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Citation for published version:

Plana-Paz, L, Orsini, V, Boulter, L, Calabrese, D, Pikiólek, M, Nigsch, F, Xie, Y, Roma, G, Donovan, A, Marti, P, Beckmann, N, Dill, MT, Carbone, W, Bergling, S, Isken, A, Mueller, M, Kinzel, B, Yang, Y, Mao, X, Nicholson, TB, Zamponi, R, Capodiecì, P, Valdez, R, Rivera, D, Loew, A, Ukomadu, C, Terracciano, LM, Bouwmeester, T, Cong, F, Heim, MH, Forbes, S, Ruffner, H & Tchorz, JS 2016, 'The RSPO-LGR4/5-ZNRF3/RNF43 module controls 1 liver zonation and size', *Nature Cell Biology*.
<https://doi.org/10.1038/ncb3337>

Digital Object Identifier (DOI):

[10.1038/ncb3337](https://doi.org/10.1038/ncb3337)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Nature Cell Biology

Publisher Rights Statement:

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1 **The RSPO-LGR4/5-ZNRF3/RNF43 module controls liver zonation and size**

2

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20

21 Key words: Wnt, lineage tracing, liver regeneration, liver development

22

1 **LGR4/5 receptors and their cognate RSPO ligands potentiate Wnt/ β -Catenin signalling**
2 **and promote proliferation and tissue homeostasis in epithelial stem cell**
3 **compartments. In the liver, metabolic zonation requires a Wnt/ β -Catenin signalling**
4 **gradient, but the instructive mechanism controlling its spatiotemporal regulation is**
5 **not known. We have identified a novel role for the RSPO-LGR4/5-ZNRF3/RNF43**
6 **module as master regulator of Wnt/ β -Catenin-mediated metabolic liver zonation.**
7 **Liver-specific LGR4/5 loss-of-function (LOF) or RSPO blockade disrupted hepatic**
8 **Wnt/ β -Catenin signalling and zonation. Conversely, pathway activation in**
9 **ZNRF3/RNF43 LOF mice or with recombinant RSPO1 protein expanded the hepatic**
10 **Wnt/ β -Catenin signalling gradient in a reversible and LGR4/5-dependent manner.**
11 **Furthermore, we show that LGR4+ hepatocytes throughout the lobule contribute to**
12 **liver homeostasis and regeneration. Likewise, recombinant RSPO1 protein increased**
13 **liver size and improved liver regeneration, whereas LGR4/5 LOF caused the opposite**
14 **effects, resulting in hypoplastic livers. Together, the RSPO-LGR4/5-ZNRF3/RNF43**
15 **module controls metabolic liver zonation and acts as a hepatic growth/size rheostat**
16 **during development, homeostasis and regeneration.**

17

18 Hepatic zonation and organ size control are required for physiological liver function,
19 including metabolization of a wide range of endogenous products and xenobiotics.
20 Hepatocyte function is determined by its position along the porto-central axis of the
21 liver lobule, which creates a metabolic zonation in the liver. Complementary metabolic
22 pathways occur within non-overlapping liver zones, thus maintaining optimal metabolic

1 homeostasis ¹⁻⁵. Physiological liver size is tightly controlled by the concerted growth of
2 hepatocytes and other hepatic cells during development, homeostasis and regeneration
3 ⁶⁻⁸. Recent studies have highlighted the Wnt/ β -Catenin pathway as a major regulator of
4 liver zonation, development and regeneration ^{4, 9-11}. Wnt pathway activation stimulates
5 cytoplasmic stabilization and nuclear translocation of β -Catenin, which subsequently
6 associates with transcriptional regulators to control β -Catenin target gene expression
7 (*e.g.* Axin2 and leukocyte cell-derived chemotaxin 2 (Lect2)) and expression of other
8 Wnt/ β -Catenin-dependent genes (*e.g.* Glutamine synthetase (GS) and Cyp2e1) ¹². β -
9 Catenin-deficient mouse livers exhibit defects in metabolic liver zonation ¹⁰. The
10 Adenomatous polyposis coli (APC) protein antagonizes β -Catenin activity, since APC
11 deletion activates β -Catenin signalling and thereby expands the hepatic Wnt activity
12 gradient ^{9, 13}. Deletion of β -Catenin impaired hepatocyte proliferation during liver
13 development and regeneration, whereas transgenic β -Catenin overexpression or APC
14 deletion results in hyperplastic livers and hepatocellular carcinoma due to increased
15 hepatocyte proliferation ¹⁴⁻¹⁹. However, the instructive molecular cues for
16 spatiotemporal control of Wnt/ β -Catenin signalling in liver zonation, development and
17 regeneration have remained elusive.

18 R-Spondin (RSPO)1-4 ligands potentiate Wnt/ β -Catenin signalling via Leucine-rich repeat
19 containing G protein-coupled receptors 4-6 (LGR4-6) ²⁰⁻²³. In the absence of RSPO
20 proteins, Wnt signal transduction is negatively regulated by cell-surface transmembrane
21 E3 ubiquitin ligases, Zinc and ring finger 3 (ZNRFB3) and its homologue Ring finger 43
22 (RNF43), which both promote Wnt receptor turnover. Upon stimulation, RSPO proteins

1 bind to LGR4/5 receptors as well as the ZNRF3/RNF43 transmembrane ubiquitin ligases,
2 causing the latter to become cleared from the plasma membrane which leads to
3 increased Wnt signalling^{24, 25}. We and others have shown that RSPO-LGR4/5-
4 ZNRF3/RNF43 signalling plays an essential role in developing and maintaining various
5 epithelial tissue stem cell compartments by regulating tissue homeostasis^{21, 23, 26, 27}. The
6 liver has a remarkable plasticity with an intrinsically high capacity for context-dependent
7 regeneration in response to diverse injuries and during homeostasis²⁸⁻³². LGR5 has been
8 reported to be exclusively expressed around the portal vein only following injury^{33, 34},
9 whereas undamaged livers have persistent high Wnt/ β -Catenin activity around the
10 central vein^{1, 35}. This is in contrast to other tissues where cells with high Wnt/ β -Catenin
11 activity express LGR4 and LGR5 to mediate RSPO-mediated Wnt/ β -Catenin signalling^{21,}
12^{23, 26, 27}. A potential role of RSPO-LGR4/5-ZNRF3/RNF43 signalling in the regulation of
13 hepatic Wnt/ β -Catenin activity during liver development, homeostasis and regrowth has
14 remained unclear.

15 Through integrated *in vivo* approaches comprising lineage tracing, tissue-specific LGR4/5
16 LOF, pathway activation via RSPO1 injections or ZNRF3/RNF43 LOF and RNA expression
17 analyses we have identified a novel role for the RSPO-LGR4/5-ZNRF3/RNF43 module in
18 controlling metabolic liver zonation. We further show that the RSPO-LGR4/5-
19 ZNRF3/RNF43 module acts as a hepatic growth/size rheostat during development,
20 homeostasis and regeneration and that recombinant RSPO1 protein administration
21 accelerates liver regrowth.

22

1 LGR4 and LGR5 are co-expressed in pericentral hepatocytes with high Wnt activity

2 To gain a detailed resolution of the hepatic Wnt/ β -Catenin signalling gradient regulating
3 metabolic liver zonation, we assessed Wnt/ β -Catenin activity in different hepatic zones
4 using immunostaining, *in situ* hybridization (ISH) and transgenic reporter mice.
5 Expression of GS, a Wnt/ β -Catenin-dependent gene and key enzyme in the ammonia
6 detoxification pathway¹⁰ (Figure 1a, 1c) as well as β -Catenin reporter activity in Tcf/Lef-
7 Venus mice³⁶ (Figure 1b, 1c), were restricted to the first layers of hepatocytes around
8 the central vein. Similarly, ISH showed that Axin2 mRNA was predominantly expressed
9 in these cells (Figure 1d, 1e). Expression of CYP2E1, another metabolic enzyme and
10 Wnt/ β -Catenin-dependent gene³⁷, extended into the parenchyma adjacent to the
11 central veins (Figure 1a, 1c). This suggests differential expression of Wnt/ β -Catenin-
12 dependent genes along the centro-portal Wnt/ β -Catenin signalling gradient possibly
13 because of different inductive thresholds or transcriptional regulation for these genes.
14 To explore whether the RSPO receptors LGR4 and LGR5 and the ubiquitin ligases ZNRF3
15 and RNF43 contribute to the regulatory network that establishes the Wnt/ β -Catenin
16 signalling gradient and liver zonation, we performed a detailed expression analysis.
17 Using ISH staining in wild type mice, we found Lgr5 expression mainly restricted to
18 pericentral hepatocytes with high Wnt activity. Almost all hepatocytes adjacent to the
19 central vein, mono- and bi-nucleated, expressed Lgr5. The number of Lgr5-positive
20 hepatocytes slightly decreased in the 2nd and 3rd pericentral layers, whereas only a
21 negligible minority of parenchymal hepatocytes expressed Lgr5. Lgr4, which was
22 coexpressed with Lgr5 in pericentral hepatocytes, was expressed in virtually all

1 hepatocytes across the liver lobule (Figure 1f, 1g; Supplementary Figure 1a, 1b). *Znrf3*
2 was broadly expressed in hepatocytes throughout liver (Figure 1h, 1i), whereas mice
3 that express LacZ from the *Rnf43* locus (Supplementary Figure 2a) showed that *Rnf43*
4 expression was restricted to central vein hepatocytes (Figure 1j, 1k), similar as seen for
5 *Lgr4* and *Lgr5*, respectively. To further explore the expression profiles and roles of *Lgr4*
6 and *Lgr5* in the liver, we performed lineage tracing experiments using mice expressing
7 tamoxifen (TAM)-inducible Cre (CreERT2) from the *Lgr4* (*Lgr4ki* mice) or *Lgr5* (*Lgr5ki*
8 mice) loci ²⁶, crossed with Rosa26 (R26)- β -Galactosidase (β -Gal; LacZ) ³⁸, R26-tdTomato
9 (tdTOM) or R26-enhanced green fluorescent protein (EGFP) reporter mice
10 (Supplementary Figure 3a). Ten-day lineage tracing in *Lgr4ki*/R26-LacZ mice labelled
11 hepatocytes throughout the liver, confirming broad LGR4 expression (Figure 2a,
12 Supplementary Figure 3b). Ten-month LGR4 lineage tracing in these mice showed a
13 similar expression pattern with small LacZ⁺ hepatocyte clones consisting of 1-5 cells, in
14 line with low rates of homeostatic cell division ⁵ (Figure 2b, 2c, Supplementary Figure
15 3b). Similarly sized clones of LGR4 lineage-traced pericentral, parenchymal and
16 periportal hepatocytes are indicative of similar proliferation rates within the 3 different
17 liver zones (Figure 2c). Ten-day lineage tracing in *Lgr5ki*/R26-LacZ mice confirmed that
18 LGR5 expression is restricted to pericentral hepatocytes (Figure 2d, Supplementary
19 Figure 3c). Eighteen-month lineage tracing and distributions analysis across different
20 zones indicated that LGR5⁺ hepatocytes remained at the central vein as small clones
21 consisting of 1-5 cells and do not radiate out into the parenchyma (Figure 2e, 2f,
22 Supplementary Figure 3c, 3d). Co-expression of *Axin2* and *Lgr5* in virtually all of the

1 LGR5 lineage-traced hepatocytes, as measured by ISH, confirmed active Wnt signalling
2 in these cells similar as in untraced neighbouring pericentral hepatocytes
3 (Supplementary Figure 3e-g). Moreover, Ki67 staining in the LGR5 lineage-traced
4 hepatocytes 48 h post-partial hepatectomy (PH) indicated that the proliferation capacity
5 of these cells was similar compared to untraced neighbouring pericentral hepatocytes
6 (Supplementary Figure 3h-j). A comparative 7-day EdU proliferation analysis in Lgr5ki
7 and wt mice showed that the absence of one of the Lgr5 alleles did not impair
8 homeostatic proliferation of pericentral hepatocytes (Figure 2i, 2j). Ten-day lineage
9 tracing in Lgr4ki/R26-EGFP mice (Figure 2g) and Lgr5ki/R26-tdTOM mice (Figure 2h) and
10 co-staining of labelled cells with hepatocyte markers Hepatocyte Nuclear Factor 4 Alpha
11 (HNF4 α) or GS, respectively, confirmed that the traced cells were indeed hepatocytes. In
12 gut and skin, LGR4 is more broadly expressed than LGR5, and LGR5 expression is
13 restricted to tissue stem cells with high Wnt activity. Concurrently, LGR5 is a Wnt target
14 gene itself ³⁹⁻⁴¹. Similarly, we observed a broad expression of LGR4 in hepatocytes
15 throughout the liver, while LGR5 expression was restricted to pericentral hepatocytes
16 with high Wnt activity. Despite persistent high Wnt activity and LGR5 expression,
17 pericentral hepatocytes rarely proliferated and did not exhibit increased proliferation
18 rates when compared to those of other hepatic zones, as indicated both by EdU
19 labelling (Figure 2i, 2k-m and Supplementary Figure 3k-n) and Ki67 staining (Figure 2n-
20 p). This is in contrast to actively cycling LGR5+ cells in tissue stem cell compartments of
21 other organs ²⁷, but consistent with similar homeostatic proliferation rates across
22 different liver zones reported earlier ⁴². In undamaged livers, the majority (>80%) of

1 proliferating cells were found in the liver parenchyma (Figure 2m, 2p, and
2 Supplementary Figure 3n), itself the largest hepatic zone, indicating that parenchymal
3 hepatocytes account for the majority of new hepatocytes during liver homeostasis. This
4 is consistent with previous reports showing that the majority of proliferating
5 hepatocytes reside in the parenchyma and that hepatocytes throughout the lobule
6 contribute to liver homeostasis without zonal dominance⁴²⁻⁴⁴.

7

8 LGR4 and LGR5 receptors are essential for metabolic liver zonation

9 To delineate whether LGR4 and LGR5 are mediating the instructive cues that establish
10 the hepatic Wnt/ β -Catenin signalling gradient and liver zonation, we generated liver-
11 specific Lgr4 knock-out (KO) (Lgr4LKO), Lgr5KO (Lgr5LKO) and Lgr4/5 double KO
12 (Lgr4/5dLKO) mice using Albumin (Alb)Cre-mediated deletion (Supplementary Figure
13 2b). Mice of all 3 genotypes were born at normal Mendelian ratios and viable with
14 normal lifespans (data not shown). Lgr4LKO and Lgr4/5dLKO, but not Lgr5LKO mice
15 showed reduced liver weight when compared to control mice (Figure 3a). Loss of Lgr4
16 and Lgr5 mRNA in the liver was confirmed by reverse transcription polymerase chain
17 reaction (RT-PCR) (Figure 3b) and ISH (Figure 3c). While no overt changes were observed
18 in Lgr5LKO mice, Wnt signalling was abrogated in Lgr4/5dLKO and Lgr4LKO mice, as
19 indicated by loss of Axin2 and Lect2 expression. Lgr5 mRNA expression was absent in
20 Lgr4LKO mice (Figure 3b, 3c), similar as we previously reported for the developing
21 intestinal epithelium²⁶. This suggests a dominant role for LGR4 over LGR5, and that loss
22 of Lgr5 expression is a result of abrogated Wnt signalling. GS staining was severely

1 reduced in Lgr4LKO and completely lost in Lgr4/5dLKO mice, whereas Lgr5LKO mice had
2 slightly elevated GS expression when compared to control mice (Figure 3d, 3e).
3 Likewise, LGR4 and LGR4/5 deletion resulted in impaired CYP2E1 expression (Figure 3f,
4 3g). Early postnatal analysis of Lgr4/5dLKO mice showed reduced GS expression already
5 at postnatal day (P)2 which remained low on P10 and P30, whereas it was increased in
6 control mice from P2 to P10 (Supplementary Figure 4a, 4b). P2, P10 and P30 Lgr4/5dLKO
7 mice showed functional hepatocyte and biliary cell differentiation during liver
8 development, as evidenced by normal distribution of HNF4 α + hepatocytes, SRY (Sex
9 Determining Region Y)-Box 9 (SOX9)+/Cytokeratin 19 (CK19)+ biliary ducts and absence
10 of SOX9 in hepatocytes (Supplementary Figure 4c, 4d), excluding the possibility that
11 compromised hepatocyte differentiation during liver development accounts for the
12 impaired metabolic zonation. To gain further insights into the mechanistic
13 consequences of liver-specific LGR4/5 deletion, we performed high-throughput RNA
14 sequencing (RNA-Seq) on total liver RNA samples isolated from control and Lgr4/5dLKO
15 mice. Expression of Wnt target genes (*e.g.* Axin2, Lect2 and Tumor Necrosis Factor
16 Receptor Superfamily, Member 19 (Tnfrsf19; Troy)) as well as gene sets for Wnt
17 signalling and metabolic processes were significantly downregulated in livers with
18 combined LGR4 and LGR5 deletions (Supplementary Figure 5a, 5b, Supplementary Table
19 1, 2). Comparative expression analysis of genes encoding for metabolic enzymes⁴⁵
20 revealed that pericentrally expressed metabolic genes were downregulated, whereas
21 few periportal genes (Hsd17b13 and Hal) were upregulated in Lgr4/5dLKO compared to
22 control mice (Figure 3h, Supplementary Figure 5a). RT-PCR analysis confirmed

1 downregulation of pericentral metabolic genes (Glul, Cyp2e1 and Cyp1a2) and
2 upregulation of Hsd17b13 and Hal (Figure 3i, 3j). This indicates that loss of hepatic
3 Wnt/ β -Catenin signalling in Lgr4/5dLKO mice results in loss of metabolic liver zonation.
4 RT-PCR and immunostaining confirmed that periportal metabolic genes, that were
5 upregulated in β -Catenin KO mice or mice with virus-mediated DKK1 overexpression^{9,13},
6 were not upregulated in Lgr4/5dLKO compared control mice (Figure 3j; Supplementary
7 Figure 5c, 5d). It is possible that inactivation or deletion of Wnt pathway components at
8 different hierarchical levels result in distinct signalling output events which might
9 account for these differences; *e.g.* LGR4/5 deletion was also shown to impair non-
10 canonical Wnt signalling, in contrast to β -Catenin deletion or DKK1 overexpression^{22,46}.
11 Moreover, downregulated gene sets involved in cell cycle regulation further suggest a
12 role for LGR4 and LGR5 in controlling hepatic cell proliferation (Supplementary Figure
13 5b). Together, we identified an essential role for LGR4 and LGR5 receptors in spatial
14 regulation of the centro-portal Wnt/ β -Catenin signalling gradient and metabolic liver
15 zonation.

16

17 RSPO controls liver zonation via LGR4 and LGR5 receptors

18 Overexpression of the extracellular domain (ECD) of ZNRF3 (ZNRF3ECD) was shown to
19 block endogenous RSPO function by blocking RSPO1-induced membrane accumulation
20 of Frizzled proteins²⁴. To analyse whether RSPO proteins control the hepatic Wnt/ β -
21 Catenin signalling gradient, we generated BAC-transgenic mice that allow doxycycline
22 (DOX)-induced ubiquitous expression of the ZNRF3ECD (Znrf3ECD mice) and crossed

1 these mice with Axin2-LacZ mice to obtain double transgenic Znr3ECD/Axin2-LacZ mice
2 (Figure 4a). Znr3ECD/Axin2-LacZ mice fed with DOX-containing diet showed reduced
3 LacZ expression when compared to those with normal diet, indicating that loss of
4 endogenous RSPO function impaired the hepatic Wnt/ β -Catenin activity gradient (Figure
5 4b, 4c). Likewise, GS (Figure 4d, 4e) and CYP2E1 (Figure 4f, 4g) expression was
6 dramatically reduced in Znr3ECD/Axin2-LacZ mice fed with DOX-containing diet. We
7 next generated mice that allowed for the inducible combined deletion of ZNRF3 and
8 RNF43 using TAM (Znr3/Rnf43dfl;R26CreERT2 mice) (Figure 4h). TAM injections
9 resulted in a dramatic increase in GS (Figure 4i, 4j) and CYP2E1 (Figure 4k, 4l) expression
10 in Znr3/Rnf43dfl;R26CreERT2 mice when compared to Znr3/Rnf43dfl;R26CreERT2 mice
11 without TAM, with CYP2E1 expression even expanding into portal vein hepatocytes
12 (Figure 4k). This indicates that the LGR4/5-ZNRF3/RNF43 complex is a key regulator of
13 the hepatic Wnt/ β -Catenin signalling gradient and metabolic liver zonation.

14 To further study the effect of RSPO in liver zonation, we administered recombinant
15 RSPO1 (10 mg/kg) or PBS twice weekly to Lgr4/5dLKO and control mice for 1 week, 2
16 weeks and 2 weeks followed by 4 weeks off-treatment (wash-out) (Figure 5a). 2 weeks
17 RSPO1 injection in adult wild type mice and 2 weeks RSPO1 injection in TCF/LEF-Venus
18 mice markedly increased Axin2 mRNA (Figure 5b, 5c) and Wnt/ β -Catenin reporter
19 expression (Figure 5d, 5e), respectively. Importantly, RSPO1 injections expanded Wnt/ β -
20 Catenin signalling into periportal hepatocytes, indicating that all cells across the liver
21 lobule are competent to respond to Wnt pathway activation and that Wnt receptors and
22 ligands must be available throughout the liver. Likewise, RSPO injections increased GS

1 protein (Figure 5f, 5g) and CYP2E1 protein (Figure 5h, 5i) expression in control mice but
2 not in Lgr4/5dLKO mice when compared to PBS injected mice after 1 week. After 2
3 weeks, additional RSPO1 injections further expanded GS and CYP2E1 expression, which
4 were reduced to PBS control levels after additional 4 weeks off-treatment (Figure 5f-i).
5 This further indicates that RSPO1 controls hepatic Wnt/ β -Catenin signalling in an
6 LGR4/5-dependent manner to confer spatiotemporal regulation of the centro-portal
7 Wnt/ β -Catenin activity gradient and metabolic liver zonation. It also indicates that
8 sustained signalling through the RSPO-LGR4/5-ZNRF3/RNF43 module is essential to
9 maintain this zonation. Notably, RSPO1 injections or ZNRF3/RNF43 deletions did not
10 result in immediate ectopic GS expression throughout the liver but caused progressing
11 expansion along the centro-portal axis, similarly to what was observed in *Apc* mutant
12 mice¹³. This suggests the possibility that other mechanisms contribute to regulating
13 zoned GS expression; *e.g.* YAP activity in periportal hepatocytes may restrict GS
14 expression, as it was recently shown that YAP is expressed in a porto-central gradient
15 and can negatively regulate GS^{47, 48}. In summary, our data suggest that Wnt receptors
16 and ligands are distributed throughout the liver lobule and that the RSPO-LGR4/5-
17 ZNRF3/RNF43 module is essential for hepatic Wnt signalling and metabolic liver
18 zonation.

19

20 LGR4 and LGR5 deletions impair postnatal liver development

21 Reduced liver weight and downregulation of gene sets involved in cell cycle regulation
22 indicated impaired growth control in Lgr4/5dLKO mice. Analysis of early postnatal

1 development revealed impaired hepatocyte proliferation in *Lgr4/5dLKO* mice at P2 and
2 P10, whereas no significant difference was observed at P30 with overall reduced
3 proliferation rates (Supplementary Figure 6a-c), as well as in adult mice (data not
4 shown) when compared to control littermates. Notably, impaired proliferation was most
5 evident in pericentral hepatocytes with high Wnt activity. Consequently, liver weight
6 was significantly reduced in P10 and P30 *Lgr4/5dLKO* mice when compared to control
7 mice, while it was comparable at P2 among both genotypes (Supplementary Figure 6d).
8 To study whether LGR4 and LGR5 are important for controlling proliferation during
9 embryonic liver development we stained E16.5 embryos with full-KO for LGR4, LGR5 and
10 LGR4/5 as well as wild type littermates²⁶ for Ki67. We did not observe any differences in
11 the proliferative capacity of the liver between the different genotypes (Supplementary
12 Figure 6e, 6f), indicating that LGR4 and LGR5 are dispensable for embryonic liver growth
13 at E16.5. This suggests that reduced liver weight in *Lgr4/5dLKO* mice is a consequence of
14 impaired hepatocyte proliferation during later stages of liver development.

15

16 RSPO controls liver size and regeneration via LGR4/5 receptors

17 Impaired liver development and liver size in *Lgr4/5dLKO* mice suggested that RSPO-
18 LGR4/5 signalling might act as a hepatic growth/size rheostat. To further investigate this
19 mechanism, we subjected *Lgr4LKO*, *Lgr5LKO*, *Lgr4/5dLKO* and control mice to PH (Figure
20 6a). 2 days post-PH, liver weight was significantly reduced in *Lgr4LKO* and *Lgr4/5dLKO*
21 but not in *Lgr5LKO* mice when compared to controls (Figure 6b). Detailed proliferation
22 analysis by Ki67 immunostaining revealed that *Lgr4LKO*, *Lgr5LKO* and *Lgr4/5dLKO* mice

1 showed impaired proliferation in pericentral hepatocytes (Figure 6c). In contrast,
2 proliferation of parenchymal (Figure 6d) and periportal (Figure 6e) hepatocytes was only
3 impaired in Lgr4LKO and Lgr4/5dLKO but not in Lgr5LKO mice when compared to control
4 mice. Zonal differences in proliferation indicate that LGR receptors are required for
5 proliferation in their respective expression zones. Interestingly, during the peak
6 regenerative response post-PH in control mice, hepatocyte proliferation rates were
7 generally lower around the central vein when compared to portal vein and parenchyma
8 (Figure 6c-e). RT-PCR for Axin2 showed activation of the Wnt pathway during liver
9 regeneration (d2 post-PH) in control mice and confirmed lack of Axin2 expression in
10 Lgr4/5dLKO mice (Figure 6f). Moreover, Axin2 ISH showed a global increase in Wnt/ β -
11 Catenin signalling that extended to periportal hepatocytes at d2 post-PH
12 (Supplementary Figure 7a, 7b), similar as seen in mice with RSPO1 injections.

13 The above findings indicate that LGR4/5 LOF impaired hepatocyte proliferation during
14 liver regeneration as a consequence of abrogated Wnt signalling. It was recently shown
15 that liver progenitor markers, including Lgr5, were upregulated d2 post-PH in periportal
16 hepatocytes³⁴. While RNA-Seq analysis confirmed upregulation of progenitor markers
17 (Krt7, Tnfrsf12a) in control mice d2 post-PH when compared to naïve controls, Lgr5
18 expression was not increased (Supplementary Figure 7c). ISH analysis confirmed that
19 Lgr5 was neither upregulated nor expressed in periportal hepatocytes of control mice at
20 d2 post-PH. Similarly, Lgr4 ISH signals were not increased d2 post-PH (Supplementary
21 Figure 7d-f). RNA-Seq of Lgr4/5dLKO compared to control livers at d2 post-PH further
22 showed no change in liver progenitor marker expression (Sox9, Afp, Tnfrsf12a, Krt7,

1 Krt19; Supplementary Figure 7g), suggesting that these cells did not contribute to
2 impaired regeneration in Lgr4/5dLKO mice. RNA-Seq analysis further revealed that
3 expression of Wnt target genes and genes regulating cell cycle progression and
4 metabolism were reduced in Lgr4/5dLKO compared to control livers at d2 post-PH
5 (Figure 6g). Likewise, gene sets implicated in cell cycle regulation and metabolism were
6 downregulated in livers of regenerating Lgr4/5dLKO mice (Figure 6h). To study whether
7 Lgr4/5LKO mice suffer from delayed liver regeneration, we additionally assessed
8 hepatocyte proliferation during later stages of liver regeneration at d4 and d7 post-PH
9 by Ki67 immunostaining (Figure 6i). Both at d4 and d7 hepatocyte proliferation was
10 significantly increased in Lgr4/5LKO when compared to control mice (Figure 6j), similar
11 to the delayed onset of proliferation seen in β -Catenin KO mice¹⁴. Despite increased
12 proliferation at later phases of liver regeneration, Lgr4/5dLKO mice showed significant
13 impairment in the magnitude and effectiveness of the regenerative response, resulting
14 in a smaller final liver to body weight ratio compared to control mice (Figure 6k). This
15 indicates that LGR4/5 are essential for the Wnt/ β -Catenin-mediated early regenerative
16 response which is required for establishing a normal liver to body weight ratio during
17 regeneration, similar to our observations during liver development and homeostasis.
18 Lineage tracing in Lgr5ki/R26-LacZ mice following PH indicated that pericentral LGR5+
19 hepatocytes do not extensively populate the liver during regrowth (Supplementary
20 Figure 7h, 7i). Together, these findings highlight an important role for LGR4 and LGR5 in
21 hepatocyte proliferation and liver size control during regeneration, with a dominant role
22 for LGR4. Moreover, complete absence of Wnt/ β -Catenin signalling in mice with LGR4/5

1 deletions established that the RSPO-LGR4/5-ZNRF3/RNF43 module is an essential
2 mediator rather than a sole potentiator of Wnt/ β -Catenin activity.

3 To study the role of RSPO in hepatic size control, we administered recombinant RSPO1
4 (10 mg/kg) or PBS twice weekly to *Lgr4/5*dLKO and control mice for 1 week, 2 weeks and
5 2 weeks followed by 4 weeks off-treatment (see scheme in Figure 5a). 1 week RSPO1
6 treatment in control mice significantly increased hepatocyte proliferation in all 3 liver
7 zones when compared to PBS-injected control mice, with the strongest proliferative
8 response in periportal hepatocytes. In *Lgr4/5*dLKO mice, RSPO1 did not increase
9 hepatocyte proliferation (Figure 7a, 7b). Consequently, RSPO1 treatment in control mice
10 resulted in increased liver weight when compared to PBS-injected mice, further
11 increasing with prolonged treatment and decreasing upon RSPO1 withdrawal. In
12 contrast, *Lgr4/5*dLKO mice did not gain liver weight following RSPO1 treatment (Figure
13 7c). Together, this indicates that RSPO1 increases liver size in a reversible and *Lgr4/5*-
14 dependent manner. Likewise ZNRF3/RNF43 deletion in *Znrf3/Rnf43*dfi;*R26CreERT2* mice
15 resulted in a dramatic increase in hepatocyte proliferation 11 days post-TAM injections
16 when compared to *Znrf3/Rnf43*dfi;*R26CreERT2* mice without TAM (Figure 7d, 7e),
17 showing a similar zonal profile as in RSPO1-injected mice. Increased proliferation of
18 hepatocytes throughout the liver following ZNRF3/RNF43 deletions highlights the
19 importance of these E3 ubiquitin ligases in restricting hepatocyte proliferation during
20 liver homeostasis and suggests broad availability of Wnt receptors and ligands
21 throughout the liver. Proliferation induced by RSPO1 injections or ZNRF3/RNF43
22 deletions was most evident in periportal hepatocytes, whereas Wnt/ β -Catenin activity

1 was higher in pericentral and parenchymal hepatocytes in RSPO1-injected mice. It is
2 therefore possible that other pathways might cooperate with Wnt/ β -Catenin to mediate
3 this phenotype.

4 Finally, we assessed the potential of recombinant RSPO1 for improving liver
5 regeneration. We therefore injected control mice with 10 mg/kg RSPO1 on 2
6 consecutive days, subjected the mice to PH and monitored liver regrowth by MRI (Figure
7 7f). While liver weight was not changed during the early phase (d2) of regeneration in
8 either PBS- or RSPO1-injected mice, the latter showed a significant increase in liver
9 weight at d7 post-PH (Figure 7g). Consistently, an MRI time-course showed increased
10 liver size in RSPO1-injected mice during late phase regeneration (d6, d7) beyond levels
11 of PBS-injected mice, whereas it was not changed before PH or at early stages of liver
12 regeneration (Figure 7h, i). This indicates that increased RSPO1 levels overrule liver size
13 control during regeneration. Most importantly, RSPO1 treatment accelerated liver
14 regeneration since RSPO1-treated mice reached the final liver volume of control mice
15 more rapidly. Together, RSPO-LGR4/5-ZNRF3/RNRF43 signalling acts as a hepatic
16 growth/size rheostat during development, homeostasis and regeneration.

17

18 **Discussion**

19 Our data support a novel role for RSPO-LGR4/5-ZNRF3/RNF43 signalling in controlling
20 spatiotemporal regulation of the hepatic Wnt/ β -Catenin activity gradient and metabolic
21 liver zonation. Impaired liver zonation in Lgr4/5dLKO mice is phenocopied in Znrf3ECD
22 mice, whereas RSPO1 administration expanded the hepatic Wnt gradient in a reversible

1 and LGR4/5-dependent manner. A recent study suggested that Wnt9b confers Wnt/ β -
2 Catenin activity restricted to the central vein³¹. We now found that RSPO1 injections or
3 ZNRF3/RNF43 deletions expanded Wnt/ β -Catenin activity to periportal hepatocytes,
4 indicating that Wnt receptors and ligands are available throughout the liver. Moreover,
5 Wnt9b deletion only reduced Wnt/ β -Catenin activity³¹ while LGR4/5 deletion resulted
6 in complete loss of hepatic Wnt/ β -Catenin signalling. This indicates that the RSPO-
7 LGR4/5-ZNRF3/RNF43 module is not just potentiating Wnt activity but is essential for
8 functional Wnt/ β -Catenin signalling in the liver. While our data suggest that RSPO
9 proteins confer local restriction of hepatic Wnt activity, their source and distribution
10 remain to be identified.

11 The strongest increase in RSPO-induced proliferation around the portal vein was not
12 paralleled by highest Wnt/ β -Catenin activity in the region, indicating that other
13 pathways might contribute to this phenotype. It was recently shown that YAP
14 expression shows an inverse gradient to Wnt/ β -Catenin activity in the liver⁴⁷. Moreover,
15 Wnt/ β -Catenin activation promotes YAP signalling in other tissues⁴⁹. It is possible that
16 Wnt/ β -Catenin-mediated YAP activation may contribute to periportal hepatocyte
17 proliferation.

18 In a variety of tissue stem cell compartments, LGR4+ and LGR5+ cells are actively cycling
19 and give rise to more mature cells with a high turnover³⁹⁻⁴¹. Unlike other LGR5+
20 epithelial cells, pericentral LGR5+ hepatocytes did neither show overt proliferation nor
21 gave rise to hepatocytes distant to the central vein, either under homeostasis conditions
22 within an observation period of 18 months or during a 7-day post-PH liver regeneration

1 period. Moreover, our EdU and Ki67 analyses in wild type mice could not detect
2 increased proliferation of pericentral hepatocytes when compared to those of other
3 liver zones. Our findings did not support the results of a recent study that was published
4 during revision of our manuscript³¹. The authors proposed that pericentral hepatocytes
5 comprise liver stem cells that repopulate large parts of the liver during homeostasis,
6 whereas their potential during regeneration was not assessed³¹. Another recent study
7 suggested that periportal hepatocytes with low SOX9 expression levels are liver stem
8 cells with high regenerative capacity during homeostasis and drug-induced liver damage
9 caused by zonal injury at the central vein²⁹. It has been speculated that the site of injury
10 determines the site of regeneration and that hepatocytes in different hepatic zones may
11 mutually support liver regeneration in opposing sides²⁸. Studying homeostatic
12 hepatocyte renewal or regenerative response to PH, a paradigm model for liver
13 regeneration that is not biased by zonal injury, we now show that LGR4+ hepatocytes
14 throughout the liver contribute to liver homeostasis and regeneration. While the
15 majority (>80%) of proliferating hepatocytes during homeostasis was found in the
16 parenchyma, proliferation rates between the 3 zones were similar. During the peak
17 regenerative response post-PH, pericentral hepatocytes proliferated less than
18 parenchymal and periportal hepatocytes. Our findings are consistent with previous
19 reports showing no zonal domination during liver homeostasis and reduced proliferation
20 of pericentral hepatocytes following PH^{42, 50}. This supports that parenchymal
21 hepatocytes can have similar regenerative capacity as pericentral or periportal
22 hepatocytes. Our data add to the emerging concept that there might not be just one

1 liver stem cell compartment but that cells in different liver zones show increased
2 regenerative potential depending on the injury^{28, 32}.

3 Despite recent advances in identifying cellular compartments that contribute to liver
4 regeneration and maintenance^{28-30, 32, 34, 44}, the detailed mechanisms that confer
5 spatiotemporal control of proliferation to maintain proper liver size remained elusive.
6 We now show that increased RSPO-LGR4/5-ZNRF3/RNF43 signalling overrules the stop
7 signals during liver regrowth or homeostasis, whereas LGR4/5 deletions result in
8 hypoplastic livers. Likewise, LGR4/5 deletions impair liver regeneration, whereas RSPO1
9 injections accelerate regeneration. This supports the notion that the RSPO-LGR4/5-
10 ZNRF3/RNF43 module acts as rheostat controlling liver growth and size. Our data
11 further highlight important roles of ZNRF3 and RNF43 in restricting proliferation in the
12 liver, consistent with *Rnf43* mutations observed in liver tumours⁵¹.

13 Recently, periportal LGR5+ progenitor cells with bipotential progenitor characteristics
14 were identified following CCl4 liver damage or diets that resulted in oval cell response in
15 mice³³. In contrast to our study, no LGR5+ cells were found in livers of naïve mice. It is
16 possible that different genetic backgrounds between the mice used in our study (pure
17 C57Bl/6) and those used in the above study (mixed C57Bl/6-Balb/c F1), different TAM
18 dosing regimens or partial hepatic transgene silencing, as previously reported^{33, 52},
19 could explain these differences.

20 In contrast to LGR5, which was restricted to pericentral hepatocytes with high Wnt/ β -
21 Catenin pathway activity, LGR4 was broadly expressed in hepatocytes throughout the
22 liver. Moreover, LGR5 has been described as a Wnt/ β -Catenin target³⁹⁻⁴¹. LGR5 deletion

1 impaired pericentral hepatocyte proliferation post-PH, and combined deletion of LGR4
2 and LGR5 further impaired metabolic liver zonation than LGR4 deletion alone. This
3 indicates that LGR5 is functionally relevant rather than just a marker of cells with high
4 Wnt/ β -Catenin activity. In contrast to LGR4 deletion, LGR5 deletion alone did not result
5 in impaired Wnt/ β -Catenin signalling. Loss of LGR5 expression as a consequence of LGR4
6 deletion further suggests a dominant role of LGR4 over LGR5, as previously shown in
7 other tissues²⁶.

8 Our findings highlight novel roles for RSPO-LGR4/5-ZNRF3/RNF43 module in regulating
9 Wnt/ β -Catenin-mediated metabolic liver zonation and in acting as a growth/size control
10 rheostat during liver development, homeostasis and regeneration. We further show
11 that LGR4+ hepatocytes throughout the 3 liver zones contribute to homeostasis and
12 regrowth. The growth-promoting effect of ectopic RSPO1 in livers highlights its potential
13 use for regenerative therapies.

14

15

16 **Materials and Methods**

17 Immunostainings, ISH, RT-PCR and partial hepatectomy were essentially performed as
18 described⁵³⁻⁵⁵. Detailed information on MRI, RNA-Seq, recombinant RSPO1 protein
19 generation, mouse model design, image analysis and statistical analysis is available in
20 the supplementary online material and methods. The raw RNA-sequencing reads
21 are available in the NCBI Short Read Archive under the accession number SRP055521.

22

1 **Acknowledgements**

2 We thank Johann Wirsching, Carole Manneville, Marianne Lemaistre, Michel Haffner,
3 Bettina Leonhard, Svenja Ley, Ning Guo, Tanya Lewis, Mei Li, Martin van de Velde,
4 Daniel Breustedt, Hong Lei, Ryan Boomer, Stefan Zurbruegg, Ludovic Perrot, Francisco
5 Cordoba, Sven Schuierer, Ana Arif and Julien Gfeller for technical assistance. For helpful
6 discussion and critical reading of the manuscript we thank Elizabeth Wiellette, Gabi
7 Hintzen, Bill Dietrich, Dong Liu, Frederic Bassilana, Christian Parker, Klaus Seuwen and
8 Jeff Porter. This work was supported by the Leverhulme Trust ECF-2012-262 to Luke
9 Boulter, SNF grant 310030B_147089 to Markus Heim, MRC Niche hub grant
10 MR/K026666/1 to Stuart Forbes and the Novartis Institutes for BioMedical Research
11 Program Office.

12

13 *Conflict of interest statement.* All authors except SF, LB, LMT, MHH, DC and MTD are
14 employed by and/or shareholders of Novartis Pharma AG.

15

16 **Figure legends**

17 **Figure 1. Lgr4 and Lgr5 are co-expressed in pericentral hepatocytes.** **a**, GS and CYP2E1
18 co-staining in control mice. **b**, Venus expression in Tcf/Lef-Venus mice. **c**, Percent of
19 hepatocytes expressing GS, CYP2E1 and Tcf/Lef-Venus in 3 cell layers around central and
20 portal veins and in the parenchyma. n = 5 mice per group. **d, f, h**, Axin2 (**d**), Lgr4 and
21 Lgr5 (**f**) and Znr3 (**h**) ISH in control mice. **e, g, i**, Axin2 (**e**), Lgr4 and Lgr5 (**g**), and Znr3 (**i**)
22 ISH quantified in the indicated liver zones. n = (e) 5 mice where 4225 cells from 75

1 images were quantified, (g) 4 mice per group where 3380 cells from 60 images were
2 quantified, and (i) 5 mice where 4341 cells from 75 images were quantified. **j**, LacZ
3 staining in Rnf43-LacZ mice. **k**, Percent of hepatocytes expressing Rnf43-LacZ in the
4 indicated liver zones. n = 4 mice. cv, central vein; pv, portal vein. Scale bars, (a, b) 100
5 μm , (magnification in a) 20 μm , (magnification in b) 50 μm , (d, f, h) 20 μm ,
6 (magnifications in d, f, h) 10 μm , (j) 100 μm and (magnifications in j) 50 μm .

7

8 **Figure 2. Lgr4 and Lgr5 lineage tracing during liver homeostasis.** **a, b**, LacZ staining in
9 Lgr4ki/R26-LacZ mice showing LGR4+ hepatocytes after 10 days (**a**) or 10 months (**b**) of
10 tracing. Arrowheads indicate clusters of LGR4+ hepatocytes. **c**, Clone size distribution of
11 Lgr4ki/R26-LacZ+ hepatocytes. n = 3 mice. **d, e**, LacZ staining in Lgr5ki/R26-LacZ mice
12 showing LGR5+ hepatocytes after 10 days (**d**) or 18 months (**e**) of tracing. Arrowheads
13 (in b, e) indicate doublet cells. **f**, Clone size distribution of Lgr5ki/R26-LacZ+ hepatocytes.
14 n = 4 mice. **g**, EGFP and HNF4 α co-staining in Lgr4ki/R26-EGFP mice, showing
15 EGFP+/HNF4 α + hepatocytes (arrowheads). **h**, tdTOM and GS co-staining in Lgr5ki/R26-
16 tdTOM mice, showing tdTOM+/GS+ hepatocyte (arrowhead). **i**, Scheme depicting EdU
17 injections in WT and Lgr5ki mice. **j**, EdU+ pericentral hepatocytes quantified in WT and
18 Lgr5ki mice. n = 6 mice where 2195 (WT) and 1591 (Lgr5ki) cells were quantified. **k**, GS
19 and EdU co-staining in control mice. Arrowheads point at EdU+ hepatocytes. **l**, EdU+
20 hepatocytes quantified in liver zones of control mice (percentage of EdU+ hepatocytes
21 among total number of hepatocytes in respective zones). n = 6 mice where 2195 (CV),
22 3051 (PA) and 2555 (PV) cells from 90 images per zone were quantified. **m**, Distribution

1 of EdU+ hepatocytes in the indicated liver zones. n = 6 mice where 1296 cells were
2 quantified. **n**, GS, biliary marker CK19 and Ki67 co-staining in control mice. Arrowheads
3 point at Ki67+ hepatocytes. **o**, Ki67+ hepatocytes quantified in liver zones of control
4 mice (percentage of Ki67+ hepatocytes among total number of hepatocytes in
5 respective zones). **p**, Distribution of Ki67+ hepatocytes in the indicated liver zones. n = 4
6 mice where 891 cells from 60 images were quantified. cv, central vein; pv, portal vein.
7 ns, not significant. Scale bars, (a, b, d, e) 50 μ m and (g, h, k, n) 20 μ m.

8

9 **Figure 3. Lgr4 and Lgr5 receptors control liver zonation.** **a**, Relative liver weight of the
10 indicated mice. **b**, mRNA expression of different genes in the indicated mice. n = 5 mice
11 per group. **c**, Lgr4 and Lgr5 ISH in the indicated mice. **d**, GS staining in the indicated
12 mice. **e**, GS+ hepatocytes quantified in the indicated mice. **f**, CYP2E1 staining in the
13 indicated mice. **g**, CYP2E1+ staining quantified in the indicated mice. **h**, Heatmap
14 showing RNA expression of metabolic genes in livers of the indicated mice (yellow, high
15 expression; blue, low expression). **i**, **j**, mRNA expression of pericentral (**i**) or periportal (**j**)
16 metabolic genes in livers of the indicated mice. cv, central vein. n = 5 mice per group. *,
17 P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; *****, P<0.00001. ns, not significant.
18 Scale bars, (c) 20 μ m, (d, f) 200 μ m and (magnifications in d, f) 20 μ m.

19

20 **Figure 4. ZNRF3 and RNF43 control liver zonation.** **a**, Scheme depicting Znrf3ECD/Axin2-
21 LacZ mice that were given DOX or vehicle control on d1 and analysed on d7. **b**, **d**, **f**, LacZ
22 (**b**), GS (**d**) or CYP2E1 (**f**) staining in Znrf3ECD/Axin2-LacZ mice +/- DOX. **c**, **e**, **g**, LacZ+ (**c**),

1 GS+ (e) or CYP2E1+ (g) staining quantified in Znr3ECD/Axin2-LacZ mice +/- DOX. h,
2 Scheme depicting Znr3/Rnf43df1;R26CreERT2 mice that were given TAM or vehicle
3 control. i, k, GS (i) or CYP2E1 (k) staining in Znr3/Rnf43df1;R26CreERT2 mice +/- TAM. j,
4 I, GS+ (j) or CYP2E1+ (l) staining quantified in Znr3/Rnf43df1;R26CreERT2 mice +/- TAM.
5 cv, central vein; pv, portal vein. **, P<0.01; ***, P<0.001. Scale bars, (b) 100 μm,
6 (magnifications in b) 50 μm, (d, f, i, k) 200 μm and (magnifications in d, f, i, k) 50 μm.

7

8 **Figure 5. RSPO1 controls liver zonation via Lgr4 and Lgr5 receptors.** a, Scheme
9 depicting control, Lgr4/5dLKO and Tcf/Lef-Venus mice that were injected i.v. with RSPO1
10 or PBS for the indicated times. b, Axin2 ISH in control mice +/- RSPO1. c, Axin2 ISH
11 quantified in control mice +/- RSPO1 in the indicated liver zones. n = 5 mice per group
12 where 3392 cells (PBS) and 3774 cells (RSPO) from 75 images each were quantified. d,
13 Venus expression in Tcf/Lef-Venus mice +/- RSPO1. e, Percent of Venus-expressing
14 hepatocytes in Tcf/Lef-Venus mice +/- RSPO1 in the indicated liver zones. n = 4 mice
15 (PBS) and 5 mice (RSPO). f, h, GS (f) and CYP2E1 (h) staining of control and Lgr4/5dLKO
16 mice +/- RSPO1 for the indicated times. g, i, GS+ (g) or CYP2E1+ (i) staining quantified in
17 control and Lgr4/5dLKO mice +/- RSPO1 for the indicated times. *, P<0.05; **, P<0.01;
18 ***, P<0.001; ****, P<0.0001; ns, not significant. Scale bars, (b) 20 μm, (magnifications
19 in b) 10 μm, (d) 100 μm, (magnifications in d) 50 μm and (f, h) 200 μm.

20

21 **Figure 6. Loss of Lgr4 and Lgr5 results in impaired liver homeostasis and regeneration.**
22 a, Scheme depicting mice that were subjected to partial hepatectomy (PH) and analysed

1 at different time-points post-PH. **b**, Relative liver weight of the indicated mice at d2
2 post-PH. **c**, **d**, **e**, Ki67 staining and pericentral (**c**), parenchymal (**d**) and periportal (**e**)
3 hepatocyte proliferation of the indicated mice at d2 post-PH. **f**, Axin2 mRNA expression
4 in livers of the indicated mice. n = 5 mice (control + PH and Lgr4/5dLKO + PH), 6 mice
5 (control - PH) and 7 mice (Lgr4/5dLKO - PH). **g**, Volcano plot showing genes differentially
6 expressed in livers of the indicated mice. **h**, Gene sets downregulated in livers of the
7 indicated mice. **i**, Ki67 staining in livers of the indicated mice at d4 and d7 post-PH. **j**,
8 Liver cell proliferation quantified in the indicated mice at d4 and d7 post-PH. **k**, Relative
9 liver weight of the indicated mice at d2, d4 and d7 post-PH. *, P<0.05; **, P<0.01; ***,
10 P<0.001; ns, not significant. Scale bars, 100 μ m.

11

12 **Figure 7. RSPO1 promotes hepatocyte proliferation and liver regeneration via Lgr4 and**
13 **Lgr5 receptors.** **a**, Ki67 staining in livers of the indicated mice, showing Ki67+
14 hepatocytes (arrowheads). **b**, Hepatocyte proliferation quantified in liver zones of the
15 indicated mice upon RSPO1 or PBS injection. **c**, Relative liver weight of the indicated
16 mice. **d**, Ki67 staining in livers of the indicated mice, showing Ki67+ hepatocytes
17 (arrowheads). **e**, Hepatocyte proliferation quantified in liver zones of the indicated mice.
18 **f**, Scheme depicting control mice that were injected i.v. with RSPO1 or PBS on two
19 consecutive days and subjected to PH. **g**, Relative liver weight of the indicated mice. **h**,
20 Representative MRI liver sections of the indicated mice. **i**, Liver volume growth curve of
21 the indicated mice following RSPO1 or PBS injection and PH. n = 6 male mice per group.

1 *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, not significant. Scale bars, (a, d)
2 50 µm and (h) 6 mm.

3

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Figure 1
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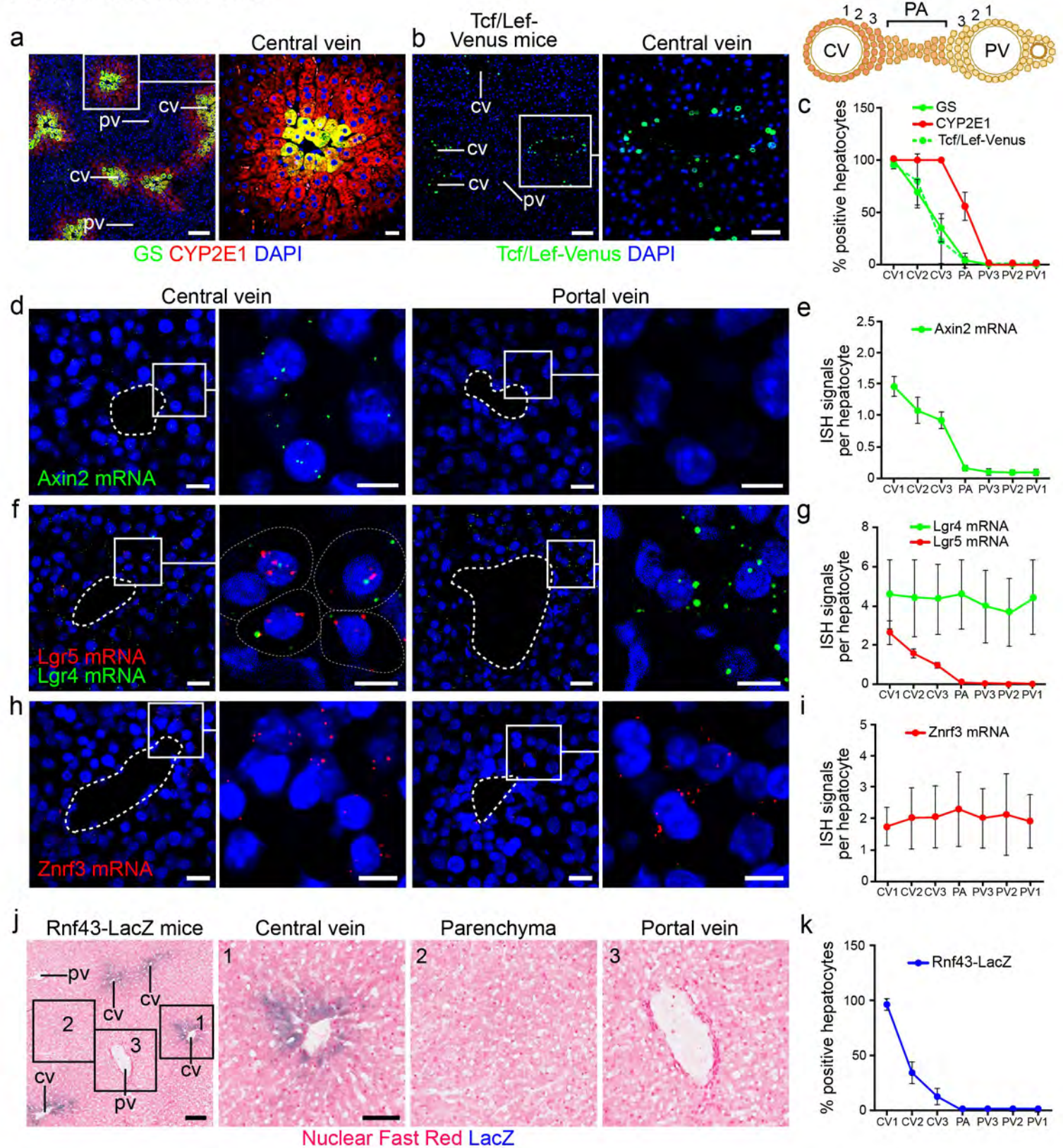


Figure 2

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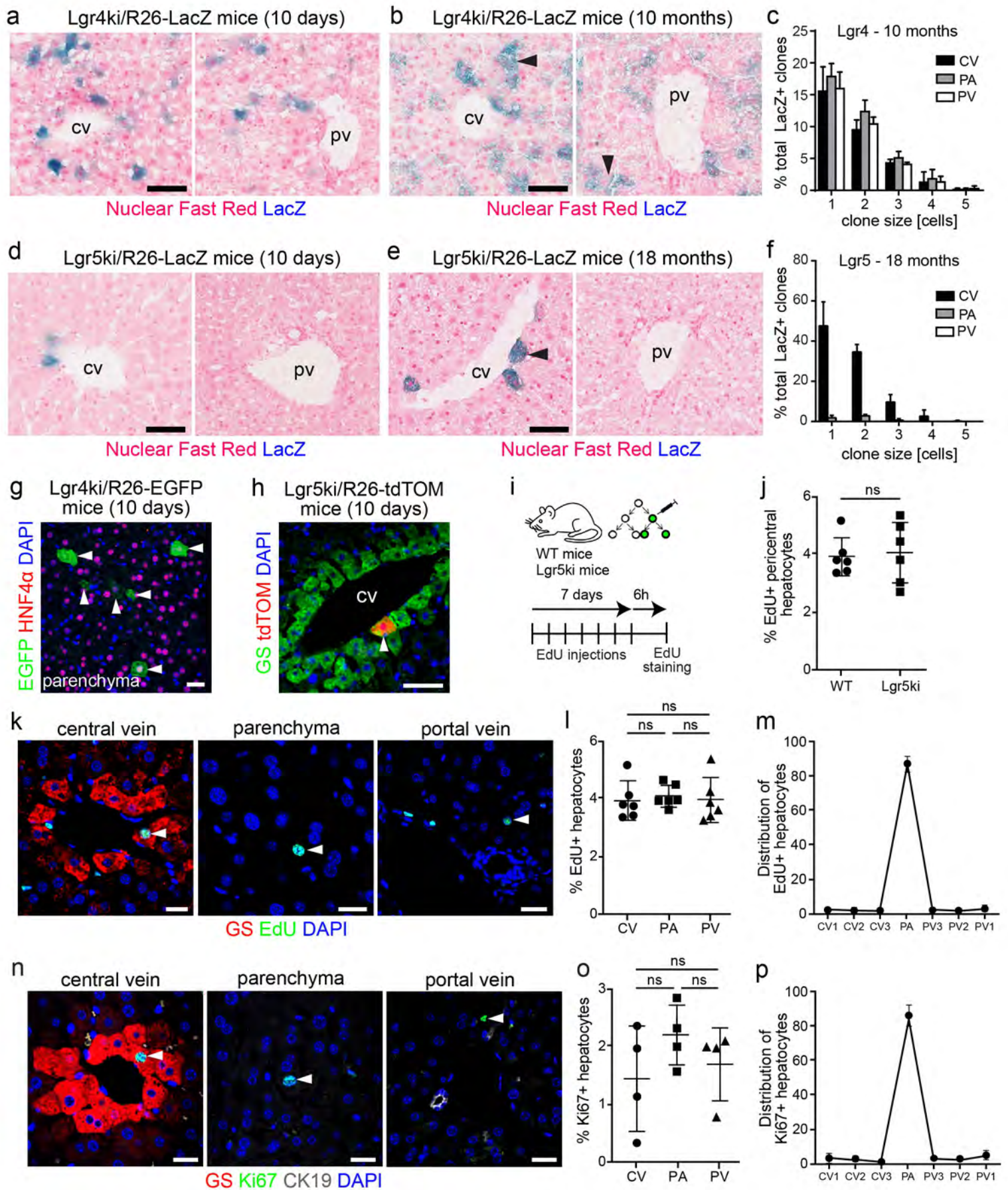


Figure 3
Planas-Paz & Orsini et al.

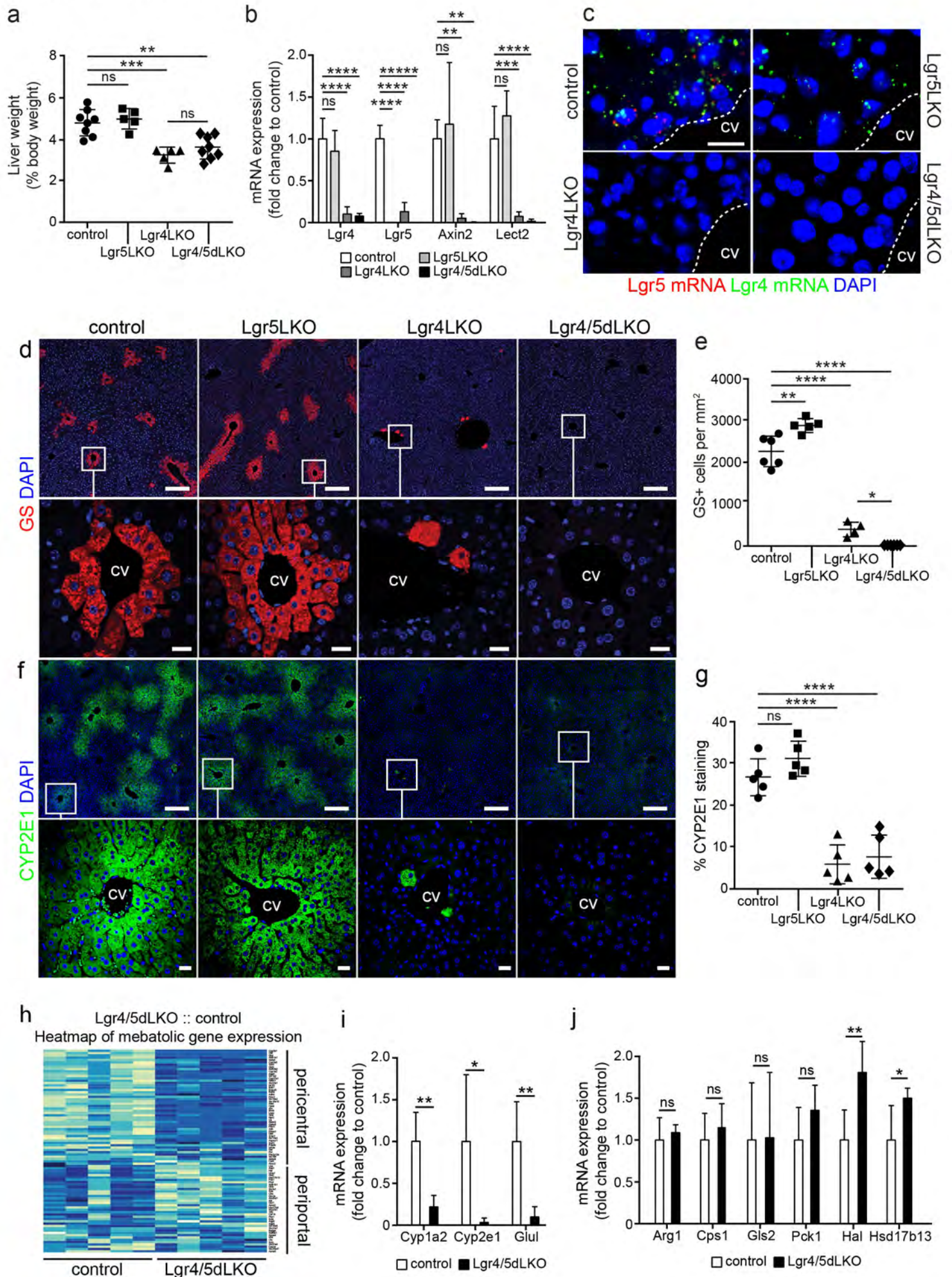


Figure 4
Planas-Paz & Orsini et al.

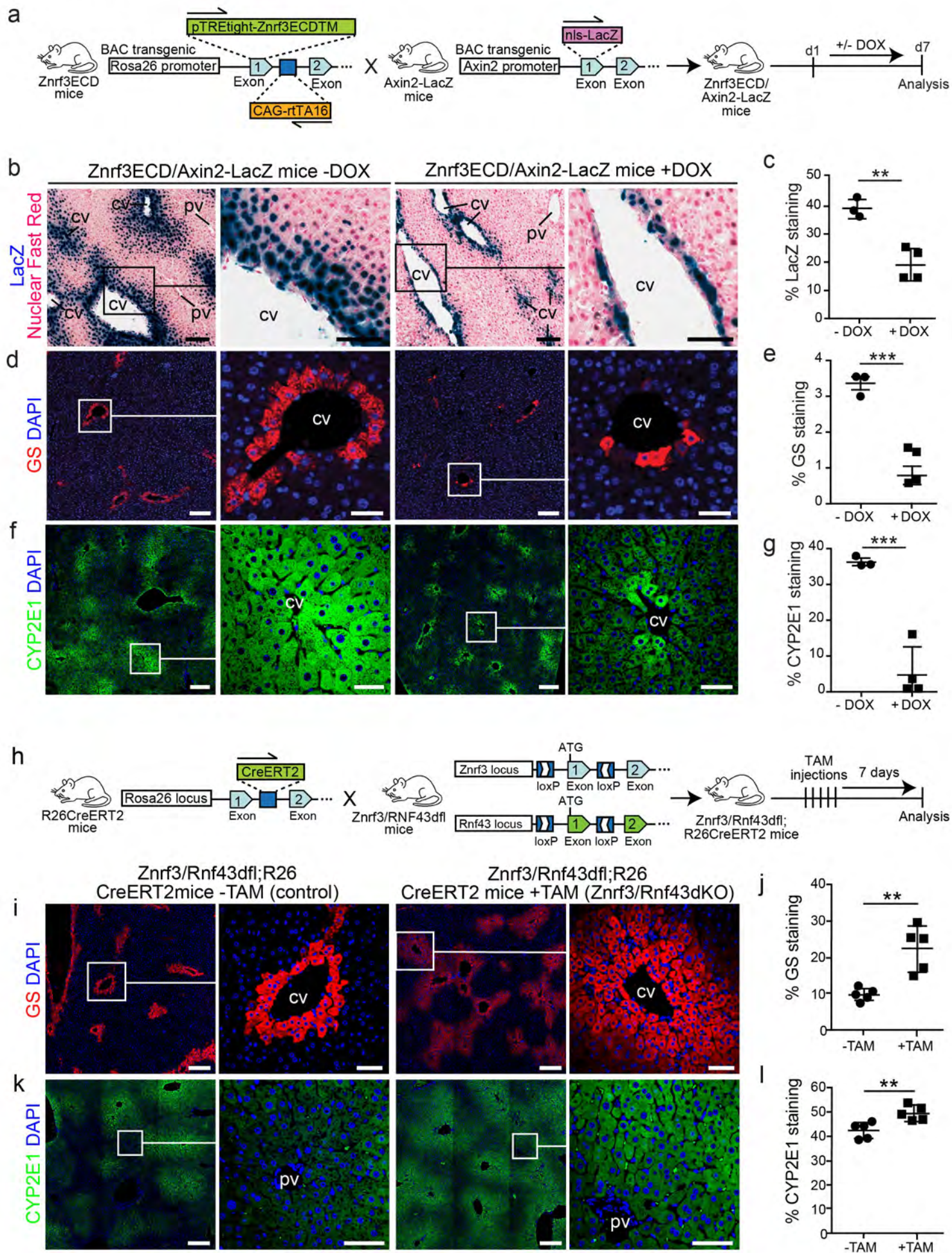


Figure 5
Planas-Paz & Orsini et al.

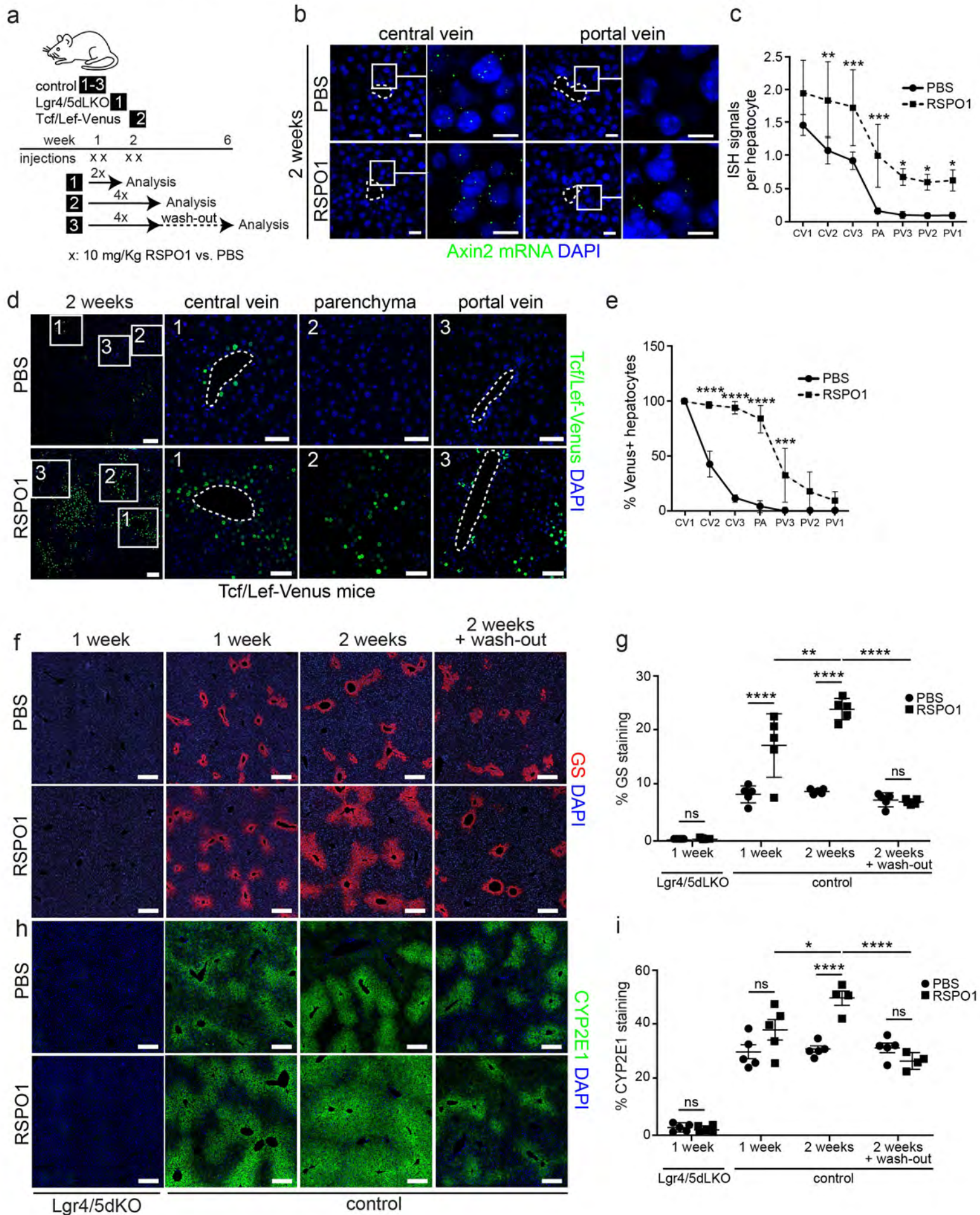


Figure 6
Planas-Paz & Orsini et al.

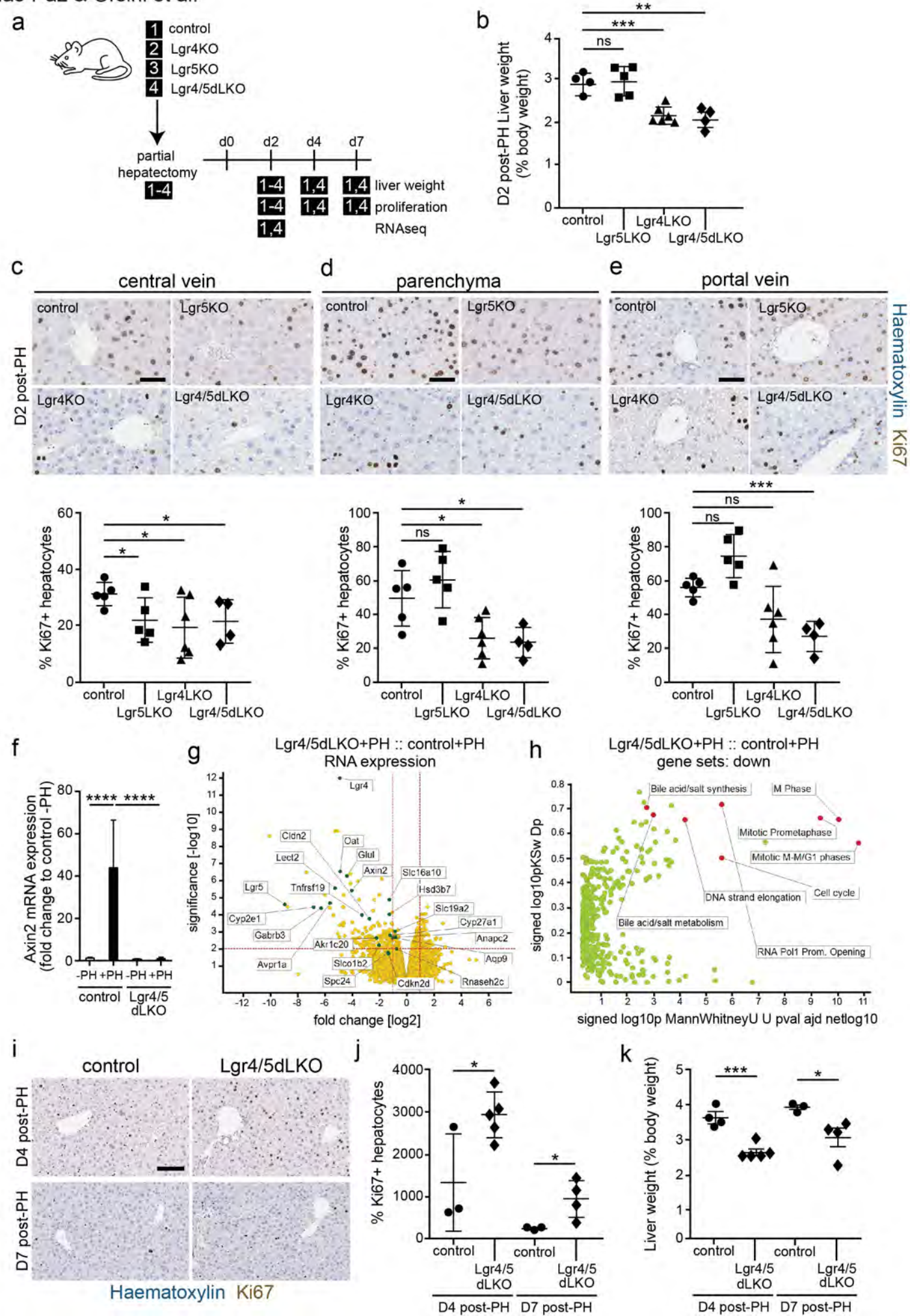
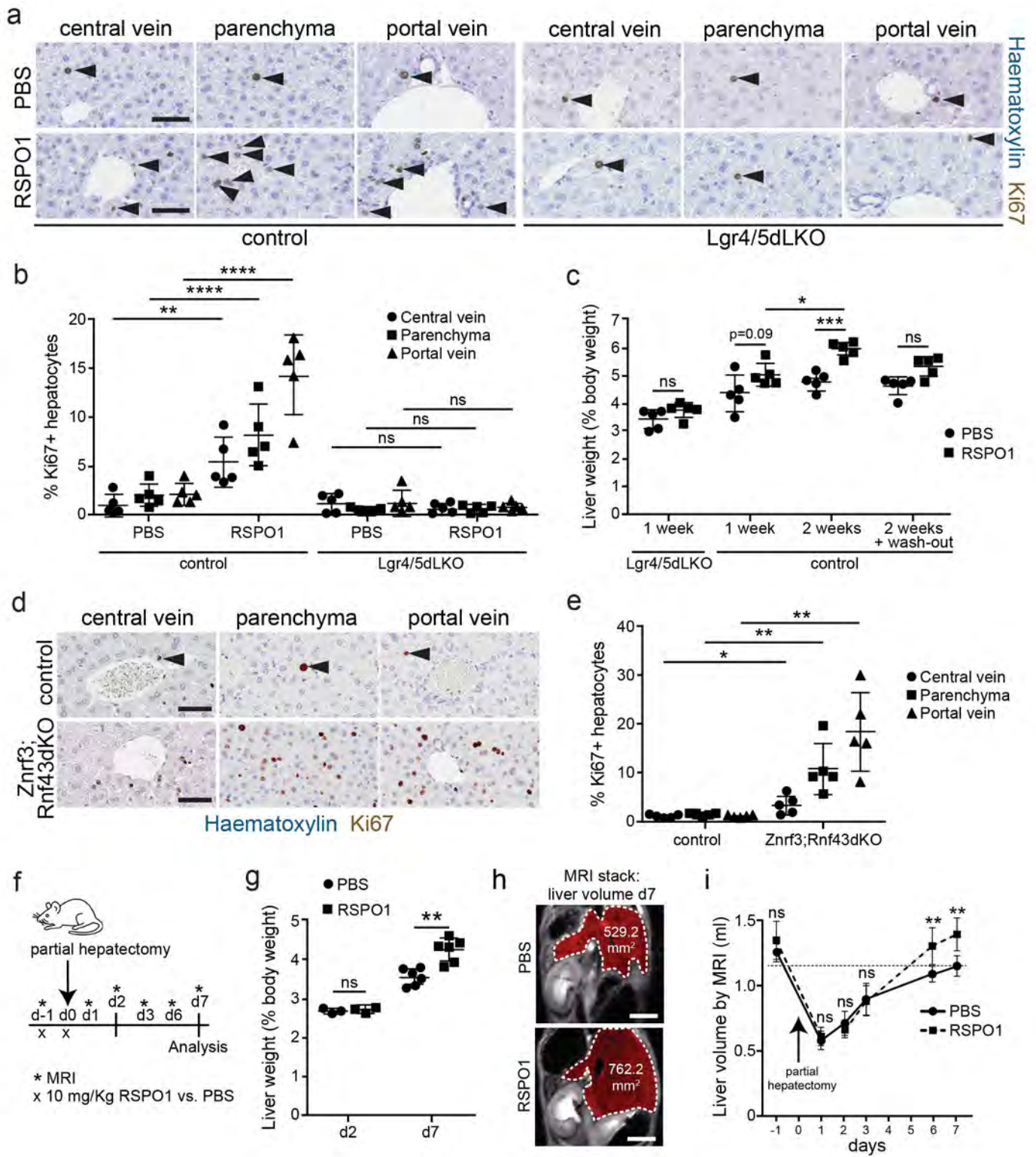
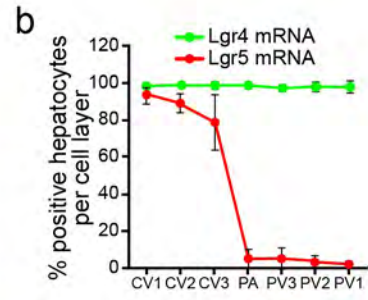
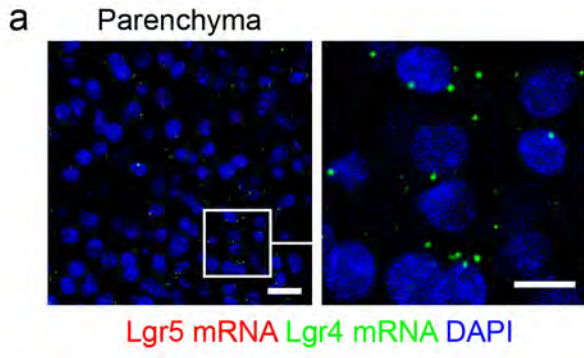


Figure 7
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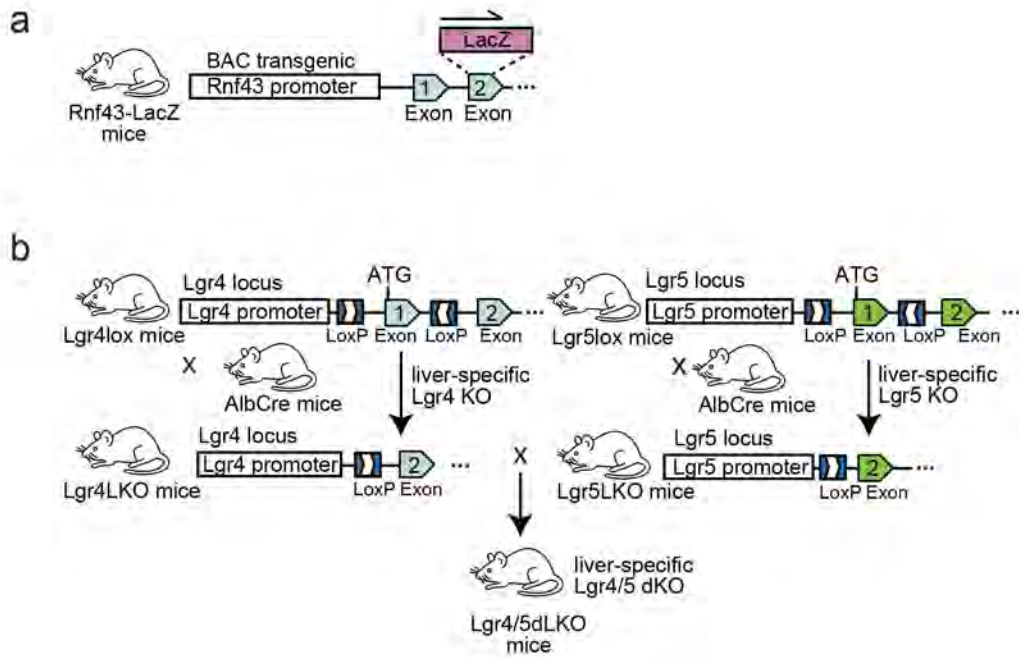
Supplementary Figure 1

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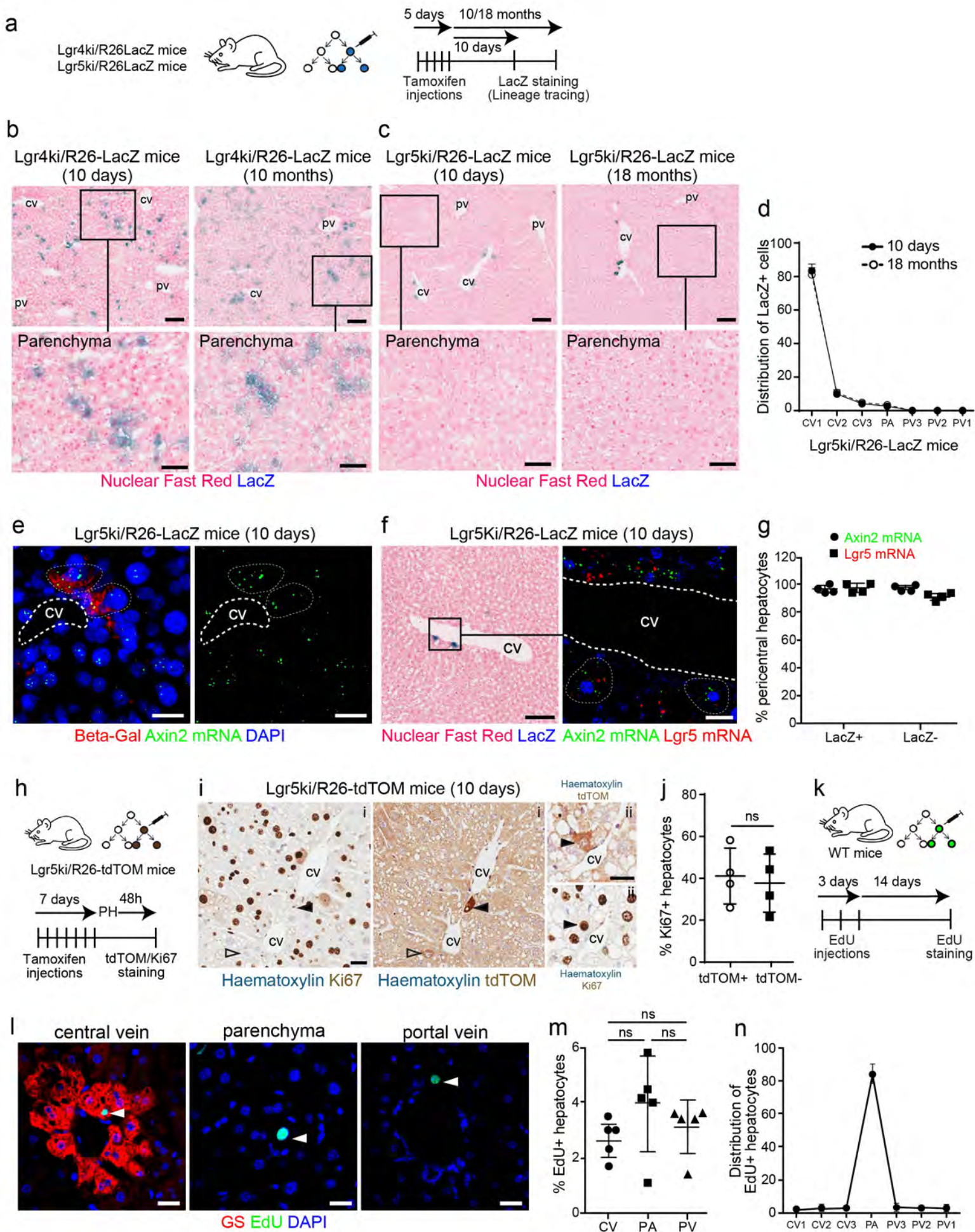
Supplementary Figure 2

Planas-Paz & Orsini et al.



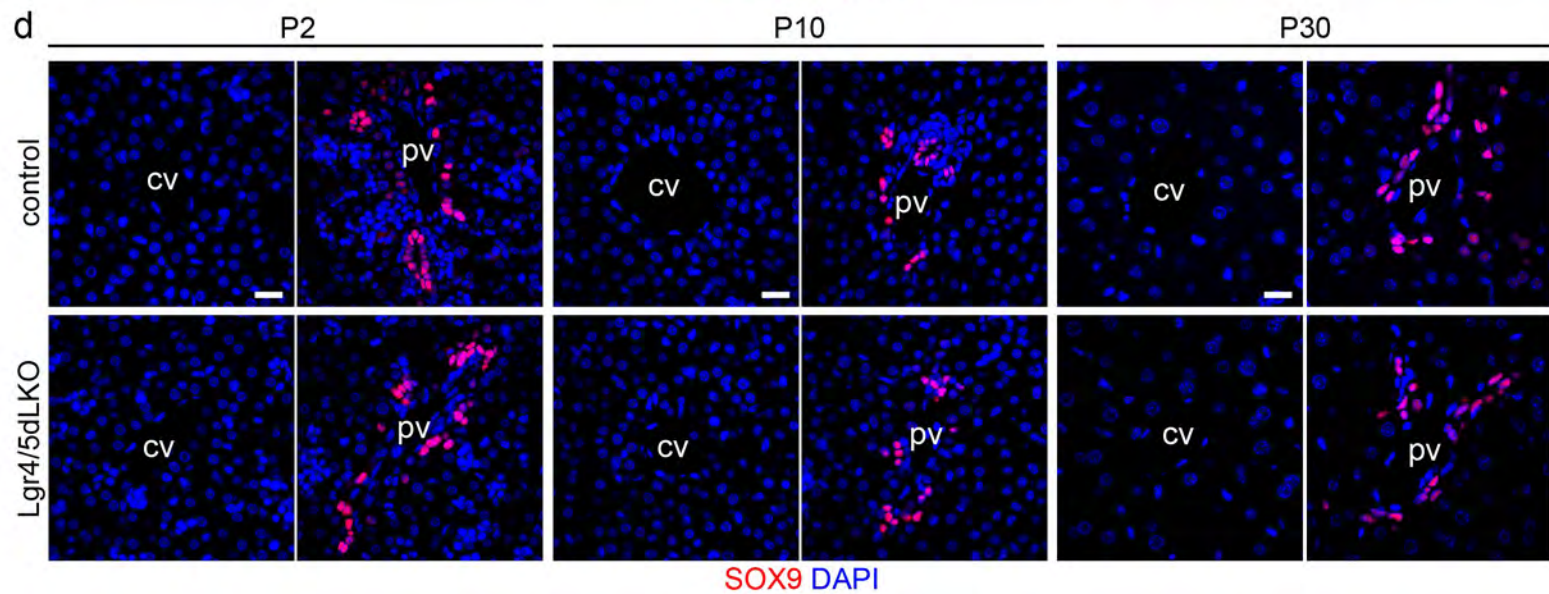
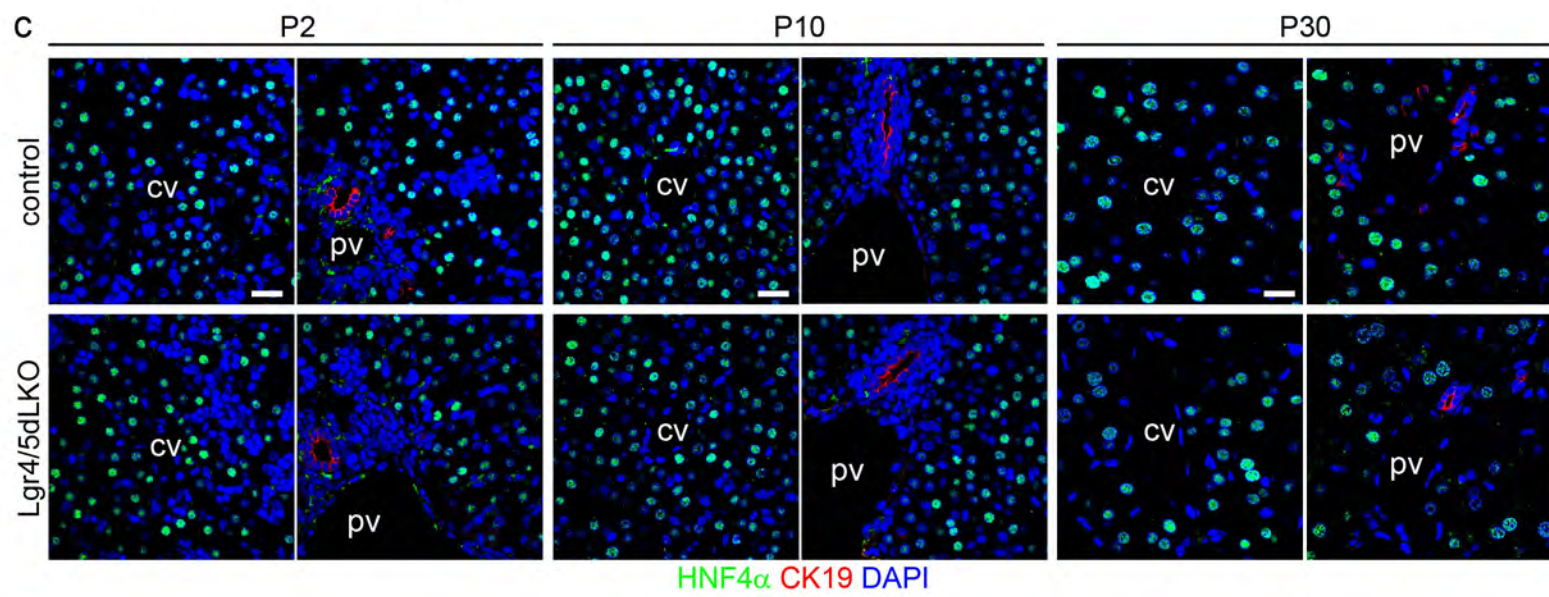
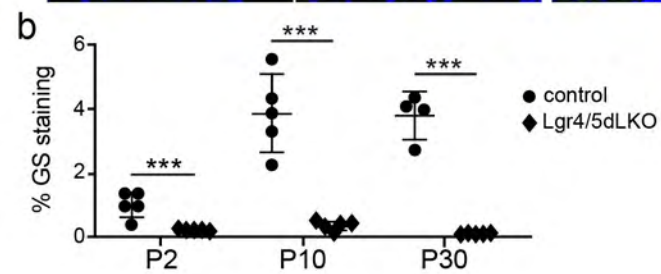
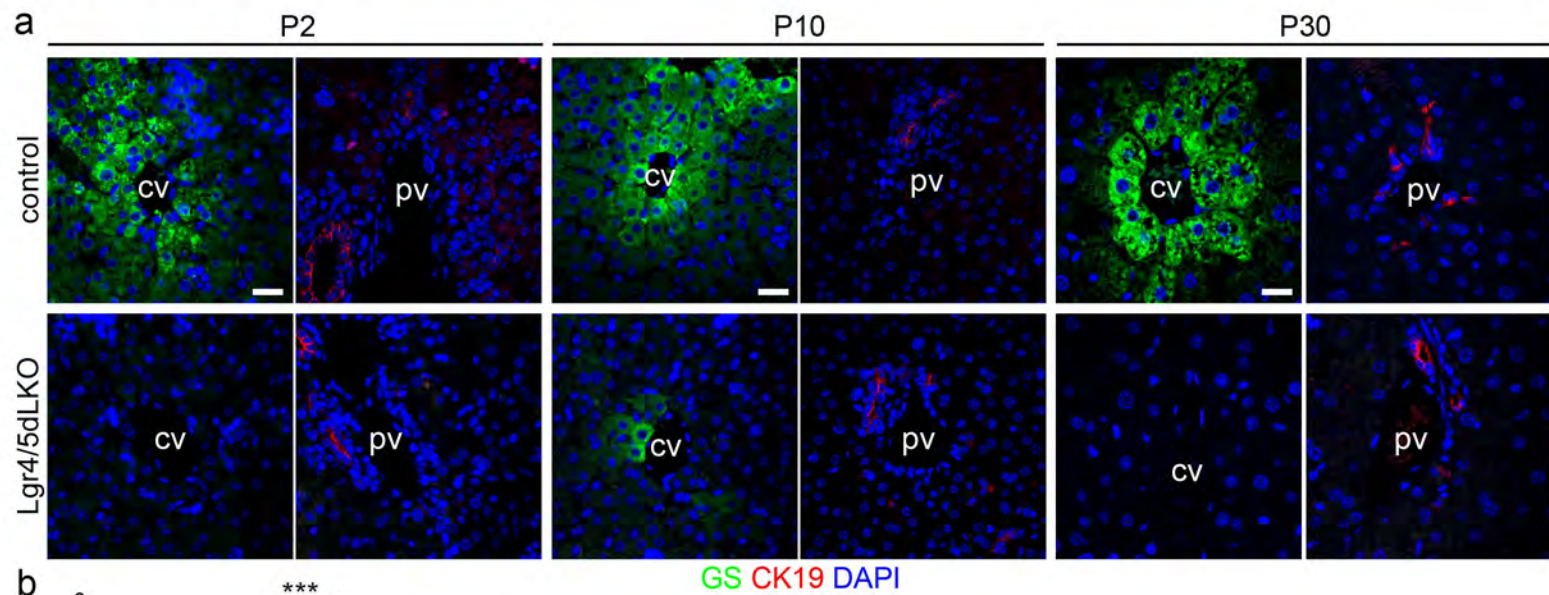
Supplementary Figure 3

Planas-Paz & Orsini et al.



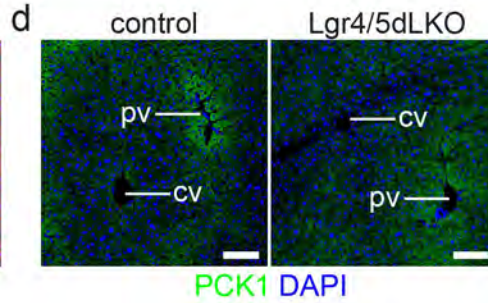
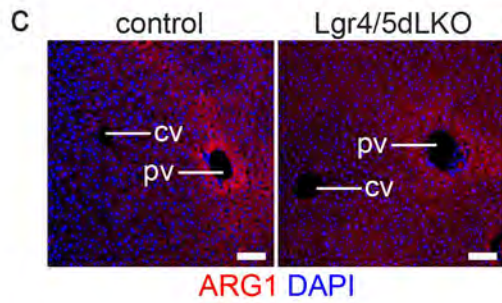
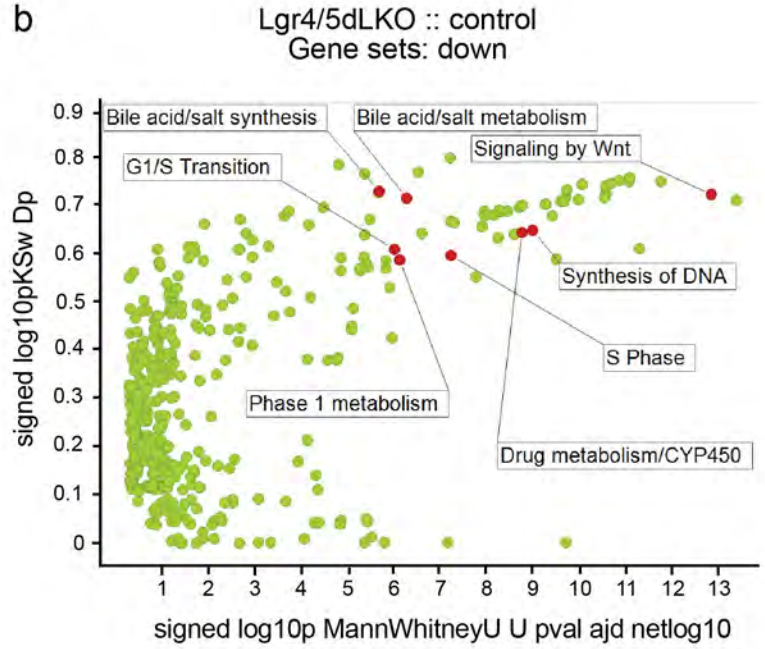
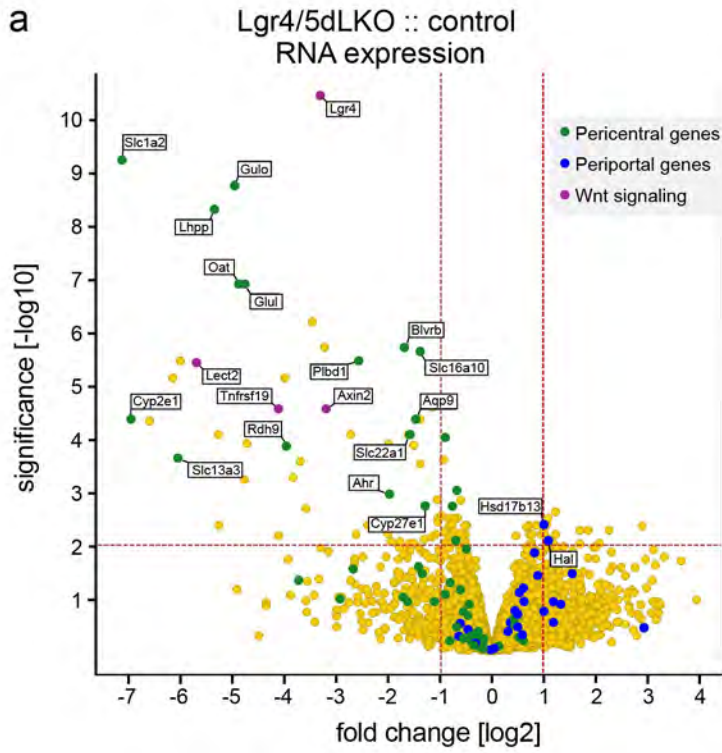
Supplementary Figure 4

Planas-Paz & Orsini et al.



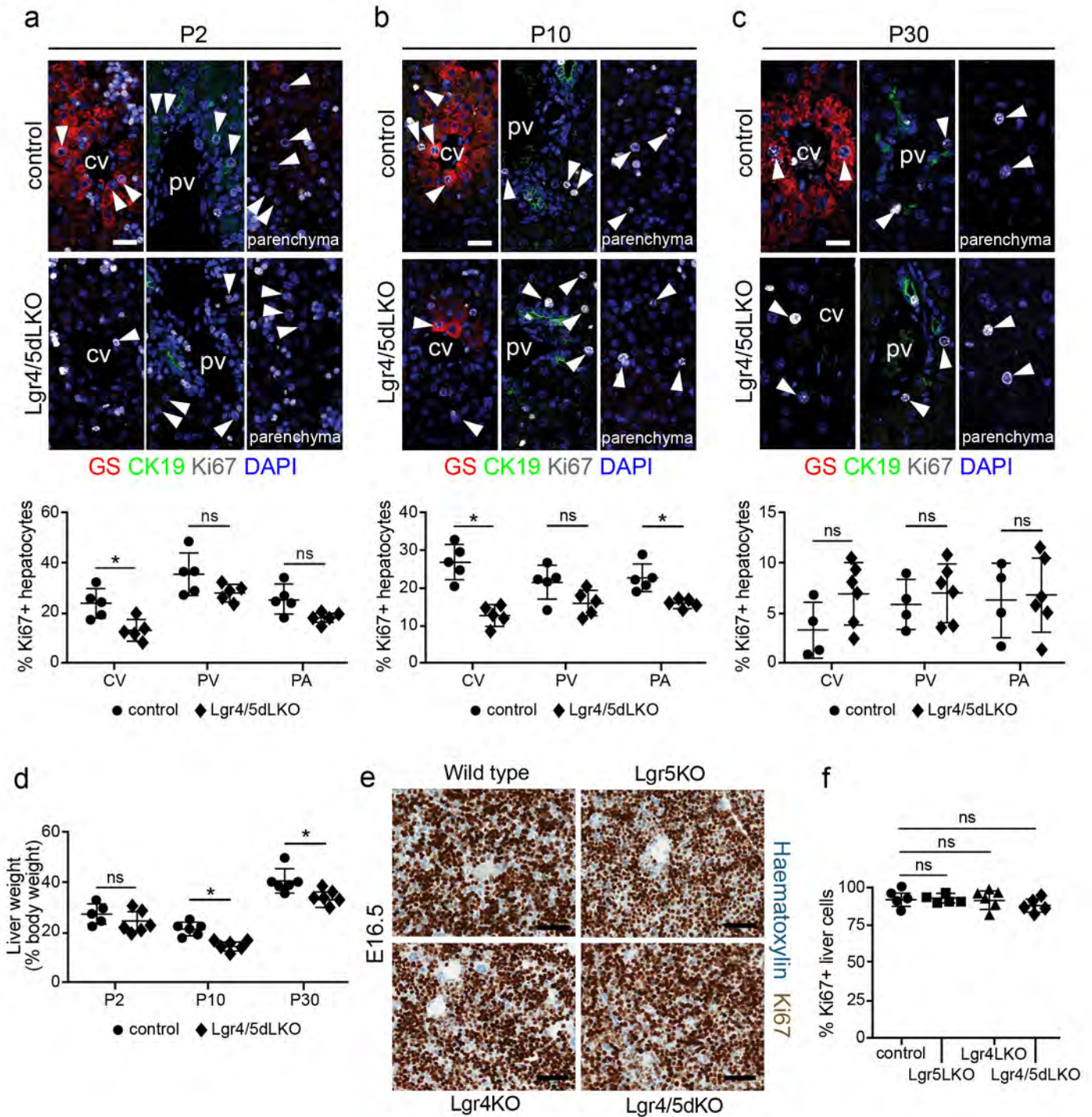
Supplementary Figure 5

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Supplementary Figure 6

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Supplementary Figure 7

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