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Experimental and Translational Hepatology

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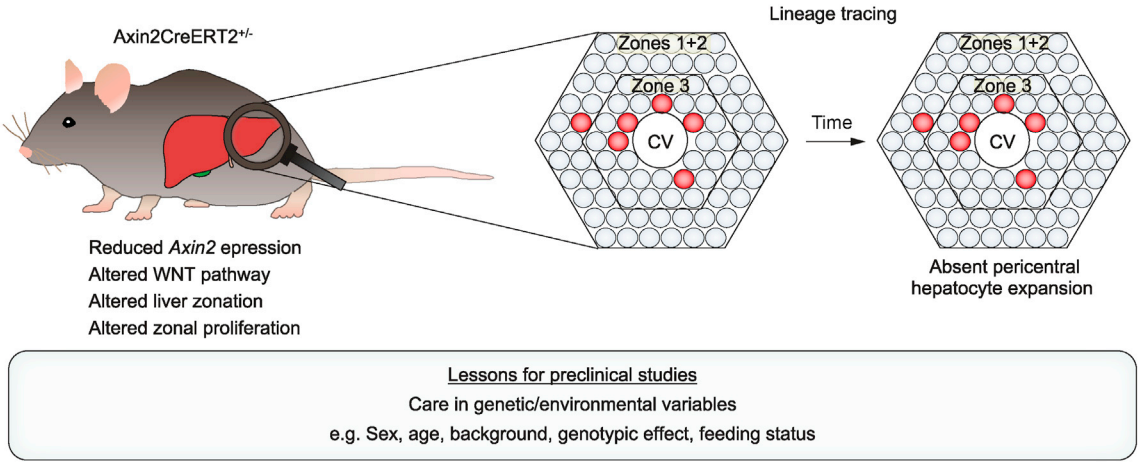
Graphical abstract



Highlights

- Pericentral hepatocytes do not play a dominant role in physiological hepatocyte regeneration.
- The Wnt pathway is suppressed by Axin2 haploinsufficiency.
- Mouse genetics and environmental conditions should be rigorously controlled in preclinical models.

Impact and implications

Understanding the source of cells which regenerate the liver is crucial to harness their potential to regrow injured livers. Herein, we show that cells which were previously thought to repopulate the liver play only a limited role in physiological regeneration. Our data helps to reconcile differing conclusions drawn from results from a number of prior studies and highlights methodological challenges which are relevant to preclinical models more generally.

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Absent expansion of AXIN2+ hepatocytes and altered physiology in Axin2CreERT2 mice challenges the role of pericentral hepatocytes in homeostatic liver regeneration

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Background & Aims: Mouse models of lineage tracing have helped to describe the important subpopulations of hepatocytes responsible for liver regeneration. However, conflicting results have been obtained from different models. Herein, we aimed to reconcile these conflicting reports by repeating a key lineage-tracing study from pericentral hepatocytes and characterising this Axin2CreERT2 model in detail.
Methods: We performed detailed characterisation of the labelled population in the Axin2CreERT2 model. We lineage traced this cell population, quantifying the labelled population over 1 year and performed in-depth phenotypic comparisons, including transcriptomics, metabolomics and analysis of proteins through immunohistochemistry, of Axin2CreERT2 mice to WT counterparts.
Results: We found that after careful definition of a baseline population, there are marked differences in labelling between male and female mice. Upon induced lineage tracing there was no expansion of the labelled hepatocyte population in Axin2CreERT2 mice. We found substantial evidence of disrupted homeostasis in Axin2CreERT2 mice. Offspring are born with sub-Mendelian ratios and adult mice have perturbations of hepatic Wnt/β-catenin signalling and related metabolomic disturbance.
Conclusions: We find no evidence of predominant expansion of the pericentral hepatocyte population during liver homeostatic regeneration. Our data highlight the importance of detailed preclinical model characterisation and the pitfalls which may occur when comparing across sexes and backgrounds of mice and the effects of genetic insertion into native loci.

Introduction

Understanding how the liver regenerates is a key biological question. Hepatocytes are the principle regenerative population in the liver.[1] The liver architecture compromises a spectrum of hepatocyte phenotypes across the liver lobule – spanning from the periportal (zone 1) to pericentral (zone 3) separated by a midlobular area (zone 2). All hepatocytes across the liver lobule have the ability to regenerate following surgical resection.[1] However, significant debate is ongoing as to whether specific subpopulations of hepatocytes are particularly responsive to the subtle regenerative cues present during hepatocyte turnover during homeostasis – in the absence of injury. Understanding whether this occurs and what the mechanisms are which promote such regeneration in any subpopulation of hepatocytes primed for proliferation may shed important insights, which could be harnessed for regenerative therapies.

Lineage tracing applies genetic tagging to a restricted population in order to track its descendants over time. Recently, numerous lineage-tracing studies tracking subpopulations of hepatocytes in adult mice have reported conflicting results, using a variety of hepatocyte-based reporting systems in mice during homeostasis.[1–7] The first significant lineage-tracing study on a distinct subpopulation of hepatocytes in homeostasis reported hyper-proliferation of self-renewing pericentral hepatocytes which subsequently expanded across the liver lobule.[6] This study used a CreERT2 construct knocked into the endogenous *Axin2* locus; hereafter termed Axin2CreERT2. This study was supported by a more recent comprehensive lineage-tracing analysis across multiple models which, whilst showing expansion of this population in

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the Axin2CreERT2 model, demonstrated subtle phenotypic differences between these and wild-type (WT) mice.[4] Other studies, using different markers to identify pericentral hepatocytes (*e.g.* LGR5(7), glutamine synthetase [GS][4]), have not shown expansion of a related lineage traced population over time. Typically, these studies have employed transgenes inserted randomly within the genome, using a bacterial artificial chromosome (BAC) approach, whilst others have specifically knocked in Cre recombinase-activating systems in the non-coding regions of endogenous genetic loci, *e.g*. 3' untranslated regions, to preserve native gene function. In one such approach using a BAC AxinCreERT2 transgene,[3] a resulting lineage-tracing study once again did not show population expansion in homeostasis. Additionally, a further BAC-based *Lgr5* transgene showed no expansion of pericentral hepatocytes from puberty through to adulthood in homeostasis.[8] Recent studies examining the zonal origin of proliferating cells[5] and tracing cells from across the lobule[4,9] have shown consistent results, suggesting that hepatocytes within zone 2 are most prone to expansion during adult homeostasis.

Herein, we aim to reconcile these discrepancies by re-evaluating lineage tracing in the Axin2CreERT2 knock-in model and exploring the physiological consequences of this mutant allele. We were unable to find evidence of expansion of an Axin2CreERT2-labelled population and show that this population, whilst showing a propensity to label pericentral hepatocytes *vs*. other hepatocytes, is spread throughout the lobule and thus is not zonally restricted. Finally, we report that this allele results in profound perturbation of the Wnt pathway and physiology in the mouse.

Materials and methods

Details regarding the materials and the methods used are described in the supplementary information. Reagents used in this study are listed in the CTAT table.

Results

Absence of expansion from an Axin2^{+} population in homeostasis

In order to define a baseline labelled population in the Axin2CreERT2$^{+/WT}$ mouse model we administered mice with 4 mg tamoxifen, which does not affect Wnt target gene expression in WT mice (Fig. S1A,B). We analysed labelling efficiency at three time points within a week after induction (Fig. 1A). We observed maximal labelling of pericentral hepatocytes after 5 days (Fig 1B,C), with significantly less labelling in male compared to female mice. For simplicity, we hereon refer to quantitative data for female mice, but trends were similar in both sexes. We then compared different reporters previously employed in the Axin2CreERT2$^{+/WT}$ mouse models; mTmG(6) and LSL-RFP(4) (Fig. S2A). Here, we observed equivalent labelling in the hepatocyte population with each reporter (Fig. S2B-E). Next, we studied the zonal restriction of hepatocyte labelling using hepatic zones defined by expression of GS and E-cadherin (Fig. 1D). Zonal analysis showed a progressive increase in labelling in zone 3 from day 2, plateauing from day 5 to day 7 when 35% of hepatocytes in zone 3 were labelled in the LSL-RFP model (Fig. 1E) with equivalent labelling in the mTmG model (Fig. S2f). However, only approximately half of all labelled hepatocytes were contained within zone 3 (Fig. S2G); itself comprising consistently 6-8% of all hepatocytes (Fig. SH). In the remaining liver lobule (zones 1 and 2), 1.5-3.5% of hepatocytes were also labelled (Fig. 1B open arrows, Fig. S2I), including hepatocytes within zone 1 (Fig. 1F,G); albeit to a lesser extent than in other liver zones (Fig. S2J). Consistent with recombination of the reporter in Axin2CreERT2$^{+/WT}$ across the lobule, *Axin2* was expressed throughout the lobule in WT mice but with a gradient of expression from highest in zone 3 to lowest in zone 1 (Fig. 1H). We assessed whether reporter labelling was dependent upon zonal location and/or *Axin2* expression and observed a dominant effect of zonal location when comparing zones 1+2 to zone 3. However, there was also evidence of *Axin2* expression affecting reporter labelling within zones 1+2 (Fig. S2K). Therefore, consistent with existing data,[4] whilst this labelling system preferentially labels zone 3 hepatocytes, the labelling is not restricted to these pericentral hepatocytes, as was reported in the original study;[6] with 53.6% of labelled hepatocytes residing in zones 1 and 2 combined. Furthermore, as incomplete labelling occurs at early time points, 5-7 days (but not as early as 2 days) is a more appropriate baseline for lineage tracing in this model.

To further explore labelling outside zone 3 we performed a depletion experiment by destroying the GS zone using the hepatotoxin carbon tetrachloride (CCl_4). Here, we increased the labelling of hepatocytes using a repeated tamoxifen dosing regimen with CCl_4 administered 1 week after the start of induction (Fig. S3A). Twenty-four hours post CCl_4 administration, there was efficient destruction of zone 3 hepatocytes but only a partial depletion of RFP-labelled hepatocytes (Fig. S3B,C). Labelled hepatocytes were preserved into the recovery phase with some reconstituted GS hepatocytes bearing the reporter 4 days post CCl_4 (Fig. S3D). We further tested this using a single induction regimen of 4 mg tamoxifen and administered CCl_4 or corn oil 7 days after induction and analysed liver tissues 24 h post CCl_4 (Fig. S3E). As with the high tamoxifen-labelling regimen, we see depletion of zone 3 hepatocytes and a partial loss of RFP-labelled hepatocytes compared to corn oil-treated animals (Fig. S3F,G), as well as retention of labelled hepatocytes outside zone 3 (Fig. S3H). Therefore, using a well characterised toxin to functionally define the pericentral zone, we again observe genetic labelling outside zone 3.

For long-term lineage-tracing studies, with the rationale of maximising restriction of hepatocyte labelling to zone 3, we used a lower dose of tamoxifen (Fig. 1I) and further compared labelling with LSL-RFP *vs.* the original mTmG reporter.[6] The 3 mg tamoxifen dose achieved comparable labelling of hepatocytes (~7% in LSL-RFP model; Fig. 1J) to that previously published in the mTmG model at day 7 using 4 mg.[6] Again, we observed progressive labelling from day 2 to 7 and therefore used day 7 as a baseline. At day 7, no areas of confluent hepatocyte labelling were observed. Lineage tracing was then performed in otherwise untreated mice for up to 1 year. During this time, across all lobes, no overall expansion of the labelled hepatocyte population occurred in either female or male mice when evaluating either hepatocytes or total labelled liver area (Fig. 1J-L and Figs. S4 and S5A). Some patches of confluent labelling of hepatocytes were observed at 200 and 365 days but these were infrequent (approximately 3/lobe) and occurred overwhelmingly in female mice. Additionally, we observed no expansion of the labelled population with lineage tracing to 90

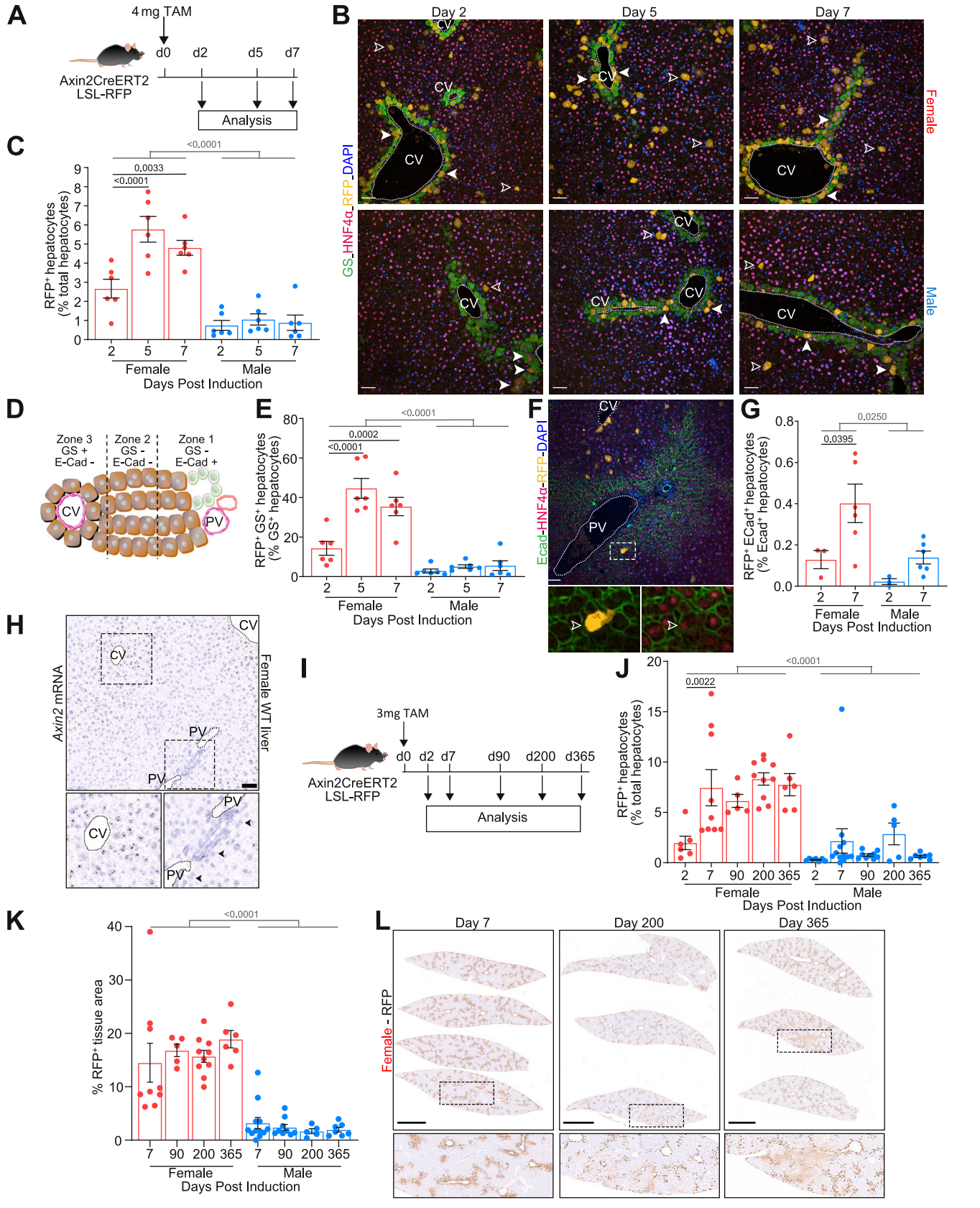

Fig. 1. Axin2^{+} hepatocytes do not preferentially contribute to homeostatic liver regeneration. (A) Axin2CreERT2$^{+/WT}$ progeny of Axin2CreERT2$^{+/WT}$ mice crossed with LSL-RFP$^{+/+}$ reporter mice were induced with 4 mg tamoxifen and analysed at 2, 5 and 7 days post induction. (B) Representative microscopic images highlighting central perivenular (GS; green), labelled (RFP; orange) hepatocytes (HNF4α; red) and nuclei (DAPI; blue) are shown for female and male mice at each time point post tamoxifen induction, demonstrating preferential labelling of hepatocytes around the CV (solid arrows), but also hepatocytes outside of this area (open arrows). Dashed

days in the Axin2CreERT2$^{+/WT}$ mTmG model (Fig. S5A-C). Therefore, whilst some localised expansion events are observed, no overall expansion of the labelled hepatocyte population occurred in Axin2CreERT2$^{+/WT}$ mice.

Given the previous report of increased proliferation and self-renewal of zone 3 hepatocytes,[6] we examined both proliferation in WT mice, using both young and aged mice of both sexes, and retention of labelled cells within zone 3 in the Axin2CreERT2$^{+/WT}$ lineage-tracing models. Consistent with other reports,[4,5,7] in WT mice, we did not find hyper-proliferation of hepatocytes within zone 3 relative to other zones and found zone 2 to be more proliferative (Fig. S6A-E). In the lineage-tracing model, within zone 3 we observed stable proportions of labelled hepatocytes over time, demonstrating retention, but no expansion, of labelled pericentral hepatocytes in the Axin2CreERT2$^{+/WT}$ mice independent of the reporter employed (Fig. S6F-H).

Wnt pathway is altered in homeostasis in the presence of the Axin2CreERT2 allele

As Axin2 is part of the Wnt/β-catenin pathway we hypothesised that this knock-in allele of Axin2CreERT2 may cause a haploinsufficiency phenotype. We began by examining the physiological effects of the presence of this allele in uninduced mice. Breeding Axin2CreERT2$^{+/WT}$ mice, we were unable to generate homozygote pups (0/14 at weaning; with four dying perinatally). In heterozygote x WT matings, we obtained significantly fewer heterozygote pups at weaning than predicted (Fig. 2A, Fig. S7A). Despite this, uninduced heterozygote mice assessed during adulthood have equivalent body weight (data not shown), relative liver weight (Fig. S7B), hepatocyte proliferation (Fig. S7C) and liver biochemistry (Fig. S7D-F) as WT littermates.

Next, we assessed the Wnt pathway in Axin2CreERT2$^{+/WT}$ mice. Herein, we used Wnt reporter mice (Tcf/Lef:H2B-GFP, Fig. 2B) expressing GFP in Wnt/β-catenin responsive cells.[10] *Axin2* expression was reduced in uninduced Axin2CreERT2$^{+/WT}$ mice (Fig. 2C-E, Fig. S8A). Expression of *Axin2* was again zonated but not zonally restricted in both genotypes (Fig. 2D).

Given Axin2's role in the β-catenin destruction complex we next assessed the Wnt pathway in the liver. Using cell fractionation (Fig. 2F), we observed increased nuclear β-catenin in Axin2CreERT2$^{+/WT}$ mice compared to WT littermates (Fig. 2G, Fig. S8B) suggestive of hyper-activation of the Wnt/β-catenin pathway. We then examined Wnt/β-catenin pathway activation. First, in an unbiased approach, we performed a transcriptomic analysis. We found numerous dysregulated genes (Fig. 2H, Fig. S8C), with expression of Wnt pathway targets GS (*Glul*) and a pseudogene of GS (*Glns-ps1*) being the most altered in uninduced Axin2CreERT2$^{+/WT}$ mice compared to WT mice. A reduction of GS was confirmed by quantitative reverse-transcription PCR (Fig. 2I) and at the protein level (Fig. 2J,K). Additionally, a reduction was seen across a larger panel of Wnt pathway transcriptional targets (Fig. 2I-O and Fig. S8D). We sought to validate these findings with the *in vivo* Wnt pathway reporter system and found reduced Tcf/Lef-driven GFP reporter in uninduced Axin2CreERT2$^{+/WT}$ mice compared to WT littermates (Fig. 2P,Q). Finally, as β-catenin dysregulation has been described to affect free fatty acid metabolism in the liver,[11] we performed an untargeted metabolomic analysis on liver tissue from uninduced Axin2CreERT2$^{+/WT}$ mice compared to WT mice. The resulting principal component analysis segregated Axin2CreERT2$^{+/WT}$ from WT tissue (Fig. 2R). Consistent with the dysregulation of *Srebf1* expression[12] (Fig. 2H), the levels of several acyl carnitines were significantly decreased in Axin2CreERT2$^{+/WT}$ (Fig. S8E and Table S1). Taken together, our data demonstrate altered physiology in mice possessing the Axin2CreERT2 allele with profound changes in the Wnt signalling pathway in the liver, particularly in female mice.

As we observed changes in the Wnt pathway in Axin2-CreERT2 mice, but did not observe changes in liver size nor hepatocyte proliferation generally, we returned to perform a more detailed examination of hepatocyte proliferation across the lobule. Uninduced WT and Axin2CreERT2 mice were administered BrdU and tissue samples were taken 2 h later. Hepatocyte proliferation was measured within each of zones 1-3. In WT mice, we observed reduced proliferation in zone 3 (Fig. S8F), as we observed (Fig. S6D) and others have reported previously.[4,5,7] In Axin2CreERT2 mice, however, there was altered proliferation across the zones with comparatively greater proliferation within a constricted zone 3 (Fig. S8F and Fig. 2J,K). Therefore, the changes occurring in the Wnt pathway in Axin2CreERT2 animals are also associated with altered and abnormal zonal contribution to homeostatic hepatocyte proliferation in this model.

white line = border of CV, scale bar = 50 μm. (C) Quantification of RFP$^+$ hepatocytes over the time course compared to day 2; 21-44 (median = 31) high power fields in n = 6 mice at each time point separated by sex, two-way ANOVA, mean ± SEM, total hepatocytes 219,768/225,318/228,551 quantified at days 2/5 and 7, respectively. (D) We defined hepatocytes in liver zones as follows; zone 1 (periportal region) GS$^-$/Ecad$^+$, zone 2 GS$^-$/Ecad$^-$ and zone 3 (pericentral region) GS$^+$/Ecad$^-$. (E) Quantification of the proportion of zone 3 hepatocytes that are RFP$^+$, n = 6 mice at each time point, two-way ANOVA, mean ± SEM. (F) Microscopic images showing periportal (Ecad; green), labelled (RFP; orange) hepatocytes (HNF4α; red), in a female mouse 2 days post induction; dashed white line = border of CV and PV, open arrow highlights a RFP-labelled hepatocyte within the Ecad$^+$ zone 1, scale bar = 50 μm. (G) Quantification of the proportion of zone 1 hepatocytes labelled with RFP$^+$ over time post induction; 11-20 (median = 15) high power fields in n = 3 and 6 mice at days 2 and 7, respectively, for both sexes, two-way ANOVA, mean ± SEM, total hepatocytes 49,668/107,642 quantified at days 2 and 7, respectively. (H) RNA *in situ* hybridisation for *Axin2* in WT female murine liver demonstrating a zonated pattern of *Axin2* expression across the hepatic lobule; arrows highlight periportal hepatocytes expressing *Axin2*, scale bar = 50 μm. (I) Long-term lineage tracing for up to 1 year was performed post induction of Axin2CreERT2$^{+/WT}$ LSL-RFP$^{+/-}$ mice with 3 mg tamoxifen. (J) Quantification of RFP$^+$ hepatocytes over time compared to day 7 baseline; 17-69 (median = 30) high power fields analysed in n ≥5 mice at each time point, separated by sex and total hepatocytes 277,826/417,545/270,315/209,895/169,662 quantified at days 2/7/90/200 and 365, respectively, see Fig. S4 for examples of cell registration. No expansion from day 7 to 90, 200 or 365 days post induction; *p* values = 0.8482/0.6716 (day 90), 0.9203/0.9795 (day 200) and 0.9989/0.6659 (day 365) in females/males, respectively; two-way ANOVA, mean ± SEM. N numbers at days 2, 7, 90, 200 and day 365 were 6/7, 9/12, 5/9, 10/5 and 6/7 for females/males, respectively. (K) Additional quantification of RFP$^+$ expansion from 7 days as quantified by tissue area in whole liver tissue areas; numbers at days 7, 90, 200 and 365 were 9/12, 5/9, 10/5 and 6/7 mice for females/males respectively. *p* values *vs*. day 7 = 0.7546/0.9720 (day 90), 0.9195/0.9035 (day 200) and 0.2365/0.9237 (day 365) in females/males, respectively; two-way ANOVA, mean ± SEM, note these data are generated by using the same mice as in Fig. 1J. (L) Representative low power microscopic images of whole lobes from individual female mice stained with RFP are shown for day 7, 200 and 365; scale bars = 2.5 mm. Statistical comparisons between female *vs.* male mice are reported as the interaction factors from two-way ANOVA. CV, central vein; Ecad, E-cadherin; GS, glutamine synthetase; LSL, lox-stop-lox; PV, portal vein; RFP, red fluorescent protein; TAM, tamoxifen.

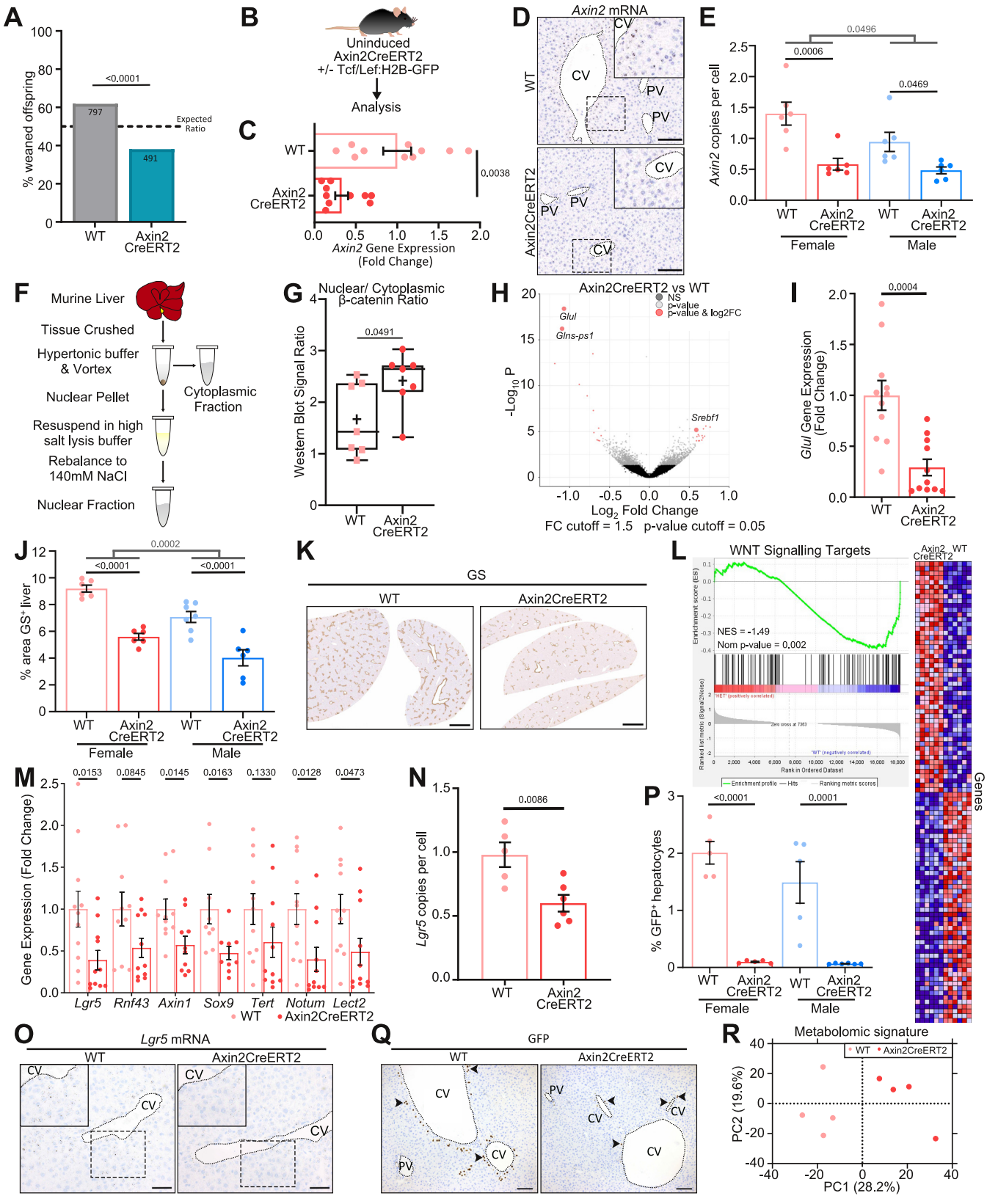

Fig. 2. Axin2CreERT2 knock-in construct alters liver physiology. (A) Expected Mendelian ratios of offspring weaned were not observed from WT x Axin2CreERT2$^{+/WT}$ mating's (1,288 mice from 80 matings over 6 years); chi-squared test. (B) For all homeostatic experiments, tissue was harvested from uninduced Axin2CreERT2 wild-type (referred to as WT) and heterozygous mice, either with a Tcf/Lef:H2B-GFP$^{+/-}$ reporter (n = 6 mice each) or without (n = 5 mice each). (C) *Axin2* gene expression from whole liver by qRT-PCR in female mice; n = 10 WT and 10 Axin2CreERT2$^{+/WT}$ mice, two-tailed Mann-Whitney *U*, mean ± SEM. (D) Representative images of RNA *in situ* hybridisation for *Axin2* in female livers comparing uninduced Axin2CreERT2$^{+/WT}$ *vs.* WT, with quantification in (E); scale bar = 100 μm, n = 6 per cohort, two-way ANOVA,

Presence of the Axin2CreERT2 allele does not affect liver regeneration

In view of the important role of Wnt/β-catenin in liver regeneration[13] and the dysregulation of this pathway in mice possessing the Axin2CreERT2 allele, we next examined whether the regenerative response is altered in these animals. To address this, we used the archetypical 70% partial hepatectomy regenerative model in uninduced mice (Fig. 3A), with analyses at either peak regeneration (day 2) or at a recovery time point (day 7). This model causes global parenchymal loss rather than the zonal-specific damage caused by most hepatotoxins. Using the Tcf/Lef:H2B-GFP reporter we also investigated Wnt responses. Here, recovery of liver weight, indicative of regeneration post partial hepatectomy, was independent of genotype in both sexes (Fig. 3B,C). Peak and total hepatocyte proliferation responses were also equivalent (Fig. 3D,E). Tcf/Lef-based reporting after partial hepatectomy was not altered in either control or Axin2CreERT2$^{+/WT}$ mice (Fig. 3F). Finally, taking an unbiased approach, we performed global hepatic transcriptomic analysis and found that highly similar transcriptional profiles occurred in both genotypes induced by partial hepatectomy (Fig. 3G-I) with no evidence of specific dysregulation of the Wnt/β-catenin pathway. Therefore, we conclude that, whilst differences in physiology and the Wnt/β-catenin pathway exist in homeostasis, we find no evidence of an altered regenerative response in Axin2CreERT2$^{+/WT}$ adult animals in response to partial hepatectomy.

Discussion

This study helps to reconcile the conflicting hepatocyte lineage-tracing studies. Crucially, over time, there is no increased expansion of the labelled population overall, particularly when taking into account the delayed labelling which occurs within the first week post tamoxifen. Consistent with previous reports,[4,6] we find, through the presence of confluent labelling in rare patches, evidence of expansion of some cells within the overall population labelled by Axin2CreERT2 (Fig. S4). Hence, within the labelled hepatocyte population, some rare expansion events occur, but these are balanced by the loss of labelled cells elsewhere. Our data argue against a dominant expansion of cells from within the pericentral zone in homeostasis, as previously proposed in the Axin2CreERT2 model,[6] and are consistent with other lineage-tracing studies, including those using zonally enriched Wnt/β-catenin pathway members in adult[3,4,7] and juvenile mice,[8] recent studies examining sites of proliferation[4,5,7] and lineage tracing elsewhere in the hepatic lobule.[4,9]

Despite theoretical differences between our Axin2CreERT2 and the published Axin2 BAC-transgene,[3] due to the site of knock-in and loss of endogenous regulatory elements, we were unable to find discrepancies in lineage tracing between these two models. Both the Axin2CreERT2 and the Axin2 transgenic Cre[3] models contain an intact native *Axin2* gene; however, in the Axin2CreERT2 model, this is in a haploinsufficient state. Thus, an additional model using Cre knock-in in the 3'untranslated region of *Axin2* may produce a physiologically stable model from which to lineage trace from the endogenous *Axin2* locus. We exclude the reporter as a reason for the discrepancy, between our findings and those of Wang *et al.*,[6] in lineage tracing and the Wnt/β-catenin phenotype related to the Axin2CreERT2 allele. Whilst we observe a consistent phenotype whilst backcrossing our mouse models, variations in mouse background between groups may underlie the differential ability to lineage trace. Our inability to lineage trace calls into question the biological importance of Axin2^{+} hepatocytes as a source for homeostatic liver regeneration. Whilst our study is unable to resolve the identity of those rare cells within the labelled population that expand, it is consistent with recent reports of cells undergoing preferential expansion within zone 2(4, 5), an area which is also labelled using the Axin2CreERT2 system that is not, as was originally reported,[6] zonally restricted. Due to the lack of zonal-specific labelling, which we and others[4] have observed, we are unable to define whether the expansion events represented by confluent labelling in isolated patches occur from a pericentral origin or from within zone 2. However, the pattern of labelling does imply that there are vascular relationships to the expansion, as margins of confluent labelled areas typically associate with vascular territories.

This study furthermore highlights the impact of haploinsufficiency when using a genetic knock-in within a functional locus. It was previously acknowledged that such haploinsufficiency may have an impact but upon examining hepatocyte proliferation, including within zone 3 specifically, no phenotypic alteration was demonstrated between Axin2-CreERT2 and WT mice.[6] This is in agreement with our data on both homeostatic and regenerative proliferation. Whilst we see

mean ± SEM. (F) Schematic for isolating nuclear and cytoplasmic protein fractions. (G) Quantification of nuclear to cytoplasmic β-catenin ratio in uninduced WT and Axin2CreERT2$^{+/WT}$ mice, each fraction is normalised to β-actin and GAPDH respectively; n = 7 WT mice and 7 Axin2CreERT2$^{+/WT}$ mice, unpaired *t* test, box plot with centre line showing the median values (IQR), for WT 1.43 (1.08-2.37) and Axin2CreERT2$^{WT/-}$ 2.64 (2.20-2.72), whiskers represent minimum and maximum values, '+' = mean. (H) Volcano plot displaying differentially expressed genes from RNA-sequencing analysis of whole female livers of uninduced Axin2CreERT2$^{+/WT}$ compared to WT mice; n = 6 per cohort, relevant specific genes are highlighted. (I) *Glul* gene expression validated from whole liver by qRT-PCR in female mice, n = 11, two-tailed Mann-Whitney *U* test, mean ± SEM. (J) Quantification of GS positivity by tissue area in whole liver from female and male mice, with representative low power microscopic images of whole lobes from individual female mice (K); n = 6/6 and 7/6 in WT/Axin2CreERT2$^{+/WT}$ mice for females and males, respectively, two-way ANOVA, mean ± SEM, scale bars = 1.5 mm. (L) Gene set-enrichment analysis plot demonstrating downregulation of Wnt signalling target genes in uninduced female Axin2CreERT2$^{+/WT}$ mice compared to WT and respective heat map of the top 50 features for each genotype, n = 6 mice per cohort. Hits mark the position of genes in published datasets. (M) Gene expression of Wnt target genes from whole livers of uninduced WT and Axin2CreERT2$^{+/WT}$ female mice by qRT-PCR; n = 9-11 mice, two-tailed Mann-Whitney *U* (or *t* tests if normally distributed), mean ± SEM. (N) Quantification of RNA *in situ* hybridisation for hepatic *Lgr5* demonstrating a reduction in uninduced Axin2CreERT2$^{+/WT}$ mice compared to WT female mice, with representative microscopic images (O), n = 5 WT and 6 Axin2CreERT2$^{+/WT}$, unpaired *t* test, mean ± SEM, scale bars = 100 μm. (P) Using the Tcf/Lef:H2B-GFP marker allele as a canonical Wnt pathway reporter, GFP^{+} hepatocytes were quantified from IHC sections stained with GFP in uninduced WT and Axin2CreERT2$^{+/WT}$ mice, with representative microscopic images (Q); n = 5 mice per cohort, except for male Axin2CreERT2$^{+/WT}$ mice where n = 6, two-way ANOVA, mean ± SEM, scale bars = 100 μm. (R) PC analysis of the untargeted metabolomics of liver tissue from uninduced WT and Axin2CreERT2$^{+/WT}$ mice; n = 4 per cohort. Statistical comparisons between female *vs.* male mice are reported as the interaction factors from two-way ANOVA. CV, central vein; FC, fold change; GFP, green fluorescent protein; GS, glutamine synthetase; H2B, histone H2B; IHC, immunohistochemistry; NaCl, sodium chloride; NES, normalised enrichment score, Nom, nominal; PC, principal component; PV, portal vein; WT, wild-type.

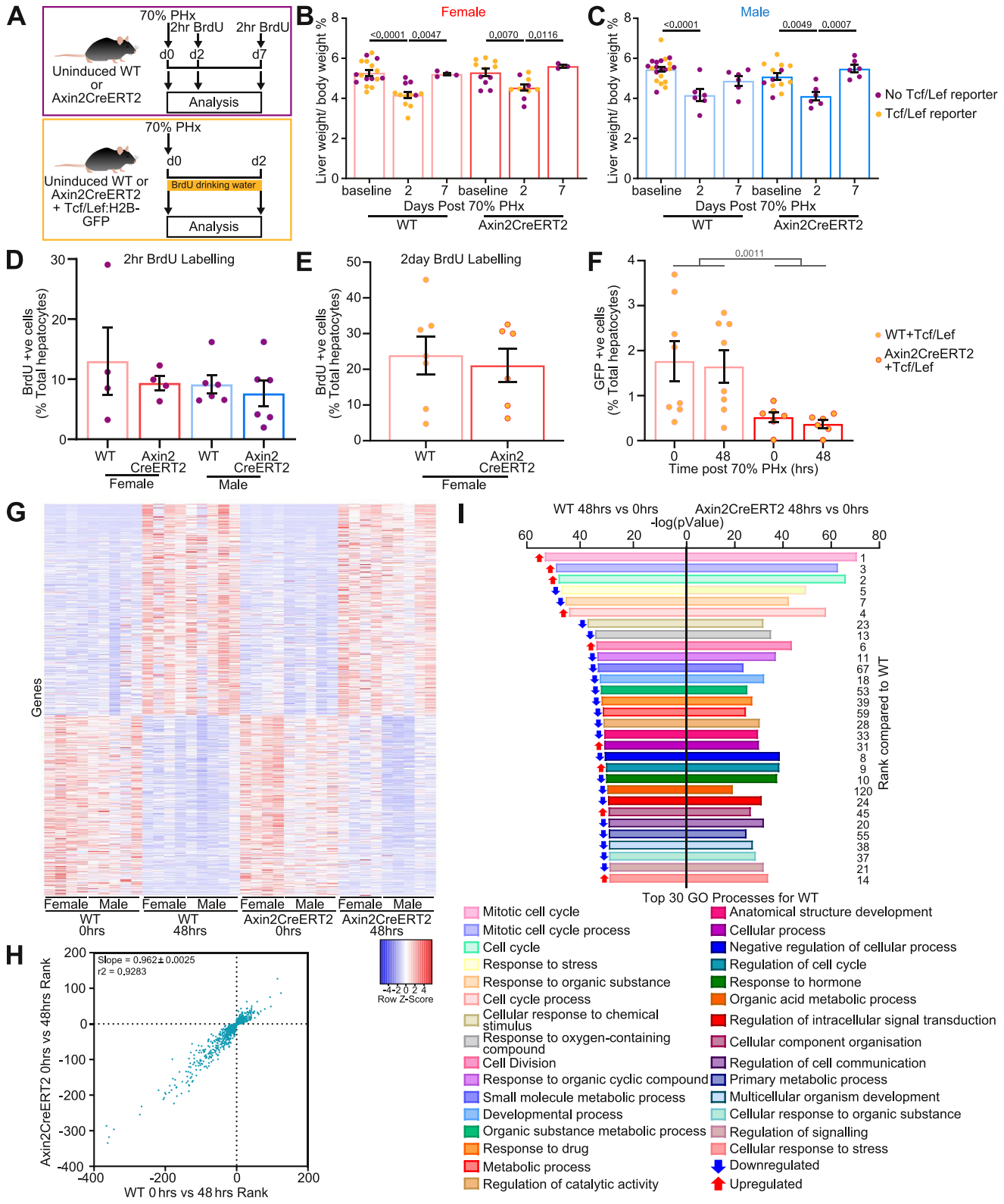

Fig. 3. Liver regeneration is not affected by the presence of the Axin2CreERT2 construct. (A) Liver regeneration in response to 70% PHx in uninduced WT and Axin2CreERT2$^{+/WT}$ mice (purple) or in mice also harbouring the Tcf/Lef:H2B-GFP reporter (orange). Mice were either administered BrdU 2 h before sampling or for 2 days in the drinking water after surgery, respectively. Tissue was analysed at 2 or 7 days post-surgery. Liver weight-to-body weight ratios were calculated for (B) female and (C) male WT and Axin2CreERT2$^{+/WT}$ mice at baseline and at days 2 and 7 post PHx; n = 17/21, 12/6 and 4/6/in WT mice and 11/13, 10/6, and 3/6 in uninduced Axin2CreERT2$^{+/WT}$ mice at baseline, day 2 and day 7 for females/males, respectively, two-way ANOVA, mean ± SEM. Hepatocyte proliferation was quantified from IHC sections stained with BrdU at day 2 post-surgery following either 2 h BrdU pulse (D) or continuous application after surgery (E); n = 4 female mice and 6 male mice per cohort (D) and 7 WT and 6 Axin2CreERT2+/WT mice (E), two-way ANOVA and two-tailed Mann-Whitney *U*, respectively, mean ± SEM. (F) Percentage of GFP^{+}

no overall difference in hepatocellular proliferation between these genotypes, we observe heightened proliferation within zone 3 in Axin2CreERT2 mice, consistent with a previous analysis.[4] We propose that as zone 3 is the area of highest Axin2 expression in the WT liver, this reduction of Axin2 in AxinCreERT2 mice is responsible for heightened proliferation within this zonal region. Similarly, zone 3 which has relatively reduced proliferation in the WT liver, is the area of highest tonic Wnt pathway activation and our data suggests that the Wnt pathway actively suppresses homeostatic proliferation in the WT liver. This alteration to proliferation in the absence of injury may underpin the observations by others of expansion of this labelled population of hepatocytes.

Upon deeper characterisation of the Axin2CreERT2 model than previously performed,[4,6] we show profound changes in the Wnt pathway. In the chronic *Axin2* haploinsufficient state, these include effects on development and metabolism but also both in *Axin2* expression itself and the Wnt pathway, which Axin2 regulates.[14] Notably, we observed an uncoupling of canonical Wnt signalling in these animals. Reduced Axin2 is expected to lead to impaired β-catenin degradation and consequently high nuclear β-catenin, resulting in over-expression of canonical Wnt pathway targets. In chronic *Axin2* haploinsufficiency, the latter becomes uncoupled, with instead reduced expression of canonical Wnt pathway targets. Notably this effect is more prominent in female mice. Therefore, it appears that there is significant compensation in the regulatory networks of Wnt signalling in those animals surviving to adulthood. We propose that this compensation underlies the normal proliferation in these animals in the chronic adapted state compared to the well-documented dysfunctional regeneration observed after acute Wnt/β-catenin signalling perturbation.[15]

We also found that sex effects are also relevant to lineage tracing. We show that comparisons between male and female mice are inaccurate, with differences both in stable labelling of a baseline population and in lineage tracing, which is strongly influenced by sex. This may be related to greater baseline labelling and/or heightened dysregulation of the Wnt pathway in females. Therefore, it is imperative to report the sex of animals used in such studies, consistent with guidelines for reporting in animal studies,[16] and to compare within but not between the sexes.

Finally, our study highlights some important points with respect to the experimental conduct and reporting of such studies in the future. We believe that, particularly when using endogenous loci for knock-in studies, rigorous phenotyping of the mutant model is required to rule out subtle alterations in homeostatic and regenerative physiology. We additionally highlight the implications of other variables when interpreting studies in preclinical models; these include but are not limited to sex, age, background, feeding status and other environmental factors, as highlighted by other recent studies.[17,18] Overall, whilst preclinical models offer a unique scientific platform to mechanistically study physiology and disease, it is crucial to understand their limitations and to ensure that a single variable is tested between experimental groups.

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Abbreviations

BAC, bacterial artificial chromosome; CCl_4, carbon tetrachloride; GS, glutamine synthetase; WT, wild-type; RFP, red flourescent protein; GFP, green flourescent protein; mTmG, membrane-targeted tandem dimer Tomato membrane-targeted green fluorescent protein.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

SM performed the animal studies, partial hepatectomy, designed and performed experiments, analysed the data, wrote the manuscript and produced figures. MM designed experiments, performed animal experiments, administered anaesthetic during partial hepatectomy and provided discussion. CRL performed experiments and analysed data. GLS performed and analysed the Western blots. PJW performed Western blots, sequenced, and analysed the *Axin2* gene. AH and RS performed bioinformatic analysis. WC and AK performed RNA and Sanger sequencing respectively. CN performed IHC and ISH. LO-J and FB optimised and performed multiplex ISH-IF staining, deep learning algorithms and acquired data. IRP processed multiplex ISH-IF data. JVV, ST and DS performed metabolomics and analysed the data. TD performed partial hepatectomy. ASR and CK assisted with animal studies. OJS provided resources, discussion and acquired funding. JLQ and MB provided resources. TGB designed experiments, analysed data,

hepatocytes responding to the Tcf/Lef:H2B-GFP Wnt reporter before and 2 days after 70% PHx were quantified in uninduced WT and Axin2CreERT2$^{+/WT}$ mice from IHC sections stained with GFP; n = 8 WT and 6 Axin2CreERT2$^{+/WT}$ mice, two-way ANOVA, mean ± SEM. (G) Heat map displaying differentially expressed genes identified by RNA-sequencing analysis of whole livers from uninduced Axin2CreERT2$^{+/WT}$ and WT mice following 70% PHx. This is using paired resected (0 h, during surgery) and regenerated tissues (48 h post-surgery) from both sexes, n = 9 mice per cohort (4 female and 5 male). (H) Ranking of differentially expressed genes in WT and Axin2CreERT2$^{+/WT}$ mice (combined sexes, n = 9 for each genotype [4 female and 5 male] based on their π-score; linear regression slope = 0.962 ± 0.0025). (I) Top 30 significant GO cellular processes, ranked by *p* value, altered in WT tissue after 70% PHx. The equivalent GO processes are shown for uninduced Axin2CreERT2$^{+/WT}$ mice and their respective ranking compared to WT; (n = 9 per cohort). Arrows show directionality of change following PHx. Statistical comparisons between female *vs.* male mice are reported as the interaction factors from two-way ANOVA. BrdU, Bromodeoxyuridine; GFP, green fluorescent protein; GO, gene ontology; H2B, histone H2B; IHC, immunohistochemistry; PHx, partial hepatectomy; WT, wild-type.

wrote the manuscript and acquired funding. All authors read the manuscript and provided critical comments.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Supplementary data

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