Single-cell spatial transcriptomics reveals a dynamic control of metabolic zonation and liver regeneration by endothelial cell Wnt2 and Wnt9b

Graphical abstract

Highlights

- Conserved expression of Wnt2 and Wnt9b in liver endothelial cells is identified
- Wnt2 and Wnt9b deletion from endothelial cells affects gene expression in zone 3
- Endothelial Wnt2 and Wnt9b deletion affects liver regeneration after hepatectomy
- Late treatment with Wnt agonist rescues regeneration after acetaminophen overdose

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In brief
Hu et al. show conserved expression of Wnt2 and Wnt9b in liver endothelial cells whose deletion led to abrogation of expression of zone 3 genes, appearance of zone 1 genes in this area, and blunted liver regeneration. Wnt agonist stimulated regeneration in acute liver injury model.
Single-cell spatial transcriptomics reveals a dynamic control of metabolic zonation and liver regeneration by endothelial cell Wnt2 and Wnt9b

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SUMMARY

The conclusive identity of Wnts regulating liver zonation (LZ) and regeneration (LR) remains unclear despite an undisputed role of β-catenin. Using single-cell analysis, we identified a conserved Wnt2 and Wnt9b expression in endothelial cells (ECs) in zone 3. EC-elimination of Wnt2 and Wnt9b led to both loss of β-catenin targets in zone 3, and re-appearance of zone 1 genes in zone 3, unraveling dynamicity in the LZ process. Impaired LR observed in the knockouts phenocopied models of defective hepatic Wnt signaling. Administration of a tetravalent antibody to activate Wnt signaling rescued LZ and LR in the knockouts and induced zone 3 gene expression and LR in controls. Administration of the agonist also promoted LR in acetaminophen overdose acute liver failure (ALF) fulfilling an unmet clinical need. Overall, we report an unequivocal role of EC-Wnt2 and Wnt9b in LZ and LR and show the role of Wnt activators as regenerative therapy for ALF.

INTRODUCTION

The Wnt-β-catenin signaling pathway plays fundamental roles in tissue development, homeostasis, repair, regeneration, and tumorigenesis.1,2 β-Catenin transcriptional activity is controlled by Wnt proteins. Once secreted with the help of the cargo protein Wntless (Wls), Wnt proteins bind to cell surface receptor Frizzled (FZD) and co-receptors low-density lipoprotein receptor-related protein 5/6 (LRP5/6). This leads to inactivation of the destruction complex composed of APC-GSK3β-Axin and allows β-catenin nuclear translocation and target gene expression.2

The Wnt-β-catenin signaling pathway and the RSPO-LGR4/5-ZNRF3/RNF43 axis are well-known regulators of pericentral gene expression and in turn of metabolic liver zonation (LZ) and also contribute to liver regeneration (LR) after partial hepalectomy.3–6 LZ is a function of the hepatocytes exhibiting differential gene expression based on their location along the portal-central axis of a hepatic lobule.7 Such heterogeneity allows hepatocytes located within each zone to perform specific metabolic, synthetic, and xenobiotic functions, improving efficiency through division of labor. LZ also allows compartmentalization of certain injuries such that the remaining cells could proliferate to restore liver mass and function.8 LR after partial hepalectomy (PH) is also a distinctive feature of an adult liver, which is evolutionarily conserved, and allows the remnant liver to grow after surgical resection.9,10 Mice with liver-specific knockout of β-catenin (β-catenin-LKO) or liver-specific double-knockout of LRP5 and LRP6 (LRP5-6-LDKO) have disrupted pericentral LZ as seen by loss of pericentral expression of glutamine synthetase, CYP2E1, and CYP1A2; and delayed LR due to impaired cyclin D1 expression.11–14 Hepatic endothelial cells (ECs) are the main source of Wnt ligands in an adult murine liver.15–17 Like β-catenin-LKO and LRP5-6-LDKO, mice with EC Wls deletion (EC-Wls-KO) lack pericentral LZ and show delayed LR. While 19 Wnt ligands are expressed in various cell types of the liver, their distinct pathophysiological roles in vivo are less well understood.18 In murine livers, Wnt2 and Wnt9b are present at the central venous ECs and hepatic sinusoidal ECs near the central vein.19 PH induces a 45-fold upregulation in Wnt2 and 18-fold upregulation Wnt9b expression in ECs at 12 h.17 Upregulation of Wnt2 and Wnt9b is also apparent in other hepatic regeneration settings.20,21 While these correlative studies suggest roles of these two Wnts in the liver, conclusive proof requires direct genetic evidence.

While mechanisms of LR have been studied for several decades, there are no targeted therapies available to stimulate
LR in acute or chronic liver injuries. Since the Wnt-β-catenin pathway contributes to both LZ and LR, it is a promising candidate pathway for drug development for regenerative medicine.\textsuperscript{22,23} Recently F\textsuperscript{14,15,17}L6\textsuperscript{1,3} (abbreviated FL6.13), a water-soluble tailored tetrameric antibody consisting of two pan-FZD paratopes and two anti-LRP6 paratopes was developed through rational design.\textsuperscript{24} FL6.13 promotes FZD-LRP6 clustering to stabilize receptor conformations compatible with robust β-catenin activation at nanomolar concentrations in both murine and human cell lines, organoids, and \textit{in vivo}.

Here, we analyzed liver cell-specific expression of Wnt gene transcripts across different species using an existing single-cell RNA (scRNA) sequencing database that allowed us to identify Wnt2 and Wnt9b in ECs.\textsuperscript{25} We generated Wnt2 and Wnt9b double floxed mice to breed to lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1)-Cre mice and generated EC-Wnt2-KO, EC-Wnt9b-KO, and EC-Wnt2-9b-DKO mice. The EC-Wnt2-9b-DKO mice exhibited perturbed metabolic LZ as revealed using 100-gene single-cell spatial transcriptomics by Molecular Cartography, which was rescued by FL6.13. The EC-Wnt2-9b-DKO mice showed delayed LR after PH, which was rescued by FL6.13. FL6.13 also induced β-catenin-dependent pericentral gene expression and cell proliferation program, and successfully promoted LR in murine model of surgical resection and rescued delayed acetaminophen (APAP) overdose-induced liver injury, thus fulfilling a major unmet clinical need.

**RESULTS**

**Endothelial cell expression of Wnt2 and Wnt9b is evolutionarily conserved**

Since the Wnt-β-catenin signaling pathway is important for liver function, we queried expression of various Wnt genes in different liver cells through a searchable scRNA sequencing database from cells within livers of various species.\textsuperscript{25} In the human dataset, 18 out of 19 WNTs were detected in various hepatic cell types (Figure S1). ECs were the predominant source of Wnt2, WNT2B, and WNT9B, although the signal of WNT9B was weak (Figures 1A and S1). In monkey, WNT2, WNT2B, WNT3, WNT9A, and WNT9B were highly expressed among 16 detected WNTs (Figures 1A and S2). ECs expressed high levels of WNT2 and WNT9B, while WNT2B was mainly expressed by Kupffer cells. In the pig, 9 WNTs were detected (Figures 1A and S3). ECs in porcine liver also expressed very high levels of WNT2 and WNT9B. WNT2B was expressed at a very low level. In mice, 15 Wnts were detected, of which Wnt2 and Wnt9b were highest in ECs (Figures 1A and S4). Even in mice with NALFD, Wnt2 and Wnt9b were still predominantly expressed in ECs, suggesting preservation of their expression even in pathological states (Figure S5). Altogether, as could be seen from analysis in multiple species (Figure 1A), ECs are uniformly the predominant source of Wnt2 and Wnt9b and only EC expression of Wnt2 and Wnt9b appears to be evolutionarily conserved.

ECs are a heterogeneous population across the liver lobule.\textsuperscript{26,27} By using Uniform Manifold Approximation and Projection (UMAP) for dimension reduction, we observed pericentrally zonated expression of Wnt2 and Wnt9b in humans (Figure S6) and mice (Figure 1B). Zonal expression of Wnt2 and Wnt9b was also confirmed in another murine single-nuclei RNA sequencing database (Figure S7). Consistently, Wnt9b was expressed mainly by central venous ECs, while Wnt2 was expressed more broadly by both central venous ECs and sinusoidal ECs (Figures 1B and S6).

To further validate these observations, we applied Molecular Cartography (Resolve Biosciences), which allows 100-plex spatial mRNA analysis on wild-type C57BL/6 mouse liver. Genes encoding for various components of the Wnt pathway, and genes that are known to be zonated, were spatially resolved by Molecular Cartography (Table S3). We identified central-portal zonation of hepatocytes using location of zonated genes (pericentral: Glul, Cyp2e1; midzonal: Igfbp2; periporal: Cyp2f2) and also identified cholangiocytes through Cldn7 expression (Figure 1C). Of the 19 Wnts, only Wnt2 and Wnt9b were pericentrally zonated (Figure 1C). To confirm the cellular source of Wnt2 and Wnt9b, we mapped gene expression of cell-specific markers, such as Pecam1 for ECs, Lyz2 for macrophages, and Lrat for hepatic stellate cells (HSCs). Wnt2 and Wnt9b predominantly colocalized with Pecam1, while some overlap was evident with Lyz2 and Lrat, confirming ECs to be the major Wnt2- and Wnt9b-expressing cells in zone 3 of the murine liver (Figures 1D and S8).

Altogether, these data suggest spatially confined expression of Wnt2 and Wnt9b in ECs, which, along with Rspo3 from ECs and HSCs, might be instructing pericentral Wnt-β-catenin activity to contribute to metabolic LZ.\textsuperscript{28,29}

**Hepatic EC deletion of Wnt2 and Wnt9b in mice**

Wnt2\textsuperscript{lox/lox}\textsuperscript{flox/flox} mice with loxP sites flanking exon 2 of the murine Wnt2 gene were generated using CRISPR-Cas9 as discussed in STAR Methods (Figure S9A). Wnt9b\textsuperscript{lox/lox}\textsuperscript{flox/flox} mice and Lyve1-Cre mice were obtained from Jackson laboratories.\textsuperscript{17,30} Rosa-stop\textsuperscript{lox/lox}\textsuperscript{flox/flox}-EYFP mice were bred to these strains to fate-trace the expression and activity of Cre-recombinase transgene. After strategic breeding (discussed in STAR Methods), we successfully generated EC-Wnt2-9b-DKO mice that were identified by the simultaneous presence of Cre, floxed Wnt2, floxed Wnt9b, and Rosa-stop alleles in the genomic DNA PCR (Figure S9B).

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**Figure 1. Endothelial cell expression of Wnt2 and Wnt9b is evolutionarily conserved**

(A) Violin plots showing expression levels of WNT2 and WNT9B in human, monkey (male Cynomolus macaques), and pig (female piglets) livers, and Wnt2 and Wnt9b in mice (C57BL/6) livers among different hepatic cell types, with highest expression evident in endothelial cells across species. Cartoons were created with BioRender.com.

(B) Feature plots showing expression of Wnt2 and Wnt9b among all hepatic ECs in mice. While Wnt9b is almost exclusively expressed in central vein endothelial cells, Wnt2 is expressed more widely in both central vein endothelial cells as well as in liver sinusoidal endothelial cells (LSECs) toward zones 2 and 3.

(C) Molecular Cartography showing expression in hepatocytes of Glu and Cyp2e1 (zone 3), Igfbp2 (zone 2), Cyp2f2 (zone 1), and Cldn7 (cholangiocytes), along with Wnt2 and Wnt9b, which are pericentrally zonated. CV, central vein; PV, portal vein.

(D) Molecular Cartography of Wnt2 and Wnt9b along with markers of specific cell types showing Pecam1+ ECs, but not Lyz2+ macrophages or Lrat+ hepatic stellate cells are the main source of Wnt2 and Wnt9b.
While Lyve1 is mainly expressed by midzonal hepatic sinusoidal ECs in an adult murine liver, Lyve1-Cre recombines floxed alleles in both hepatic sinusoidal ECs and vascular ECs, likely due to expression of Lyve1 sometime during development in all ECs. To confirm, GFP expression in livers from EC-Wnt2-9b-DKO mice was seen in both hepatic sinusoidal and central venous ECs by immunohistochemistry (IHC) and immunofluorescence (Figures S9C and S9D).

To examine the impact of Wnt2, Wnt9b, or combined deletion from hepatic ECs, we assessed serum of mice from each genotype for transaminases and examined hepatic histology. Liver transaminases and histology from all genotypes were insignificantly different from the controls (Figure S9 E and not shown). Liver weight to body weight ratio (LW/BW) was around 22% and significantly lower in the EC-Wnt2-KO and EC-Wnt9b-KO mice when compared with the controls and was even lower in EC-Wnt2-9b-DKO mice, similar to previously reported hepatic genetic KO of various components of the Wnt-β-catenin pathway (Figure S3F).

**EC-derived Wnt2 and Wnt9b control expression of key β-catenin target genes in baseline liver**

Since loss of β-catenin or LRP5-6 from hepatocytes or Wls from ECs resulted in loss of Wnt-β-catenin target genes in pericentral zone, we next examined their expression in EC-Wnt2-KO, EC-Wnt9b-KO, and EC-Wnt2-9b-DKO mice. In males, by IHC and by western blot, single deletion of Wnt2 or Wnt9b led to partial but consistent loss of pericentral expression of GS, CYP2E1, and CYP1A2 (Figures 2A and 2B). Minimal difference in their expression was evident in females (Figures 2A and 2B). Interestingly, both male and female EC-Wnt2-9b-DKO mice exhibited complete loss of pericentral GS expression and had minimal residual CYP2E1 and CYP1A2 (Figure 2A). Lack of CYP2E1 and CYP1A2 led to absence of APAP metabolism to NAPQI and hence EC-Wnt2-9b-DKO mice showed normal serum transaminases compared with controls (not shown). In fact, EC-Wnt2-9b-DKO phenocopied β-catenin-LKO, LRP5-6-DLKO, and EC-Wls-KO mice, proving that, among the 19 Wnts, Wnt2 and Wnt9b are sufficient for maintaining baseline expression of key pericentral hepatocyte targets of the Wnt-β-catenin pathway and their dual deletion cannot be compensated by other mechanisms (Figure 2B).

Cyclin D1 is a β-catenin target gene that is mainly expressed in hepatocytes in the midzone of a quiescent liver. Like in various models of Wnt pathway disruption in the liver, cyclin D1 was decreased in both single and double KO mice in both males and females, but more profoundly in males and in DKOs (Figure 2B).

**Single-cell spatial transcriptomic profiling reveals a critical role of EC Wnt2 and Wnt9b in dynamic control of metabolic zonation**

To evaluate zonation changes in-depth, we applied Molecular Cartography to study the single-cell spatial expression of 100 genes (Table S3). Two pipelines were used for the analysis of the control and EC-Wnt2-9b-DKO mice (Figure S10). First, to obtain the genetic signature at single-cell level, we used QuPath to outline single hepatocytes and obtained the expression of genes per cell. Comparable numbers of cells were obtained from control and DKO livers (Figure 3 A). Next, using expression of 16 known zonated genes, a UMAP was generated, which identified six distinct hepatocyte populations (Figure S10). Clusters 1 and 2 represented hepatocytes with expression of pericentral genes, while clusters 4, 5, and 6 represented cells expressing periportal genes (Figure 3A). Intriguingly we observed a dramatic enrichment of cells in cluster 4, 5, and 6 at the expense of cells exhibiting pericentral genes in EC-Wnt2-9b-DKO, indicating an overall shift of gene expression from pericentral to periportal in these livers (Figure 3A).

Next, to obtain the spatial signature, we divided the liver lobe evenly into nine segments (pericentral to periportal: S1 to S9) using landmark genes (Figure S10). Gene expression density (gene
A. Genetic Signature

- Cyp2x1
- Cyp2f2

Pericentral (C1, C2)
Periportal (C4, C5, C6)

Control
EC-Wnt2-9b-DKO

B. Spatial Signature

- S1
- S2 Pericentral (PC)
- S3
- S4
- S5 Midzonal (Mid)
- S6
- S7
- S8 Periportal (PP)
- S9

C. Control vs. EC-Wnt2-9b-DKO

D. Expression of Genes:
- Cyp2x1
- Axin2
- Glis2
- Etsx2
- Ass1
- Ptc1
- Notch1

E. UMAP_1 vs. UMAP_2

F. UMAP_1 vs. UMAP_2

G. (Legend on next page)
counts per area) were quantified within each zone and averaged across the defined pericentral-to-periportal regions thus allowing us to compare gene expression across the lobule using line plots (Figure S10). To combine the genetic signature and spatial signature, we then identified hepatocytes located within the nine segments based on their position (x and y axis) on the slides and applied this information back to the UMAP to track their localization (Figure S10). As expected, in controls, pericentral cells were seen mainly in clusters 1 and 2 and periportal cells in clusters 4, 5, and 6. In EC-Wnt2-9b-DKO mice, most pericentral and all midzonal and periportal cells were in clusters 4, 5, and 6, exhibiting periportal genetic signature (Figure 3B).

We next visualized molecular cartography expression of specific pericentral Wnt targets (Glul, Lect2, Axin2, Cldn2, Cyp1a2, Oat, Cyp2e1, and Rgn) and of periportal genes (Fbp1, G6pc, Pck1, Gls2, Arg1, Ass1, Cps1, and Cyp2f2) (Figure 3C). Most pericentral Wnt-β-catenin target genes in hepatocytes were dramatically downregulated or absent in EC-Wnt2-9b-DKO livers, including Glul, Oat, Cyp2e1, Axin2, Cldn2, Cyp1a2, Cyp7a1, Lect2, Lgr5, Prodh, Rgn, Rnf43, and Tbx3 (Figures 3D and S11A), supporting these β-catenin-TCF4 targets to be under direct control of paracrine Wnt2 and Wnt9b ligands from the neighboring ECs. Intriguingly, not all pericentral genes were affected and paradoxically some genes, such as Alad, C6, Cpox, Cyp27a1, Gstm1, and Lrp5, were upregulated, suggesting their expression to be controlled by other pericentral regulators or may be a consequence of compensation to the Wnt-β-catenin pathway disruption (Figure S11B). Interestingly, we observed de novo ectopic expression of periportal genes in zone 3 with concomitant decrease in their periporal expression (Fads1, Cbb, Hsd17b13, Igf1, Pck1, and Pigr) (Figures 3E and S11C), without any change in their baseline periportal expression (Gls2, Cps1, Elovl2, Arg1, Cpt2, Cyp8b1, Lp6, Ndufb10, Uqcrh, and Vtn) (Figures 3E and S11D), or with concurrent increase in their periportal expression (Ass1, Atp5a1, Cyp2f2, Fbp1, Igfals, and Sox9) (Figures 3E and S11E).

To assess if such dynamism in the process of LZ was more global and not limited to one model, we queried expression of selected zonally expressed genes by Molecular Cartography in β-catenin-LKO, LR55-6-LDKO, and EC-Wls-KO livers. All models showed similar alterations with gain of periportal gene expression in zone 3 hepatocytes along with alterations in their native zone 1 expression (Figure S12).

Zone 2 hepatocyte proliferation has been shown to be at least in part driven by the IGFBP2-mTOR-CCND1 axis. Indeed, Igbp2 and Ccnd1 were both expressed in midzone in the control liver, with highest expression of Ccnd1 in segment 4 and highest expression of Igbp2 in segment 5 (Figure 3G). In EC-Wnt2-9b-DKO mice, Ccnd1 level was overall decreased (average fold change = 0.84, p = 1.00654 × 10^{-10}), while Igbp2 was marginally increased (average fold change = 1.09, p = 1.72255E-10). Importantly, there was decrease of Ccnd1 in midzone but an increase in periportal hepatocytes along with an increase in Igbp2 (Figures 3F and 3G). The loss of Ccnd1 from midzone in DKO suggests its expression is also normally controlled by EC Wnt2 and Wnt9b and from midzonal Igbp2, and the latter may become the dominant regulator in the absence of Wnts. High expression of Ccnd1 evident in periportal cells is a technical artifact due to inadvertent inclusion of Ccnd1-positive biliary cells during cell outlining by the QuPath. Midzonal expression of Pon1 showed a similar shift (Figure 3G).

Collectively, we observe “periportalization” of the liver lobule in all Wnt-β-catenin pathway disrupted livers. Furthermore, the spatiotemporal changes in the expression of multiple zonated genes in hepatocytes in all zones upon elimination of Wnt2 and Wnt9b that is basally expressed only in zone 3 ECs, or β-catenin signaling that is active only in zone 3 hepatocytes, underscores the overall dynamic nature of LZ. Thus, LZ appears to be a net impact of both active transcription and repression of genes in the same cells.

**EC-derived Wnt2 and Wnt9b contribute to normal LR after PH**

The Wnt-β-catenin pathway has also been shown to play an important role in regulating hepatocyte proliferation after PH. Loss of β-catenin or LR55-6 from hepatocytes or Wls from...
ECs, resulted in decreased cyclin D1 expression in hepatocytes leading to notably lower numbers of hepatocytes in S-phase and decreased proliferation at 40 h, with eventual recovery at 72 h.\textsuperscript{13,14,17} To investigate the role of Wnt2 and Wnt9b from ECs in this process, we subjected male and female EC-Wnt2-KO, EC-Wnt9b-KO, and EC-Wnt2-9b-DKO mice to PH. Male EC-Wnt2-KO and EC-Wnt9b-KO mice exhibited a profound decrease in cyclin D1 (Figure 4A). This decrease was less conspicuous in females, likely due to basally higher cyclin D1 levels (Figures 2B and 4A). Importantly, like β-catenin-LKO, LRP5-6-LDKO, and EC-Wls-KO, cyclin D1 was barely detectable in the EC-Wnt2-9b-DKO mice in both genders, suggesting that the two Wnts are collectively required for normal cyclin D1 upregulation during LR (Figure 4A).

We next assessed the localization of cyclin D1-positive hepatocytes by IHC. In controls, periportal and midzonal hepatocytes were cyclin D1 positive, while one to two layers of hepatocytes around the central vein remained negative (Figure 4B). In single KOs, cyclin D1-positive hepatocytes were concentric around the central vein except for one to two layers of hepatocytes immediately around the vessel (Figure 4B). In EC-Wnt2-9b-DKO mice, cyclin D1 was weak but more diffusely expressed. These observations suggest that the concentric pattern of cyclin D1 in the EC-Wnt2-KO mice may be due to reciprocal increase in Wnt9b in pericentral neighborhood and vice versa in the EC-Wnt9b-KO mice (Figure 4B). But their combined loss prevented such localization of cyclin D1. Unlike males, female single KO lacked any peculiarities in cyclin D1 levels or localization, and only DKO mice showed a dramatic decrease in cyclin D1-expressing hepatocytes (Figure 4B). To study the consequence of cyclin D1 decrease we compared LR in controls, single, and double KO livers at 40 h by Ki67, a marker of S-phase, and BrdU, an indicator of cell proliferation, injected to mice 5 h before euthanasia. Like cyclin D1, proliferating hepatocytes were mainly localized around the perportal and midzonal region in the controls. Single KO mice had sparsely positive cells, and DKO mice were completely negative for Ki67 and BrdU labeling at 40 h (Figures 4B and 4C). These observations were consistent in both genders. Therefore, there is a notable deficit in LR in EC-Wnt2-KO and EC-Wnt9b-KO mice, which is even
worse in EC-Wnt2-9b-DKO mice phenocopying β-catenin-LKO, LRP5-6-LDKO, and EC-Wls-KO mice. 13,14,17

FL6.13 rescues metabolic LZ and delayed LR in Wnt-deficient mice

Since FL6.13, the tailored tetravalent antibody has recently been shown to engage the FZD-LRP6 receptor in the absence of natural Wnt ligands, we next investigated if it could activate β-catenin signaling and rescue both the pericentral gene expression or metabolic LZ as well as LR after PH in the EC-Wnt2-9b-DKO and in EC-Wls-KO mice. 17 Four doses of control IgG or FL6.13 were injected into 8-week-old male KO or control mice every other day for a week as described in STAR Methods (Figure 5A). PH was performed on day 8 and the resected livers were processed for analysis. Regenerating livers were also harvested at 24 h post PH. Mice were given 1 mg/mL BrdU in drinking water throughout the study to label all proliferating cells during the process (Figure 5A). Pretreatment with FL6.13 restored pericentral expression of GS and pericentral and midzonal expression of CYP2E1 in both models (Figure 5B). FL6.13 was also efficient in inducing pan-zonal expression of cyclin D1 except in the most immediate one to two layers of pericentral hepatocytes (Figure 5B). Concomitantly, hepatocyte proliferation as seen by enhanced BrdU incorporation was evident in both groups of KOs at baseline (Figure 5B). At 24 h after PH, a profound increase in hepatocyte proliferation was observed in EC-Wnt2-9b-DKO and EC-Wls-KO mice by BrdU incorporation and LW/BW recovery, which was even greater than the control animals dosed with control IgG, suggesting rescue and even shift to the left in LR kinetics with FL6.13 (Figures 5B and 5C). This enhanced proliferation in response to FL6.13 in both KOs was associated with continued increase in cyclin D1 in most hepatocytes pan-zonally.

To address the specificity of the response by FL6.13, we next treated LRP5-6-LDKO mice lacking Wnt co-receptors in hepatocytes. 14 These mice have been shown previously to also phenocopy β-catenin-LKO in both lacking pericentral Wnt-β-catenin targets and delayed LR. When these mice were treated with FL6.13 (Figure 5A), there was no change in pericentral gene expression of GS and CYP2E1, which continued to be absent in these mice (Figure 5B). Similarly, FL6.13 was unable to restore either cyclin D1 expression or BrdU incorporation either at baseline or 24 h post PH (Figure 5B). This was also reflected by a
deficient LW/BW at 24 h post PH (Figure 5C). This study shows the requirement of intact Wnt co-receptors for FL6.13 to stimulate the Wnt-β-catenin signaling in vivo.

**FL6.13 induces pericentral gene expression at baseline**

To characterize Wnt-β-catenin activation by FL6.13 in greater depth, we tested the effect of four doses of FL6.13, given every 48 h, on the wild-type mice, as described in STAR Methods. Twenty-four hours after the last injection, the mice were euthanized, and livers were processed for Molecular Cartography using the same set of probes (Table S3). Two pipelines were applied for analyses (Figure S14). Zonal distribution of hepatocytes was identified based on differentially expressed genes (Figure S14) and confirmed by tracing back the localization and zonation of hepatocytes on the slides (Figure S13). The genetic signature and spatial signature overlapped quite well on the UMAP for the current analysis, unlike the EC-Wnt2-9b-DKO mice that lacked Wnt-β-catenin target genes in zone 3 hepatocytes precluding overlap (Figure S13). Five different clusters were identified, which enabled reconstruction of the metabolic zones (pericentral: clusters 1 and 2; midzonal: cluster 3; periporal: clusters 4 and 5) (Figure 6A). A notable increase of the proportion of C3 and C4 was noted after FL6.13 treatment (Figure 6B), which was marked by the ectopic expression of pericentral Wnt target genes, such as Lect2, Cyp2e1, Rgn, Cyp1a2, Gstm1, and Cltn2 (Figures 6C and S15A). Interestingly, some pericentral genes were not induced by FL6.13 using spatial single-cell analysis, including transcription factor Tbx3, heme synthesis enzymes Alad and Cpox, 33,34 bile acid synthesis enzyme Cyp27a1, 34 and complement pathway gene C6 (Figure S15B).

This indicated that FL6.13 selectively activates some but not all pericentral genes. Unlike de-repression of periportal genes in the EC-Wnt2-9b-DKO mice, there was no concomitant decrease in the expression of periporal genes, including complement pathway genes (C6b, Vtn), Fc receptor (Pigr), lipid metabolism gene (Hsd17b13), oxidative phosphorylation genes (Uqcrh, Atp5a1), gluconeogenesis genes (G6pc, Fbp1, Pck1), glutamine catabolism gene (GLs2), urea cycle genes (Arg, Cps1), and hormone (Igf1) (Figures 6C and S16A). Representative images from Molecular Cartography showed that FL6.13 induced an expansion of pericentral markers (Lect2, Cyp2e1, Rgn) to both midzonal and periporal regions, while the expression of periportal markers (Arg1, Ass1, Cps1) remained unaltered (Figure 6D).

We also validated some of these findings by qPCR. Indeed, most pericentral Wnt-β-catenin target genes, such as Glul and Axin2, were significantly induced by FL6.13 treatment, whereas periportal genes were not affected (Figures 6E and 6F). Not all pericentral genes were increased by FL6.13. Levels of Tbx3 and Lgr5 were unchanged after FL6.13 treatment (Figure 6E).

To visualize zonation changes at the protein level, we stained pericentral markers (CYP2E1 and RGN) and periportal markers (CYP2F2 and PIGR) in control and FL6.13-treated livers. Expansion and ectopic expression of CYP2E1 and RGN was observed at the midzone and in the periportal hepatocytes after FL6.13 treatment, while areas of CYP2F2 and PIGR immunostaining were similar as controls and in periportal hepatocytes (Figure 6G).

Altogether, these data indicate a selective and potent effect of FL6.13 in inducing expansion of most pericentral Wnt-β-catenin target genes but not at the expense of periportal gene expression.
FL6.13 induces Ccnd1 gene expression, hepatocyte proliferation, and promotes LR after PH
Since FL6.13 effectively promoted LR after PH in the EC-Wnt2-9b-DKO and EC-Wls-KO mice through stimulation of Ccnd1 expression, we next queried its impact on normal baseline liver after four doses of FL6.13 given every 48 h. A notable increase in Ccnd1 mRNA and cyclin D1 protein was observed after FL6.13 treatment (Figure 6H). Interestingly, induction of cyclin D1 was not evenly distributed across the liver lobule, but only around the periportal region (Figures 6I and 6J), like observations with another Wnt agonist and RSP01.35 BrdU incorporation was significantly induced after FL6.13 treatment, indicating that periportal hepatocytes entered S-phase upon Wnt activation and cyclin D1 expression (Figures 6J and 6K). Collectively, FL6.13 could activate the hepatic Wnt-β-catenin pathway, induce expansion of pericentral metabolic LZ through midzone and even periportal, without impairing expression and localization of periporal genes, and while provoking proliferation of periportal and midzonal hepatocytes. These data also suggest that hepatocytes within different zones in the liver have distinct responses to exogenous Wnt stimulation.

Considering the strong effect of FL6.13 in inducing hepatocyte proliferation, as a proof-of-concept for therapeutic intervention, we next investigated the effect of FL6.13 on LR using the PH model. Four doses of control IgG or FL6.13 were i.p. injected into 8-week-old male C57BL/6 mice. PH was performed on day 8 and regenerating livers were harvested 24 h later. Mice were given 1 mg/mL BrdU in drinking water to label proliferating cells throughout the process (Figure S16B). In control IgG-treated mice, cyclin D1 was expressed mainly at the midzone at 24 h post PH, and very few hepatocytes were BrdU positive (Figure 6L). Pretreatment with FL6.13 induced dramatic expansion of cyclin D1 toward pericentral region and 6-fold increase in periportal BrdU incorporation (Figures 6L and 6M). Proliferative advantage led to accelerated LR and contributed to significantly greater recovery of LW/BW at 24 h (Figure 6N). These results demonstrate a potent effect of FL6.13 pretreatment in promoting LR leading to faster recovery of hepatic mass.

Late treatment with FL6.13 promotes liver repair after APAP injury by promoting Wnt-β-catenin activation and hepatocyte proliferation
To test the clinical applicability of FL6.13 to promote repair in a model of acute liver insult, we evaluated its efficacy in APAP overdose-induced hyperacute liver injury. APAP is a widely used analgesic and antipyretic, but it accounts for 46% of acute liver failure (ALF) in the United States.36 Patients with APAP overdose either progress to liver failure requiring transplant or may recover spontaneously. N-Acetyl cysteine (NAC) is the only dose either progress to liver failure requiring transplant or may recover spontaneously. N-Acetyl cysteine (NAC) is the only reported to induce severe liver injury with mild hepatocyte proliferation during the recovery process.37,38 Indeed, in the control group cyclin D1 was weakly expressed only by the first layer of hepatocytes surrounding pericentral necrotic regions at 48 h post APAP. Hepatocytes were negative for Ki67 demonstrating lack of G1 to S-phase transition at this time (Figure S17D). Interestingly, FL6.13 treatment did not induce cyclin D1 and Ki67 at 48 h after APAP overdose (Figure S17D). Comparable immune cell infiltration, important for phagocytosis of necrotic cells and subsequent liver repair, was evident in both control IgG and FL6.13-treated mice, shown by IHC of CD45 (Figure S17D).39 CYP2E1 plays an essential role in APAP metabolism to generate N-acetyl-p-benzoquinone imine (NAPQI), a highly reactive metabolite that leads to hepatotoxicity.40 Since FL6.13 was shown to induce pericentral and periportal CYP2E1 expression in wild-type mice (Figure 6G), it is likely that 12 h post 600 mg/kg APAP injection, remnant APAP in mice was inadvertently metabolized to NAPQI by FL6.13-induced CYP2E1 expression, leading to a more severe liver injury. Our data suggest that early intervention with a Wnt agonist may have unintended consequences and may worsen APAP-induced liver injury due to induction of CYP2E1.

Next, we investigated if late treatment of FL6.13 could have any therapeutic benefit through promoting regeneration, especially when APAP has already been metabolized. Such opportunity represents an unmet clinical need since there are no therapies currently available if NAC fails to prevent ALF when patients arrive for clinical intervention late after APAP overdose. Single dose of control IgG or FL6.13 was administrated at 32 h post 600 mg/kg APAP injection, and mice were sacrificed at 60 h (Figure 7A). Grossly, livers displayed normal color and appeared healthier in the FL6.13 group (Figure 7B). FL6.13-treated mice showed significantly reduced serum ALT, while AST trended favorably as well (Figure 7C). The control group showed pericentral necrotic areas at 60 h post APAP injection with increased expression of cyclin D1 in peri-necrotic hepatocytes (Figure 7D). Ki67-positive hepatocytes were seen surrounding necrotic regions and in hepatocytes in the periportal region (Figure 7D). FL6.13-treated mice exhibited a significantly reduced serum ALT, while AST showed a trend toward increased expression of cyclin D1 in peri-necrotic hepatocytes (Figure 7D). FL6.13-treated mice exhibited a significantly reduced necrotic area, which was associated with a profound increase in cyclin D1-expressing hepatocytes pan-zonally, indicating that these cells are all able to enter the cell cycle across the liver lobule (Figures 7D and 7E). Many periportal hepatocytes, as well as pericentral hepatocytes, were Ki67 positive in the FL6.13-treated group, which was significantly more than the isotype control-treated group (Figures 7D and 7F). FL6.13 treatment showed a more localized immune cell response around necrotic regions, as seen by staining for CD45 (Figure 7D). Altogether, delayed Wnt agonism promotes liver repair after acute APAP injury by inducing hepatocyte proliferation that occurs locally and periportal and may be “pushing” hepatocytes toward the central vein to fill the space created by the cleared necrotic debris by
the immune cells. Thus, these data extend the application of FL6.13 as delayed regenerative treatment choice in APAP-associated ALF and other relevant indications.

**DISCUSSION**

Liver has a unique capacity to regenerate after PH.\(^9,10\) This allows surgical resection of part of liver for oncological indications and permits living donor and split-liver transplantation. LZ uniquely allows the hepatocytes located in various zones to perform specialized functions and also limits some injuries and disease processes to specific zones.\(^7,8\) Many studies show β-catenin as key regulator of LZ and LR.\(^5\) In view of this, it is important to answer, “Who regulates the regulator?” While Wnt2 and Wnt9b have been shown to be expressed in the central venous and pericentral sinusoidal ECs,\(^19\) and their upregulation observed in various liver injuries,\(^17,20,21\) their unequivocal roles in LZ and LR have not been established. Through genetic elimination of Wnt2 and Wnt9b from ECs, we provide conclusive evidence that these molecules play additive roles in instructing LZ and in driving hepatocyte proliferation after PH, despite a broader Wnt2 expression. We observed gender differences such that females were able to compensate singular Wnt2 or Wnt9b loss better than males, which requires further investigation. Also, Wnt2 and Wnt9b are conserved in their high expression in hepatic ECs in multiple species, suggesting that these two Wnts may be most physiological for use in hepatic regenerative medicine for regulating LZ and LR, just like Wnt7a, Wnt7b, and Wnt10a, have been shown to be relevant during cholestasis.\(^41\) Finally, based on analysis of human scRNA sequencing data from hepatic ECs, and location of β-catenin targets GLUL, AXIN2, and CYPs in zone 3 hepatocytes, it is likely that the same WNTs are also playing a role in human LZ.\(^7,42,43\)

Although previous studies have hinted at periportal genes being ectopically expressed in zone 3 hepatocytes upon β-catenin inhibition, the conclusions have been limited by lack of sensitive
technical tools. Molecular Cartography enabled us to evaluate single-cell spatial expression of 100 genes on the same slide to analyze changes after genetic or pharmacological intervention. Such profiling of EC-Wnt2-9b-DKO livers revealed induction of many periporal genes de novo in pericentral hepatocytes. Intriguingly, for some genes, this change occurred simultaneously with increase, decrease, or no change in their normal baseline zonal expression. Loss of Wnt2 and Wnt9b or Wntless from hepatic ECs, or loss of β-catenin or LRP5-6 from hepatocytes, all lead to similar absence of pericentral genes while simultaneously de-repressing and altering periporal gene expression. This suggests LZ to be a highly dynamic process constituted by simultaneous transcriptional activation and repression of genes in each zone. Periportalization of EC-Wnt2-9b-DKO mice led to resistance to APAP injury, as shown previously for other genetic mouse models of perturbed Wnt-β-catenin signaling. Interestingly, administration of Wnt agonist FL6.13 to a control animal induced zone 3 gene expression in periporal hepatocytes, but it did not result in loss of zone 1 genes. One possible explanation for the differences could be permanent genetic elimination versus transient effect brought about by administration of an exogenous molecule. Another possibility is the differential zonal dynamism of gene expression in hepatocytes residing in zone 3 versus zone 1. It is conceivable that, by default, all hepatocytes expressed periporal genes; however, hepatocytes in zone 3 acquire their zone 3 identity by exposure to neighborhood signals, such as from ECs, which led to repression of periporal genes while activating pericentral genes. Indeed, hepatocytes derived from differentiation of liver stem cells ex vivo, show spontaneous zone 1 expression, while zone 3 gene expression needed to be forced by β-catenin activation. While further studies are needed to address how zone 3 hepatocytes shift to periporal identity in the EC-Wnt2-9b-DKO mice, previous studies have also shown that HNF4α inhibits pericentral β-catenin targets. In the absence of β-catenin, TCF4 associated with HNF4α could possibly bind to HNF4α-responsive elements, inducing the expression of periporal genes, and could be an explanation for our observations.

What regulates Wnt2 and Wnt9b expression and secretion at baseline in the ECs in zone 3, and what stimulates their immediate-early upregulation and release after PH, remains unknown. Being a critical pathway in modulating both hepatocyte metabolism and proliferation, activating the Wnt-β-catenin to promote hepatic function and restore mass is an attractive therapeutic strategy. We utilized FL6.13, a tetravalent antibody described recently, which induces FZD-LRP6 engagement to induce β-catenin activation. FL6.13 rescued pericentral LZ in EC-Wnt2-9b-DKO and EC-Wls-KO mice but not in LRP5-6-LDKO mice. FL6.13 expanded pericentral Wnt target genes in zone 1 and zone 2 but not at the expense of periporal genes. It simultaneously induced Ccd1 expression, allowing hepatocytes to enter the cell cycle and in this way stimulated liver repair without impacting metabolic function. Such duality of Wnt agonism to induce cell proliferation while maintaining metabolic function could benefit treatment of acute and chronic liver insufficiency. While other agonists of Wnt signaling have been generated, our molecule directly engages Wnt receptor and co-receptor and hence is likely more potent and specific.
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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-GS | Sigma-Aldrich | Cat#G2781; RRID: AB_259853 |
| Rabbit polyclonal anti-CYP2E1 | Sigma-Aldrich | Cat#HPA009128; RRID: AB_1078613 |
| Mouse monoclonal anti-CYP1A2 | Santa Cruz Biotechnology | Cat#sc-53241; RRID: AB_629359 |
| Rabbit monoclonal anti-Cyclin D1 | Abcam | Cat#ab134175; RRID: AB_2750906 |
| Rat monoclonal anti-CD45 | Santa Cruz Biotechnology | Cat#sc-53665; RRID: AB_629093 |
| Rabbit monoclonal anti-Ki67 | Cell Signaling Technology | Cat#12202S |
| Rat monoclonal anti-BrdU | Accurate Chemicals | Cat#BD0030A |
| Mouse monoclonal anti-CYP2F2 | Santa Cruz Biotechnology | Cat#sc-374540; RRID: AB_10987684 |
| Rabbit monoclonal anti-GFP | Cell Signaling Technology | Cat#2956S |
| Mouse monoclonal anti-RGN | Santa Cruz Biotechnology | Cat#sc-390098 |
| Goat polyclonal anti-PIGR | R and D Systems | Cat#AF2800; RRID: AB_2283871 |
| Rat monoclonal anti-CK-19 | DSHB | Cat#TROMA-III; RRID: AB_2133570 |
| Goat polyclonal anti-CD31 | R and D Systems | Cat#AF3628; RRID: AB_2161028 |
| **Bacterial and virus strains** | | |
| AAV.TBG.PI.Cre.rBG | addgene | Cat#107787-AAV8 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Control IgG | Clevers et al. | N/A |
| FL6.13 | Clevers et al. | N/A |
| Acetaminophen | Sigma-Aldrich | Cat#A7085 |
| 5-Bromo-2'-deoxyuridine (BrdU) | Sigma-Aldrich | Cat#B5002 |
| **Critical commercial assays** | | |
| TRIzol™ | Thermo Scientific | Cat#15996026 |
| RNesy Mini Kit | Qiagen | Cat#74104 |
| Power SYBR® Green PCR Master Mix | Applied Biosystems | Cat#4367660 |
| Nonfat dry milk | Cell Signaling Technology | Cat#9999 |
| SuperSignal® West Pico Chemiluminescent Substrate | Thermo Scientific | Cat#34080 |
| VECTASTAIN® Elite® ABC-HRP Kit, Peroxidase | Vector Laboratories | Cat#PK-6101 |
| DAB Substrate Kit, Peroxidase (HRP) | Vector Laboratories | Cat#SK-4100 |
| **Deposited data** | | |
| Raw and processed spatial single-cell data | This paper | GSE199463 |

### Experimental models: Organisms/strains

| Mouse: C57BL/6J | The Jackson Laboratories | JAX: 000,664; RRID: IMSR_JAX:000,664 |
| Mouse: B6; 129P2-Lyve1tm1.1(MOPC31/13EGFP/Cre)Cyp/J | The Jackson Laboratories | JAX: 012,601; RRID: IMSR_JAX:012,601 |
| Mouse: B6.129X1-Gt(Rosa26)Sortm1(EYFP)Cck/J | The Jackson Laboratories | JAX: 006,148; RRID: IMSR_JAX:006,148 |
| Mouse: Wnt9btm1.2(Rosa26)Cok/J | The Jackson Laboratories | JAX: 008,469; RRID: IMSR_JAX:008,469 |
| Mouse: Wnt2tm1.1(Rosa26)Cok/J | This paper | N/A |
| Mouse: EC-Wnt9b-KO on C57BL/6J | This paper | N/A |
| Mouse: EC-Wnt2-KO on C57BL/6J | This paper | N/A |
| Mouse: EC-Wnt2-9b-DKO on C57BL/6J | This paper | N/A |
| Mouse: EC-Wls-KO on C57BL/6J | Russell and Monga | N/A |
| Mouse: Lrp5tm1.1(Rosa26)Cok/Lrp6tm1.1(Rosa26)Cok | This paper | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Satdarshan P. Monga (smonga@pitt.edu).

Materials availability
Mouse lines and all unique reagents used in this study are available from the lead contact with a completed Material Transfer Agreement.

Data and code availability
Spatial single-cell data has been deposited into NCBI GEO database with accession ID: GSE199463. Raw and processed data can be downloaded by https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE199463. The software and algorithms for data analyses used in this study are published and referenced throughout the STAR Methods section. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
All animal husbandry and experimental procedures, including animal housing and diet, were performed under the guidelines and approval of the National Institutes of Health and the Institutional Animal Care and Use Committee at the University of Pittsburgh. Mice were fed regular chow in standard caging and kept under a 12-h light–dark cycle with no enrichment. Lyve1-cre mice were purchased from Jackson Laboratories. Wnt9b\textsuperscript{floxflox} mice were reported before.\textsuperscript{30} Wnt2\textsuperscript{exon-2-floxed} mice were generated by insertion of \textit{LoxP} sites flanking exon 2, through use of CRISPR/Cas9 technology as described elsewhere.\textsuperscript{52} Briefly, using single-guide RNAs (sgRNAs), double-strand breaks flanking exon 2 were targeted to optimal CAS9 target sites within introns 1 and 2. A synthetic floxed allele was generated as a single-stranded-oligodeoxynucleotide (ssODN) template for homology-directed repair (HDR). The HDR template was a megamer, 1166bp ssDNA oligo (Integrated DNA Technologies), encoding exon 2 flanked by \textit{LoxP} sites with adjacent EcoR1 sites for genotyping and 5’ AND-3’ homology arms homologous to the genomic sequences up-and downstream of the 5’ AND-3’ CAS9 cut sites in Introns 1 and 2. Homologous recombination was achieved by microinjection of a mixture of Cas9 protein (0.3 \textmu M), I1 & I2 sgRNAs (21.23 ng/\textmu L each) and the ssODN (10 ng/\textmu L) into the pronuclei of fertilized embryos (C57BL/6J, The Jackson Laboratory). The injected zygotes were cultured overnight, the next day the embryos that developed to the 2-cell stage were transferred to the oviducts of pseudopregnant CD1 female recipients. Offspring were genotyped by PCR and RFLP analysis to confirm insertion of 5’ and 3’ \textit{loxP} sites. Founder mice, M14 and M15, were back-crossed with C57BL/6J wildtype (WT) mice for two generations and selected for the presence of Wnt2\textsuperscript{floxflox} allele to generate N2 mice, which were then bred with Wnt9b\textsuperscript{floxflox}, Rosa-stop\textsuperscript{floxflox-EYFP}, and Lyve1-cre\textsuperscript{1/−} mice. Genotyping primers and PCR products are listed in Tables S1 and S2.

Lyve1-cre\textsuperscript{1/−}; Wnt2\textsuperscript{floxflox}; Wnt9b\textsuperscript{floxflox} Rosa-stop\textsuperscript{floxflox-EYFP} mice are hereafter referred to as EC-Wnt9b-KO. Lyve1-cre\textsuperscript{1/−}; Wnt2\textsuperscript{floxflox}; Wnt9b\textsuperscript{floxflox} Rosa-stop\textsuperscript{floxflox-EYFP} mice are hereafter referred to as EC-Wnt2-KO. Lyve1-cre\textsuperscript{1/−}; Wnt2\textsuperscript{floxflox}; Wnt9b\textsuperscript{floxflox} Rosa-stop\textsuperscript{floxflox-EYFP} mice are hereafter referred to as EC-Wnt2-9b-DKO. The rest of mice were used as littermate controls (Control). EC-Wls-KO mice were described before.\textsuperscript{17} LRP5-6-LDKO mice were generated by i.p. injection of 1 x 10\textsuperscript{12} genome copies of AAV8-TBG-Cre (Addgene, 107,787-AAV8) per mouse to LRP5-6 double floxed mice described before.\textsuperscript{14}
METHOD DETAILS

Two-third or partial hepatectomy
Two to three-month-old male and female Control, EC-Wnt2-KO, EC-Wnt9b-KO, EC-Wnt2-9b-DKO mice were subjected to PH as describe before. Animals were sacrificed at 40 h post-PH. Serum and liver tissues were harvested for further analysis.

FL6.13 treatment
Two to three-month-old male wildtype, EC-Wnt2-9b-DKO, EC-Wls-KO, LRP5-6-LDKO mice were treated with 5 mg/kg control IgG or FL6.13 every other day as described elsewhere, for one week followed by PH. Animals were sacrificed at 24 h post-PH. 1 mg/mL BrdU was given in drinking water. Serum and liver tissues were harvested for further analysis.

Acetaminophen study
8-week-old male C57BL/6 mice were ordered from Jackson lab. Mice were given food and water from 6 p.m. to 9 p.m. in dark room and then fasted from 9 p.m. to 9 a.m. next day. 600 mg/kg APAP was intraperitoneal (i.p.) injected, and food were given back. 12 h or 32 h post APAP injection, mice were randomly grouped and were i.p. injected with 5 mg/kg control IgG or FL6.13 in 0.9% saline. Mice were sacrificed at 48 h or 60 h. Serum and liver tissues were collected for further analysis.

scRNA sequencing data analysis
scRNA sequencing data were published before and were analyzed at: https://www.livercellatlas.org/index.php.

Molecular cartography™
Detailed protocol of tissue processing, probe design, imaging, signal segmentation and barcoding was discussed before. Probes used in this study are listed in Table S3. One animal per group was used. Molecular Cartography images were performed in ImageJ using genexyz Polylux tool plugin from Resolve BioSciences to examine specific Molecular Cartography signals.53

Single-cell spatial transcriptomic analysis was performed according to the workflow in the Figures S10 and S13. Two bioinformatic pipelines were applied to study the data. For the first pipeline, gene counts were quantified per cell based on the cell identification from QuPath software.54 Slide control, slide EC-Wnt2-9b-DKO, and slide FL6.13 treatment were integrated by R package Seurat.55 For quality control, cells with less than 10 gene-count were filtered out. Non-hepatocyte cells were defined by the cells with any expression of the five non-hepatocyte makers (Lrat, Pecam1, Ptprc, Lyz2, and Adgre1) and were removed from further analysis when comparing control and FL6.13 treatment (Figure S13). After pre-processing, dimension reduction method principal component analysis (PCA) and uniform manifold approximation and projection (UMAP)56 were performed on hepatocytes based on 16 zonated markers (pericentral markers: Cldn2, Lect2, Glui, Oat, Cyp1a2, Cyp2e1, Gstm1, Rgn; midzonal markers: Pon1; periportal markers: Gls2, G6pc, Fbp1, Hsd17b13, Vtn, Cyp2f2, Pigr). Eventually, cells were grouped into distinct clusters and annotated by the zonated genes. Feature plots and violin plots were generated by Seurat to visualize gene expression at cell-level and cluster-level.

For the second pipeline, a line was drawn from center of central vein (marking pericentral region) to portal vein (marking periportal region) based on respective landmark genes (Cyp2e1, Glui, and Sox9). Then an upper-bound and a lower-bound line were drawn parallel to the central line with 500-pixel extension. Bounded by these two lines, perpendicular lines were drawn to separate the region between pericentral and periportal into 9 equal segments. In total, 7, 9, and 9 pericentral-to-periportal regions were identified in slide control, slide EC-Wnt2-9b-DKO, and slide FL6.13 treatment, respectively. Gene counts and expression density (gene counts per area) were quantified within each segment and averaged across the defined pericentral-to-periportal regions. Cells located in these 9-segments were also identified based on their X, Y positions in the slides and traced back to the UMAPs to show the histological location of cells.

Tissue sections
Mouse liver samples were frozen in liquid nitrogen as recommended by the Molecular Cartography protocol. Frozen samples were sectioned with a cryostat (Leica, CM 1850-3-1) and 10μ thick sections were placed within the capture areas of cold Resolve BioSciences slides. Samples were then sent to Resolve BioSciences on dry ice for analysis. Upon arrival, tissue sections were thawed and fixed according to Molecular Cartography protocol with MF1 for 30 min at 4°C. After fixation, sections were washed twice in 1x PBS for two min, followed by one min washes in 70% Ethanol at room temperature. Fixed samples were undergoing an alcoholic series starting with an incubation in isopropanol for 1 min, followed by 95% and 75% ethanol. The samples were used for Molecular Cartography™ (100-plex combinatorial single molecule fluorescence in-situ hybridization) according to the manufacturer’s instructions Day 1: Molecular Preparation Protocol for mouse or human liver, starting with the aspiration of ethanol and the addition of buffer DST3 followed by tissue priming and hybridization. Briefly, tissues were primed for 30 minutes at 37°C followed by overnight hybridization of all probes specific for the target genes (see below for probe design details and target list). Samples were washed the next day to remove excess probes and fluorescently tagged in a two-step color development process. Regions of interest were imaged as described below and fluorescent signals removed during decolorization. Color development, imaging and decolorization were repeated for multiple cycles to build a unique combinatorial code for every target gene that was derived from raw images as described below.
**Probe design**

The probes for 100 genes were designed using Resolve’s proprietary design algorithm. Briefly, the probe-design was performed at the gene-level. For every targeted gene all full-length protein coding transcript sequences from the ENSEMBL database were used as design targets if the isoform had the GENCODE annotation tag “basic”. To speed up the process, the calculation of computationally expensive parts, especially the off-target searches, the selection of probe sequences was not performed randomly, but limited to sequences with high success rates. To filter highly repetitive regions, the abundance of k-mers was obtained from the background transcriptome using Jellyfish. Every target sequence was scanned once for all k-mers, and those regions with rare k-mers were preferred as seeds for full probe design. A probe candidate was generated by extending a seed sequence until a certain target state.

**RNA isolation and qPCR**

Whole liver was homogenized in TRIzol (Thermo Scientific, 15,596,026) and nucleic acid was isolated through phenol-chloroform extraction. RNA was reverse transcribed into cDNA using Superscript III (Invitrogen, 18,080-044). Real-time PCR was performed...
in technical duplicate on a StepOnePlus Real-Time PCR System (Applied Biosystems, 4,376,600) using the Power SYBR Green PCR Master Mix (Applied Biosystems, 4,367,660). Target gene expression was normalized to housekeeping genes Rn18s, and fold change was calculated utilizing the ΔΔ-Ct method. Primers are listed in Table S4.

**Protein isolation and western blot**

Snap frozen liver samples were homogenized in RIPA buffer with fresh proteinase and phosphatase inhibitor. The concentration of the protein was determined by the bicinchoninic acid assay. Protein sample was prepared with loading buffer (Bio-Rad, 1,610,737) with 5% 2-Mercaptoethanol (Bio-Rad, 161-0710) and subjected to electrophoresis. Protein sample was separated on pre-cast 7.5% or 4-20% polyacrylamide gels (Bio-Rad) and transferred to the PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were stained with Ponceau-S and blocked for 30 min with 5% nonfat dry milk (Cell signaling, 9999) or 5% BSA in Blotto buffer (0.15M NaCl, 0.02M Tris pH 7.5, 0.1% Tween in dH2O), and incubated with primary antibodies at 4°C overnight at the following concentrations: GS (Sigma, G2781, 1:2000), CYP2E1 (Sigma, HPA009128, 1:1000), CYP1A2 (Santa Cruz Biotechnology, sc-53241, 1:1000), Cyclin D1 (Abcam, ab134175, 1:1000). Membranes were washed in Blotto buffer and incubated with the appropriate HRP-conjugated secondary antibody for 60 min at room temperature. Membranes were washed with Blotto buffer, and bands were developed utilizing SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34,080) and visualized by time-gradient autoradiography.

**Immunohistochemistry**

Livers were fixed in 10% buffered formalin for 48-72 h prior to paraffin embedding. Blocks were cut into 4μm sections, deparaffinized, and washed with PBS. For antigen retrieval, samples were microwaved for 12 min in pH = 6 sodium citrate buffer (CD45), or in pH = 9 Tris-EDTA buffer (BrdU), or were pressure cooked for 20 min in pH = 6 sodium citrate buffer (CYP2E1, CYP1A2, Ki67, CYP2F2), or in pH = 9 Tris-EDTA buffer (GFP, Cyclin D1, RGN, PIGR). For BrdU, slides were then incubated with 2M HCl for 1 h at room temperature and washed with 0.5M Borax for 5 min. GS staining doesn’t need antigen retrieval. Samples were then placed in 3% H2O2 for 10 min to quench endogenous peroxide activity. After washing with PBS, slides were blocked for 10 min. The primary antibodies were incubated at the following concentrations in PBS: CD45 (Santa Cruz Biotechnology, sc-53665, 1:100), BrdU (Accurate Chemicals, OBT0030A, 1:75), GS (Sigma, G2781, 1:3000), CYP2E1 (Sigma, HPA009128, 1:100), CYP1A2 (Santa Cruz Biotechnology, sc-53241, 1:100), Ki67 (Cell signaling, 12,202, 1:500), CYP2F2 (Santa Cruz Biotechnology, sc-374540, 1:100), GFP (Cell signaling, 2956, 1:100), Cyclin D1 (Abcam, ab134175, 1:200), RGN (Santa Cruz Biotechnology, sc-390098, 1:100), PIGR (R&D Systems, AF2800, 1:100) for 1 h at room temperature. Samples were washed with PBS three times and incubated with the appropriate biotinylated secondary antibody (Vector Laboratories) diluted 1:250 in antibody diluent for 15 min at room temperature. Samples were washed with PBS three times and sensitized with the Vectastain ABC kit (Vector Laboratories, PK-6101). Following three washes with PBS color was developed with DAB Peroxidase Substrate Kit (Vector Laboratories, SK-4100), followed by quenching in distilled water. Slides were counterstained with hematoxylin (Thermo Scientific, 7211), dehydrated to xylene and coverslips applied with Cytoseal XYL (Thermo Scientific, 8312-4). Images were taken on a Zeiss Axioskop 40 inverted brightfield microscope.

**Immunofluorescence**

For triple staining of CK19, CD31, and GFP, paraffin sections were deparaffinized and pressure cooked in pH = 9 Tris-EDTA buffer for 20min. Slides were permeabilized with 0.3% Triton X-100 in PBS for 20 min at room temperature and then blocked with 5% normal donkey serum in 0.3% Triton X-100 in PBS (antibody diluent) for 30 min at room temperature. Antibodies were diluted as follows: CK19 (DSHB, TROMA-III, 1:10), CD31 (R&D Systems, AF3628, 1:100), GFP (Cell signaling, 2956, 1:100), in antibody diluent and incubated at 4°C overnight. Samples were washed three times in 0.1% Triton X-100 in PBS (wash solution) and incubated with the proper fluorescent secondary antibody (Alexa Fluor 488/555/647, Invitrogen) diluted 1:300 in antibody diluent for 2 h at room temperature. Samples were washed three times and incubated with DAPI (Sigma, B2883) for 1 min. Samples were washed three times and mounted with gelvator. Images were taken on a Nikon Eclipse Ti epifluorescence microscope or a Zeiss LSM700 confocal microscope and were analyzed with ImageJ.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical comparison between two groups was done with the unpaired Student’s t test. Multiple-group comparison was done with one-way ANOVA. The statistical details for each experiment can be found in the figure legends. GraphPad Prism version 9.0 was used for graph generation, and a p value of less than 0.05 was considered significant. The bars represent means ± SEM ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.