (Suppl. Fig.1a – noTAM - in the manuscript). Upon TAM injection, nuclear Cre was only detected in OPN⁺ cholangiocytes but not in any other liver cells (Fig.1a – TAM - in the manuscript).

Tamoxifen drives the expression of the reporter protein in cholangiocytes and not elsewhere.

OPN-iCreER^{T2};Rosa26R^{YFP} mice were sacrificed 3days after the last injection of tamoxifen, together with mice that did not receive tamoxifen. They were analyzed for OPN, ck19 and YFP expression: YFP expression was only found in cholangiocytes in mice injected with the tamoxifen (Suppl. Fig. 1 in the manuscript). Similar observations are made in OPN-iCreER^{T2};Rosa26R^{mT/mG} mice (Suppl. Fig.1c-d and Suppl. Fig. 2 in the paper).

Similarly, the livers of OPN-iCreER^{T2};Rosa26R^{YFP} mice who received 2 injections of TAM at post-natal days 21 and 23 (n= 6; 3M/3F) or no Tam injection (n=5; 2M/3F) were examined at 4 weeks post TAM injection. 2 sections across each liver lobe were screened in each liver: YFP was not detected outside the Ck19⁺ biliary cell population. The same livers were used to determine the efficiency of the system for labelling the cells of the biliary compartment and it was found that >85% of the biliary cells were tagged (Suppl. Fig. 1b in paper).

To analyse the specificity of the OPN-iCreER^{T2} further we took advantage of the Rosa26R^{mT/mG} reporter mice as they express membrane fluorescent proteins facilitating FACS analysis and FACS sorting procedures. One month after TAM injections, livers from tamoxifen-treated OPN-iCreER^{T2};Rosa26R^{mT/mG} mice were processed for FACS analysis⁵. Both, non-parenchymal cells and hepatocytes were analyzed. We used a FACSAriaII (BD) to isolate LSECs (CD32⁺F4/80⁻UV⁻PI⁻), macrophages (F4/80⁺CD32⁻UV⁻PI⁻), HSCs (UV⁺F4/80⁻CD32⁻PI⁻) and BECs (EpCAM⁺UV⁻CD45⁻PI⁻) from the non-parenchymal cell population. Hepatocytes were resuspended in 2mM EDTA + 1% FBS. All cell suspensions were analyzed for mTomato and mGFP expression using a FACSAriaII (BD). The gating strategy is detailed in the Suppl. Fig. 2 in the revised manuscript. Livers of 3 females and 3 male mice were analyzed. The majority of the cells in the EpCAM⁺ fraction were GFP⁺. No GFP⁺ cell types were found outside this EpCAM⁺ cell fraction (Suppl. Fig. 1c-d and 2 in the paper).

As no fluorescence protein expression is observed in the absence of Tamoxifen, even in aged and female mice, together our data strongly support that in a healthy liver Tam treatment is required to turn on YFP expression in OPN-iCreER^{T2};Rosa26R^{YFP} or mGFP in OPN-iCreER^{T2};Rosa26R^{mT/mG} mice. When this occurs fluorescent tag expression is restricted to OPN expressing cholangiocytes and no other cell type in the liver. Of note, in the experiments described in the paper TAM was injected at day 21 and 23 in otherwise healthy mice, naïve for other manipulations. Induction of liver injury started 4 weeks after, which is a sufficiently long to obtain a complete washout of Tamoxifen from the system⁶.

In pathologic states and ongoing liver injury, literature reports that besides biliary cells and cells of the ductular reaction various cell types express OPN, such as inflammatory cells (mainly lymphocytes)⁷ and injured hepatocytes⁸. In the long term CCl₄ model used in our study clusters of OPN⁺ hepatocytes were readily seen in the liver (Fig.1 for the reviewers and Suppl. Fig. 3 in the paper). To evaluate whether “ectopic and disease-driven” Osteopontin expression drives the cre activation, also during a long term injury, 4 females and 6 male OPN-iCreER^{T2}; Rosa26R^{YFP} mice without tamoxifen were treated using the same CCl₄ scheme as used in our study and analyzed for OPN and YFP: YFP expression was NEVER detected whether in cholangiocytes, hepatocytes or inflammatory cells (Fig. 1 for the reviewers). Some authors have described OPN gene expression in activated stellate cells/myofibroblasts during fibrogenesis^{9,10} but immunodetectable expression of OPN in activated-HSCs was not confirmed in publications by others^{9,10} nor in our hands in this model in other injury models we previously tested. Hence activated-HSCs co-expressing α SMA and YFP were not observed in injured OPN-iCreER^{T2};Rosa26R^{YFP} (Figure 6 for the reviewers).

In our hands and using the protocols described, we did not evidence leakage and therefore this OPN-iCreER^{T2} line proves to be an efficient and adequate system to follow the fate of cholangiocytes. Besides, we would like to point out that key observations were **duplicated in a totally unrelated model** in which all hepatocytes were labelled using AAV8-TBG-Cre (Fig. 3 in the paper).

We would like also to point out that the DR-derived hepatocytes population emerging in the periportal area during injury, as we identified using this system, comprises cells that are smaller, have a lower degree of ploidy, are more proliferative and express less stress response genes (see RNAseq data), further supporting that are not simply a fraction of the native hepatocytes.

2. SENESCENCE MARKERS: We provide now evidence that senescent markers are observed after CCl₄ treatment (from week 6 to the end of the experiment) in native hepatocytes and lasts during all the injury, showing that:

- In hepatocytes isolated from controls and CCl₄-treated livers, senescent cells expressing P21 are readily found after 6 weeks of treatment but not in controls and not in 4wks-treated livers.
- Senescence persists in the liver of mice chronically exposed to CCl₄ livers as demonstrated by the expression of associated-secretory phenotype (SASP) genes after 16 weeks of treatment and by western blot analysis of P21 on protein extracts from whole livers.

In our samples, the drop in proliferation coincides with a steep up-regulation of p21 and with a progressive rise in the expression of p16 which culminates at 16wk time point (Fig. 1G in the paper). At 4 weeks, while hepatocytes actively proliferate, expression of p21 and p16 is similar to that of control livers.

We tried several p21 antibodies on paraffin embedded liver in 3 different labs: one specialized in analysis of animal models, one specialized in cell cycle and one human pathology lab. Unfortunately, we were not able to obtain convincing IHC staining for p21 on the harvested liver.

Thus, to be able to analyze the senescence in the hepatocytes, we isolated fresh hepatocytes from CTL, 4wk- and 6wk CCl₄-treated wild type mice. A total of 7 mice were used (CTL n=2; 4w CCl₄ n=2; 6w CCl₄ n=3). Part of the isolated hepatocytes were cultured for 24 hours in DMEM/F12 + 10% FBS and used for immunofluorescence detection; the remaining were used to extract proteins for western blot analysis.

Senescence can only be detected in isolated hepatocytes from 6w CCl₄-treated mice:

We analyzed the isolated hepatocytes for HNF4 α and p21 expression as well as for SA- β -gal activity (Fig 12 for the reviewers). In all the groups >96% of the cultured cells were HNF4 α ⁺, confirming the high purity of the isolated population of hepatocytes. To evaluate the senescence, we first stained the cultured hepatocytes for p21: in the CTL as well as in the 4w CCl₄-treated hepatocytes, p21 was not expressed, while 4% of the 6w CCl₄-treated hepatocytes were p21⁺ (now Fig. 1 H-I in the revised manuscript). Similarly, SA- β -gal activity could only be visualized in the hepatocytes isolated from 6w CCl₄-

treated mice (Fig. 13 for the reviewers). To confirm these results, we also extracted proteins from CTL, 4w and 6w CCl₄ isolated hepatocytes and evaluated the p21 expression by western blot analysis. p21 was found expressed only in hepatocytes isolated from mice treated for 6w with the CCl₄ (Fig. 14A for the reviewers). To the best of our knowledge, there is not, except perhaps for p21, a specific marker of final senescence (negative in apoptosis, autophagy, or other cell degenerative process) that can be evaluated by means of IHC.

Senescence persists in CCl₄-treated mice as demonstrated by the expression of senescence associated-secretory phenotype (SASP) genes:

Using RNAseq data, we examined the expression of the SASP factors to evaluate the senescence also at 16w CCl₄ vs CTL: all the SASP genes were higher expressed in the 16w CCl₄ treated mice (Fig. 15 for the reviewers), giving confirmation of the senescence in the CCl₄-treated mice. Importantly, we also showed (Fig. 6D) that genes related to DNA damage and senescence (Fridman_senescence) are enriched in native hepatocytes vs DR-derived hepatocytes. For confirmation, we also extracted the protein from the OPN-CreER^{T2}CTL and treated mice and performed a p21 western blot analysis demonstrating that p21 increased during all the injury (now Suppl. Fig. 5 in the revised manuscript and Fig. 14 for the reviewers).

We provide a point-by-point response to the specific comments raised by the reviewers.

We are now proposing a revised version of the manuscript taking into account the reviewers' comments: The changes are underlined in the revised version that is now also enriched with new figures (Fig. 1H and 1I; Suppl. Fig. 5; Suppl. Fig. 14). Details of the changes made is described in the point-by-point response to the reviewers.

We believe that, by answering the editors' and reviewers' comments, we strengthen our message about the emergence of clones of "young" and resistant hepatocytes of biliary origin during chronic liver injury.

We thank you for considering our manuscript for publication in the Journal of Hepatology.

In the name of all authors

Isabelle A. Leclercq

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1) Submission

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| f) All authors to complete and upload an ICMJE conflict of interest form. | Y |
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2) Materials and methods

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| c) Identify animal species, number of animals used, strain, sex and age. | In the CTAT form and in methods and caption of each figure |
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b) Explain the sample size and how this size provides an adequate power to detect a pre-specified effect size.

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a. Protein, DNA and RNA sequences

Y

b. Microarray data

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Y – Access code in the CTAT

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CTAT methods

Tables for a “Complete, Transparent, Accurate and Timely account” (CTAT) are now mandatory for all revised submissions. The aim is to enhance the reproducibility of methods.

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1.1 Antibodies

Name	Supplier	Cat no.	Clone no.
γ -H2AX	Cell Signaling	9718	20E3
GFP	Abcam	Ab6673	
Ck19	Developmental Studies Hybridoma Bank (University of Iowa)	TROMA III	
Glutamine synthetase	BD Biosciences	610517	6
Ki67	Abcam	Ab16667	
CyclinD1	ThermoFisher	RB-9041-P0	
PHH3	Cell Signaling	9701	
β -catenin	BD Biosciences	610154	14
α -fetoprotein	Abcam	Ab46799	
HNF4 α	R&D system	PP-H1415	
CPS1	Abcam	Ab3682	
CYP2E1	Abcam	Ab28146	
P21	Santa-cruz biotechnology	Sc-6246	F-5
CEACAM	Lifespan Biosciences	LS-C106710	CC1

1.2 Cell lines

Name	Citation	Supplier	Cat no.	Passage no.	Authentication test method

1.3 Organisms

Name	Supplier	Strain	Sex	Age	Overall n number
OPN-iCreER ^{T2} , Rosa26-YFP	Available in the laboratory	CD1	M/F	>21days	90
OPN-iCreER ^{T2} , Rosa26-mTmG	Available in the laboratory	CD1	M/F	>21days	45
OPN-iCreER ^{T2} , Rosa26-Confetti	Available in the laboratory	C57	M/F	>21days	46 (8 in the paper)
Rosa26-YFP	Available in the laboratory	CD1	M/F	>21days	13
Rosa26-mTmG	Available in the laboratory	C57	M/F	>21days	10
CD1	Charles river		M/F	>21days	7

1.4 Sequence based reagents: primers

Name	Supplier	Forward	Reverse
Sox9	Invitrogen	CGGCTCCAGCAAGAACAAG	GCGCCCACACCATGAAG
Fn14	Invitrogen	GACCTCGACAAGTGCATGGA	CGCATCCCAGGCAGAAGT
EpCam	Invitrogen	CTGTCATTTGCTCCAAACTGGCGT	CGTTGCACTGCTTGGCTTTGAAGA
NCAM	Invitrogen	CCCAAATCACCTATGTAGAGAATCA	GGTCTCCGGAGGCTTCACA
Notch1	Invitrogen	GATGGCCTCAATGGGTACAAG	TCGTTGTTGTTGATGTCACAGT
Notch2	Invitrogen	TCTATCCCCGTCGATTTCG	GATGTGATCATGGGAGAGGATGT
Jagged1	Invitrogen	AACGACCGTAATCGCATCGTACTGC	CAGCCAAAGCCATAGTAGTGGTCATCAC
Hey1	Invitrogen	CCGACGAGACCGAATCAATAAC	TCAGGTGATCCACAGTCATCTG
RPL19	Invitrogen	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT
36B4	Invitrogen	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
β-Gus	Invitrogen	CTCATCTGGAATTTGCGCGA	GGCGAGTGAAGATCCCCTTC
P16	Invitrogen	CGTACCCCGATTTCAGGTGAT	TTGAGCAGAAGAGCTGCTACGT
P21	Invitrogen	CGGTGGAACCTTTGACTTCGT	CAGGGCAGAGGAAGTACTGG
CHK1	Invitrogen	GGATCACCATCCCAGACATT	AGACTCTGACATACCACCTGATGT
CHK2	Invitrogen	ATCGAGAGAAGCCGACACA	CAATGTAATAATCTTCCGCATCA
FANCI	Invitrogen	CTTTCTAAAATGACAAACCAGCAC	TGTCTGATCATCTCGTGGATT
GADD45	Invitrogen	GATGTGGCTCTGCAGATCCA	ATGTCGTTCTCG-CAGCAAAA
RAD51	Invitrogen	CGAGGGTTCAACACAGACC	CTGTCTACAATAAGCAGTGCATACC
BRCA1	Invitrogen	TTCACCAACATGCCCAAAG	AGCTCCTTACCACGGAAG

1.5 Biological samples

Description	Source	Identifier

1.6 Deposited data

Name of repository	Identifier	Link
Gene Expression Omnibus database	GSE114436	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114436 Here the token: ixabqsyszpgbnqh

1.7 Software

Software name	Manufacturer	Version
Image J	NIH Image	1.46v
Leica software	Leica Microsystem	
Kallisto	Lior Pachter's lab at UC Berkeley	V0.43.1
FastQC	Illumina	V0.11.5
R/BIOCONDUCTOR (package edger)		V3.20.9
RStudio	RStudio	1.1.473
R/BIOCONDUCTOR (package tximport)		V1.4.0
GSEA	Broadinstitute	3.0
Prism 5/7	GraphPad	5/7

1.8 Other (e.g. drugs, proteins, vectors etc.)

Tamoxifen	Sigma	T5648-5G
AAV8-TBG-Cre	PennVector Core	
DEN	Sigma	N-0258
Carbon Tetrachloride	Sigma-Aldrich	319961

1.9 Please provide the details of the corresponding methods author for the manuscript:

rita.manco@uclouvain.be

2.0 Please confirm for randomised controlled trials all versions of the clinical protocol are included in the submission. These will be published online as supplementary information.

N/A

Point by point response to the reviewer's comments

Reviewer 1:

1. The main point in this study is to follow the fate of biliary/DR cells. The authors used Osteopontin-iCreERT2 (OPN-Cre) mice to label biliary cells during chronic liver injury (CCl₄). However, although OPN normally exists in the bile duct epithelium physiologically in the liver, liver pathological expression of OPN was confirmed in Kupffer cells, hepatic macrophages and hepatic stellate cells (HSCs) in necrotic areas after carbon tetrachloride intoxication (1). In supplementary figure 3, OPN has appeared non-biliary expression under CCl₄ treatment compared with CK19 staining by consecutive section. Therefore, there's no sufficient evidence to support that OPN-Cre specifically label biliary cells, especially when mice received carbon tetrachloride. The author should carefully consider this point or the conclusion is not credible.

In the Suppl. Fig 3 are shown OPN-iCreERT²;Rosa26R-YFP mice that have NOT received tamoxifen injection but have undergone only chronic CCl₄ treatment (3 times per week for 6, 8 and 16 weeks). Indeed, as the reviewer pointed out, in an injured liver beside cholangiocytes of the bile ducts and of the ductular reaction, OPN was readily observed by immunohistochemistry in inflammatory cells as well as in hepatocytes. OPN staining appeared as intracytoplasmic granules in some portal hepatocytes and it appeared as a stronger and more regular cytoplasmatic staining in hepatocytes surrounded by inflammatory cells, some of which being also OPN+ (Suppl. Fig. 3 in the paper and Fig. 1 for the reviewers). YFP immunostaining (both DAB and fluorescence detection) was performed on consecutive sections from these livers. Sections in all liver lobes of 4 females and 6 male (noTAM) OPN-iCreERT²;Rosa26R-YFP treated only with CCl₄ were examined. Not a single YFP positive cell (whether cholangiocytes, inflammatory cells or hepatocytes) was found. (Suppl. Fig. 3 in the paper and Fig. 1 for the reviewers). Thus, in the absence of tamoxifen, OPN expression (whether physiologic or pathologic) does not drive transgene expression.

In order to not be mistaken in the interpretation of the results, two important points must be considered: the genetic of the mouse model and the timeline of the experiments.

- 1- *GENETIC MODEL: The OPN-iCreERT²;Rosa26R-YFP carries out 2 transgenes (Fig. 2A for the reviewers), one with the Cre-recombinase iCreERT² that is under the control of the OPN promoter and the second that has a reporter gene (in this study, YFP, mTmG or the confetti) located after a loxP-flanked STOP cassette. Important, the CRE-recombinase is a modified Cre-recombinase (iCre) that is activated by Tamoxifen and not by other (natural) estrogens. With this setting, two are the possible scenarios:*
 - a. in the absence of TAM, the Cre-recombinase is expressed only in the OPN+ cells (being cholangiocytes in a healthy liver or other liver cells in a diseased liver), but is inactive, located in the cytoplasm, unable to cut the STOP cassette and thus unable to turn on the expression of the reported gene (Fig. 2B and 3 (NoTAM) for the reviewers);*
 - b. Only upon exposure to TAM, the Cre-recombinase expressed in OPN+ cells will be translocated into the nucleus, activated and it will cut the STOP cassette. Thus, the reported gene will be expressed (Fig. 2C and 3 (TAM) for the reviewers). Of note, in a healthy liver, the OPN is expressed only in the biliary cells (Fig. 4 for the reviewers), which means that in a healthy liver the Cre-recombinase is only expressed in the OPN+ biliary cells. We can clearly state this because:*
 - *after TAM in otherwise untreated mice (so, OPN only expressed in the biliary cells), the reporter gene was only expressed in the biliary cells (Suppl. Fig. 1b-c-d and Suppl. Fig. 2 in the paper).*

- in noTAM OPN-iCreER^{T2};Rosa26R-YFP mice, the reporter gene (YFP, mG or Confetti) has NEVER been detected in CTL (no CCl₄) as well as in CCl₄ treated mice (Suppl. Fig. 1b (noTAM), Suppl. Fig. 3 in the paper; Fig. 1 for the reviewers).
Moreover, in injured OPN-iCreER^{T2};Rosa26R-YFP, activated-HSCs co-expressing α SMA and YFP were not observed (Fig. 6 for the reviewers).

2- **TIMELINE:** It may take a few days for the organism to eliminate TAM, but no trace of tamoxifen have been found 4 weeks after the injection (Reinert et al., Plos One, 2012, DOI: 10.1371/journal.pone.0033529). Thus, if injury is induced before tamoxifen clearance, transgene expression may also occur in injured hepatocytes, reactive inflammatory or stellate cells or any other cell type with pathological expression of OPN. In the experiments described in the paper, TAM was injected at postnatal day 21 and 23 in otherwise healthy mice, naïve for other manipulations. Induction of liver injury started always minimum 4 weeks after the last injection, which is a sufficiently long time to obtain a complete washout of Tamoxifen from the system, which means that the CRE-recombinase that could become expressed in cells with pathological expression of OPN will not be activated. In support to this, no YFP+ hepatocytes, inflammatory cells or HSC were found in the liver of OPN-iCreER^{T2};Rosa26R-YFP mice that received a single CCl₄ high dose 4 weeks after Tam injection (Español-Suner, Gastroenterology, 2012 DOI: 10.1053/j.gastro.2012.08.024).

Hence, in OPN-iCreER^{T2};Rosa26R-YFP mice the Cre recombinase will be activated and translocate into the nucleus to excise the stop cassette in front of the reporter gene allowing expression of fluorescence protein **if and only if** the two conditions of osteopontin promotor activation **and** presence of tamoxifen are met **at the same time**.

Given (1) that YFP was not detected in OPN+ cells (whether cholangiocytes, hepatocytes or other cell types) in no TAM- OPN-iCreER^{T2};Rosa26R-YFP mice (being naïve of insult or (repeatedly) exposed to CCl₄), and (2) that, no cells other than cholangiocytes (and in particular no hepatocyte) expressed YFP in the liver of TAM- OPN-iCreER^{T2};Rosa26R-YFP mice whether naïve or after a single dose of CCl₄ (Español-Suner, Gastroenterology, 2012 DOI: 10.1053/j.gastro.2012.08.024), we have absolutely no argument to support leakiness.

In addition, when hepatocytes are traced using the AAV8-TBG-Cre system, patches of small hepatocytes (not traced as of hepatocyte origin) arise in the liver. The characteristics of the cells, the location, kinetics of appearance and of growth of the patches are similar to those traced as of DR origin in OPN-iCreER^{T2};Rosa26R-YFP mice.

Thus during chronic injury, the liver is repopulated by clonal expansion of small hepatocytes with specific characteristics of “undamaged hepatocytes” ascribed to their “youth” or at least up to then preserved from the ambient toxic damaging environment. Our data (as other data in the literature) collectively support a biliary origin for these cells, refute a hepatocyte origin, and bring no support for a non-parenchymal cell origin (an extrahepatic origin has not been explored).

2. The authors showed quantification of the Ki67 index and PHH3 index in YFP+ and YFP- hepatocytes by immunofluorescence staining. However, the authors need to use hepatocyte markers to localize hepatocytes in order to make the results more convincing.

In Fig 7 and 8 for the reviewers we showed representative double IF for ki67/HNF4 α and PHH3/HNF4 α . For quantification, we identified hepatocytes on the basis of cell and nuclear morphology : They are readily distinguishable from NPC and infiltrating immune cells by their size, shape and large round nuclei. We stained consecutive sections for ki67 and YFP or for PHH3 and YFP (Fig. 9 for the reviewers): on scanned sections, we delimited YFP area (Fig 9 for the reviewers), reported it on the ki67 (or PHH3) section and quantified Ki67+ (or PHH3+) hepatocytes in the YFP+ and YFP- populations, as already mentioned in the Methods section. For each liver slide, 5 fields (10x magnification) were counted.

As shown in representative images in Fig.10 for the reviewers, hepatocyte nuclei are clearly distinguishable from NPC nuclei and brown Ki67⁺ nuclei are clearly distinguishable from Ki67⁻ nuclei. Because of inconsistent quality of the triple IF staining (YFP/Ki67/HNF4a) within a single section and between different slides, the methods we chose was in our hand more reliable for quantification.

3. In supplementary figure 4, the author should provide control groups to be consistent with figure 1e.

As the reviewer suggested, we added a representative picture of a liver section from CTL OPN-CreER^{T2};Rosa^{YFP} stained for Ki67 IHC.

4. Most of the quantification data does not come with representative immunofluorescence or immunohistochemistry figures. It is difficult to discern which marker used and what experimental procedures are used, which makes the experiment is difficult to track.

We refer to answer to question 2. Moreover:

- *representative pictures for γ H2AX can be found in supplementary Fig. 16,*
- *in Fig. 11 for the reviewers, you can find representative pictures for TUNEL and ploidy analysis. The ploidy analysis has also been added as Suppl. Fig. 14 in the revised manuscript.*

5. The authors need to explain the meaning of the red arrow and the white arrow in figure 7e.

We added now in the Figure's legend the meaning of the arrows. Specifically, the red arrows indicate the YFP⁺ Ki67⁺ DR-derived hepatocytes. On the contrary, the white arrows point to the YFP⁻ Ki67⁺ native hepatocytes.

6. The authors claimed that the emergence of YFP⁺ hepatocytes-like cells after 6wks CCl₄ coincided with a marked drop in proliferative activity of native hepatocytes as assessed by Ki67 index (Fig. 1e and Suppl. Fig. 4), as well as with an increased expression of p16 and p21 Senescence markers and of the checkpoint kinases 1 (CHK1) and 2 (CHK2) (Fig. 1g). However, figure 1g showed p16 expression was only significantly increased in 16wks CCl₄ treatment; p21 expression was only significantly increased in 6 wks CCl₄ treatment; changes of CHK1 and CHK2 expression were not statistically significant.

As shown by others, p21 appear as an early event in the senescence program while p16 is rather representative of later events (Alcorta et al., PNAS 1996, 93(24): 13742–13747).

To strengthen the analysis of senescence, we performed additional experiments.

- a. *we isolated hepatocytes from CTL, 4 and 6 weeks CCl₄-treated mice. >96% of the isolated cells expressed HNF4 α (Fig. 12A-B for the reviewers), showing the purity of these population. After 24 hours in culture, we performed p21 immunocytochemistry: CTL and 4 weeks CCl₄-treated hepatocytes were found negative for p21, while 4% of 6 weeks CCl₄-treated hepatocytes were p21⁺ (now Fig. 1H-I in the revised manuscript). Similarly, the SA- β -gal activity was only detected in the 6 weeks CCl₄-treated hepatocytes (Fig. 13 for the reviewers). Moreover, from these populations we also extracted the proteins for western blot analysis. p21 was only detected in the 6 weeks CCl₄-treated hepatocytes (Figure 14A for the reviewers). These data are now included in the manuscript as figure 1H-I, in place of the CHK1/2 PCR graph because we indeed found the P21 data as more relevant. For your information, we also evaluated P21 protein expression in whole livers by western blot in some samples (Suppl. Fig. 5 in the revised*

manuscript and Fig. 14 for the reviewers). Thus, we confirmed hepatocyte senescence concomitant to a drop in hepatocyte proliferation at the 6 weeks' time-point.

- b. Using RNAseq data, we examined the expression of the SASP genes to evaluate the senescence in hepatocytes from 16w CCl₄ livers vs CTL: the expression of all the SASP genes was higher in the 16w CCl₄ treated mice than in controls (Fig. 15 for the reviewers), confirming the senescence in the CCl₄-treated mice. Importantly, we also showed (Fig. 6d) that genes related to DNA damage and senescence (Fridman_senescence) are enriched in native hepatocytes vs DR-derived hepatocytes.*

Reviewer2:

1. one minor factual point in the intro...the Raven paper also uses p21 over expression and multiple injury models- not all sub-acute!

Thank you for the comment. We now changed the text in the manuscript, adding: “, or the overexpression of p21 in conjunction with induction of injury using DDC, MCD and TAA²⁴”.

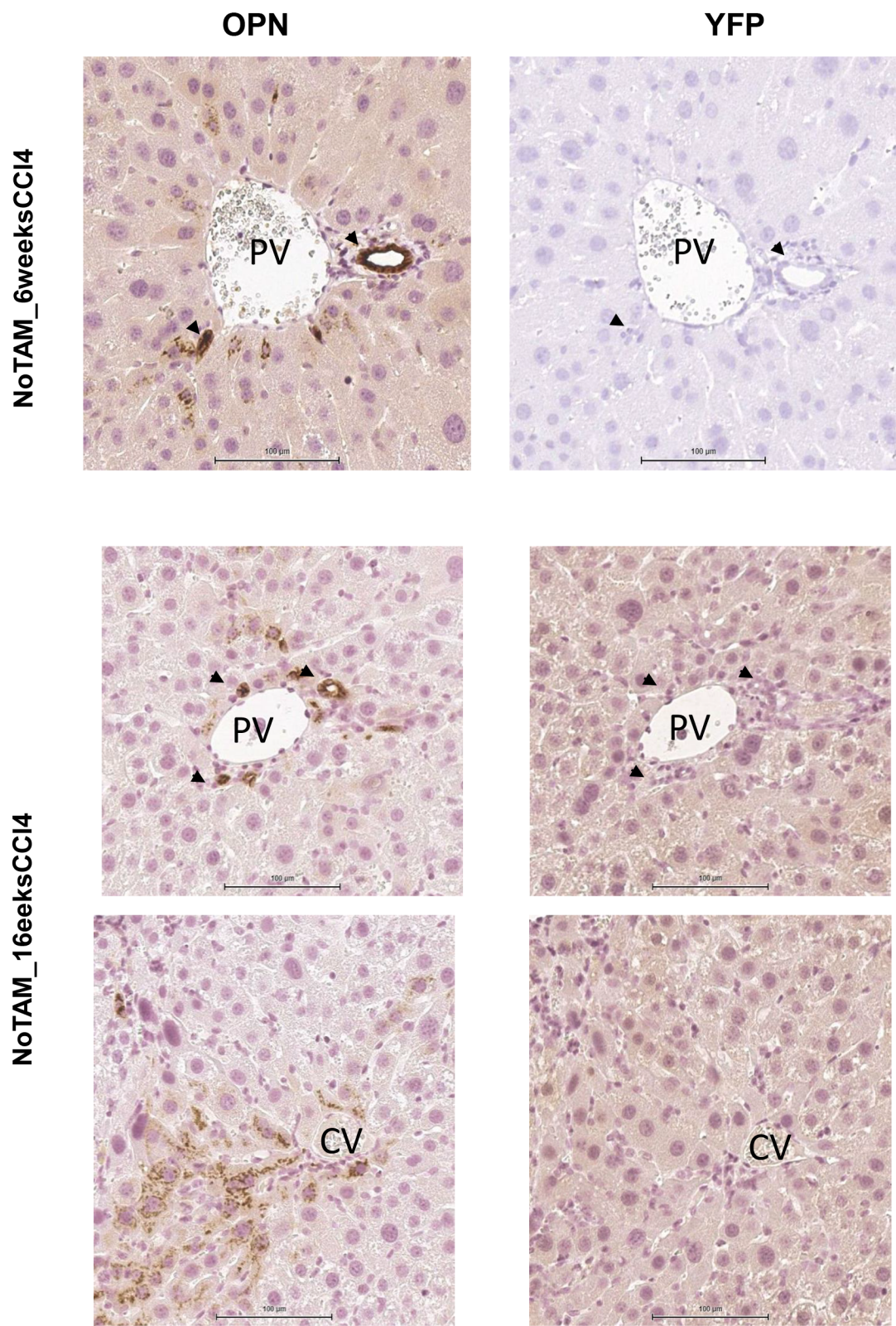


Figure 1. OPN-iCreERT²;Rosa26R^{YFP} that did not receive tamoxifen were treated for 6 or 16 weeks with CCl₄ (3x/week). Representative images of liver stained for OPN and YFP. Scale bar: 100μm
PV: portal vein; arrowhead: bile duct. CV: central vein

Figure 2 for the reviewers

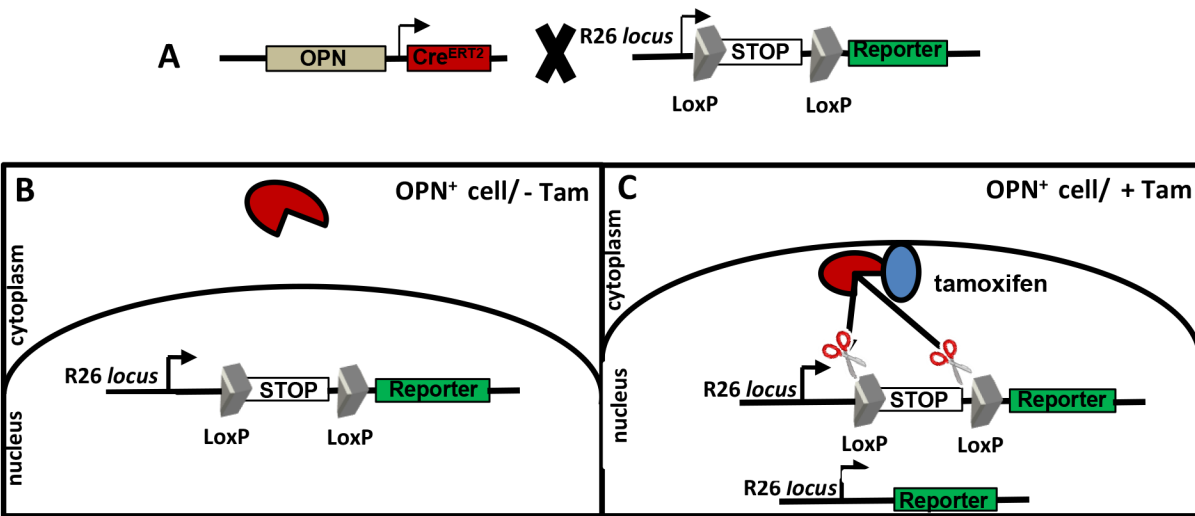


Figure2: The Cre-loxP system in OPN-CreER^{T2};reporter mouse model. (A) representation of the two transgenes present in the model; (B) without Tam, the Cre recombinase is located in the cytoplasm of the OPN positive cells, where it is not active; (C) after Tam administration, the Cre-recombinase will be translocated from the cytoplasm to the nucleus and will catalyze the removal of the STOP cassette, flanked by loxP sites.

Figure 3 for the reviewers

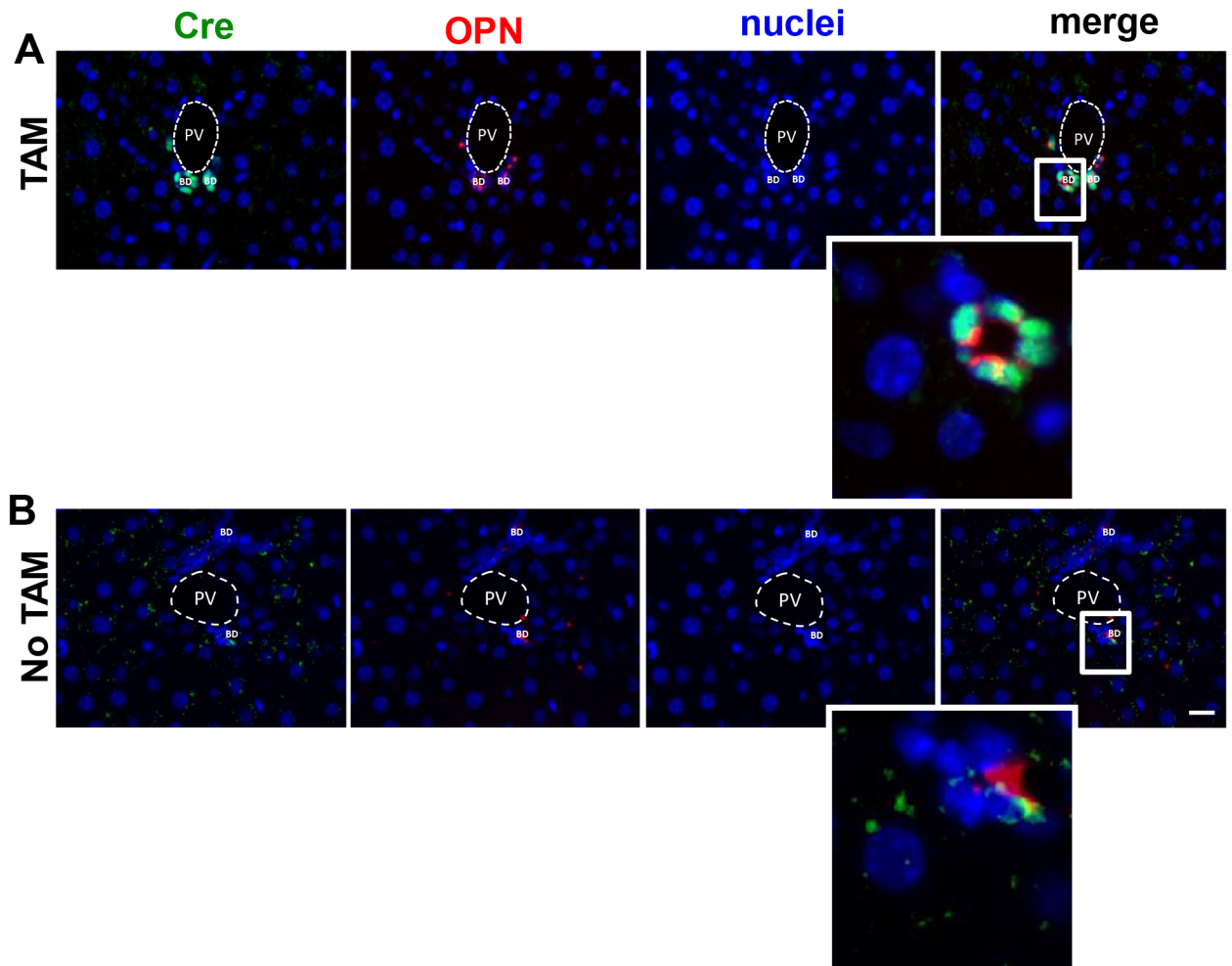


Figure 3. Nuclear Cre recombinase is expressed only in OPN⁺ cells and only after tamoxifen injections. Representative images of liver sections from OPN-iCreER^{T2} with (A) or without (B) tamoxifen stained for Cre and OPN. Scale bar: 20μm
PV: portal vein; BD: bile duct

Figure 4 for the reviewers

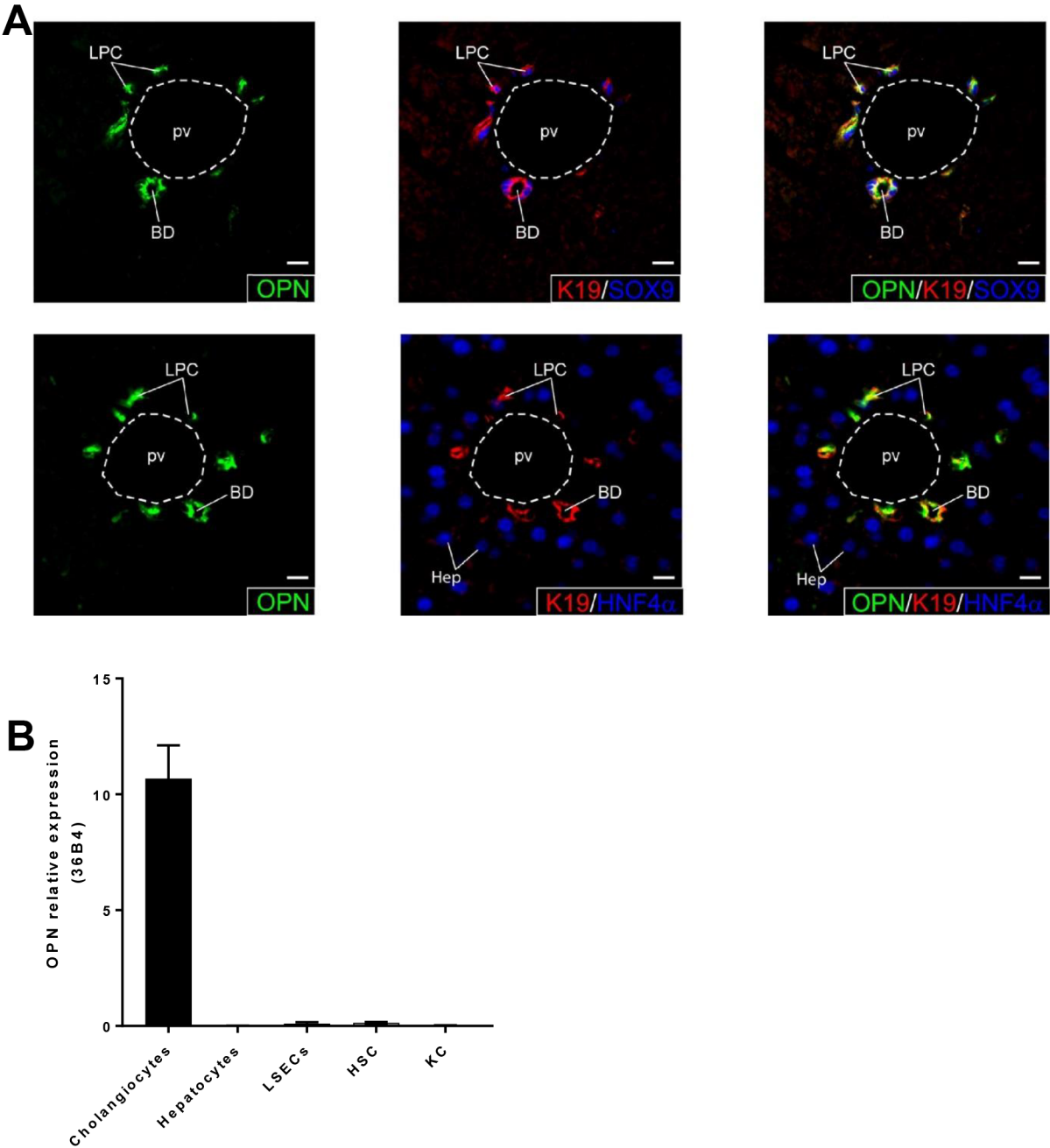


Figure 4. Osteopontin is expressed in cholangiocytes of the bile ducts and liver progenitor cells in healthy mouse livers. Liver was harvested from untreated **3 month old** adult male and female C57B1/6j mice and stained for OPN, ck19, Sox9 and HNF4 α . OPN expression is only observed in ck19⁺ and Sox9⁺ cells, and never in HNF4 α ⁺ cells. (A) Representative pictures from a female mouse liver. LPC, liver progenitor cells; size bar=20 μ m. (B) Osteopontin gene expression in cholangiocytes, hepatocytes, LSECs, HSC and KC sorted from TAM-treated OPN-iCreER^{T2};Rosa26R^{mT/mG} mouse livers.

Figure 5 for the reviewers

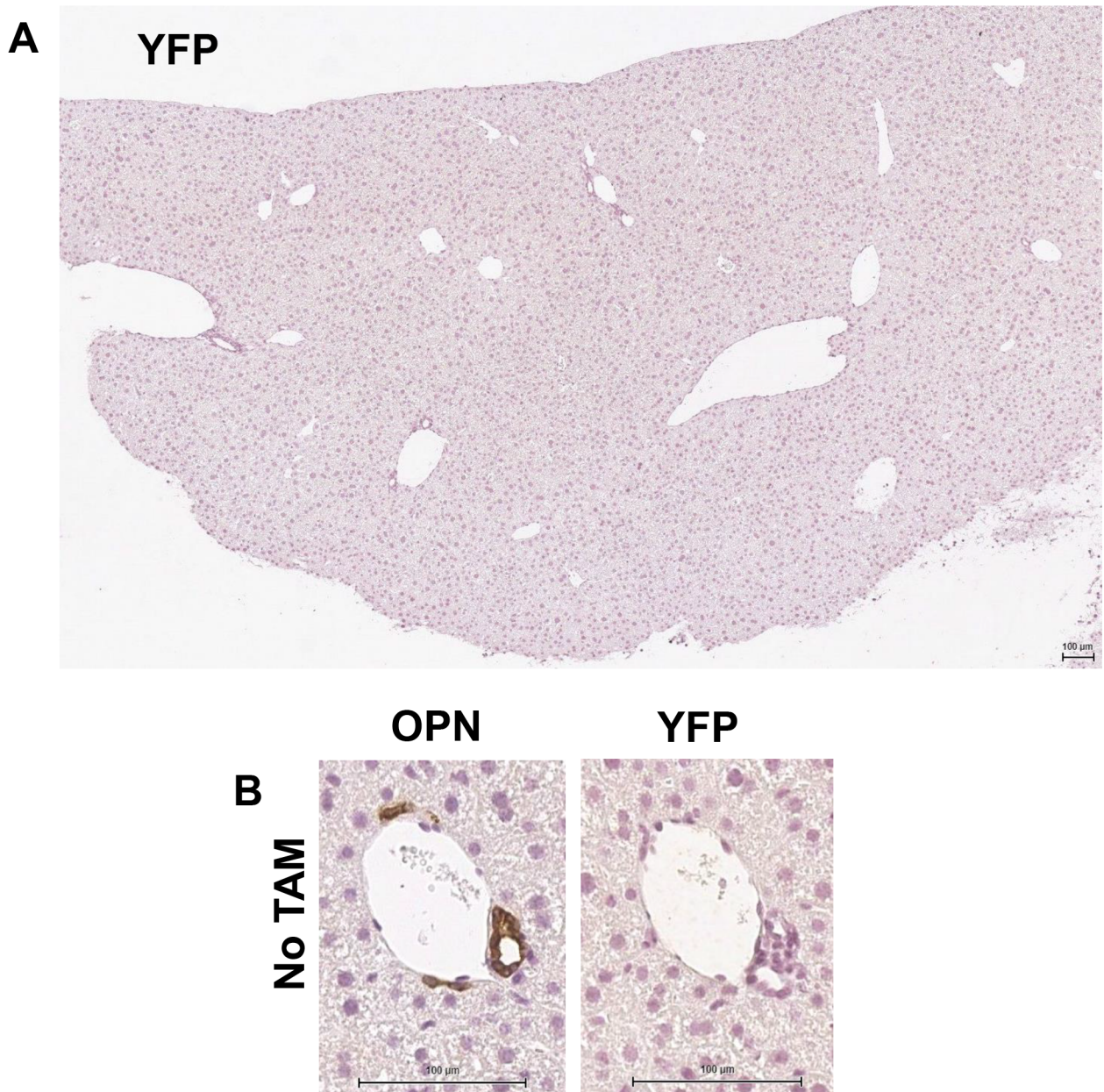


Figure5. (A) Low power view of a liver section from a 3 month old female OPN- $iCreER^{T2};Rosa26R^{YFP}$ without Tamoxifen injection stained with YFP antibody. (B) Representative pictures of consecutive sections from the same mouse liver stained for Osteopontin (OPN) and YFP. Scale bar =100 μm.

Figure 6 for the reviewers

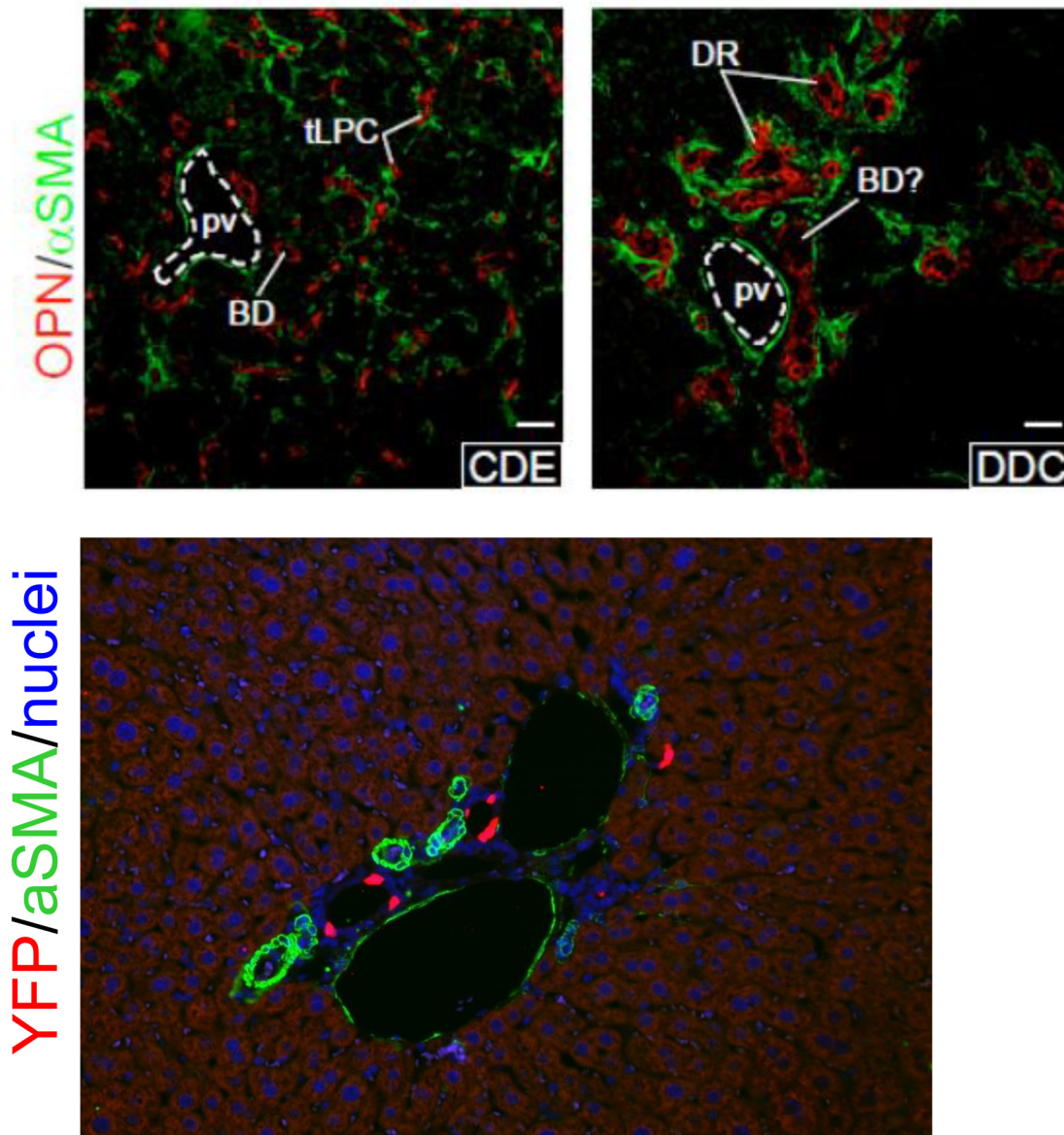
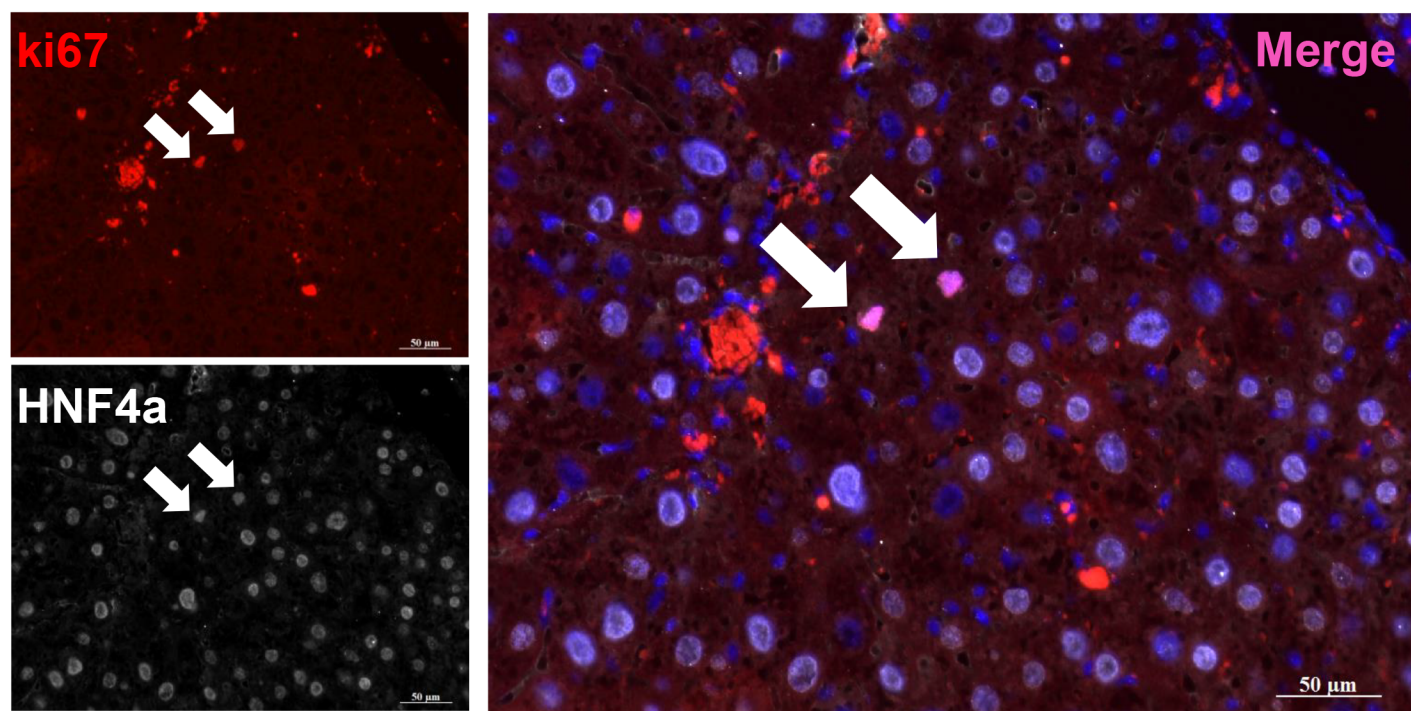


Figure 6. Tamoxifen-treated OPN-iCreERT²; Rosa26R^{YFP} mice received CDE or DDC diet for 3weeks. No OPN expression was found in αSMA⁺ cells upon liver injury. Representative pictures stained for (A) OPN and αSMA and (B) YFP and αSMA. Scale bar 20 μm. tLPC: transit-amplifying cells, DR: ductular reaction.

Figure 7 for the reviewers

8w



16w

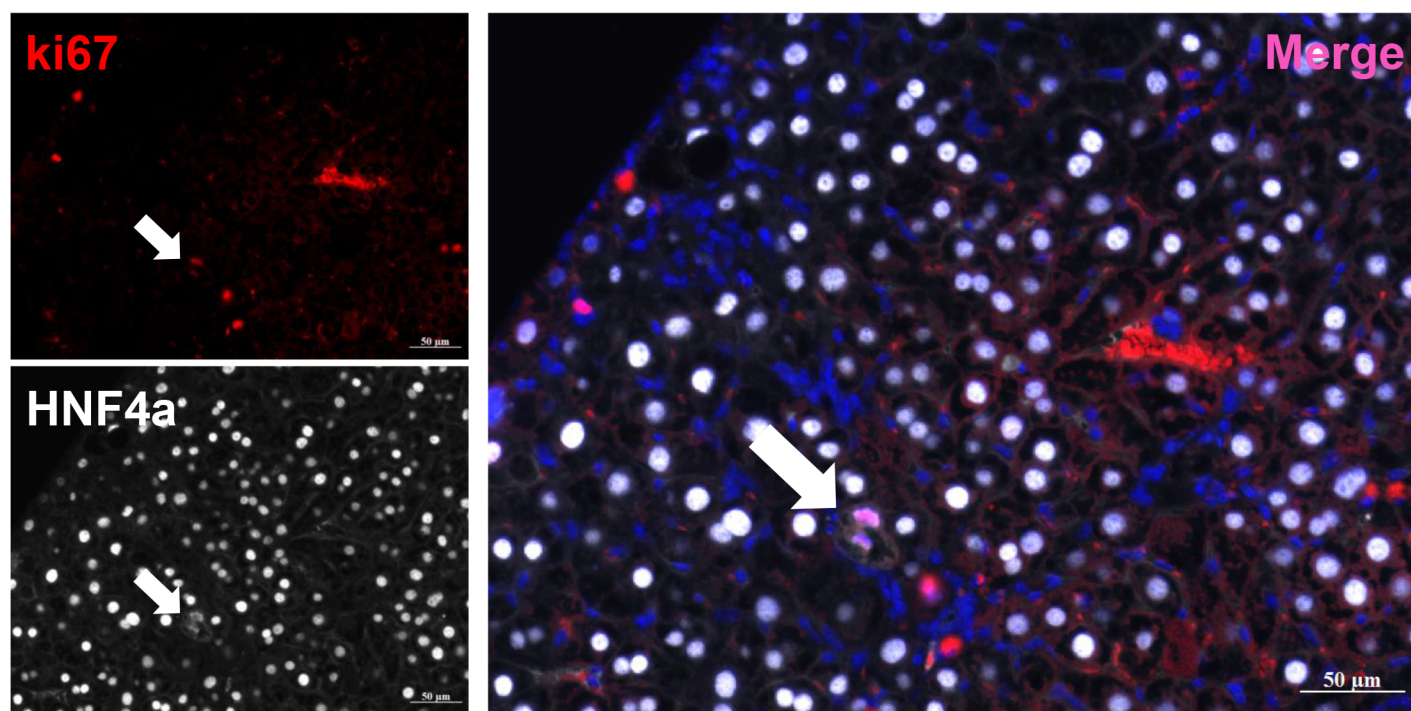
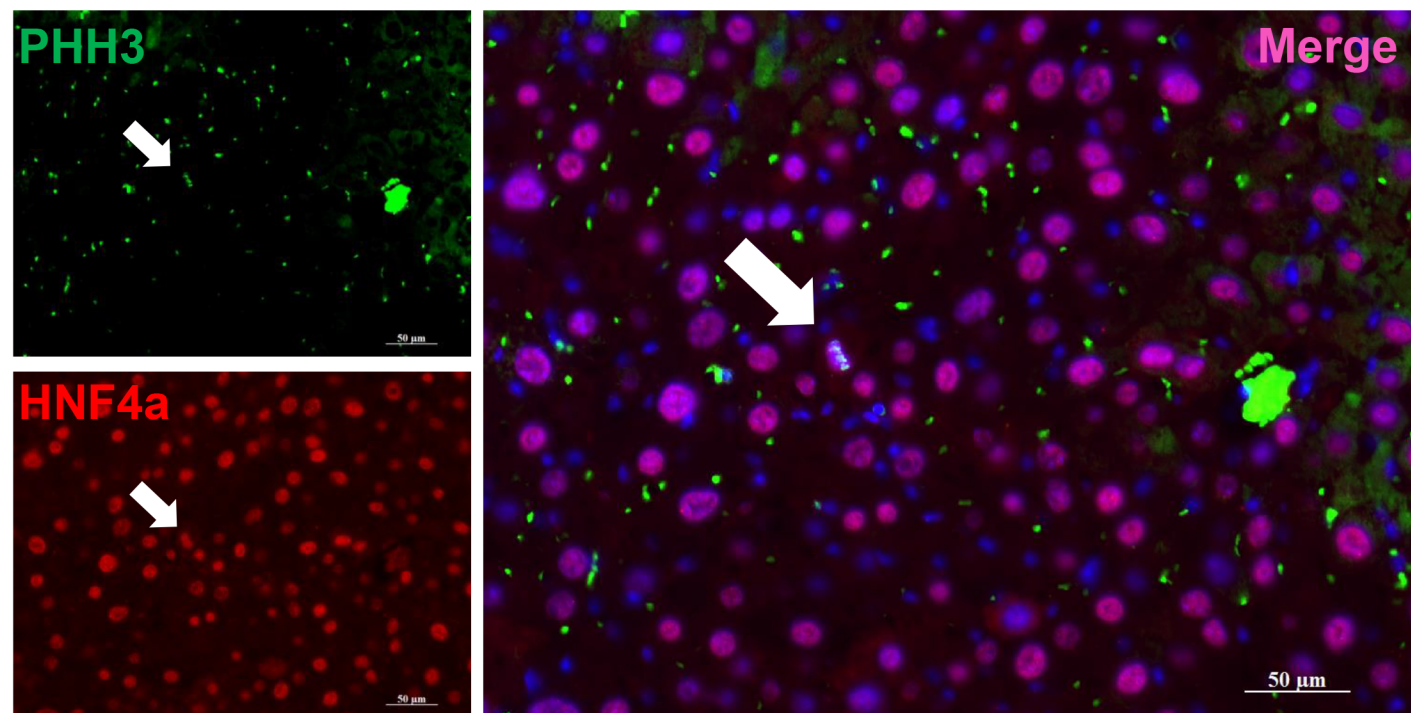


Figure 7. Representative images of liver from OPN-iCreERT²;Rosa26R^{YFP} mice treated for 8 and 16weeks with CCl₄. stained for Ki67/HNF4a. White arrows point to the ki67+ hepatocytes. Scale bar: 50µm

Figure 8 for the reviewers

8w



16w

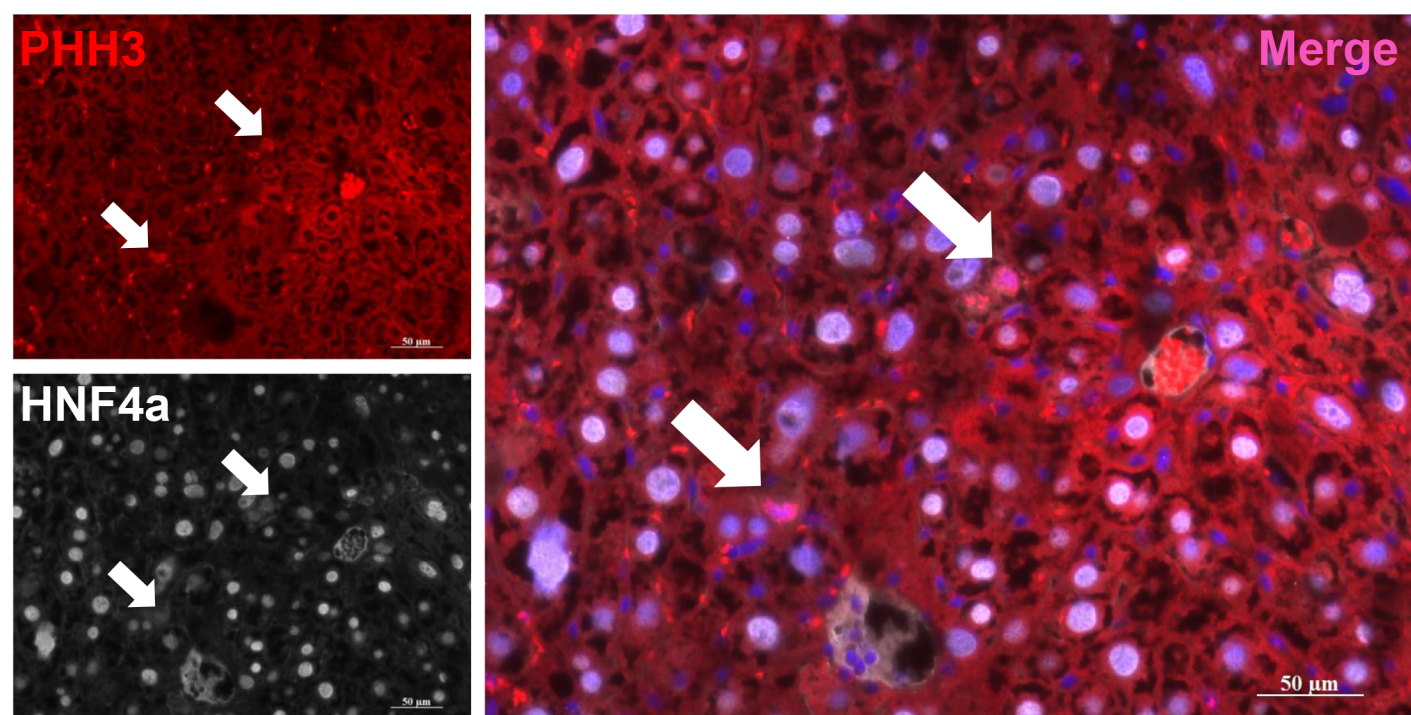
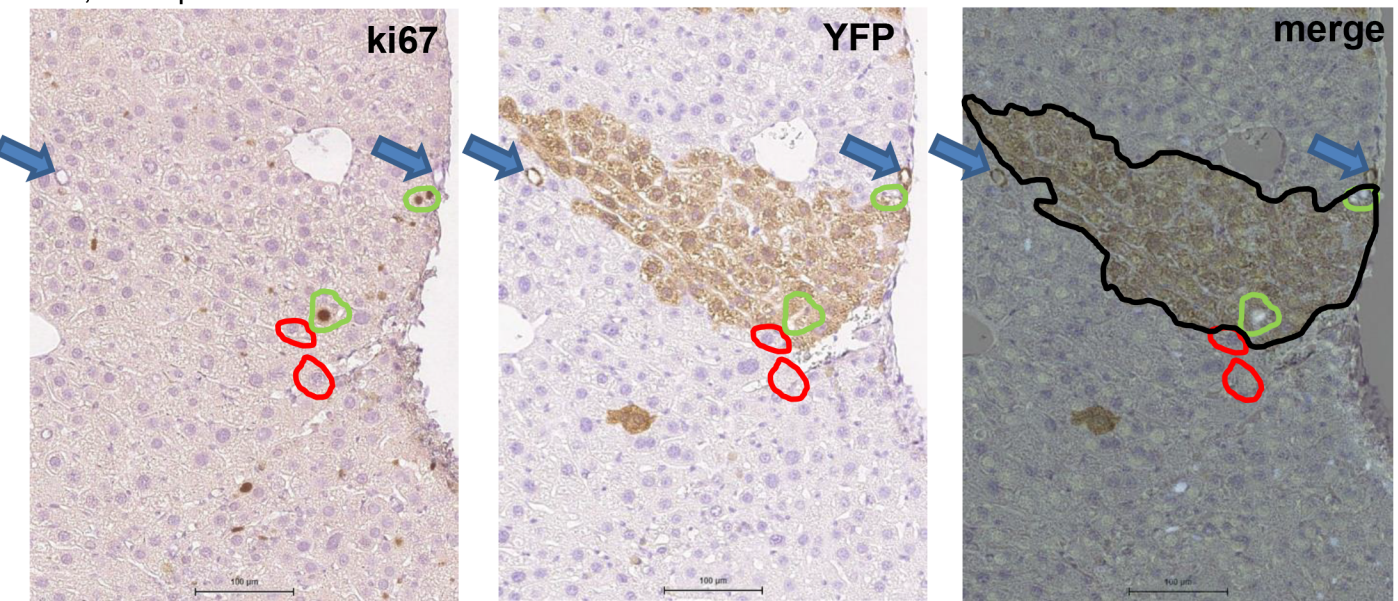


Figure 8. Representative images of liver from OPN-iCreERT²;Rosa26R^{YFP} mice treated for 8 and 16weeks with CCl₄. stained for PHH3/HNF4a . White arrows point to the PHH3+ hepatocytes. Scale bar: 50 μ m

Figure 9 for the reviewers

A

Ki67; 8w: 4µm slides



B

PHH3; 8w: 4µm slides

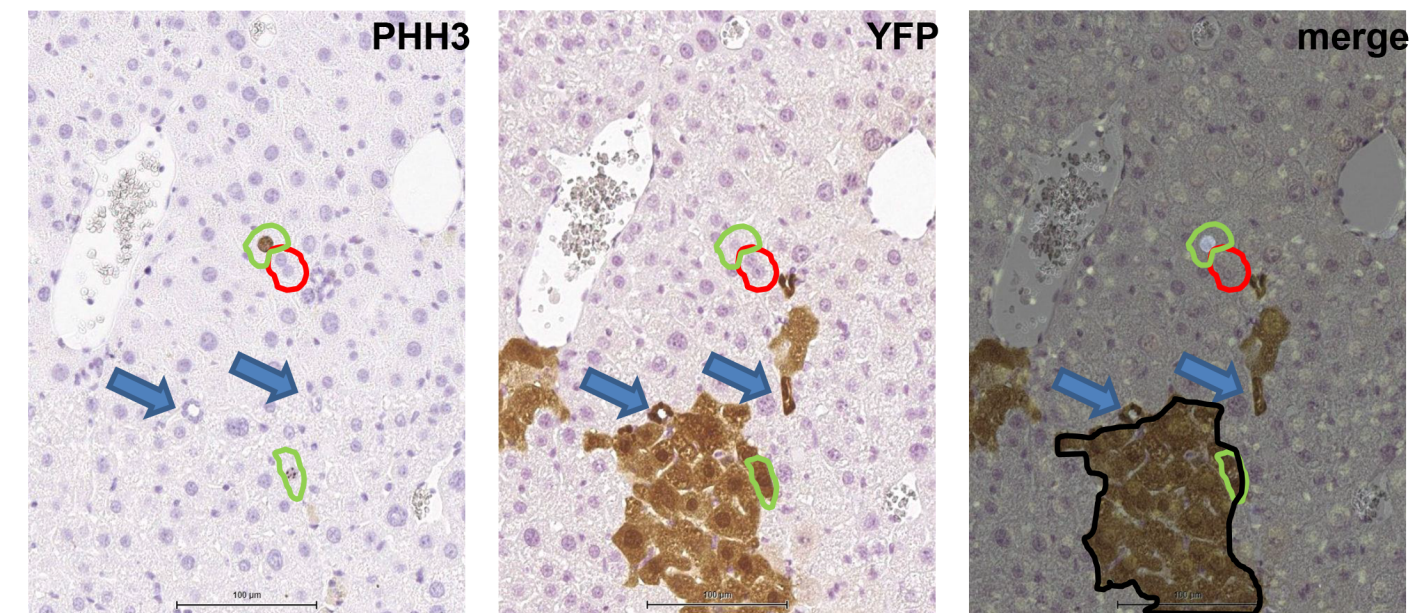


Figure 9. Representative images of consecutive 4 µm slides from liver of OPN-*iCreERT²*; *Rosa26R^{YFP}* mice treated for 8 with CCl₄. stained for (A) ki67/YFP and (B) PHH3/YFP. In Green the ki67 and PHH3+ hepatocytes, in red the nearby ki67- hepatocytes. The YFP+ area was delineated in black. Scale bar: 100µm

Figure 10 for the reviewers

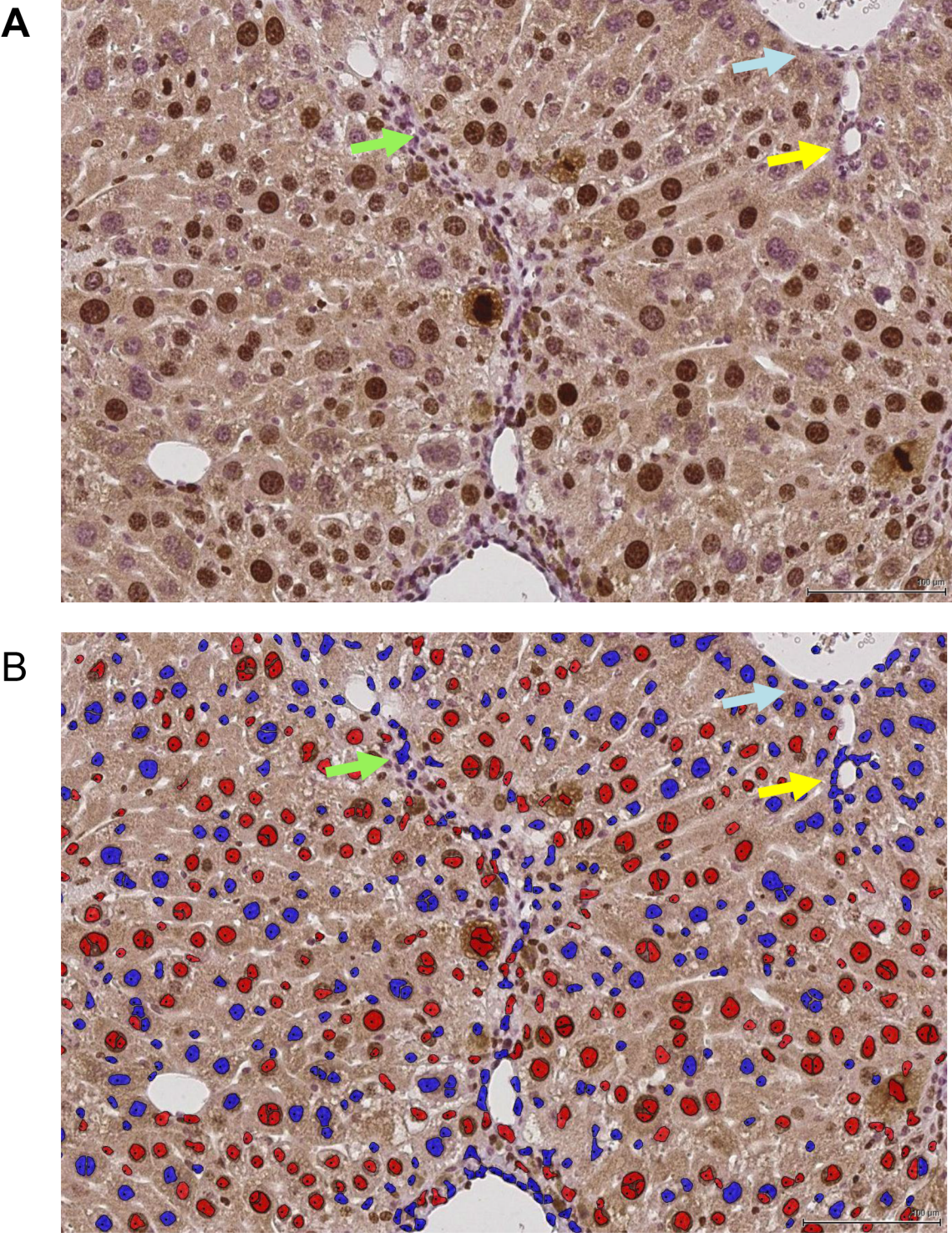
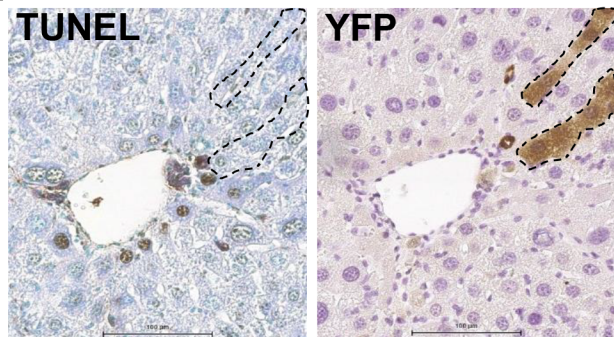


Figure 10. (A) Representative images of liver from OPN-iCreERT²;Rosa26R^{YFP} mice treated for 4 weeks with CCl₄. stained for Ki67. Scale bar: 100μm
(B) Example of the outcome pictures after Tissue IA software analysis. In Red: Ki67⁺ brown large hepatocytes nuclei taken into account; in blue: Ki67⁻ nuclei or small size ki67⁺ nuclei not taken into account. Green arrow: infiltrating immune cells; yellow arrow: cholangiocytes; light-blue arrow: LSECs.

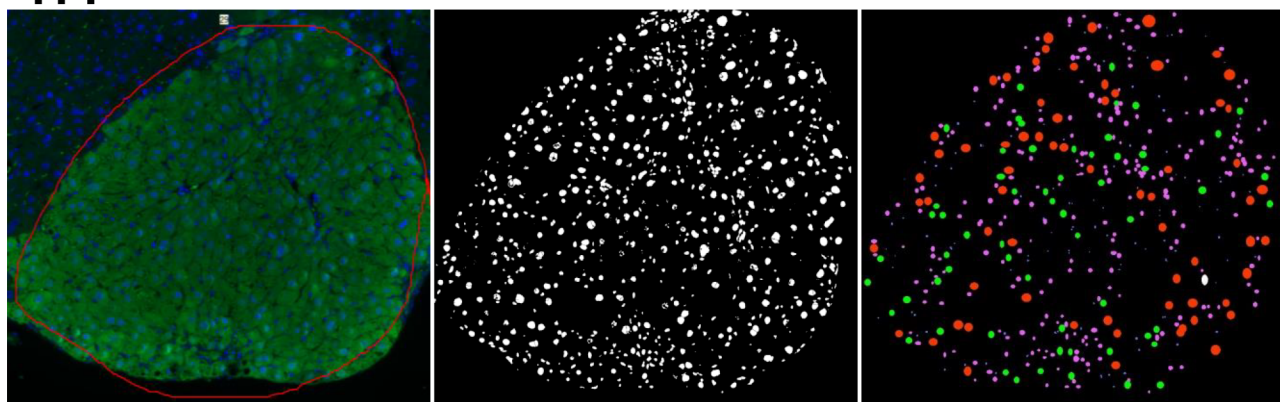
Figure 11 for the reviewers

A

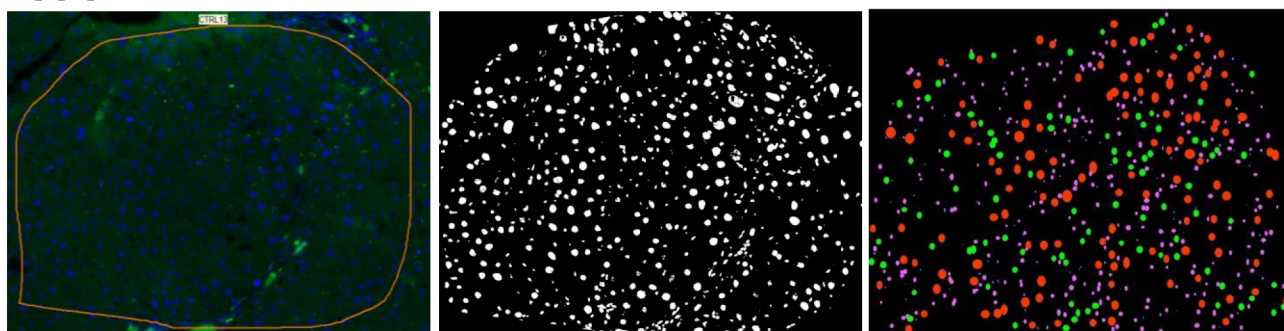


B: ploidy analysis

YFP+



YFP-



2N
4N
8N

Figure 11. (A) Representative images consecutive 4 μm slides from liver of CCl₄-treated OPN-iCreER^{T2};Rosa26R^{YFP} for TUNEL. Scale bar: 100μm
(B) Example of the ploidy analysis on YFP stained 3 μm slides.

Figure 12 for the reviewers

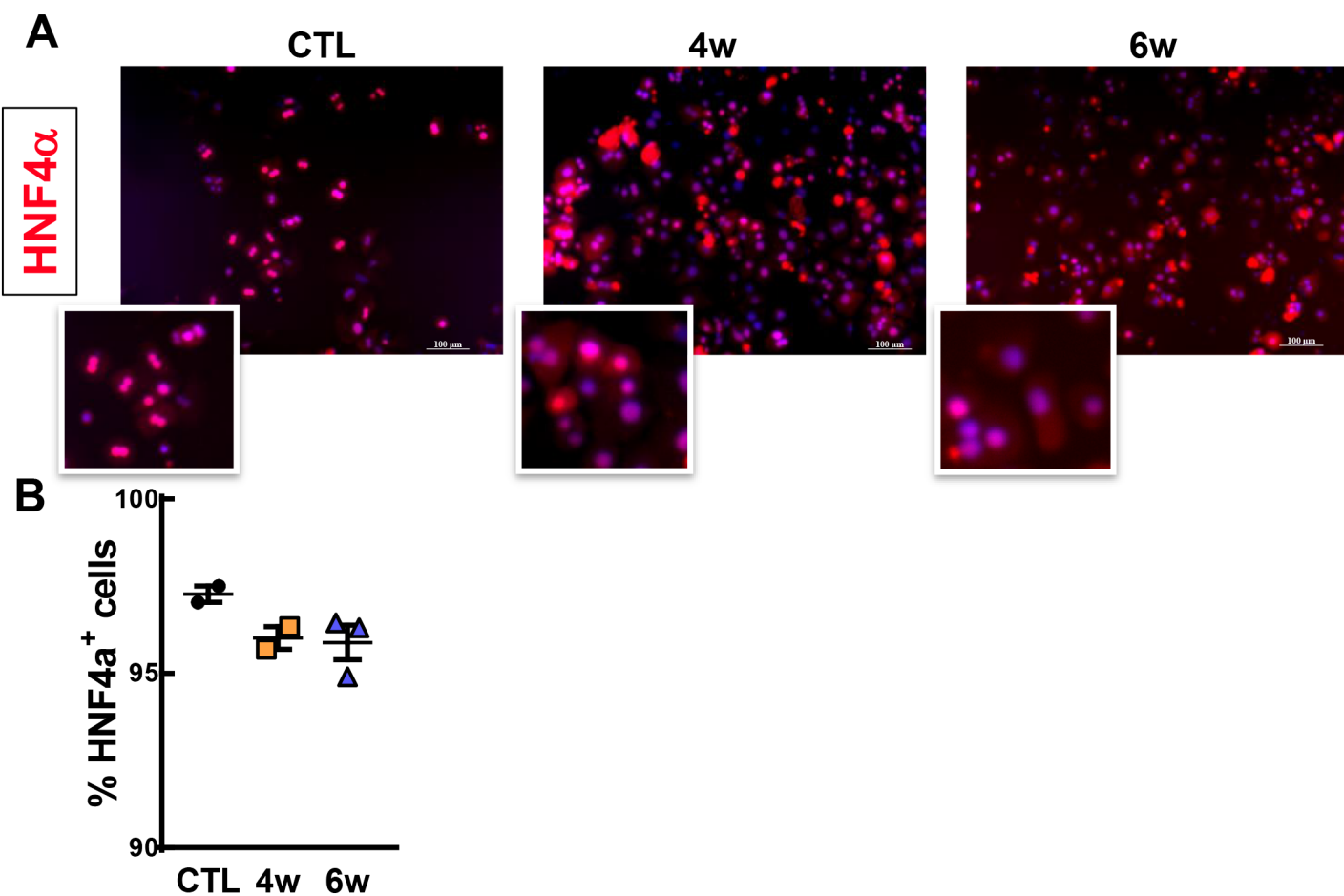


Figure 12. (A) Representative images of isolated hepatocytes stained for HNF4a. Scale bar: 100 μ m
(B) quantification of the HNF4a⁺ cells

Figure 13 for the reviewers

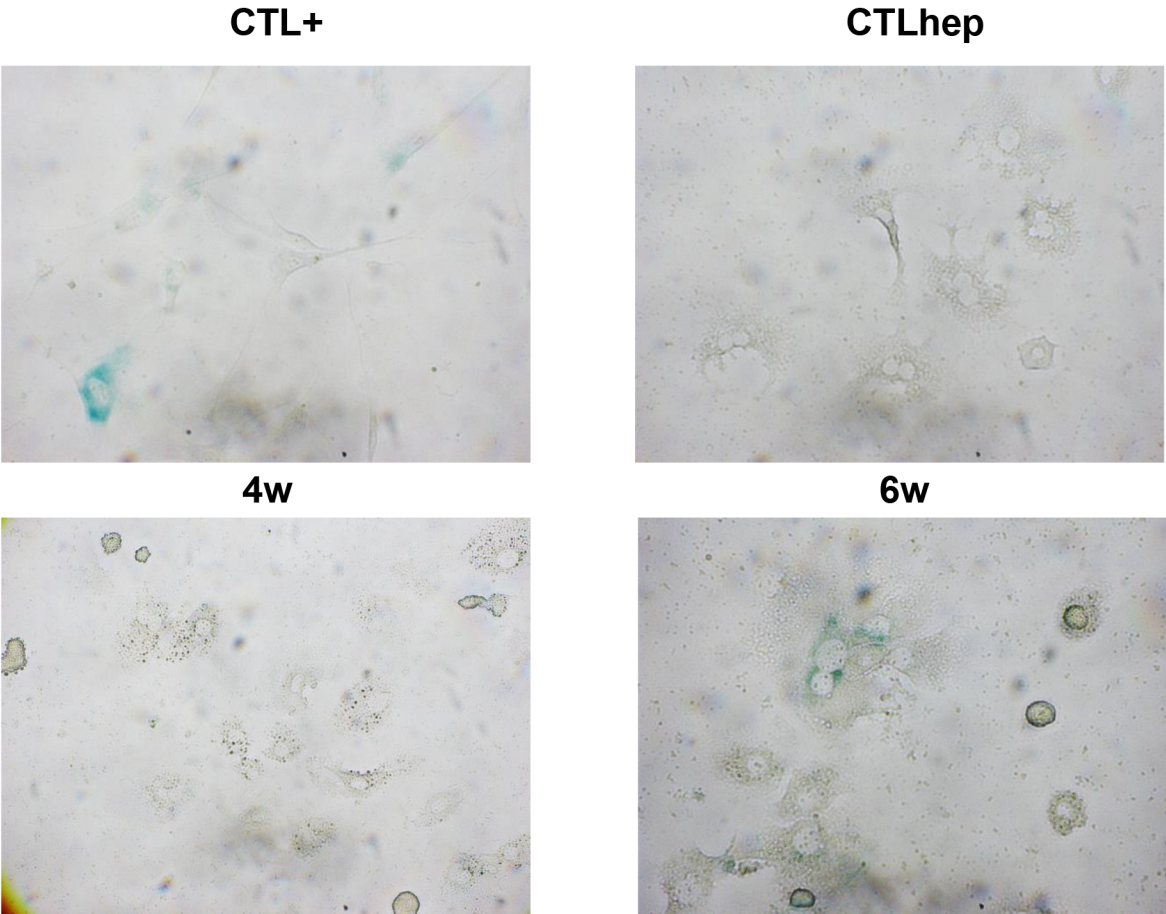
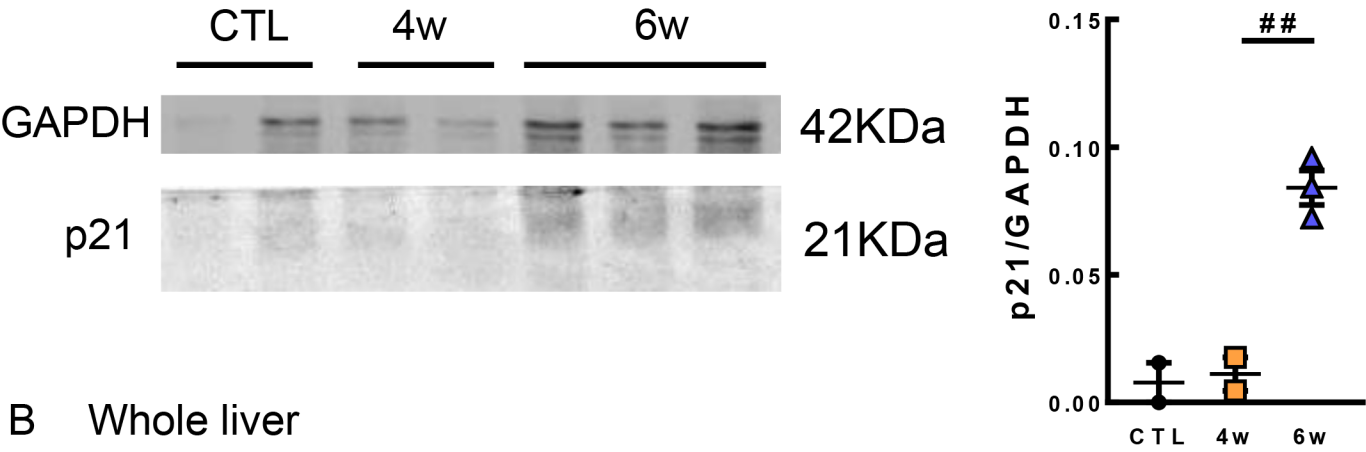


Figure 13. (A) Representative images of isolated hepatocytes stained for SA-b-gal activity. CTL+ is represented by mesenchymal stem cells

Figure 14 for the reviewers

A Isolated hepatocytes



B Whole liver

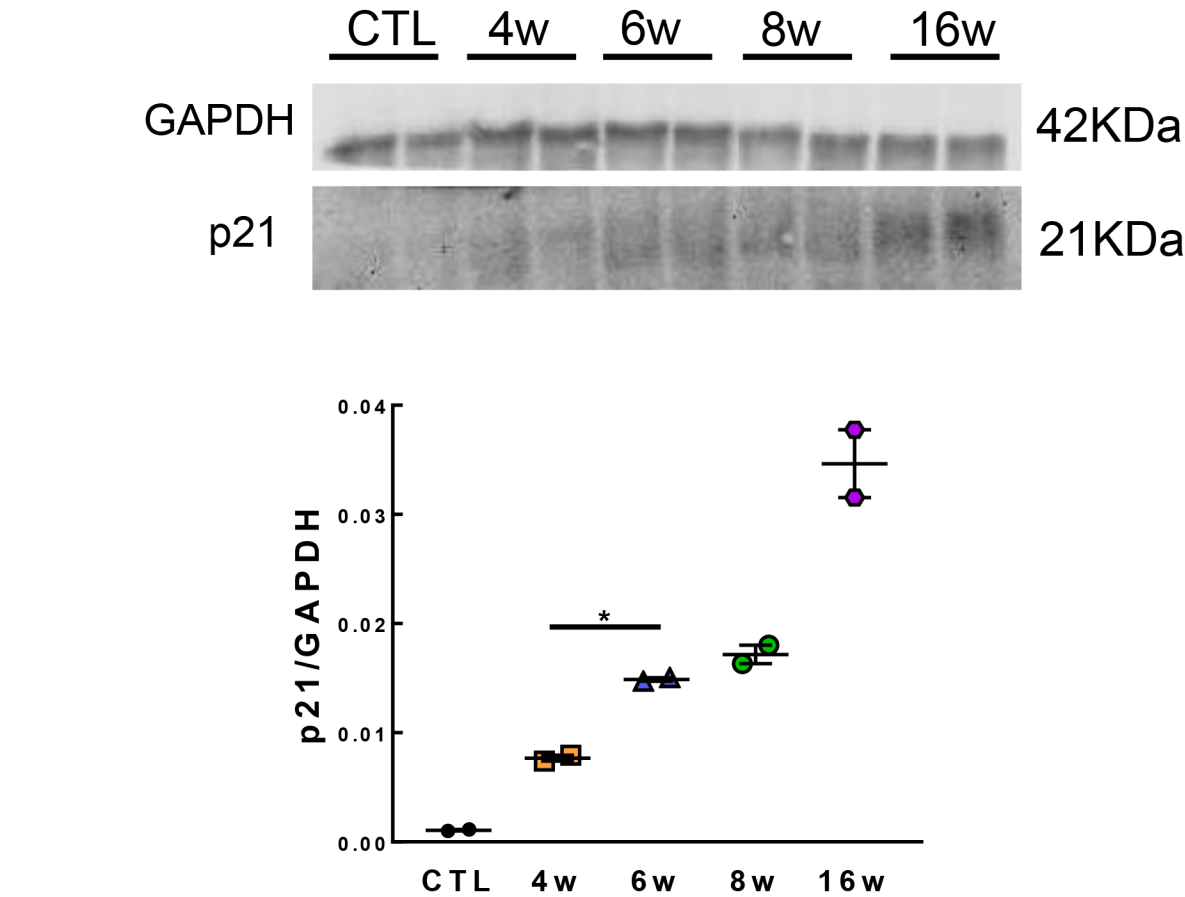
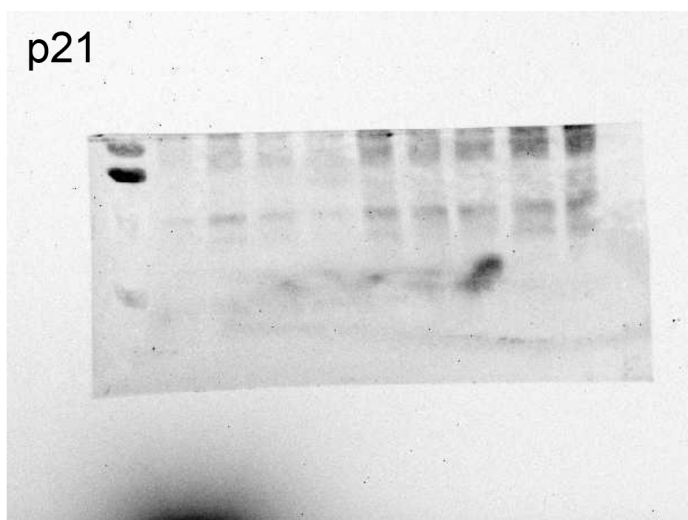
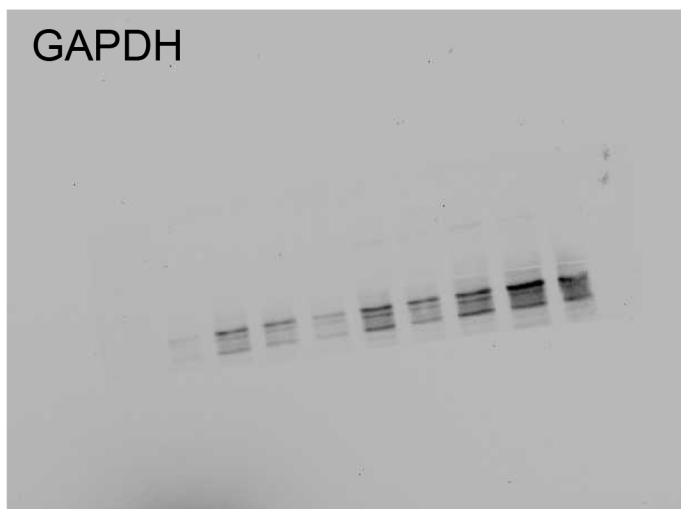


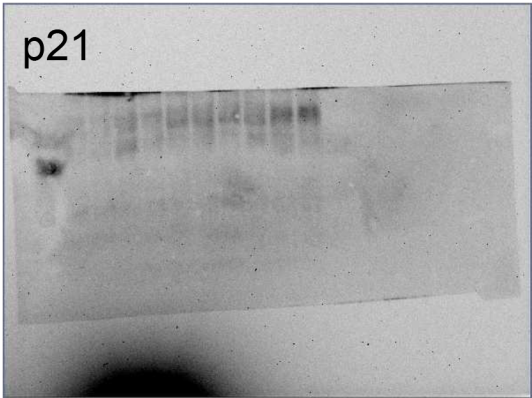
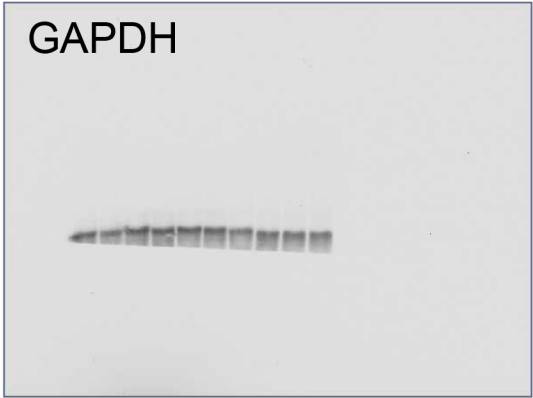
Figure 14. (A) Representative western blot using protein extracted from wild-type CTL mice and 4 and 6 weeks CCl₄-treated mice of p21. GAPDH was used as housekeeping protein and the graph of densitometry quantification of the WB. (B) Representative Western Blot using protein extracted from OPN-iCreER^{T2};R26R-YFP CTL mice and injected for 4, 6, 8 and 16 weeks with CCl₄ of p21. GAPDH was used as housekeeping protein, plus the graph of densitometry quantification of the WB.

Isolated hepatocytes



Entire western blot membrane for Fig. 14 A

Whole liver



Entire western blot membranes for Fig. 14 B

Figure 15 for the reviewers



Figure 15. SASP genes expression in CTL and 16w CCl₄-treated mice

Reactive cholangiocytes differentiate into proliferative hepatocytes with efficient DNA repair in mice with chronic liver injury

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COMPETING PERSONAL AND FINANCIAL INTERESTS: The authors declare no competing personal or financial interests.

AUTHORS' CONTRIBUTION: R.M. designed, conducted the experiments, analyzed and discussed the data, L-A.C. analyzed and discussed the data, S.V. and LA. vG. generated, analyzed and discussed the FACS analysis, analyzed and discussed the RNAseq data, M.B-N. and C.D. generated, analyzed and

discussed the ploidy data, C.S. generated the HIC score and discussed the data, J.A., B.B. and JL Gala analyzed and discussed the RNAseq data, Y.H. discussed the hypothesis, study design and data, I.L. designed the experiments, conducted the study, and analyzed and discussed the data; R.M. and I.L. wrote the original manuscript. All authors read and edited the manuscript.

Key words: lineage tracing; mouse model; regeneration; cell therapy

Electronic word count: main text 5839

Number of figures: 8 main figures

ABSTRACT:

Background and Aim: Chronic liver diseases are characterized by expansion of the small immature cholangiocytes – a mechanism named ductular reaction (DR) – which have the capacity to differentiate in hepatocytes. We investigated the kinetics of DR differentiation into hepatocytes as well as several important features as functional maturity, clonal expansion and resistant to stress of the newly formed hepatocytes in mice with long-term liver damage.

Methods: We tracked cholangiocytes using osteopontin-iCreER^{T2} and hepatocytes with AAV8-TBG-Cre. Mice received carbon tetrachloride (CCl₄) for >24weeks to induce chronic liver injury. Livers were collected for reporter proteins, cell proliferation and death, DNA damage, and nuclear ploidy analysis; hepatocytes were isolated for RNAseq.

Results: During liver injury we observed a transient DR and the differentiation of DR-cells into hepatocytes as clones that expanded to occupy 12% of the liver parenchyma by week 8. By lineage tracing, these new hepatocytes derived from cholangiocytes but not from native hepatocytes. They had all features of mature functional hepatocytes. In contrast to the exhausted native hepatocytes, these newly emerged hepatocytes had higher proliferation, less apoptosis, a lower proportion of highly polyploid nuclei and had better eliminated DNA damage.

Conclusions: In chronic liver injury, DR-cells differentiate into stress resistant hepatocytes that repopulate the liver. The process might account for the observed parenchymal reconstitution in livers of patients with advanced-stage hepatitis and can be a target for regenerative purpose.

Electronic word count: 227

LAY SUMMARY: During chronic liver disease, while native hepatocytes are exhausted and genetic instable, a subset of cholangiocytes clonally expand to differentiate into young, functional and robust hepatocytes. Targeting this cholangiocyte cell population could allow regeneration and rescue function in patient with chronic liver insufficiency.

INTRODUCTION

Persistent injury of the hepatic tissue leads to fibrosis, which eventually evolves to cirrhosis, the end-stage of any chronic liver diseases. Cirrhosis is characterized by distortion of hepatic architecture, regenerative nodules and hepatocyte dysfunction and is associated with life-threatening complications such as hepatocellular insufficiency and hepatocellular carcinoma (HCC)[1]. Liver cirrhosis is estimated to cause around 170,000 deaths annually[2]. So far, liver transplantation represents the only curative therapeutic solution for many chronic liver diseases.

In chronic liver diseases, extension of the fibrotic scars correlates with the presence of “Ductular reaction” (DR) [3][4]. This term refers to proliferation of small immature cholangiocytes, located at the most proximal branches of the biliary tree [5][6]. DR-cells (also referred to as oval cells or liver progenitor cells) express hepatocytes (CK8, CK18) and cholangiocytes (OV-6, CK7, CK19)[7–9] proteins and have the potential to differentiate into either of these two liver epithelial lineages[10]. Studies on human chronic liver diseases, including chronic viral hepatitis, auto-immune hepatitis and cirrhotic alcoholic or non-alcoholic fatty liver diseases, have highlighted substantial DR and the emergence of cells intermediate in size and immunophenotype between DR-cells and hepatocytes[3][11]. Several studies report such intermediate cells represent more than half of the hepatocyte pool in the cirrhotic liver [8,9,12]. A morphological continuum between DR, intermediate cells and hepatocytes may be interpreted as a gradual differentiation of DR in hepatocytes or as a de-differentiation of hepatocytes with acquisition of biliary traits (metaplasia). This conundrum is hard to resolve by the observation of human material. For this reason several (inducible) lineage tracing mouse strains tagging either cholangiocytes/DR cells or hepatocytes have been used in the last decade in attempt to unravel the origin, the dynamics and the fate of DR cells in various dietary, chemical or genetic rodent models of liver injury. The results of these studies remain conflicting. Studies by us and other authors, in which the fate of DR cells or hepatocytes was followed upon hepatocellular injury caused by a choline-deficient and ethionine-supplemented (CDE) diet, support the *in vivo* capability of DR to differentiate into hepatocytes, although in discrete proportion

($<2,5\%$)[13,14][15]. Furthermore, DR cells isolated from CDE livers largely underwent hepatocyte differentiation when transplanted *in vivo* into a compromised liver, with an improvement of both liver architecture and function[16]. In zebrafish the biliary compartment is also capable of generating functional hepatocytes[17]. On the other hand, studies using the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet as model of cholangiocytic injury failed to demonstrate DR contribution to the hepatocyte pool[18][19] and other works even support a de-differentiation of traced hepatocytes into biliary-like cells[20–22]. Taken together such inconsistent data indicates that the involvement of DR cells in regeneration is conditioned by the epithelial compartment undergoing damage and is thus disease-specific; while considerable discrepancies between models and observations in human material may stem from fundamental differences in severity and chronicity of injury. Here, we aimed to analyze DR and its contribution to regeneration in a model replicating chronicity, severity and fibrotic progression seen in chronic hepatitis in humans.

Impaired hepatocyte proliferative capacity is the essential requirement for DR[23]. Recently, the capacity of the DR cells to maintain the liver parenchyma was demonstrated using genetic approaches to induce hepatocyte replicative senescence or growth arrest such as the deletion of *Mdm2*[16] to provoke p53-mediated senescence in all the hepatocytes, of $\beta 1$ -integrin[24] to inhibit hepatic growth factor signaling thereby precluding hepatocyte replication, or of β -catenin directly in the hepatocytes to impair their proliferation[25], or the overexpression of p21 in conjunction with induction of injury using DDC, MCD and thioacetamide (TAA)[24]. Such genetic models artificially cause subacute hepatocyte failure, as opposed to progressive lesions occurring during chronic liver injury. Deng et al. demonstrated thus the capability of the DR cells to differentiate into hepatocytes in a long term injury model (up to 52 weeks) using the thioacetamide (TAA) toxicant agent[26].

However, the kinetics of the response to the injury as well as several features of the DR-derived hepatocytes, such as level of maturation, clonal expansion and resistant to stress remains unanswered.

Here, we used a model of chronic liver injury mimicking in evolution and severity chronic human diseases and followed the fate of DR cells, evaluating their contribution to the pool of hepatocyte and to characterize the newly formed hepatocytes. We show that DR emerges from clonal expansion of cholangiocytes; DR cells then undergo hepatocyte differentiation and clonal proliferation.

Hepatocyte lineage tracing studies confirmed that these emerging cells are not of hepatocyte origin.

The mature and functional newly formed hepatocytes have a survival, proliferative and DNA repair advantage that favors their amplification over native hepatocytes (i.e. those arising from division of pre-existent hepatocytes).

METHODS

Animal Models

To follow the fate of biliary/DR cells we used Osteopontin-iCreER^{T2} (OPN-Cre) mice crossed with Rosa26R^{YFP} [27], Rosa26R^{mt/mG} [28] or Rosa26R^{Confetti} [29]. To achieve *Cre-LoxP* recombination, tamoxifen (T5648; Sigma) at a concentration of 30 mg/ml corn oil was injected i.p. at 100 mg/kg BW for 2 consecutive days on 21 and 23 days old OPN-Cre;Rosa26R^{YFP} and OPN-Cre;Rosa26R^{mt/mG} mice or at 175 mg/kg BW for 5 days to ≥ 40gr OPN-Cre; Rosa26R^{Confetti} mice[30]. To genetically label the hepatocytes, AAV8-TBG-Cre adenovirus was injected i.v. at a concentration of 7, 5*10¹¹ gc/mouse in Rosa26R^{YFP} or Rosa26R^{mt/mG} reporter mice[31]. We used YFP IHC or direct observation under the fluorescence microscope to analyze tagged cells, respectively (see Suppl. Information). One month after tamoxifen treatment (to ensure complete tamoxifen wash-out) chronic liver injury was induced by repeated intraperitoneal injection of carbon tetrachloride (CCl₄) 3 times per week for 4, 6, 8, 16 and 24 weeks. Starting dose of CCl₄ was of 500 µl/kg, with dose increase up to 800 µl/kg when animals gained weight. Liver were analyzed 72h after the last CCl₄ injections or after a 2 or a 4 weeks CCl₄-free recovery period. Transgenic mice that did not receive tamoxifen were used as controls. The size of the groups is specified in the figure legends.

Mice were housed at 4-5/cage, maintained at a constant temperature of 22°C, exposed at all times to a 12-h light/12-h dark cycle and had access to food and water *ad libitum*. Animal care was provided in accordance to the guidelines for humane care for laboratory animals in accordance with European regulations and in conformity with ARRIVE guidelines. The study protocol was approved by the university ethics committee for the use of experimental animals (2012/MD/UCL/026 and 2016/UCL/MD/003).

FACS sorting analysis

Livers from control OPN-Cre;Rosa26R^{mt/mG} mice were dissociated using pronase and collagenase to obtain a single cell suspension[32]. Two centrifugations of 50g were performed to separate the non-

1 parenchymal fraction from the hepatocytes. We blocked the non-parenchymal fraction using bovine
2 serum albumin for 10 min and incubated cells with the indicated antibodies for 15 min. After adding
3 propidium iodide we used a FACSAriaII (BD) to isolate LSECs (CD32⁺F4/80⁻UV⁻PI⁻), macrophages
4 (F4/80⁺CD32⁻UV⁻PI⁻), HSCs (UV⁺F4/80⁻CD32⁻PI⁻) and BECs (EpCAM⁺UV⁻CD45⁻PI⁻). Hepatocytes were
5 resuspended in PBS and immediately sorted. In each population cells were analysed for tdTomato
6 (red) and mGFP (green) fluorescence.

7 For the isolation of hepatocytes and RNA sequencing, livers from TAM-injected untreated and CCl₄
8 treated OPN-Cre;Rosa26R^{mT/mG} mice were digested using a two-step collagenase method. Solutions
9 were perfused through the portal vein. Two centrifugations of 50g were performed to separate the
10 non-parenchymal cells from the hepatocytes. The resulting hepatocyte populations were filtered
11 through a 100µm filter and resuspended in 2mM EDTA + 1% FBS solution and sorted by a FACSAriaIII
12 (BD) according to the presence of mTomato and mGFP fluorescence. Dead cells were excluded by
13 ToPro3 staining. Sorted cells were lysed and the RNA was extracted using Qiagen RNeasy Mini kit
14 (Promega) and processed for RNA sequencing. Hepatocytes were also isolated from CTL, 4wks and 6
15 weeks CCl₄-treated wild-type mice using the two-step collagenase method. The resulting
16 hepatocytes were cultured on 6 wells Collagens-coated dishes for 24 h in DMEM/F12 + 10% FBS and
17 used for immunofluorescence detection.

18 Polyploidy analysis

19 Immunostained liver sections (β-catenin/YFP/Hoechst) were imaged with a nanozoomer 2.0,
20 Hamamatsu fluorescent microscope associated with image management software NDP view. For
21 ploidy analysis, Hoechst labelling was used to recognize hepatocytes nuclei with a roundness >0.8.
22 Nuclear area was detected automatically by a specific macro developed with ImageJ software (pixels
23 ranging from 200 to 2,500 px²). For each animal analyzed, more than 10000 nuclei were counted.

IHC score of liver tumors

Consecutive liver sections stained for ck19, GS, β -catenin and Ki67 were scored by the expert pathologist Dr. Christine Sempoux. Nodules with a diameter >1.5 mm were selected and evaluated as follow: GS was classified as normal (0) when expressed around the central vein or aberrant (1) if the expression was diffuse in the nodule or completely lost; β -catenin was evaluated as normal (1), cytoplasmatic (2) or cytoplasmatic/nuclear (3); ki67 was classified as less than 10% (1), 10-25% (2) or more than 25% (3) level. None of the nodules expressed ck19.

Statistical analysis

Data are presented as mean value $\pm$ SEM. Statistical analyses were performed using a paired two-tailed Student t-test, a one-way ANOVA followed by Bonferroni's post-hoc, a Kolmogorov-Smirnov test for rank as appropriate using Graph Pad Prism 5 software. Differences were considered significant at values of $p < 0.05$.

Additional information on methods (carcinogenetic animal model, qPCR analysis, immunohistochemistry, RNAsequencing) is provided in supplementary document.

RESULTS

DR cells differentiate into hepatocytes, contributing significantly to the liver parenchyma.

Mice were repeatedly injected with CCl₄ for up to 16 weeks (Fig. 1A) to induce repeated cycles of central necrosis and wound healing leading to progressive fibrosis. Sirius red staining revealed central bridging fibrosis after 4 weeks, extension and thickening of fibrotic bundles progressively increased with time (Fig. 1B).

During the injury, we followed the fate of biliary/DR cells using tamoxifen-inducible osteopontin-*iCreER*^{T2} (OPN-Cre) mice crossed with *Rosa26R*^{YFP} or with *Rosa26R*^{mT/mG} reporter mice to label biliary cells. Activation of the OPN promoter in cholangiocytes drives the expression of the inducible Cre recombinase. Tamoxifen binds the mutated estrogen receptor ER^{T2} and allows the activation and the translocation of the Cre-recombinase into the nucleus. Nuclear Cre-recombinase was only found in biliary OPN⁺ cells in tamoxifen-injected OPN-Cre;*Rosa26R*^{YFP} mice but not in mice that did not receive Tamoxifen (Suppl. Fig 1a). Recombination of the *Rosa26R* locus ensued and, according to the reporter strain, caused permanent expression of yellow fluorescent protein (YFP) in >85% of the cells of the biliary compartment or switched the expression of mTomato to that of mGFP in OPN-expressing cells with a 60% efficiency (suppl. Fig. 1b-d). No YFP⁺ or mGFP⁺ cells have been observed in livers harvested from tamoxifen-free OPN-Cre;*Rosa26R*^{YFP} or OPN-Cre;*Rosa26R*^{mT/mG} mice, respectively (Suppl. Fig. 1b; not shown). As reported by us[13,31] and others[33] and verified here again by cell sorting in tamoxifen treated OPN-Cre;*Rosa26R*^{mT/mG} mice (Suppl. Fig. 1c and Suppl. Fig. 2) upon tamoxifen injection reporter gene expression is restricted to the biliary compartment and does not occur in hepatocytes, stellate cells, liver sinusoidal endothelial cells or Kupffer cells. With this system, in the course of CCl₄ treatment, any YFP⁺ hepatocyte would sign its biliary origin. After 4 weeks of tamoxifen wash-out, mice were treated with CCl₄. After 4 weeks of CCl₄, rare YFP⁺ hepatocyte-like cells were observed in the vicinity of DR (Fig. 1C). As duration of liver insult increased, the number of YFP⁺ hepatocytes raised to reach a maximum at the 8 weeks' time-point, such as 12% of the liver parenchyma was occupied by DR-derived hepatocytes (Fig. 1C-D). By

contrast, in tamoxifen-free OPN-Cre;Rosa26R^{YFP} mice, no YFP⁺ cells and in particular no YFP⁺ hepatocytes were found in the course of CCl₄ treatment (Suppl. Fig. 3). Thus, DR cells give rise to hepatocyte-like cells during chronic wound healing, supporting results showed in previous study[26]. The emergence of YFP⁺ hepatocytes-like cells after 6wks CCl₄ coincided with a marked drop in proliferative activity of native hepatocytes as assessed by Ki67 index (Fig. 1E and Suppl. Fig. 4), as well as with an increased expression of p16 and p21 senescence markers ~~and of the checkpoint kinases 1 (CHK1) and 2 (CHK2)~~ (Fig. 1G). To confirm senescence in the hepatocytes, we isolated and cultured fresh hepatocytes from CTL, 4 weeks and 6 weeks CCl₄-treated mice. Senescence associated β -galactosidase activity (not shown) and P21 expression (Fig. 1H-I) were readily seen in isolated hepatocytes from 6 weeks CCl₄ samples but not from controls or 4weeks CCl₄ livers. Senescence persists in the liver for the duration of all the CCl₄ insult, as demonstrated by progressive increase in p21 protein in whole liver extracts (Suppl. Fig. 5). The YFP⁺ hepatocytes formed patches, which size increased with disease progression (Fig. 1c-f). After 6 weeks of CCl₄, a majority (>70%) of clusters were composed of 2-3 YFP⁺ hepatocyte-like cells. Over time, the proportion of small clusters declined while that of larger ones increased (Fig. 1F). The YFP⁺ hepatocytes were HNF4 α positive (Fig. 2A) and expressed hepato-specific liver enzymes according to their lobular location, such as expression of GS and CYP2E1 when pericentral or of CPS1 when periportal (Fig. 2B-C). Ceacam1 staining confirmed that YFP⁺ hepatocytes were polarized and formed bile canaliculi with adjacent hepatocytes whether YFP⁺ or YFP⁻ (Fig. 2D). Also, YFP⁺ cells stored glycogen similarly to native YFP⁻ hepatocytes (Fig. 2E). They did however not express biliary markers, such as ck19, the antigen epithelial cell adhesion molecule (EpCAM) or the cholangiocyte factor HNF1 β (Suppl. Fig.6). We isolated native (mTomato⁺) and DR-derived (mGFP⁺) hepatocytes from OPN-Cre;Rosa26R^{mT/mG} after 16wks CCl₄ (Fig. 2F; suppl. Fig 7) and compared their transcriptome by RNAseq analysis. Healthy mTomato⁺ hepatocytes from untreated OPN-Cre;Rosa26R^{mT/mG} served as controls (Fig. 2F). In the 3 populations, there was a similar high count of hepatospecific genes such as albumin (Alb),

Transthyretin (Ttr) and Cyp7a1 (Fig. 2G). They also had similar expression of Cyp2E1 and Glutamine Synthetase (Glul) (Suppl. Fig. 8).

To confirm that the new hepatocytes do not come from the division of pre-existing hepatocytes in the CCl₄ injury model[34], we performed an independent mirror-experiment in which hepatocytes' fate was analysed. Injection of AAV8-TBG-Cre adeno-associated virus to Rosa26R^{YFP} mice induced YFP expression in nearly 100% of hepatocytes and zero cholangiocytes or non-parenchymal cells (Suppl. Fig. 9). Following chronic CCl₄ injections (Suppl. Fig. 10a), patches of unlabelled HNF4α⁺ and ck19⁻ hepatocytes emerged in the liver (Fig 3A-B). Their size grew with duration injury comparably to the patches of DR-derived hepatocytes traces using the OPN-CreER^{T2};Rosa26^{YFP} system (Fig 3C). This confirms the emergence of clusters of hepatocytes not derived from division of pre-existing labelled hepatocytes.

Long term CCl₄ induces a discrete and transient Ductular Reaction.

We then evaluated the kinetics of the DR response. Ck19⁺ bile ducts, normal in number and in morphology, were present in controls as well as diseased livers. After 4 and 6 weeks CCl₄, in addition to normal bile ducts, discrete DR appeared as strings of ck19⁺ small cells irradiating the parenchymal lobule from the portal tract (Fig. 4A-B). Interestingly, in livers treated for longer duration (8-16 weeks), although the DR-derived hepatocytes increased in number (Fig. 1C-D), ck19⁺ cells were limited to bile ducts and no DR was observed (Fig.4a-b). The expression of Sox9 (a biliary transcription factor)[35], Fn14 (a cell membrane receptor that transduces mitogenic signals to DR cells)[36], Epcam and NCAM (cell adhesion molecules highly expressed in DR)[37], all genes expressed explicitly in cholangiocytes but not in hepatocytes (Suppl. Fig. 11), confirmed the transient activation of a ductular response during CCl₄ course (Fig. 4C). In keeping, expression of genes related to the Notch pathway (Notch1, Notch2, Jagged1, Hey-1), which activation is required for biliary specification[10], were concordantly expressed at higher levels in 6 weeks CCl₄ livers but no longer at later time-points (Fig. 4D).

DR cells clonally expand and differentiate into hepatocytes with a proliferative advantage.

Our observations of transient DR and of progressive rise in the size of DR-derived hepatocytes clusters suggest that few or single biliary cells expand as DR and undergo hepatocyte differentiation followed by several rounds of cell division. To test this hypothesis, we used the OPN-Cre;Rosa26R^{Confetti} mice[29] in which tamoxifen injections results in stochastic expression of one of the four fluorescent proteins (nGFP, mCFP, RFP and YFP) encoded in the confetti allele in the OPN⁺ biliary cells. In control uninjured livers, 15-20% of cholangiocytes of the bile ducts were labelled by one of the 4 fluorescent proteins (Suppl. Fig 12). After CCl₄, all cells in a given DR expressed the same fluorescent protein (Fig. 5A), and not a mosaicism of different fluorescent proteins or of tagged/untagged cells. This observation supports that DR emerges from clonal expansion of one single biliary cell.

Also, all DR-derived hepatocytes within a patch were found to express a same one fluorescent protein (Fig. 5B). No mosaicism with a different fluorescent protein or with unlabelled hepatocytes was found into such patches. The dimension of the patches was similar to the dimension of those observed when using the Rosa26R^{YFP} reporter mice (Suppl. Fig. 12b).

As also shown above in OPN-Cre;Rosa26R^{YFP} livers (Fig. 1-3), the number of tagged hepatocytes in patches is larger than the number of cells in the original DR supporting that during chronic liver injury, biliary cells undergo clonal proliferation as DR, then transformed into hepatocytes; the number of which subsequently increases due to several rounds of cell division.

If clonal expansion of differentiated hepatocytes support patches growth, we should observe transition features between DR cells and hepatocytes in nascent patches but not anymore at later stages. We measured thus the size of hepatocytes. Coherently, we found that DR-derived cells were significantly smaller than adjacent native hepatocytes at the early 6 weeks' time-point but at 16 weeks they were as large as native hepatocytes (Fig. 5C). Size of hepatocyte was also measured in the AAV8-TBG-Cre X Rosa26R^{YFP} mice confirming the increase in size of unlabelled (non-hepatocyte origin) cells with the progression of the injury (Suppl. Fig. 10b). DR-derived hepatocytes had a higher

proliferation index (% of Ki67⁺ cells) (Fig. 5D), a higher percentage of cells expressing cyclin D1 (Suppl. Fig. 13) or engaged in mitosis (phospho Histone-H3 positive) (Fig. 5E) compared with native hepatocytes, further support that proliferation of differentiated mature cells contribute to growth of DR-derived hepatocytes patches

DR-derived hepatocytes better response to stress signals.

YFP⁺ hepatocytes when located in the pericentral area (representing 10% of the YFP⁺ hepatocytes contingent - Suppl. Fig.8a) express CYP2E1 (Fig. 2C). Moreover, CYP2E1 and GS were similarly expressed at the same level in the DR-derived and native hepatocyte populations (Suppl. Fig. 8d). We therefore assumed that a similar proportion of the 2 cell populations are able to metabolize CCl₄ and undergo CCl₄-induced damage. To study the cells' response to DNA damage, we checked the expression of genes involved in the DNA repair machinery in native (mTomato-Red) and DR-derived (mGFP-Green) injured hepatocytes, sorted after 6 and 8 weeks CCl₄, and compared them with healthy control hepatocytes. The expression of BRCA1, RAD51 and FANCI (encoding for proteins that bind DNA damage site), expression of the gatekeeper GADD45 as well as of the checkpoint kinases 1 and 2 (CHK1/2) were similarly increased in the 2 populations of native and DR-derived injured hepatocytes compared to the healthy control hepatocytes (Fig. 6A). Moreover, upon CCl₄ exposure, a similar proportion of cells of the YFP⁺ and YFP⁻ population harbour nuclear γ H2AX, a protein that aggregates to DNA breaks (Fig.6B). The proportion of TUNEL⁺ apoptotic cells was however lower in DR-derived than in native hepatocyte population (Fig. 6C). Consistently, analysis from RNAseq data confirming that native population was enriched in the pathways involved in the regulation of DNA damage, apoptotic process and senescence (Fig. 6D).

Polyploidy, a feature of mature hepatocytes[38], is determined by the number of nuclei per cell (cellular polyploidy) and the DNA content for each nucleus (nuclear polyploidy). During liver development, polyploidization is mostly associated with modification of cellular polyploidy[39] whereas under stress condition nuclear polyploidy is altered[40]. Thus, in our context of CCl₄

1 treatment, we have focused on nuclear polyploidy analysis. Compared to untreated controls, YFP⁻
2 native hepatocytes display lower proportion of tetraploid nuclei (4n), and a higher proportion of
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4 highly polyploid nuclei ($\geq 8n$) as expected under CCl₄ stress condition. By contrast, YFP⁺ nuclei
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6 showed a similar polyploidy profile than untreated livers with a low proportion of highly polyploid
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8 nuclei ($\geq 8n$) (Fig. 6E and Suppl. Fig. 14). All these results suggest that although native and DR-derived
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10 hepatocytes similarly experience DNA damage in ongoing CCl₄-injury, DR-derived hepatocytes have a
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12 survival advantage over native hepatocytes with less cells driven into death or senescence.
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19 **DR-derived hepatocytes have a proliferative and DNA repair advantage upon recovery.**

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21 Thus, newly formed DR-derived hepatocytes have less stress stigmata (and are potentially
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23 genetically more stable) and the balance proliferation/death favours their amplification over native
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25 hepatocytes. To verify the repopulation advantage of YFP⁺ DR-derived cells, we exposed the mice to
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27 16 wks CCl₄ and allowed the liver to recover for 2 or 4 weeks. The parenchymal area occupied by
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29 YFP⁺ hepatocytes increased from 4.5% after 16 weeks CCl₄ to 7.5% and up to 13.5% after 2 and 4
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31 weeks recovery, respectively (Fig. 7A-B). The increase in the number of YFP⁺ hepatocytes was
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33 explained by an increase in the size of YFP⁺ clusters (Fig. 7C) and not by the number of clusters (not
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35 shown) or the cellular size (Fig. 7D). Although the total number of ki67⁺ cells decreased during
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37 recovery (Suppl. Fig. 15), the proliferative index (Fig. 7e-f) and the number of pHH3⁺ mitotic cells
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39 (Fig. 7G) was higher in YFP⁺ than in YFP⁻ hepatocytes upon recovery. γ -H2AX accumulates to form
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41 foci on damaged DNA to recruit DNA repair by homologous recombination upon injury. Indeed, as
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43 mentioned above (Fig. 6B) and as it is shown in Fig. 7H, the 2 populations of hepatocytes upon injury
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45 similarly accumulated nuclear γ -H2AX. Persistence of nuclear γ -H2AX foci once injury has resolved
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47 signs the presence of unrepaired DNA[41]. Native hepatocyte retained nuclear γ -H2AX protein upon
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49 recovery while it was rapidly cleaned in YFP⁺ hepatocytes (Fig. 7H and Suppl. Fig. 16). Collectively,
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51 these data support the concept that the increased number of DR-derived hepatocytes upon recovery
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53 from injury is achieved by effective proliferation of unstressed cells previously differentiated from
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DR, while YFP⁻ native hepatocytes harbour unrepaired DNA damage and are significantly less replicative.

DR-derived hepatocytes are not involved in the development of pre-neoplastic nodules.

The cirrhotic liver is a precancerous organ. Twenty-four weeks of CCl₄ caused macronodular cirrhosis (Fig. 8A-B). Although YFP staining revealed some cirrhotic nodules entirely composed by YFP⁺ cells or mosaic YFP⁺/YFP⁻ (arrows in Fig. 6b), the majority of nodules were nonetheless YFP⁻ (Fig. 8C).

Glutamine synthetase (GS) expression, a proxy for β -catenin activation described in preneoplastic lesions and a useful marker to identify neoplasia[42], was found in 40% of YFP⁻ nodules and only in 10% of YFP⁺ regenerative nodules (Fig. 8D-E). Moreover, in mosaic YFP⁺/YFP⁻ nodules, GS expression was restricted to YFP⁻ area while YFP⁺ DR-derived hepatocytes did not express GS (insert Fig. 8D).

Compared to normal hepatocytes in the undamaged liver, YFP⁻ native hepatocyte nuclei were more often highly polyploid (>8n) and more rarely tetraploid (4n) in the cirrhotic liver (Fig. 8F). By contrast, the level of nuclear polyploidy of DR-derived YFP⁺ hepatocytes in the cirrhotic liver was comparable to that of hepatocytes in an undamaged liver (Fig. 8F) suggesting, also at this stage of the disease, a higher protection or a better capacity of DR-derived YFP⁺ cells to manage stress induced by CCl₄ treatment. Similar to Tummala *et al.*[43], we applied to regenerative nodules a composite index based on immuno-detection of GS, β -catenin and Ki67 (IHC score) for diagnosis of malignant hepatocellular neoplasms. The index was low in all cirrhotic nodules compared to score in proven HCC[31] taken as reference (Suppl. Fig. 17d) but notably the YFP⁺ nodules had a lower score than the YFP⁻ nodules (Fig. 8G). Then, we interrogated the susceptibility of native and DR-derived hepatocytes to carcinogenesis, using the carcinogenic agent DEN (Suppl. Fig. 17a). Out of 15 mice, 12 developed macroscopic tumours. Altogether, 26 nodules were diagnosed as HCC (Suppl. Fig. 17). They were, however, all YFP⁻ and no YFP⁺ HCC were found (Suppl. Fig. 17).

DISCUSSION

It is now established from recent studies that cells from the biliary compartment give rise by proliferation to the ductular reaction and by cell differentiation to functional hepatocytes¹⁵[24][26].

The latter has been demonstrated in models in which hepatocellular injury associates with genetic abrogation of their replicative capacity¹⁵[24,25] as well as in long term TAA-injury model[26].

However, the kinetics of cell differentiation from a biliary precursor and characterization of the new hepatocytes have not up to now been investigated.

The present study was designed to test the efficiency and safety of this alternative regeneration pathway in a model of chronic hepatocellular disease. We used a model of repeated administration of the hepatotoxic CCl₄ to induce repeated rounds of parenchymal necrosis and healing that culminate to cirrhosis, mimicking in its evolution and severity chronic liver injury in humans. The use of efficient and clean genetic tracing of cholangiocytes[33] or conversely of native hepatocytes enables to monitor the relation between the ductular reaction and generation of hepatocytes from DR cells compartment or from the hepatocyte pool at various stages of disease progression. With these tools, our experiments demonstrate that ~12% of hepatocytes in the chronically ill liver derive from DR differentiation and not from replication of native pre-injury hepatocytes. In other works in the literature [14,30,44], including previous work from the lab[13], DR cells were not found to contribute to liver regeneration in short term CCl₄-induced injury. The discrepancy results from the experimental protocol and whether it impedes hepatocyte regeneration. We followed the scheme already published by our lab: 3 injections of CCl₄ per week with adaptation of the dose to the body weight. 4 weeks of CCl₄, in the current work as in Español-Suner et al[13], caused a discrete DR and no DR-derived hepatocytes were observed. In the others papers in which the expression “chronic CCl₄” was used, CCl₄ was administrated only twice a week, for 5, 6 or 8 weeks[14,30,44]. None of those papers reported the hepatocytes proliferation, but we can speculate that the dose and timing of the CCl₄ injections were not enough to inhibit hepatocytes proliferation. As evidence for this

1 hypothesis, Pu et al. reported that the periportal Mfsd2a⁺ native hepatocytes repopulate the liver
2 lobules during “chronic” CCl₄, confirming that they are not in a state of replicative senescence[34].
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4 Similar to our data, Deng et al. recently published the contribution of cholangiocytes to parenchymal
5 regeneration in a model of long lasting TAA-induced liver disease[26]. However, in the TAA model
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7 both native and cholangiocyte-derived hepatocytes had similar proliferation as far as assessed by
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9 ki67 analysis, leaving the mechanism for rising proportion of cholangiocytes-derived hepatocytes at
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11 this time unexplained. Coherent with our data, the same authors reported using the AAV8-TBG-
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13 Cre;mTmG system, that after long term (24weeks) TAA insult mTom⁺ hepatocytes composed 20% of
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15 the parenchyma, i.e. that these 20% of the hepatocytes did not derived from proliferation of native
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17 mGFP⁺ (and mTom⁻) hepatocytes. In the same model, 7% of the hepatocytes were demonstrated to
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19 be of biliary origin. The difference in number might be ascribed to the relatively poor efficiency of
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21 biliary labelling in ck19-CreER mice (70%). By using a similar approach but a much more efficient
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23 system to trace biliary cells (>85% efficiency), the patches of hepatocytes that appear after long term
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25 CCl₄ in our study were similar in size whether traced as of DR-origin or, in the mirror experiment, as
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27 of non-hepatocytic origin.
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31 In addition, we studied the temporal evolution of the DR-differentiation process, found that in CCl₄-
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33 induced injury, DR stems from clonal amplification of a discrete population of biliary cells, the
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35 identity of which remaining to be ascertained. Thereafter DR cells, or a subset of them undergo
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37 complete hepatocytic differentiation yielding functional cells, perfectly organised within the lobular
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39 architecture (Fig. 3). Coherently with studies by Lu[16] and Raven[24], initial differentiation of DR
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41 cells into hepatocytes coincided with the injury-induced drop in replication of native hepatocytes
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43 and their entry into senescence as supported by P21 expression, senescence associated β-
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45 galactosidase activity and expression of the senescence-associated gene program (SASP).
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49 Morphometric analyses revealed that early in the process, newly differentiated hepatocytes are
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51 small in size and reach a normal size with time while they amplify. Clonality experiments in confetti
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53 mice highlight that all DR-derived hepatocytes within a patch harbour one and unique fluorescent
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1 tag, identical to that expressed by adjacent DR cells signing genetic filiation. Yet when hepatocyte
2 patches expand, DR vanishes. These data suggest that during chronic liver injury, DR does not
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4 constantly re-fuel the parenchyma with newly differentiated cells, but rather when sufficient and
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6 appropriate cellular and environmental conditions are met, a limited contingent of DR cells undergo
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8 differentiation then clonal expansion to repopulate up to 12% of the liver. Further studies will be
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10 needed to determine whether the subset of DR cells that are capable to yield hepatocytes could
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12 correspond to ST14^{hi} clonogenic cholangiocytes[45], or Lgr5⁺ liver stem cells[46], or the peripheral
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14 ductule described by Kamimoto et al.[6]. Pulse labelling of DR cells during disease progression would
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16 be required to test this hypothesis[6].
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21 Our experimental system also permits to analyse separately the properties and contribution to
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23 regeneration of hepatocytes newly generated from DR cells and native hepatocytes, i.e. those
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25 already present before injury. At all times in the process, proliferative and mitotic indices are higher
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27 in the newly emerged population of hepatocytes than in native hepatocytes. Cell death by apoptosis
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29 and senescence are conversely readily seen in native hepatocytes during chronic exposure to CCl₄
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31 but not in adjacent DR-derived hepatocytes supporting a proliferative advantage of the latter over
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33 native hepatocytes. These findings suggest an explanation for sometimes extensive parenchymal
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35 reconstitution in advanced stage chronic viral hepatitis in humans[9] that has not been convincingly
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37 demonstrated in acute or mild human hepatitic injury or in most animal models of human disease.
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41 As native hepatocytes eventually become damaged and consequently senescent, the DR-derived
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43 hepatocytes then begin to repopulate owing to their untapped replicative potential[9]. This proposal
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45 was further supported by a higher proliferation rate of the DR-derived population once the injurious
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47 toxic was retrieved resulting in a significant proportional increase in the lobular area occupied by DR-
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49 derived hepatocytes and hence a decreased proportion of native hepatocytes in the lobules. As a
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51 result, DR contribute to the regeneration of a significant proportion of the parenchyma in the
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53 chronically injured liver.
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1 The ploidy pattern of DR-derived and native hepatocytes also differed. As reported elsewhere[40]
2 various stress signals, among which oxidative stress, provoke a decrease in the proportion of stable
3 tetraploid nuclei and a rise in that of highly polyploid nuclei. In a context of CCl₄ treatment, we
4 observed this precise stress-induced shift in native hepatocytes but not in DR-derived hepatocytes.
5 Indeed, the latter, although being exposed to CCl₄ and appropriately expressing CYP2E1 necessary
6 for CCl₄ activation, exhibited a nuclear polyploid pattern similar to that of liver parenchymal cells in
7 an unarmed liver. This differential ploidy pattern persisted despite continuation of CCl₄
8 administration. By maintaining a less polyploid genome, DR-derived hepatocytes would, like stem
9 cells do, retain a higher replicative potential with a lower risk to accumulate DNA damage. In
10 support to this, DR-derived hepatocytes in the healing liver retained less γ -H2AX foci and thus
11 unrepaired DNA. Moreover, when exposed to DEN, a carcinogenic agent, native but not DR-derived
12 hepatocytes underwent carcinogenic transformation, supporting stress resistance and stability of
13 the latest. Whether indeed DR-driven regeneration reduces the risk of cancer would need to be
14 confirmed in dedicated studies.

15 We provide here evidence that a population of younger and healthier hepatocytes traced from
16 cholangiocytes and not from hepatocytes emerges into an injured liver. Stimulation of DR-derived
17 regeneration *in vivo* appears therefore as a safe strategy to alleviate liver insufficiency in chronic
18 liver disease. However, several key issues are awaiting answers. Our experimental design does not
19 allow identifying the characteristics empowering (a subset of) biliary cells with reactive capacity to
20 mount the ductular reaction (the “target cells” for DR-derived regeneration). They could correspond
21 to the label retaining cells recently described by Cao et al[47]. Also, while failure of mature
22 hepatocyte to divide and regenerate the organ contributes to trigger DR and its differentiation,
23 many other processes activated during wound repair are likely to be involved such as vascular
24 changes, inflammation, modification of the ECM scaffold, etc[8,12,48]. Whether all small DR-derived
25 hepatocytes undergo clonal expansion and equally contribute to liver regeneration is not answered
26 by the present study. We observed, when liver disease becomes severe and while the number of DR-

1 derived hepatocytes increases in growing foci, a decline in the total amount of YFP⁺ cells owed to a
2 drop in the number of small foci. The absence of mosaicism in DR-derived hepatocyte foci in confetti
3 mice does not support a confluence of growing foci. We therefore suppose that a large proportion of
4 emerging small DR-hepatocytes do not survive in a stressed organ, or are not similarly exposed or do
5 not similarly respond to stimuli to enable complete differentiation and proliferation. A better
6 understanding of the mechanisms supporting DR-derived regeneration is now the next research goal
7 to identify pathways amenable to therapeutic manipulation for the treatment of liver insufficiency.
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Author names in bold designate shared co-first authorship

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Figure Legends

Figure 1. *DR cells significantly contribute to the hepatocyte pool during chronic liver injury*

(A) Schematic representation of the experimental design. (B) Liver section obtained from OPN- $iCreER^{T2};Rosa26R^{YFP}$ mice treated for 0 (n=6), 4 (n=2), 6 (n=5), 8 (n=4) and 16 (n=4) weeks CCl_4 stained with Sirius red for collagen (Scale bar: 100 μ m). (C) Representative YFP immunohistochemistry on liver sections from OPN- $iCreER^{T2};Rosa26R^{YFP}$ controls or mice treated with CCl_4 for 4, 6, 8 and 16 weeks. (Scale bar: 100 μ m) (D) Morphometric quantification of the YFP positive area; (E) Ki67⁺ hepatocyte quantification in control and treated mice. Values are expressed as means of percentage $\pm$ SEM; (F) Size of the YFP⁺ patches from the liver sections of OPN- $iCreER^{T2};Rosa26R^{YFP}$ mice treated with CCl_4 for 6, 8 and 16 weeks. (G) Genes involved in senescence and cell cycle arrest pathways were determined by RT qPCR. (H) Representative images of isolated hepatocytes stained for p21. (Scale bar: 100 μ m) (I) quantification of the p21+ hepatocytes. Values are expressed as mean $\pm$ SEM relatively to controls. *p<0,05; ***p<0,001; ****p<0,0001 by 1way ANOVA.

Figure 2. *DR-derived hepatocytes are fully mature hepatocytes*

Representative pictures of liver sections from TAM-injected-OPN- $iCreER^{T2};Rosa26R^{YFP}$ animals treated with CCl_4 during 8 weeks stained for (A) HNF4 α (red)/ YFP (green), (B) Glutamine synthetase GS (red)/YFP (green) and carbamoyl phosphate synthetase CPS1 (red)/YFP (green), (C) Cytochrome P450 2E1 (CYP2E1-red)/YFP (green), (D) carcinoembryonic antigen-related cell adhesion molecule1 (CEACAM1-red)/YFP (green) (scale bars: 50 μ m). (E) Consecutive sections stained for glycogen (periodic acid-Schiff (PAS) and YFP (brown) (Scale bar: 100 μ m). (F) Representative pictures of liver sections from TAM-injected OPN- $iCreER^{T2};Rosa26R^{mT/mG}$ mice control (Scale bar: 20 μ m) and treated for 16 weeks with CCl_4 (Scale bar: 50 μ m) (G) RNAsequencing data generated heatmap displays similar expression of mature and functional hepatocytic genes (n=5/group).

Figure 3. Patches of new hepatocytes with no-hepatocytes origin.

(A) Representative pictures of liver sections stained with YFP antibody in control mice (n=5) and mice treated with CCl₄ for 6 (n=4) and 8 (n=4) weeks (scale bar: 100μm). (B) Representative pictures of liver sections from AAV8-TBG-Cre;Rosa26R^{YFP} animals control and treated with CCl₄ for 6 and 8 weeks stained for HNF4α (red)/ YFP (green)/ck19 (white): in the CTL liver all the hepatocytes are YFP⁺, expressed HNF4α but not ck19; in CCl₄ livers YFP⁺ and YFP⁻ hepatocytes were HNF4α⁺ ck19⁻ (scale bar: 50μm). (C) Size of the YFP⁻ patches from the liver sections of AAV8-TBG-Cre;Rosa26R^{YFP} mice treated with CCl₄ for 6 and 8 weeks.

Figure 4. Chronic CCl₄ results in progressive fibrosis and discrete transient accompanying DR

(A) Liver section obtained from OPN-iCreER^{T2};Rosa26R^{YFP} mice treated for 0 (n=6), 4 (n=2), 6 (n=5), 8 (n=4) and 16 (n=4) weeks CCl₄ stained with cytokeratin 19 (ck19) for bile ducts (arrowhead) and DR (black arrows). (Scale bars: 100μm). **Insert:** higher magnifications of normal bile ducts and DR are shown. (B) Morphometrical quantification of ck19⁺ cells confirms discrete and transient DR during the progression of the injury. (C) DR markers and (D) genes of the Notch pathway were determined by RT qPCR. Values are expressed as mean ± SEM relatively to controls. *p<0,05; **p<0,01; ****p<0,0001 by 1way ANOVA.

Figure 5. Biliary cells in DR and DR-derived hepatocytes undergo clonal expansion.

Representative confocal pictures of liver sections of OPN-iCreER^{T2};Rosa26R^{Confetti} mice treated with CCl₄ for 8 weeks (n=4) showing (A) DR and (B) and DR-derived hepatocyte patches (scale bar: 20μm). (C) Size in μm² (mean ± SEM) of YFP⁺ and YFP⁻ hepatocytes in OPN-iCreER^{T2};Rosa26R^{YFP} mice. Quantification of (D) ki67⁺ and (E) phosphoHistone-H3 (PHH3)⁺ hepatocytes in YFP⁺ and YFP⁻ populations in livers from OPN-iCreER^{T2};Rosa26R-YFP mice treated for 6 (n=5), 8 (n=4) and 16 (n=4) weeks. *p<0,05 **p<0,01 **** p<0,001 by t-test in (c-e)

Figure 6. Upon injury, DR-derived hepatocytes face the same DNA-damage level, but better cope with it.

(A) Gene expression for genes involved in the DNA repair machinery in healthy hepatocytes (white bar), DR-derived hepatocytes (green bar) and native hepatocytes (red bar) sorted from TAM-treated OPN-iCreER^{T2};Rosa26R^{mT/mG} mouse livers after 6 (n=4) and 8 (n=4) weeks CCl₄. Quantification of (B) γ H2AX⁺/YFP⁺ and γ H2AX⁺/YFP⁻ hepatocytes after 8 weeks CCl₄ (C) apoptotic TUNEL⁺ hepatocytes in YFP⁺ and YFP⁻ populations in livers from OPN-iCreER^{T2};Rosa26R-YFP mice treated for 6, 8 and 16 weeks with CCl₄. (D) Gene Set enrichment analysis in mTomato (RED) native hepatocytes and mGFP (GREEN) DR-derived hepatocytes sorted from TAM-treated OPN-iCreER^{T2};Rosa26R^{mT/mG} mouse livers after 16 weeks of CCl₄ (E) Analysis of nuclear ploidy in OPN-iCreER^{T2};Rosa26R^{YFP} mice treated for 16 weeks with CCl₄ (n=4). Box plot of the percentage of the tetraploid (4n) and highly polyploid ($\geq 8n$) nuclei relative to mononuclear polyploid nuclei. *p<0,05; **p<0,01 by t-test in Mann-Whitney test.

Figure 7. DR-derived hepatocytes have a survival, proliferative and DNA repair advantage upon recovery.

OPN-iCreER^{T2};Rosa26R^{YFP} mice were treated for 16 weeks with CCl₄ and analysed 72h after last CCl₄ injection (n=4) or after 2 and 4 weeks of CCl₄-free recovery period (n=4 and n=3, respectively). (A) YFP immunohistochemistry (scale bar: 100 μ m) with (B) morphometric quantification of YFP⁺ area; (C) analysis of the size in the YFP⁺ patches and (D) of the size (in μ m²) of the YFP⁺ and YFP⁻ hepatocytes. (E) Representative picture of liver sections stained for Ki67/YFP. Red arrows indicate the YFP⁺ Ki67⁺ DR-derived hepatocytes. On the contrary, the white arrows point to the YFP⁻ Ki67⁺ native hepatocytes (Scale bar: 50 μ m). Quantification of the (F) Ki67 index and (G) PHH3 index in YFP⁺ and YFP⁻ hepatocytes; (H) Quantification of the γ H2AX⁺/YFP⁺ and γ H2AX⁺/YFP⁻ hepatocytes. *p<0,05, **p<0,01, ****p<0,0001 by 1way ANOVA (b, c, e) or t-test (d, g, h).

Figure 8. DR-derived hepatocytes in cirrhotic regenerative nodules.

(A) Representative liver harvested from OPN-iCreER^{T2};Rosa26R^{YFP} mice treated for 24 weeks with CCl₄ (n=10), (B) YFP immunohistochemistry (bar size: 1mm) with white arrows pointing towards YFP⁻ nodules, green arrows towards YFP⁺ nodules, and the white/green arrow towards a mosaic nodule. (C) Percentage of YFP⁺ and YFP⁻ nodules per liver section (one dot per liver). (D) Representative pictures of GS and YFP immunostaining on consecutive slides. (E) Analysis of GS expression in YFP⁺ and YFP⁻ regenerative nodules and (F) analysis of tetraploid (4n) and highly polyploid (≥8n) nuclei relative to mononuclear polyploid nuclei. (n=3 in CTL group; n=10 in CCl₄ group). (G) IHC score based on GS, β-catenin and Ki67 (see materials and methods) on YFP⁺ and YFP⁻ nodules. ***P<0,001 by t test (c) **p<0,01 by Mann-Whitney test (f) and * P<0,05 by Kolmogorov-Smirnov test (g).

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Figure
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Figure 1

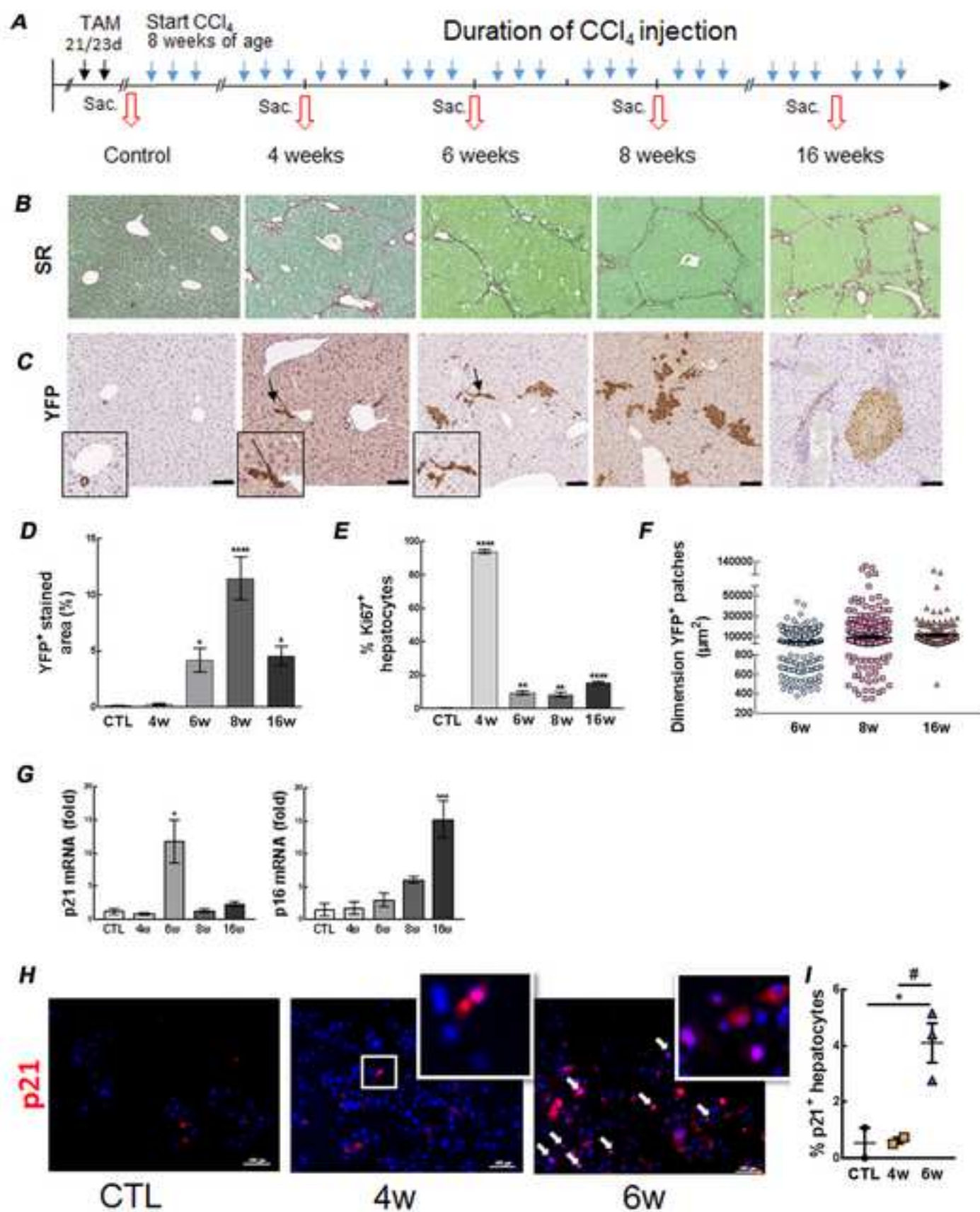


Figure 2

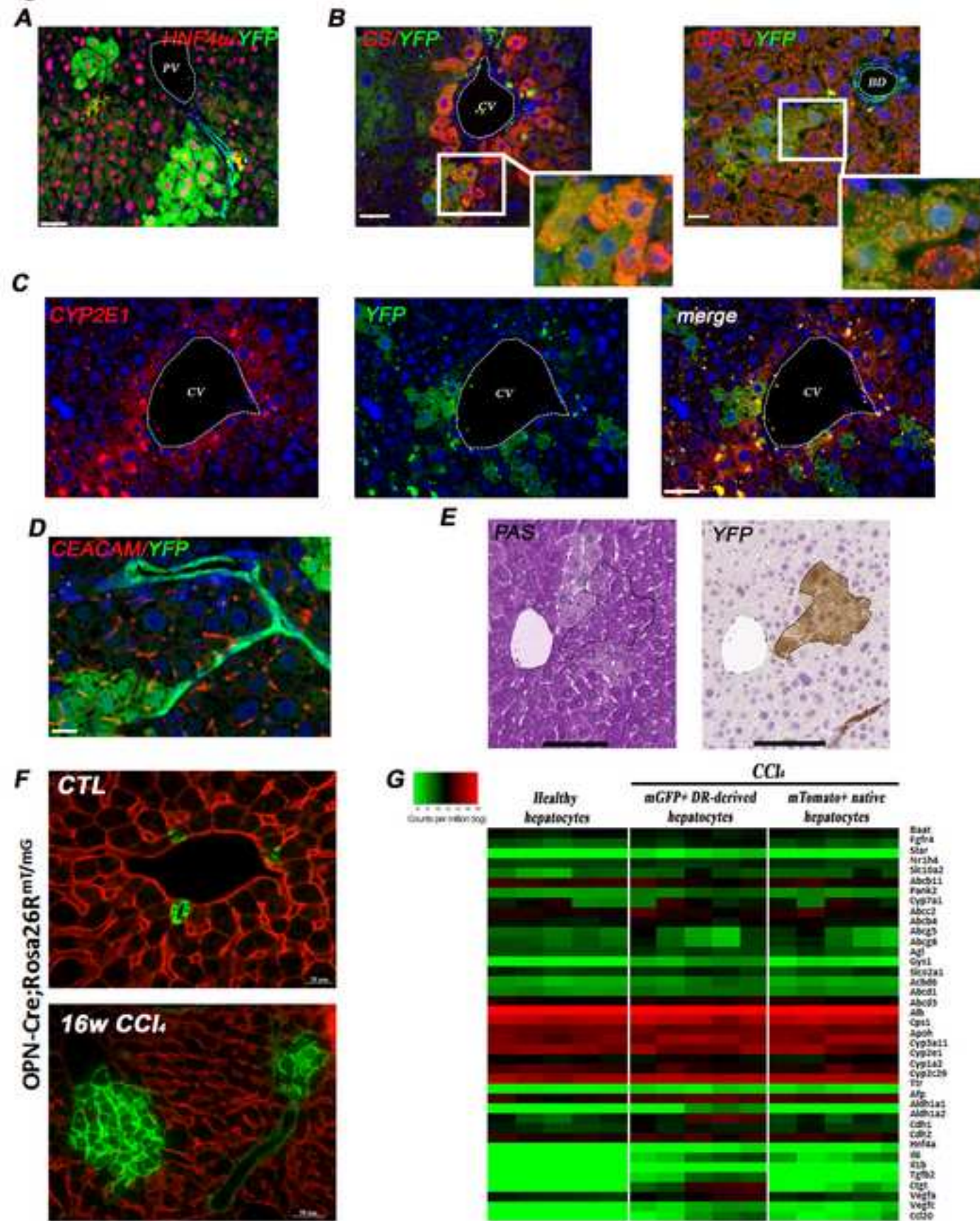


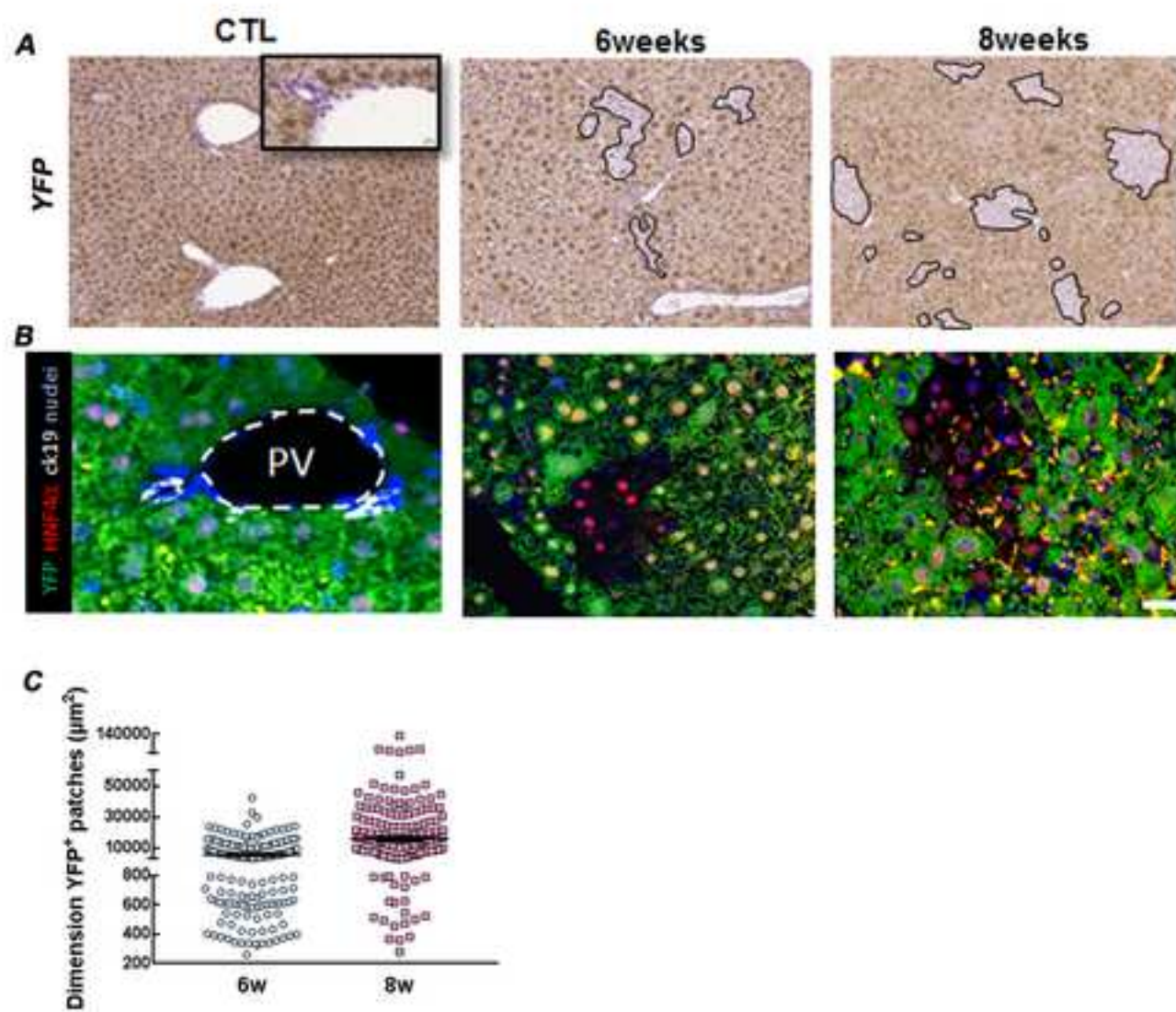
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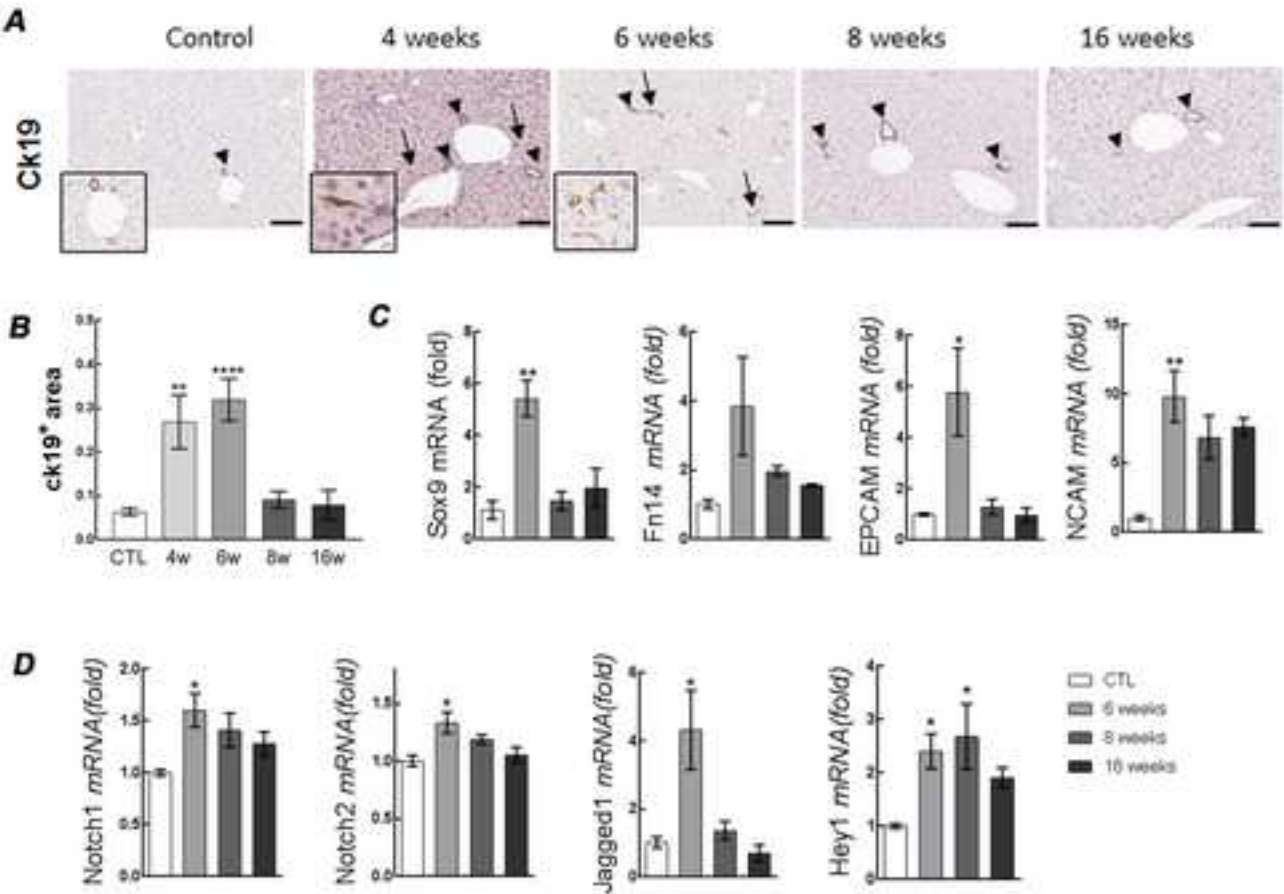
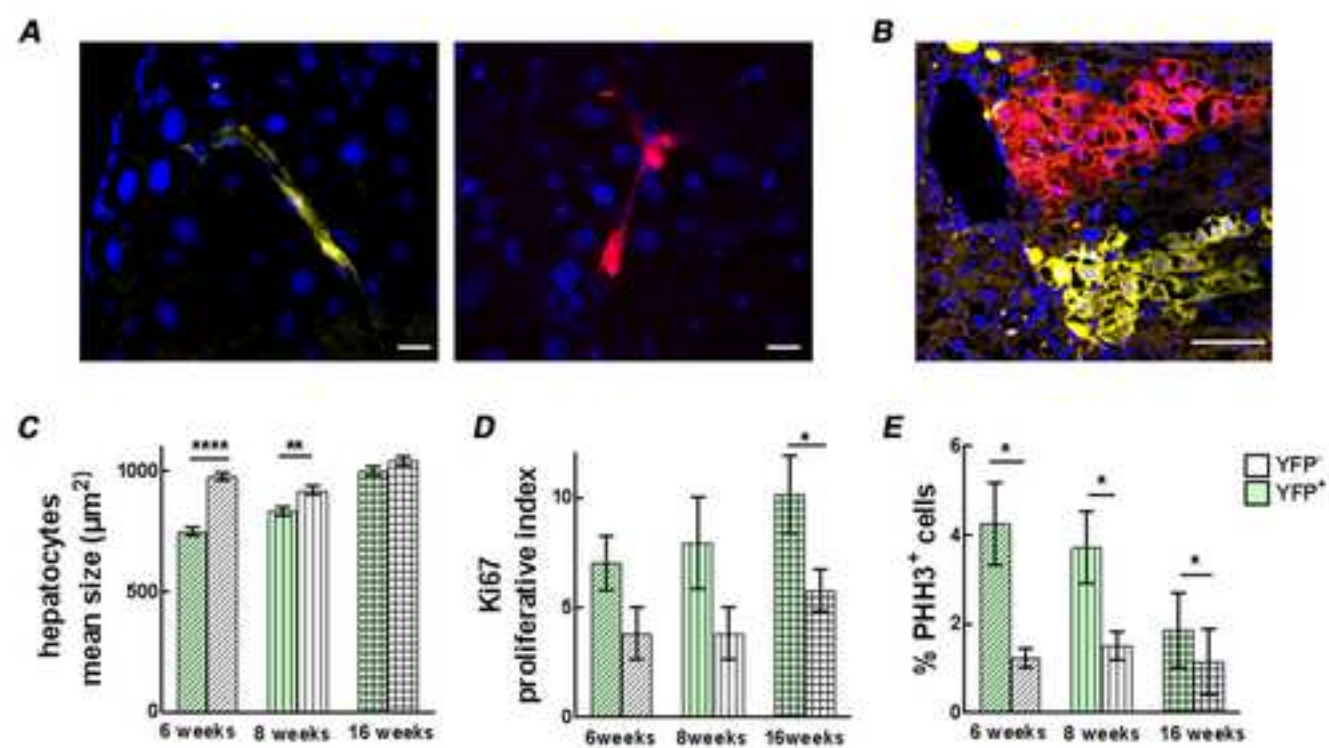


Figure 5



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Figure 6

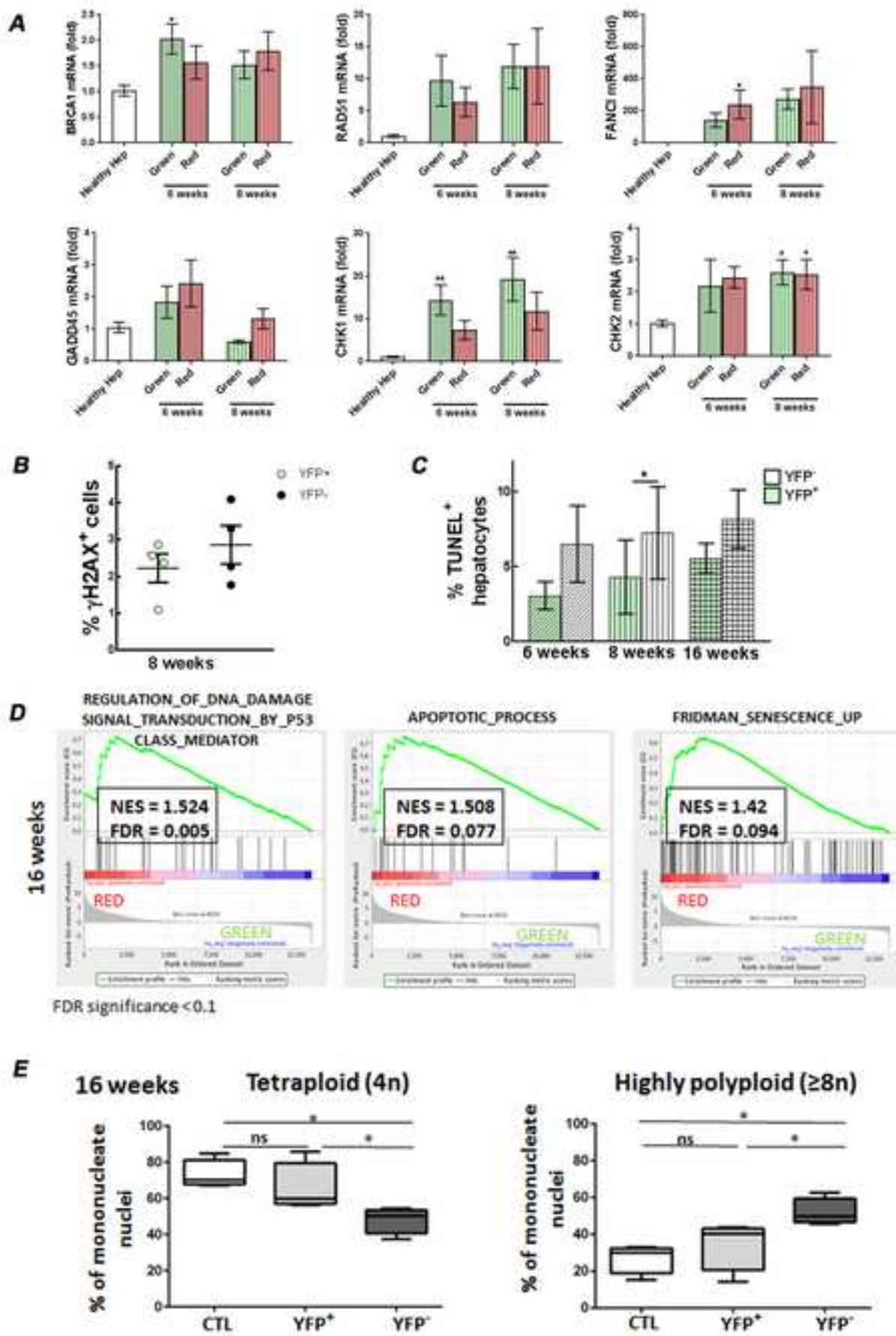


Figure 7

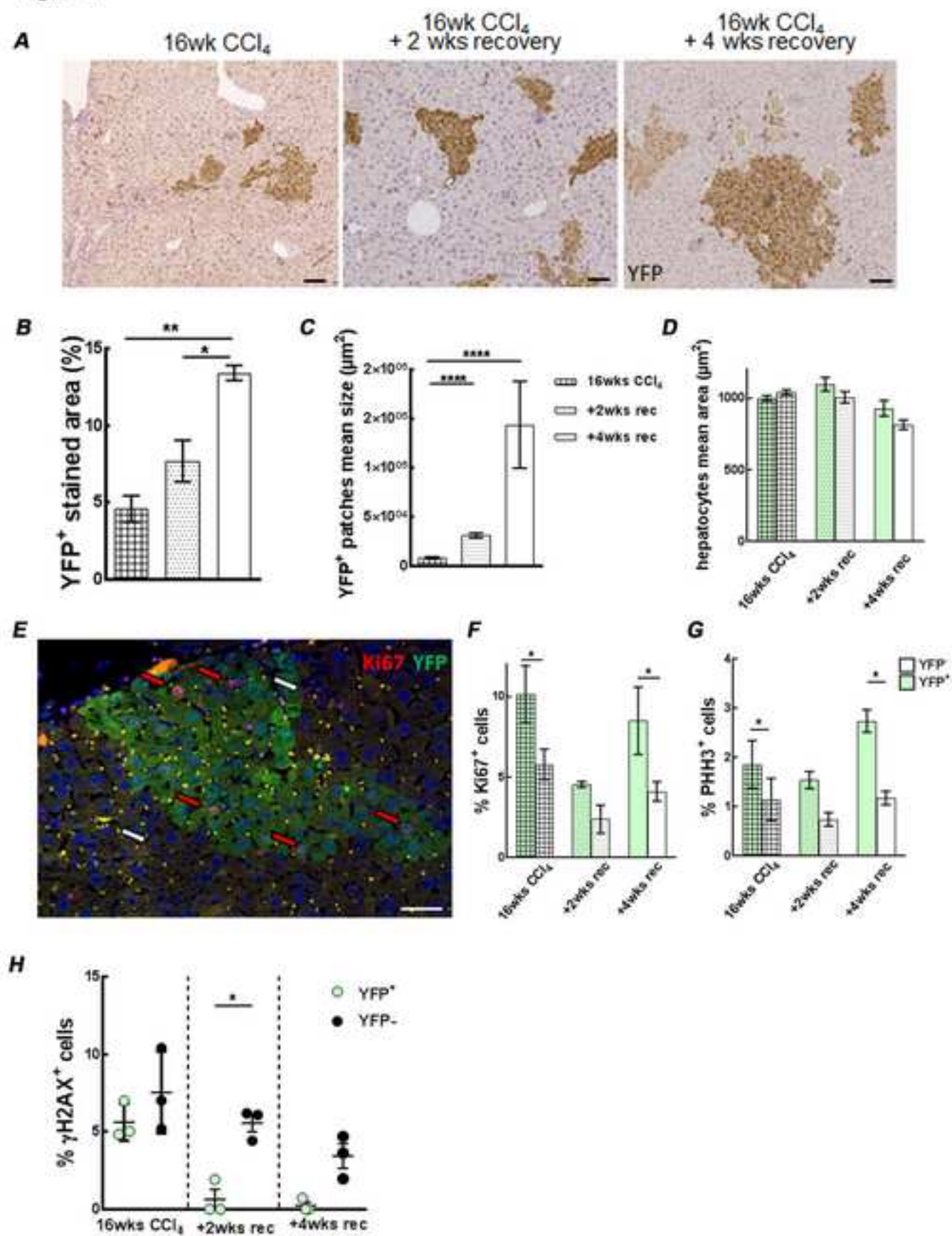
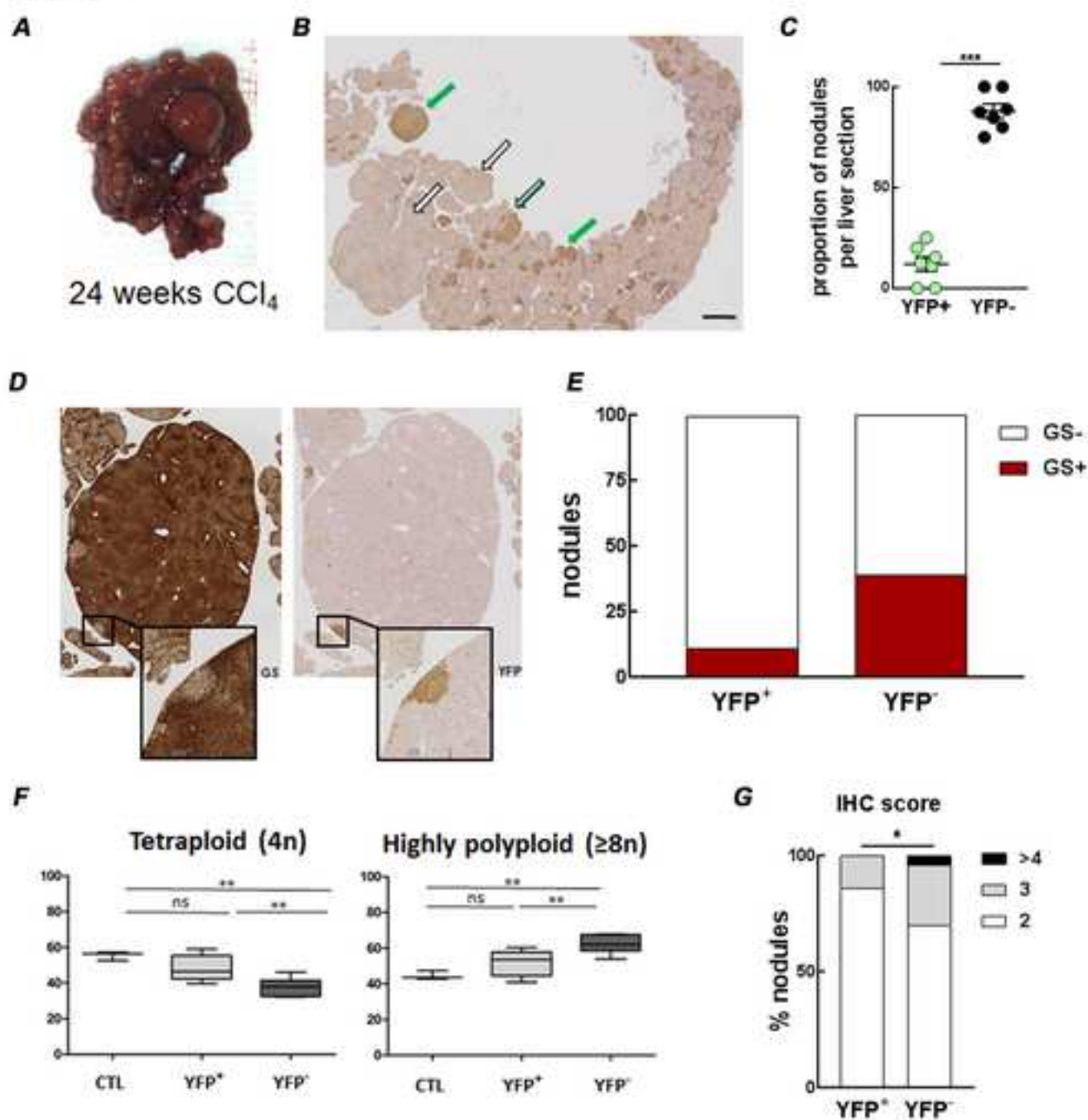
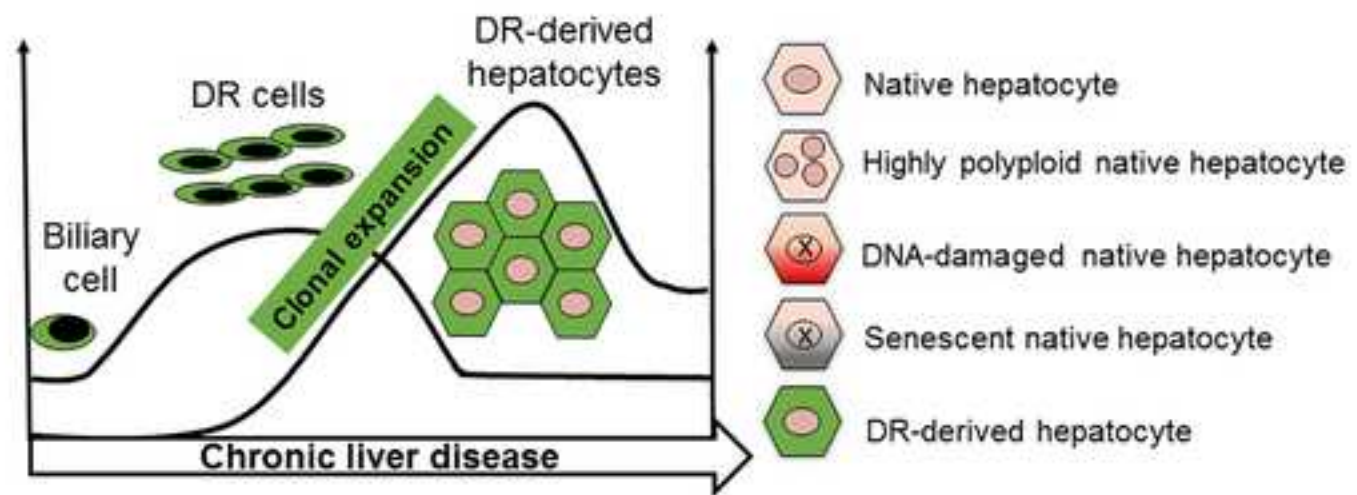
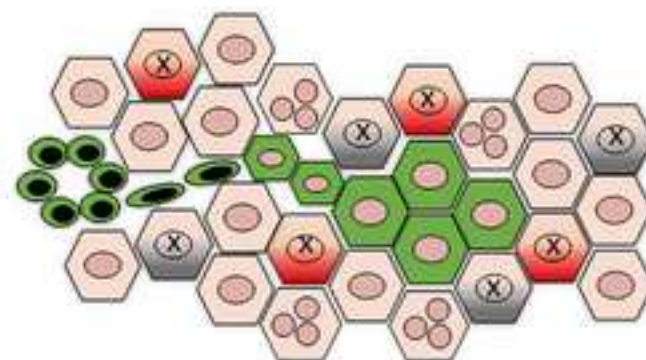


Figure 8





HIGHLIGHTS

During chronic injury,

- native hepatocytes become damaged, replicatively senescent and accumulate DNA damage.
- New hepatocytes with specific characteristics of “undamaged and young” hepatocytes emerged and clonally expand.
- Our cell tracing studies support these new hepatocytes derive from reactive cholangiocytes.

Supplementary material

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