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A time- and space-resolved nuclear receptor atlas in mouse liver

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ABSTRACT

31 The functional versatility of the liver is paramount for organismal homeostasis. Adult liver
32 functions are controlled by a tightly regulated transcription factor network including nuclear receptors
33 (NRs), which orchestrate many aspects of hepatic physiology. NRs are transcription factors sensitive
34 to extracellular cues such as hormones, lipids, xenobiotics etc. and are modulated by intracellular
35 signaling pathways. While liver functional zonation and adaptability to fluctuating conditions rely on
36 a sophisticated cellular architecture, a comprehensive knowledge of NR functions within liver cell
37 populations is still lacking. As a step toward the accurate mapping of NR functions in liver, we
38 characterized their levels of expression in whole liver from C57Bl6/J male mice as a function of time
39 and diet. *Nr1d1 (Rev-erba)*, *Nr1d2 (Rev-erbb)*, *Nr1c2 (Pparb/d)* and *Nr1f3 (Rorg)* exhibited a robust
40 cyclical expression in ad libitum-fed mice which was, like most cyclically expressed NRs, reinforced
41 upon time-restricted feeding. In a few instances, cyclical expression was lost or gained as a function
42 of the feeding regimen. NR isoform expression was explored in purified hepatocytes, cholangiocytes,
43 Kupffer cells, hepatic stellate cells and liver sinusoidal cells. The expression of some NR isoforms, such
44 as *Nr1h4 (Fxa)* and *Nr1b1 (Rara)* isoforms, was markedly restricted to a few cell types. Leveraging
45 liver single cell RNAseq studies yielded a zonation pattern of NRs in hepatocytes, liver sinusoidal cells
46 and stellate cells, establishing a link between NR subtissular localization and liver functional
47 specialization. In summary, we provide here an up-to-date compendium of NR expression in mouse
48 liver in space and time.

49

INTRODUCTION

50

51 The liver is central to metabolism by coping with qualitatively and quantitatively fluctuating
52 dietary intakes and it stores, packages and reroutes metabolic intermediates to other tissues. The liver
53 also exerts other crucial functions such as detoxification, bile acid synthesis, immune and inflammatory
54 responses and hemostasis. This versatility relies on precisely timed and spatially orchestrated activities
55 of several resident and nonresident cell types which communicate intensively to achieve organ and
56 whole body homeostasis. Within the functional unit of the liver, the hepatic lobule, the above-
57 mentioned biological processes take place into several resident, highly connected cell types
58 [hepatocytes (HC), cholangiocytes (CH), liver sinusoidal endothelial cells (LSEC), stellate cells (HSC) and
59 Kupffer cells (KC)] which are functionally specialized. An additional layer of sophistication is the
60 functional zonation of liver functions, which adapts to the centripetal blood, nutrients and oxygen flow
61 and centrifugal bile circulation (Nagy et al., 2020).

62 As sensors of the environment through their ability to bind hormones, metabolic
63 intermediates or xenobiotics, nuclear receptors (NRs) are essential relays of metabolic and endocrine
64 signals regulating transcriptional networks in hepatic cells (Soccio, 2020). NR structure allows them to
65 act directly or indirectly as transcriptional regulators (Weikum et al., 2018) and to integrate cues from
66 extracellularly activated signaling pathways (Berrabah et al., 2011). Decade-long research efforts have
67 established that NRs act in a tissue-specific manner through multiple mechanisms ranging from
68 intracellular ligand activation to transcriptional coregulator combinatorial assembly on DNA-bound
69 NR. This notion of a specific activity as a function of the site of expression is likely to be extended to
70 distinct cellular populations within a given tissue, but technical hurdles related to single-cell
71 approaches have to be solved prior to get a full appreciation of NR activity in a specific cell type.

72 In this respect, the liver is an optimal model to unravel mechanistic aspects of NR actions in
73 cellular subpopulations, as single-cell approaches have paved the way to building a functional atlas of
74 the liver lobule. Based on a thorough knowledge of liver histology, these transcriptomic analysis have
75 partially established the zonation profile of gene expression in mouse and human livers (Payen et al.,
76 2021, Droin et al., 2021, Aizarani et al., 2019, Dobie et al., 2019, Ben-Moshe et al., 2019, Ben-Moshe
77 and Itzkovitz, 2019). However, quantitative and qualitative assessments of NR expression in the liver
78 are scarce (Li et al., 2013, Gonzalez-Sanchez et al., 2017) but needed to fully appreciate their functional
79 diversity and to leverage this knowledge to define innovative therapeutic strategies. Indeed, NR
80 functions have been mostly defined in a hepatocyte background but their expression territory is more
81 diverse. For example, NUR77 encoded by the *Nr4a1* gene is known to modulate hepatic glucose and
82 lipid metabolism (Pols et al., 2008, Pei et al., 2006) and liver regeneration (Hu et al., 2014), but is

83 substantially expressed in CHs, LSECs and KCs from C57Bl/6 mouse liver (Gonzalez-Sanchez et al., 2017,
84 Li et al., 2013) in which its functions are poorly characterized. Similarly, the physiology of the
85 glucocorticoid receptor (GR), has historically been heavily characterized for its role in metabolic
86 regulations (Praestholm et al., 2020), but whose expression in all other liver resident cell types is far
87 from negligible (Gonzalez-Sanchez et al., 2017, Li et al., 2013).

88 Here, we have leveraged our different transcriptomic studies on bulk and purified liver cells to
89 provide a thorough view of NR isoforms expression in simultaneously isolated parenchymal and non-
90 parenchymal cell populations. Zonation of NR expression was also compiled from published single cell
91 RNAseq studies (Halpern et al., 2018, Bahar Halpern et al., 2017, Su et al., 2021, Dobie et al., 2019) to
92 allow for a refined appreciation of possible physiological NR functions

93

94

MATERIAL AND METHODS

95 **Animal experimentation.**

96 All experiments were approved by the Comité d’Ethique en Expérimentation Animale du Nord-
97 Pas de Calais CEEA75 in compliance with European Union regulations. To eliminate sex as a
98 confounder, only male mice were used throughout this study. C57BL6/J wild-type male mice (12-17
99 weeks) were purchased from Charles River Laboratories and housed in a temperature-controlled
100 environment (23-25°C) with a 12h/12h light-dark cycle, ZT0 being lights-on. Mice had either free
101 access to water and to a standard chow diet [Safe Diet A04](“AdLib(itum)” conditions] or access to
102 food was restricted to the active period for 2 weeks prior to euthanasia (12 hours from ZT12 to
103 ZT24)[“I(ime)-R(estricted) F(eeding) condition]. Liver samples were collected every 3 hours at ZT0,
104 ZT3, ZT6, ZT9, ZT12, ZT15, ZT18 and ZT21 for ad libitum fed mice. Livers were collected every 4 hours
105 at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20 for the time restricted feeding study.

106

107 **Multistep isolation of mouse parenchymal and non-parenchymal liver cells.**

108 This protocol was optimized to isolate simultaneously hepatocytes (HCs), Kupffer cells (KC),
109 hepatic stellate cells (HSCs), liver sinusoidal cells (LSECs) and cholangiocytes (CHs) from a single liver to
110 obtain sufficient amounts of cells for transcription studies. Livers were obtained at ZT3 from an ad
111 libitum-fed mouse.

112 **Cell isolation:** Mice (C57BL6/J male, 12-17-week-old, Charles River) were euthanized
113 by cervical dislocation at ZT3 and liver was perfused through the vena cava. After a first perfusion with
114 Wash buffer [25mM Hepes, pH7.4, 4mM EGTA in 1x Hanks' Balanced Salt solution (HBSS, Gibco-
115 ThermoFisher #14170)] at 37°C until discoloration of the liver, a second perfusion (≈50mL) was
116 performed with Dissociation buffer (25mM Hepes, pH7.4, 1mM CaCl₂ in 1xHBSS) supplemented with
117 collagenase (type IV, Sigma #C5138)(100U/mL) at 37°C. The liver was then removed and dissociated in
118 a Petri dish. The cell solution was filtered through a 70µm cell filter and centrifuged for 2 min at 50 x
119 G to collect HCs. HC pellets were washed once in 45mL Wash buffer, centrifuged for 2 min at 50 x G
120 and resuspended in FACS buffer [1x phosphate-buffered saline (PBS), pH 7.4, 0.5% bovine serum
121 albumin] supplemented with RNAsin (1:1000, Promega, #N2511) for FACS purification. The HC
122 supernatants from the first centrifugation were collected and spun again for 2 min. at 50 x G to remove
123 remaining HCs from the NPC fraction. NPC cells were then resuspended in Dissociation buffer. Seventy-
124 five % of this preparation (NPC75) was added to the non-digested liver (recovered from the initial 70
125 µm filtration), centrifuged for 5 min at 580 x G and incubated with collagenase (100 U/mL), and 0.5

126 mg/mL pronase (Sigma-Aldrich, #10165921001) and 10 µg/mL DNaseI (DNase I grade II, from bovine
127 pancreas, Sigma-Aldrich, # 10104159001) for 20 min at 37°C. The remaining 25% (NPC25) was further
128 digested with collagenase alone for 10 min at 37°C. Cellular preparations were filtered through a 70
129 µm filter and centrifuged for 5 min at 580 x G in Wash buffer at 37°C. Both NPC fractions are
130 resuspended in 1x FACS buffer supplemented with RNasin and kept on ice before cell labelling.

131 **Cell labelling:** All steps from now on were performed at 4°C in light-protected
132 conditions. Cellular fractions were spun down and resuspended in 1 mL red blood cell lysis buffer
133 (155mM NH₄Cl, 10mM NaHCO₃, 0.127mM EDTA, pH7.4) for 4 min before adding 1 ml PBS + 0.5% BSA
134 to stop lysis. Two to 3x10⁶ cells were dispatched per tube, centrifuged (5 min, 600 x G, 4°C) and
135 resuspended in 1x PBS, 0.5% Zombie Green (Biolegend, #BLE423112), 1:1000 RNasin (Promega,
136 #N2511,) for 10 min. After centrifugation, cells were suspended in mouse BD FcBlock (1:200, Blocking
137 anti-CD16/32, Becton-Dickinson #BD 553142), incubated for 15 min on ice and the antibody mix (see
138 below) added for 20 min. Cells were washed twice in 1x FACS buffer and sorted.

139 **Cell sorting:** Cells were sorted using a BD INFLUX v7 cell sorter (BDBiosciences) driven by the
140 BD FACS Software. Compensation particles were from Becton-Dickinson (BD™ CompBeads
141 Compensation Particles Anti-Rat/Hamster Ig, κ Set, # 51-90-9000949). Fluorochrome-coupled
142 antibodies (BioLegend) targeted CD31-BV421 (#BLE102424), CD45-BV510 (#BLE103138), CD326-CF594
143 (#BLE118236) F4/80-PE-Cy7 (#BLE123114) CD146-APC (#BLE134712) MHCII-AF700-(#BLE107622)
144 CD11b-APC-Cy7 (#BLE101226). Anti-CLEC4F was from R&D (#MAB2784) and coupled to CF568 using
145 the Mix-n-Stain CF568 Antibody Labeling Kit (Biotium, #BTM92235). All antibodies were used at 1:100
146 dilution. HCs sorting was performed using a 200 µm nozzle and the following settings: pressure 3.7 psi,
147 drop frequency 6.30 kHz, piezo amplitude 4.1, sample fluid pressure was adapted to reach a maximum
148 events rate of 1 000 events/sec. HCs were selected as viable large cells as visualized on FSC/SSC
149 dotplot, and subsequently gated on singlets before sorting ([Supplementary Figure 1](#)).

150 Non parenchymal cells were sorted as follows: the INFLUX cell sorter was equipped with a 86
151 µm nozzle and tuned at a pressure of 24.7 psi, a drop frequency of 48,25 kHz, a piezo amplitude of 6.7
152 and sample fluid pressure was adapted to reach a maximum events rate of 10 000 events/sec. NPCs
153 fractions (both NPC75 and NPC25, [Supplementary Figure 1B](#)) were gated for viable singlet cells as
154 visualized on FSC/SSC and FSC-W/FSC-A dotplots, respectively and live cells were then selected as
155 “Zombie Green low” events. HSCs were selected as UV⁺ granular cells out of the NPC75 fraction, taking
156 advantage of UV light excitation of retinol and retinoic acid contained in HSCs granules (Mederacke et
157 al., 2015). A “non-small HSCs” gating was applied in order to avoid sorting of degranulated or damaged
158 HSCs. LSECs were selected as UV⁻ CD45⁻ CD146^{hi} events of the NPC25 fraction as pronase digestion was

159 not compatible with CD31 detection. CHs were selected as UV⁻ CD45⁻ CD146^{low} CD326⁺ events of the
160 NPC75 fraction. KCs were selected as UV⁻ CD45⁺ F4/80⁺ CLEC4F⁺ events of the NPC75 fraction, and a
161 “non-small KCs” gating was applied in order to avoid sorting of immature or damaged KCs.

162 Sorted viable HCs were collected in 1 mL RNAlater (ThermoFisher, # 10564445) while sorted
163 viable NPCs were collected in lysis buffer and further processed for RNA extraction. Cytometry data
164 were analyzed using FlowJo v10.5.3 (FlowJo, LLC).

165

166 **Immunofluorescence on sorted liver cells.**

167 Cell preparations were deposited on a glass slide using a Cytospin 4 (ThermoScientific). Cells
168 were fixed in 4% paraformaldehyde for 10 min and washed twice in 1x Phosphate-Buffered Saline
169 (PBS). After blocking with 10% normal goat serum in 1x Tris-buffered saline (TBS) for 1 hour at room
170 temperature (RT), slides were incubated with the primary antibody for 1 hour at RT or overnight at
171 4°C. After 3 washes in 1x TBS, secondary antibodies were added at the indicated dilution in 1x TBS for
172 1 hour at RT. Slides were washed as above and prepared for microscopy.

173 Primary antibodies used were: Anti-KRT18 (C-04, Abcam, #ab668)(dilution 1:100), anti-
174 DESMIN (Y66, Abcam, #ab32362)(dilution 1:50), anti-CLEC4F (ThermoFisher, # MA5-24113)(dilution
175 1:100), anti-VECAD (ThermoFisher, # 36-1900)(dilution 1:50), anti-KRT19 (EP1580Y, Abcam, #
176 ab52625)(dilution 1:100). Secondary antibodies (ThermoFisher Scientific) were used at 1:100 dilution
177 and were goat anti-mouse AF568 (A-11004), donkey anti-rabbit AF488 (A-21206), donkey anti-mouse
178 AF555 (A-31570) and goat anti-rat AF488 (A-11006).

179

180 **RNA extraction and RT-qPCR.**

181 RNA was extracted using the Macherey-Nagel™ Mini kit Nucleospin™ (Macherey-Nagel, #
182 872061) or Qiagen RNeasy micro kit (Qiagen, # 74004), depending on abundance of cell preparations,
183 following the manufacturer’s instructions. RNA concentration and purity were assessed using a
184 Nanodrop One device (ThermoFisher Scientific) or a Qubit fluorometer (ThermoFisher Scientific) and
185 a Qubit RNA HS Assay kit (ThermoFisher Scientific, # Q32852), while RNA integrity was analyzed on a
186 Bioanalyzer 2100 (Agilent). RNA preparations with RIN<6.0 were discarded. RNAs were reverse-
187 transcribed using random primers and the High Capacity cDNA Reverse Transcription Kit
188 (ThermoFisher/Applied Biosystems, # 4368814). Quantitative PCR was performed in technical
189 triplicates from at least 3 independent biological samples using the SYBR green Brilliant II fast kit

190 (Agilent Technologies) on an Mx3005p apparatus (Agilent Technologies) or a QuantStudio 3 (Applied
191 Biosystems). Expression values obtained from mRNA levels normalized to *Rps28* (ribosomal protein
192 S28) and *Rplp0* (acidic ribosomal phosphoprotein P0) mRNA levels and were used to calculate fold
193 changes using the cycle threshold ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak, 2008). Primer sequences are
194 listed in Supplemental Table 1. PCR primer efficiencies were routinely assessed by serial 2-fold dilution
195 of a control cDNA source (in the 1 to 500-fold range), the size of the amplicon was checked by agarose
196 electrophoresis and the specificity of the PCR amplicon was systematically assessed by melting curve
197 analysis. In this study, RT-qPCR results were always confirmed with RNAseq data.

198

199 **Affymetrix array analysis.**

200 **RNA processing and array hybridization:** Gene expression from whole mouse liver (n=3) was
201 analyzed with Affymetrix GeneChip MoGene 2.0 ST arrays after RNA amplification, sscDNA labeling
202 and purification. Briefly, RNA was amplified using the GeneChip™ WT PLUS Reagent Kit (Thermo Fisher
203 Scientific, # 902280), retrotranscribed to single-stranded complementary (ssc) DNA and labeled using
204 GeneChip™ WT Terminal Labeling Kit (Thermo Fisher Scientific, # 900670), followed by hybridization
205 on the GeneChip Mouse Gene 2.0 ST Array (Affymetrix, # 902118) according to the manufacturer's
206 instructions.

207 **Data processing and analysis:** Raw data were processed on a local instance of Galaxy (Afgan
208 et al., 2018) using GIANT, a user-friendly tool suite developed in-house for microarray and RNA-seq
209 differential data analysis (Vandel et al., 2020). It consists of modules allowing to perform quality
210 control (QC), Robust Multiarray-Average method normalization, LIMMA differential analysis, volcano
211 plot and heatmaps. Signals were normalized with GIANT APTtool (v2.10.0, ThermoFisher) with options
212 "gc correction, scale intensity and rma at probeset level" followed by a \log_2 transformation. Then
213 normalized expressions were averaged per Gene Symbol (NetAffx Annotation Release 36, July, 2016)
214 and transcripts within the 10th lowest percentile were considered as technically unreliable and
215 excluded. Differential analysis was performed with GIANT limma tool [v3.36.5, (Ritchie et al., 2015)]
216 (FDR cutoff = 0.05) "

217

218 **RNA sequencing.**

219 **Library preparation and sequencing:** RNA samples (n=3) were sent to the GenomEast platform
220 for library preparation and sequencing. Briefly, RNA preparations were first depleted from unwanted,
221 abundant transcripts using Ribo-Zero Plus rRNA depletion kit (Illumina, # 20040526). cDNA synthesis,

222 3'end adenylation, adapter ligation and PCR amplification were performed using the TruSeq Stranded
223 total RNA sample preparation kit (Illumina). DNAs were sequenced using an Illumina HiSeq 4000
224 sequencer in 50 bp Single-Read following Illumina's instructions. Image analysis and base calling were
225 performed using RTA 2.7.7 and bcl2fastq 2.17.1.14. Sequencing depth was 75 million reads on average.

226 **Data processing and analysis:** Reads were preprocessed using cutadapt version 1.10 (Kechin
227 et al., 2017) in order to remove adapter, polyA and low-quality sequences (Phred quality score below
228 20). Reads shorter than 40 bases were discarded for further analysis. Remaining reads were mapped
229 onto the mm10 assembly of the *Mus musculus* genome using STAR version 2.5.3a (Dobin et al., 2013).
230 Gene expression quantification was performed from uniquely aligned reads using htseq-count version
231 0.6.1p1 (Anders et al., 2015), with annotations from Ensembl version 94 and "union" mode. Read
232 counts were normalized across samples with the median-of-ratios method (Anders and Huber, 2010),
233 suitable for inter-sample comparison. Gene expression profiles were compared using the Bioconductor
234 package DESeq2 version 1.16.1 (Love et al., 2014). P-values were adjusted for multiple testing using
235 the Benjamini and Hochberg method (Benjamini and Hochberg, 1995).

236 **Splice junctions and isoform detection:** Splice junctions were visualized in Integrative
237 Genomics Viewer (IGV, Broad Institute)(Robinson et al., 2011) using bam files of aligned reads and the
238 mm10 gene annotation track. Alignment data were visualized using the Sashimi plot function of IGV.

239

240 **Single cell data extraction.**

241 Analysis were carried out using extracted data from available datasets (GSE84490 for HC
242 zonation and GSE108561 for LSEC zonation) obtained from 6 to 16-week-old C57BL/6 male mice
243 (Halpern et al., 2018, Bahar Halpern et al., 2017). We used the ISCEBERG browser which allows analysis
244 and interrogation of single cell RNAseq data (Guille et al., 2022) for HSC zonation [GSE137720 (Dobie
245 et al., 2019)] and LSEC zonation [GSE147581, (Su et al., 2021)].

246 **HSCs, GSE137720:** Seurat (v 4.0.1) was used to analyze this dataset. Cells were obtained from
247 *Pdgfrb*-BAC-eGFP reporter mice on a C57BL/6 background (10 to 16-week-old males). According to
248 (Dobie et al., 2019), we filtered out cells expressing < 300 genes and cells expressing > 30 % of
249 mitochondrial genes. Then data were normalized and scaled using NormalizeData
250 (normalization.method=LogNormalize, scale.factor=10000) and ScaleData (features=all.genes)
251 functions from the Seurat package. We applied a batch correction using Harmony (v 0.1.0). Then
252 dimensionality reduction was achieved using Uniform Manifold Approximation and Projection (UMAP)
253 to calculate 2D coordinates (reduction="harmony",dims=1:30). SCINA (v 1.2.0) was used to characterize
254 cell populations (fibroblasts, vascular smooth muscle cells, and hepatic stellate cells) according to cell

255 markers defined in (Dobie et al., 2019). Cells distinct from HSCs were filtered out and HSCs located in
256 the periportal or pericentral areas were identified using SCINA (version 1.2.0) based on markers used
257 in the publication.

258 **LSECs, GSE147581:** Seurat (v 4.0.1) was used to analyze this dataset. Cells were obtained from
259 Cdh5-CreERT2, mT/mG mice of undefined sex. According to (Su et al., 2021), cells expressing less than
260 200 transcripts and more than 20 % of mitochondrial genes were filtered out. Data were normalized
261 and scaled as above, and Harmony (version 0.1.0) was used to apply a batch correction. UMAP
262 coordinates were calculated and clusterized with findClusters using a resolution of 0.5 to match with
263 the published analysis. Annotation with identified zonation markers was carried out using SCINA
264 (version 1.2.0).

265

266

267 **Statistical analysis.**

268 Statistical analysis was performed using GraphPad Prism (v. 9). Data are plotted as the mean
269 \pm SEM. At least 3 independent experimental replicates were obtained. Data were determined to have
270 equal variances using the F test. For 2-group comparisons, an unpaired 2-tailed *t*-test with Welch
271 correction was used. For multiple comparisons with one variable, a 1-way ANOVA followed by the
272 Tukey multiple comparison test (each group compared to every other group) was used. Multiple
273 comparisons with more than one variable were carried out using a 2-way ANOVA followed by a Tukey's
274 multiple comparison test. Cyclical patterns of gene expression were determined using JTK_Cycle
275 (version 3.1)(Hughes et al., 2010). Cyclic circadian transcripts were defined as such when having a
276 period between 21-26 hours and an adjusted p-value <0.05. All samples were incorporated in the
277 analysis (Ad libitum-fed: ZT0, ZT3, ZT6, ZT9, ZT12, ZT15, ZT18 and ZT21; TRF: ZT0, ZT4, ZT8, ZT12, ZT16,
278 and ZT20).

279

280 **Data visualization.**

281 **Bubbleplots:** Bubbleplots were generated in R studio using the ggplot2, plotly, reshape2, rcpp
282 and tidyverse packages. SVG files were modified with Inkscape v1.0 and assembled as figures using
283 CorelDraw2020. The liver trabeculae structure was adapted from a file published in a Public Library of
284 Science journal (Frevort et al., 2005) under the Creative Commons Attribution 4.0 license.

285

286 **Data availability.**

287 Affymetrix data files (“AdLib” and “TRF” data) are available under NCBI GEO
288 (<https://www.ncbi.nlm.nih.gov/geo/>) dataset numbers GSE223360 and GSE224446 respectively. RNA
289 sequencing data are available under the GEO dataset number GSE222597.

290

RESULTS

291

292

293 **Development of a liver cell type multi-step isolation protocol.**

294 In order to minimize both technical and biological biases in isolating liver cell populations, we
295 set up a protocol allowing the purification of 5 resident cell populations, i.e. HCs, LSECs, HSCs, KCs and
296 CHs from a single liver. This protocol also allowed the purification of dendritic cells and of neutrophils,
297 which were not considered further in this study. After sacrifice by cervical dislocation to avoid any side
298 effects of anesthetics, the liver was perfused with modified HBSS and dissociated with collagenase IV.
299 To enrich for specific cell populations, aliquots of the digested liver were then processed separately
300 (Figure 1). Dissociated cells were sorted based on size to yield purified HCs, whose amounts routinely
301 exceeded 20×10^6 cells per liver. Further digestion by collagenase and pronase yielded the total non-
302 parenchymal cell (NPC) fraction which was sorted using the indicated combination of antibodies
303 (Figure 1 and Supplemental Figure 1). This yielded per liver variable amounts of HSCs, KCs, LSECs and
304 CHs with numbers routinely exceeding 10^5 cells per preparation and cell type (Supplemental Figure 2).
305 Cellular homogeneity and purity were assessed by RT-qPCR and immunofluorescence using established
306 cell type-specific cellular markers (Figures 2 and supplemental Figure 3).

307

308 **Circadian rhythmicity of hepatic nuclear receptors expression.**

309 In homeostatic conditions, NR expression may vary not only as a function of nutritional and
310 hormonal cues, but also according to the day-night cycle. To assess whether time-of-the-day is a
311 critical parameter in dictating NR-encoding transcript abundance, we compared gene expression
312 patterns in C57Bl6 male mice liver fed a chow diet either *ad libitum* or under time-restricted feeding
313 for 10 days. In the latter case, food was available only during the active period (dark period for 12
314 hours, ZT12 to ZT24), while all other parameters were similar [number of mice per cage (4),
315 temperature (22-24°C), access to water]]. Transcriptomic data were obtained and analyzed using the
316 JTK package to determine gene expression periodicity (Hughes et al., 2010)(Supplemental Table 2).
317 Several NRs displayed in *ad libitum* conditions a robust 23 to 24-hours cycle [*Nr1d1(Rev-erba)*,
318 *Nr1d2(Rev-erbb)*, *Nr1c2(Pparb/d)* and *Nr1f3(Rorg)*] while *Nr2f6(Ear2)*, *Nr2b1(Rxra)*, *Nr1c1(Ppara)*,
319 *Nr2c2(Tr4)*, *Nr1h4(Fxra)* and *Nr2a1(Hnf4a)* cycled similarly albeit with a lesser amplitude (Figure 3).
320 With the exception of *Nr2a1(Hnf4a)*, the time-restricted feeding regimen did not modify the cyclic
321 expression pattern of these NRs (Figure 3 and Supplemental Table 2), while showing a trend to increase
322 the amplitude of the signal. More surprisingly, several NRs exhibited condition-specific cycling [ad

323 libitum-fed: *Nr4a2(Nurr1)*, *Nr5a2(Lrh1)*, *Nr1f1(Rora)*, *Nr3c2(Mr)*, *Nr1i2(Pxr)*, *Nr1c3(Pparg)*; time-
324 restricted feeding: *Nr2b3(Rxrg)*, *Nr1h2(Lxrb)*, *Nr3c1(Gr)*, *Nr1h3(Lxra)*, *Nr2b2(Rxrb)*, *Nr1i3(Car)*].
325 Irrespective of the functional consequences of such oscillations, these differential expression levels
326 should be considered when comparing expression levels of NRs in different conditions. We thus
327 selected ZT3 (3 hours after light-on) as a convenient reference time point to initiate liver cell type
328 isolation from ad-libitum fed mice (Figure 1). In these conditions, *Nr1d1(Rev-erba)* and *Nr1d2(Rev-*
329 *erbb)* reached their zenith, while *Nr1f3(Rorg)*, *Nr1c3(Pparg)* and *Nr1i2(Pxr)* were at their nadir.

330

331 **Nuclear receptor expression in liver cell types.**

332 NR-encoding transcripts were quantified in each cell type by RNAseq (Figure 4 and
333 Supplemental Table 3). Forty-two NRs reached a detectable level of expression (RPKM>10), and each
334 cell type was characterized by a specific NR pattern of expression, with a high level in *Nr2a1(Hnf4a)*,
335 *Nr2f6(Ear2)*, *Nr1c1(Ppara)* and *Car(Nr1i3)* mRNAs being characteristic of hepatocytes. Cholangiocytes
336 exhibited a highly restricted panel of highly expressed NRs, including only *Nr1f1(Rora)* and
337 *Nr2a2(Hnf4g)*. LSECs showed high levels in *Nr1b2* and *b3 (Rarb and g)*, *Nr2f1* and *f2 (Coup-tf1&2)*, while
338 HSCs were characterized by a high level in *Nr1h4* and *h5(Fxra and b)*, *Nr1a1(Thra)* and
339 *Nr1b1(Rara)*. Finally, *Nr2b2(Rxrb)*, *Nr4a1(Nur77)*, *Nr1c3(Pparg)* and *Nr1h3(Lxra)* elevated levels were
340 a feature of KCs. On the opposite, hepatocytes were characterized by undetectable levels in
341 *Nr4a3(Nor1)* and LSECs by the total absence of *Hnf4g*. NRs deemed to be undetectable in any cell type
342 (<10 RPKM) were *Erb(Nr3a2)*, *Dax1(Nr0b1)*, *Tlx(Nr2e1)*, *Pnr(Nr2e3)*, *Sf1(Nr5a1)*, *Rorb(Nr1f2)* and
343 *Pr(Nr3c3)*.

344

345 **Nuclear receptor isoforms expression in liver cell types.**

346 Nuclear receptor isoforms play substantially distinct physiological roles, hence determining
347 their expression level is of importance to decipher NR cell-specific functions. NR protein isotypes and
348 their corresponding isoforms were compiled from Uniprot and Protein Ontology databases (UniProt,
349 2021, Natale et al., 2017), and associated transcripts were searched in RNAseq data using the Sashimi
350 plot function from the Integrative Genome Viewer [IGV, (Katz et al., 2015, Robinson et al., 2011)].
351 Twenty out of the 42 detected NRs-encoding transcripts can be potentially expressed as distinct
352 isoforms (Figure 5 and Supplemental Table 3) out of which 11 actually displayed differential expression
353 in the 5 isolated liver cell types. These included transcripts coding for FXR α , PXR, CAR, GCNF, PPAR γ ,

354 RAR α , β and γ , ROR α , RXR β , T3R α and β , whose expression levels were qualitatively assessed as
355 described and were reported in [Figure 5](#).

356

357 **Zonation of nuclear receptor expression in hepatocytes, sinusoidal endothelial cells and stellate** 358 **cells.**

359 Functional zonation of the liver is observed along a periportal-pericentral axis and is
360 conditioned by multiple factors such as oxygen, nutrient and morphogen gradients (Panday et al.,
361 2022, Kietzmann, 2017). Specialized functions of liver cell types as a function of their spatial
362 distribution can be inferred from single-cell studies and have been molecularly detailed in recent years.
363 Expression patterns of NR-encoding genes were extracted from published datasets for mouse HCs,
364 LSECs and HSCs (Halpern et al., 2018, Bahar Halpern et al., 2017, Dobie et al., 2019, Su et al., 2021). In
365 HCs, NRs displayed distinct spatial expression patterns([Figure 6](#)), with PPAR α being equally expressed
366 along the pericentral to periportal axis, in agreement with its ability to regulate fatty acid oxidation
367 (predominantly periportal) and ketogenesis (predominantly pericentral). Other NRs also displayed an
368 even gene expression pattern along this axis (*Nr3c2(Mr)*, *Nr1f1* and *f3(Rora* and *g)*, *Nr6a1(Gcnf)*,
369 *Nr1a2(Thrb)*, *Nr2f2(Coup-tf2)*, *Nr0b2(Shp)*, while some had a dominant pericentral localization
370 [*Pparg(Nr1c3)*, *Errb(Nr3b2)*, *Nr5a2(Lrh1)*, *Nr3c1(Gr)*, *Nr2b2(Rxrb)*. Only *Nr1d2(Rev-erb β)*) and
371 *Era(Nr3a1)* were preferentially expressed in the portal area. Mining the transcriptome of LSECs
372 obtained by paired-cell RNAseq (Halpern et al., 2018) defined NR expression in this cell population
373 ([Supplemental Figure 4](#)). Thirty-seven NRs were found to have a spatially differential expression, with
374 *Nr2c1(Tr2)*, *Nr5a2(Lrh1)* and *Nr1c3(Pparg)* being almost exclusively expressed in the pericentral area.
375 Mirroring this pattern, *Pxr*, *Era* and *Errg* were exclusively detected in the periportal area, while
376 *Nr1f2(Coup-tf2)*, *Nr1h2(Lxrb)*, *Nr1c1(Ppara)*, *Nr3c1(Gr)*, *Nr1i3(Car)*, *Nr2b1(Rxra)* and *Nr3b2(Esrrib)*
377 were significantly expressed, albeit with variation, in all 4 layers. A comparison with CDH5 (VE-
378 cadherin)-expressing LSEC single-cell transcriptome data brought further elements of comparison,
379 while providing novel information about arterial (portal) and venous (central) LSECs ([Supplemental](#)
380 [Figure 5](#)). Zonation patterns matched for 50% (18 out 37) NRs, showed minimal discrepancies for 15
381 and were strikingly different for *Nr1c1(Ppara)*, *Nr3b1(Esrria)*, *Nr3b3(Errg)* and *Nr3a1(Era)*.
382 *Nr1c1(Ppara)* expression levels, which are in LSECs 4% of that found in HCs, was restricted to arterial-
383 like ECs ([Supplemental Figure 5](#)) or present all along the pericentral-periportal axis ([Supplemental](#)
384 [Figure 4](#)). Along this axis, *Nr3a1(Era)*, *Nr3b1(Esrria)* and *Nr3b3(Esrrib)* displayed an opposite gradient of
385 expression in these 2 datasets. Mouse strains, sex as it is undefined in a study (Su et al., 2021), cell
386 isolation and identification methods as well as transcript mapping procedures were different in those

387 2 studies, calling for additional strictly comparative studies to reach a consensual cartography of NRs
388 in LSECs. Of note, our LSEC purification procedure relies on a CD31/CD146 double-positive labeling to
389 obtain highly pure cell preparations, which may have nevertheless selected a particular subpopulation.

390 Finally, NR expression was mapped in the 2 identified HSC populations which locate in close
391 vicinity to the periportal (PaHSCs) or of the pericentral (CaHSC) areas ([Supplemental Figure 6](#)). With
392 the exception of *Fxra* whose expression was detected in both HSC subpopulations and slightly higher
393 in PaHSCs, the 30 quantified NR-encoding transcripts showed a markedly unbalanced expression
394 between the 2 subpopulations. *Nr2f2(Coup-tf2)*, *Nr3c1(Gr)*, *Nr2b1(Rxra)*, *Nr1f1(Rora)*, *Nr1h2(Lxrb)* and
395 *Nr2f6(Ear2)* were more preferentially expressed on PaHSCs, whereas *Nr1a1(Thra)*, *Nr4a1(Nur77)*,
396 *Nr1h5(Fxrb)*, and *Nr1b1(Rara)* transcripts were prominently localized in CaHSCs.

397

399 Nuclear receptors play critical roles in liver physiology, and establishing a precise
400 spatiotemporal atlas of their expression is mandatory to define their functions. A first study relying on
401 PCR-based detection reported the tissue distribution pattern of human NRs, using a mixed source of
402 RNAs (varying sex, age and ethnical origin, (Nishimura et al., 2004). A similar approach was applied to
403 nonreproductive tissues isolated from male 129x1/SvJ and C57/Bl6J mice at ZT0 (Bookout et al., 2006).
404 Both studies provided a first elegant assessment of the functional clustering of NRs on the basis of
405 their tissue-specific expression. Recent progress in single-cell technologies has shed some light on
406 processes driving liver functional zonation and allowed to map cell-specific expression patterns of NRs,
407 but they still lack sensitivity to identify transcript isoforms in isolated cells. Here we provide a
408 compendium of hepatic NR expression considering and minimizing whenever possible technical
409 variability, diet and time-of-the-day influences. By performing a bulk RNAseq analysis of isolated cell
410 types, NR transcript isoforms were also easily identified, as NR protein isoforms are known, at least for
411 a few cases, to bear distinct functional properties. Finally, spatial expression of NRs has been explored
412 by mining single cell transcriptomic datasets, which may provide a mean to ascribe, or rule out, novel
413 functions to NRs.

414 Our data were compared with others, which were obtained from *ad libitum*-fed C57Bl/6J male
415 (Gonzalez-Sanchez et al., 2017) or female mouse livers (Li et al., 2013) collected at non indicated times
416 and using distinct cell isolation and RNA quantification methods. While methodologies and biological
417 sources were different, these 3 sets of data identified unambiguously NRs which are never detected in
418 any liver cell types [*Nr0b1(Dax1)*, *Nr1f2(Ror β)*, *Nr2e1(Tlx)*, *Nr3e3(Pr)* and *Sf1(Nr5a1)*]. Sexual
419 dimorphism of NR gene expression was not addressed in the present study but has pathophysiological
420 relevance (Della Torre and Maggi, 2017). *Nr1d1(Rev-erba)*, *Nri3(Car)*, *Nr1i2(Pxr)*, *Nr1c1(Ppara)* indeed
421 exhibit distinct circadian patterns when comparing C57BL/6 male and female mice (Lu et al., 2013).

422 Ad libitum or time-restricted access to a high fat diet showed that besides sexually dimorphic
423 protective effects of TRF, it may also substitute to normal oscillations driven by the molecular clock
424 (Chaix et al., 2021, Chaix et al., 2018, Vollmers et al., 2009). On an obesogenic diet, TRF tends to restore
425 gene expression rhythmicity (Deota et al., 2023), which is blunted under a HFD when compared to a
426 chow diet (Eckel-Mahan et al., 2013, Hatori et al., 2012). While our data concur to show a similar trend
427 in our TRF experiment, some differences can be noted in other studies which can be ascribed to the
428 duration of the regimen in a similar genetic background [10 days vs. 49 (Deota et al., 2023) or 100 days
429 (Hatori et al., 2012)]. A sufficiently powered, strictly comparative study is required to draw formal

430 conclusions about the influence of the feeding regimen on the phase and amplitude of transcript
431 oscillations.

432 Identifying an NR-based cell type signature requires to exclude NRs with oscillating levels of
433 transcripts and strongly sensitive to feeding conditions. Since ad libitum feeding is most commonly
434 used in animal facilities, we used this experimental condition as a reference and could identify *Rev-*
435 *erba(Nr1d1)*, *Rev-erbb(Nr1d2)*, *Rorg(Nr1f3)*, *Pxr(Nr1i2)* and *Car(Nr1i3)*, *Fxra(Nr1h4)* and
436 *Pparb/d(Nr1c2)* as genes with markedly oscillating transcripts along the day-night cycle. Of note,
437 *Nr4a1(Nur77)*- and *Nr2c2(Tr4)*-encoded transcripts gained strong cyclicity in time-restricted fed mice.
438 An NR consensus signature characteristic of HC could be defined which identified, when integrating
439 isoform expression patterns. *Hnf4a1*, *Ppara* and *Thrb1* were overwhelmingly expressed in this cell
440 type, with 10x expression ratios when compared to CHs, HSCs, LSECs or KCs. In CHs, *Rora2* and *Gcnf*
441 *M2* isoforms expression were markedly higher than in other cell types (x5 to x10). In LSECs, both
442 *Nr1f2(Coup-tf2)* and *Rarg2* displayed highest levels of expression, while *Era* and *Fxra1* and *a2*
443 expression were hallmarks of HSCs. Finally, KCs displayed highest levels in *Nr3b1(Esrra)*, *Nr1h3(Lxra)*
444 and *Nr4a1(Nur77)*. Our data thus bring additional information about NR isoform expression, which
445 are in most cases in good agreement with previous reports for NRs displaying high to moderate
446 expression levels. Some minor discrepancies were observed for NRs displaying low expression levels,
447 which are reported as not expressed in PCR-based investigations, but nevertheless detected in the
448 more sensitive RNAseq assay.

449 The zoned expression of NRs calls for a more detailed consideration of NR functions deduced
450 from previous “bulk” approaches. We previously detailed the implication of the zoned expression of
451 PPARs in HCs (Berthier et al., 2021). PPAR α appears to be preferentially pericentral, together with
452 PPAR α -driven lipogenic enzymes. However, fatty acid oxidation, which is also controlled by PPAR α ,
453 mostly occurs in the oxygen-rich periportal area where a decreased, but not absent, expression of
454 *Ppara* is observed. In contrast, *Ppar γ* is uniquely expressed in pericentral HCs, in line with its pro-
455 lipogenic activities and the metabolic zonation of HCs. We note that *Rxr β* is also preferentially
456 expressed in the pericentral area. As a PPAR γ heterodimerization partner, RXR β confers increased
457 transcriptional activity to PPAR γ when compared to RXR α (Lefebvre et al., 2010). Although this remains
458 to be formally proven, this raises the possibility of cell type-specific heterodimeric combinations with
459 distinct transcriptional properties. Also consistent with the metabolic zonation of the liver, *Nr4a1*
460 (*Nur77*) is mostly expressed in the periportal area where it may exert its pro-gluconeogenic activities
461 (Pei et al., 2006). Finally, targeting a given NR in liver disease should integrate this spatial parameter.
462 NASH-induced fibrosis stems mostly from a pericentral injury, likely to activate stellate cells in this

463 area. We note that NR agonists efficiently blocking NASH and fibrosis progression, at least in rodent
464 models, display a preferential expression in central HSC (caHSC, [Supplemental Figure 6](#)).

465 Nineteen NRs detected in our study have referenced protein isoforms, a number likely to be
466 vastly underestimated (Annalora et al., 2020). Therefore splicing events could dramatically extend the
467 functional repertoire of NRs, as described for “metabolic NRs” (Mukha et al., 2021). While our study
468 was not designed to formally quantify all alternative transcripts for each NRs expressed in each liver
469 cell type, 13 NR-encoding genes were actually expressed as different isoforms. They included
470 *Nr1h4(Fxra)*, *Nr1h5(Fxrb)*, *Nr1i2(Pxr)*, *Nr1i3(Car)*, *Nr6a1(Gcnf)*, *Nr1c3(Pparg)*, *Nr1b1, 2, 3(Rara, b* and
471 *g, Nr1f1(Rora)*, *Nr2b2(Rxrb)*, *Nr1a2* and *Nr1a3(T3ra* and *b)*. Various *scenarii* were observed with
472 respect to isoform expression profiles. A single isoform could be detected per cell type (*Nr1i2, Pxr*) or
473 a single or a mix of isoforms were identified [*Nr1h4(Fxra)*, *Nr1h5(Fxrb)*, *Nr1i3(Car)*, *Nr6a1(Gcnf)*,
474 *Nr3c3(Pparg)*, *Nr1b1(Rara)*, *Nr1b2(Rarb)*, *Nr1b3(Rarg)*, *Nr1f1(Rora)*, *Nr2b2(Rxrb)*]. In most cases,
475 isoform expression is known to result from alternative promoter usage (*Fxra1, a2* vs *Fxra3, a4*; *Pparg1*
476 *vs Pparg2*; *Rara1* vs *Rara2*; *Rarg1* vs *RARG2*; *Rxrb1* vs *Rxrb2*) and in the remaining cases (*Rarb2* vs *Rarb4*
477 and *Rora1* vs *Rora2*) from alternative splicing. While the specific functions of NR isoforms has not been
478 studied in great details with a few exceptions (FXR, PPAR γ), reports generally point at distinct
479 transcriptional activities and tissue-specific expression of these variants (Mukha et al., 2021). This
480 knowledge has to be refined by investigating the role of NR isoforms in liver cell subpopulations, which
481 exert distinct roles that still remain to be explored. In this respect, the subtissular repartition of FXR,
482 which in contrast to 2 reports (Verbeke et al., 2014, Fickert et al., 2009), we and others (Gonzalez-
483 Sanchez et al., 2017, Garrido et al., 2021) found to be highest expressed in HSCs and less abundantly
484 in HCs, should be refined in light of isoform expression territories. FXR isoform functions have indeed
485 been studied by elegant approaches solely in a hepatocyte background, in which FXR α 1 and α 2 were
486 described to differentially affect bile acid and lipid metabolism (Vaquero et al., 2013, Ramos Pittol et
487 al., 2020, Correia et al., 2015, Boesjes et al., 2014). We observed that HCs mostly express FXR α 3 and
488 α 4, whereas HSCs express mostly FXR α 1 and α 2. This calls for a careful reexamination of FXR isoforms’
489 biological properties in each cell (sub)type such as CaHSCs and PaHSCs, which is currently underway in
490 our laboratory.

491 Taken as a whole, this study provides a compendium of NR expression in parenchymal and
492 non-parenchymal liver cells which calls for an in-depth investigation of NR functions in liver cell
493 populations. In addition to the multiple layers of NR activity regulation, that ranges from ligand
494 availability, dimerization, transcriptional comodulator interaction and post-translational
495 modifications, the expression territory, hence the cellular background is likely to confer specific

496 properties to NR-controlled signaling pathways, and this mapping will provide new guidance for NR-
497 based therapies.

498

499

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DECLARATIONS of INTEREST

509 The authors have nothing to declare.

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AUTHOR’S CONTRIBUTIONS

513 Author’s contributions were: Conceptualization: FPZ, BS, PL; Methodology: FPZ, AB, MV, OMC, LG, CG, DD, MBG,
514 LP; Software: LG, JCD, MJ; Validation: FPZ, OMC, JCD, CG, MJ, LP; Formal analysis: FPZ, AB, MBG, PL; Investigation:
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708

709

FIGURE LEGENDS

710

711

712 **Figure 1 – Liver cell type isolation protocol.** The sequential steps of liver cell type purification by
713 fluorescence-activated cell sorting (FACS) are shown here. A) Hepatocyte purification; B) non-
714 parenchymal cells isolation. Assessed parameters by FACS were: viability: Zombie green-negative; HCs:
715 sorted based on size; HSCs: UV-positive; LSECs: CD45-negative, CD31-positive, CD146-positive; KCs:
716 CD45-positive, Clec4F-positive, F4/80-positive; CHs: CD45-positive, CD31-negative, CD146-negative,
717 CD326-positive. Details of the complete procedure can be found in the Material & Methods section.
718 HCs: hepatocytes; NPC: nonparenchymal cells; KCs Kupffer cells; CHs: cholangiocytes; LSECs: liver
719 sinusoidal endothelial cells; HSCs: hepatic stellate cells. An example of the FACS output is shown in
720 Supplemental Figure 1.

721

722 **Figure 2. Characterization of purified liver cell types.** After FACS-based purification, RNA was
723 extracted and used for RT-qPCR assays (left panels) or cells were deposited on a glass slide using
724 Cytospin centrifugation to be further labelled with the indicated antibodies. A) HCs characterization;
725 B) HSCs characterization; C) KCs characterization; D) LSECs characterization; E) CHs characterization.

726

727 **Figure 3. Circadian expression of nuclear receptors.** Mice were fed a chow diet either ad libitum or
728 under a time-restricted regimen for 2 weeks. Liver were collected at indicated times (ZT0 being “lights-
729 on) and extracted RNAs were analyzed on Affymetrix arrays (n=3). Data were processed and gene
730 expression values for NRs were extracted and used to generate a heatmap. AdLib and TRF
731 transcriptomic data were also analyzed using the JTK_cycle R script to identify genes displaying a cyclic
732 expression with a ≈24-hour period. C: Cyclically-expressed genes, NC: non-cyclically expressed genes.

733

734 **Figure 4. NR expression in purified liver cell types.** RNA extracted from each cell type preparation
735 (n=3) was analyzed by single-end 50b RNAseq. After (pre)processing, mapping and normalization by
736 the median-of-ratios method to make counts comparable between samples, \log_2 expression values
737 were used to generate a bubble plot in which row Z-score of RPKM ($p_{adj} < 0.05$) is indicated (genes in
738 red are up-regulated, genes in blue are down-regulated) on a row-by-row basis. The size of the bubble
739 is proportional to the expression level (empty spaces indicate no significant expression).

740

741 **Figure 5. NR isoforms expression in purified liver cell types.** Gene expression data were reported from
742 Figure 4 and Uniprot-identified isoforms were indicated. Non-detected isoforms are indicated in gray.
743 (Co)Identified corresponding transcripts are indicated for each cell type and NR.

744

745 **Figure 6. NR zonation in hepatocytes.** Upper panel: Schematic organization of a liver trabeculae
746 [(adapted from Wikimedia Commons and initially published in (Frevert et al., 2005)]. Lower panel:
747 expression values for each NR were extracted from (Bahar Halpern et al., 2017) and used to compute
748 an heatmap. Only NRs displaying variable expression along the periportal-pericentral axis are shown.
749 Red: High expression, white: low expression. Note that gene expression levels are indicated for a single
750 transcript along the pericentral to periportal axis. Arrow (right to left) indicates the bile flow, arrows
751 (left to right) indicate the blood flow.

752

753 **Supplemental Figure 1. Gating strategy for liver cell type isolation.** An illustration of the flow
754 cytometric analysis/sorting is shown here (NPC25). Cells were selected as non-debris on FSC/SSC
755 scatters and singlets were gated on FSC-H/FSC-W. Live cells were selected as “low” for Zombie Green
756 staining. The polychromatic flow cytometry strategy was applied to isolate indicated liver cell types.
757 Further details can be found in the Material & Methods section as well as in Figure 1.

758

759 **Supplemental Figure 2. Numbering of purified cells.** Cell numbers obtained after the FACS procedure
760 are indicated (n=5-14). KCs Kupffer cells; CHs: cholangiocytes; LSECs: liver sinusoidal endothelial cells;
761 HSCs: hepatic stellate cells.

762

763 **Supplemental Figure 3. Cell purity assessment by gene expression profiling.** RNAs extracted from
764 each purified cell type was analyzed by RT-qPCR (n=9-16) and probed for the expression of ubiquitous
765 (*Rplp0/36b4*), common (HCs and CHs, *Ck18*) or specific (HSCs: *Dcn, Acta2, Des*; CHs: *Sox9, Epcam, Sglt1,*
766 *Prom1, Jag1*; KCs: *Cd68*; LSECs: *Cdh5*) cell markers.

767

768 **Supplemental Figure 4. NR zonation in liver sinusoidal endothelial cells.** Upper panel: Schematic
769 organization of a liver trabeculae [adapted from Wikimedia Commons and initially published in (Frevert
770 et al., 2005)]. Lower panel: expression values for each NR were extracted from (Halpern et al., 2018)
771 and used to compute an heatmap. Note that gene expression levels are indicated for a single transcript

772 along the pericentral to periportal axis. Red: High expression, white: low expression. Arrow (right to
773 left) indicates the bile flow, arrows (left to right) indicate the blood flow.

774

775 **Supplemental Figure 5. NR zonation in liver sinusoidal endothelial cells.** Upper panel: Schematic
776 organization of a liver trabeculae [adapted from Wikimedia Commons and initially published in (Frevert
777 et al., 2005)]. Lower panel: expression values for each NR were extracted from (Su et al., 2021) and
778 used to compute an heatmap. Note that gene expression levels are indicated for a single transcript
779 along the pericentral to periportal axis. Red: High expression, white: low expression. Arrow (right to
780 left) indicates the bile flow, arrows (left to right) indicate the blood flow.

781

782 **Supplemental Figure 6. NR zonation in hepatic stellate cells.** Upper panel: Schematic organization of
783 a liver trabeculae [adapted from Wikimedia Commons and initially published in (Frevert et al., 2005)].
784 Lower panel: expression values for each NR were extracted from (Dobie et al., 2019) and used to
785 generate a bubble plot in which the color gradient indicates the expression level (blue: low expression,
786 red: high expression) and the circle diameter indicates the number of cells expressing the transcript.
787 Arrow (right to left) indicates the bile flow, arrows (left to right) indicate the blood flow.

788

789 **Supplemental Table 1. List of primers used in RT-qPCR experiments.**

790

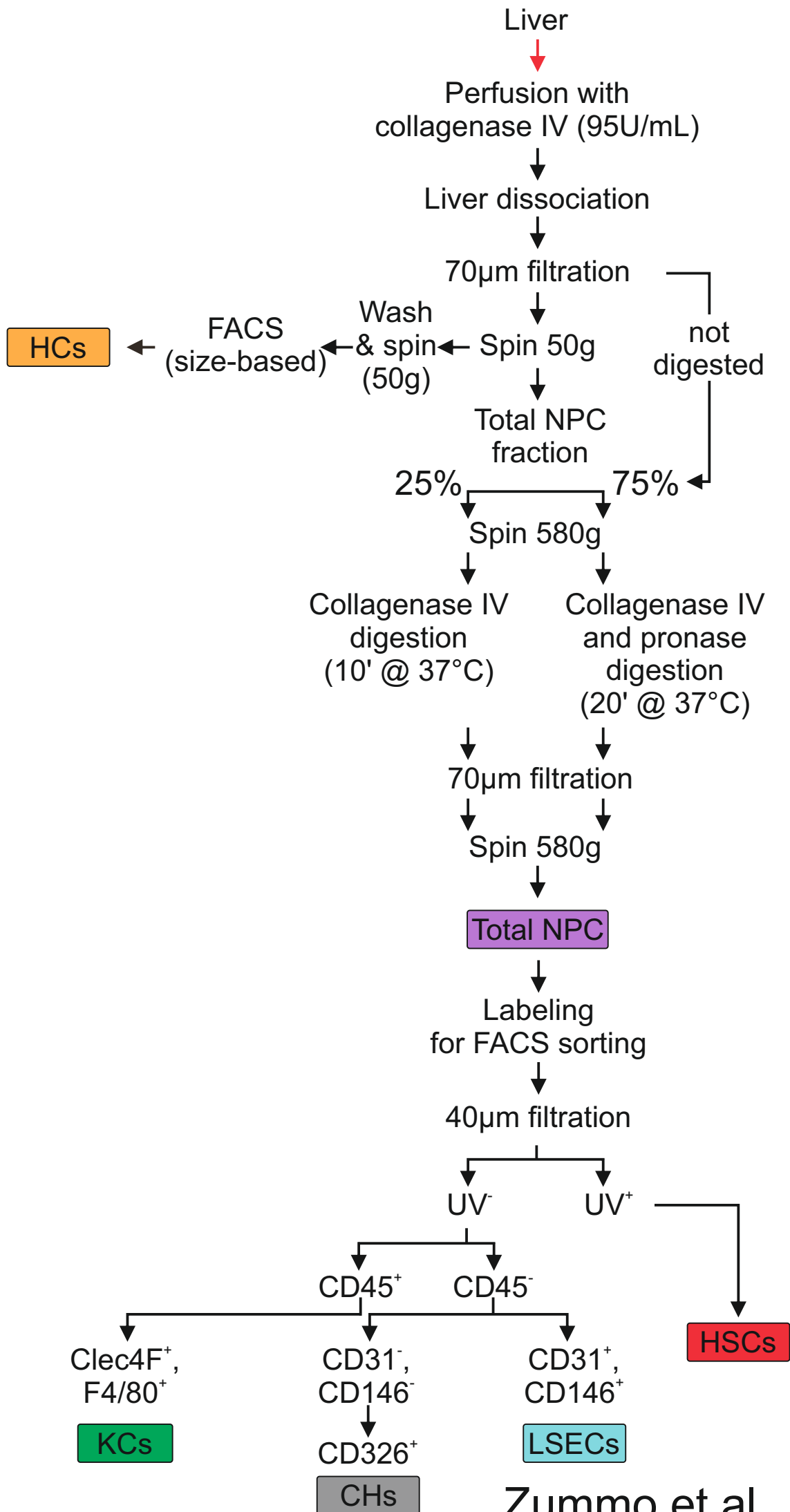
791 **Supplemental Table 2. JTK_cycle output.** Time-dependent transcriptomic data were analyzed using
792 the JTK_cycle R script. Sheet 1: JTK_cycle output for whole liver from ad libitum-fed (AdLib) mice. Sheet
793 2: JTK_cycle output for whole liver from time-restricted-fed (TRF) mice.

794

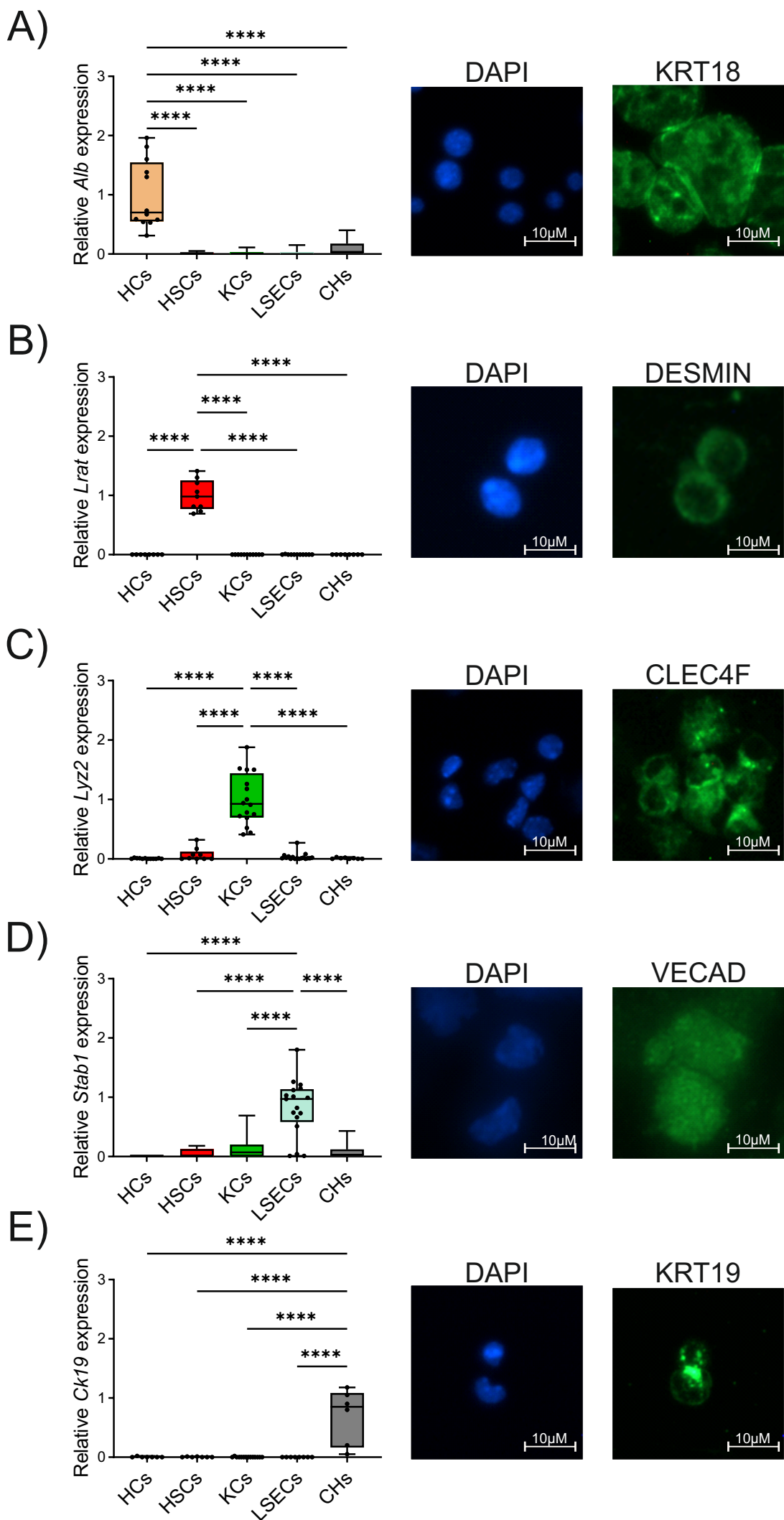
795 **Supplemental Table 3. NR gene counts and NR isoform description.** Sheet 1: Normalized average
796 (n=3) RPKMs are indicated for each mouse NR. The color scale compares NR expression level between
797 cell types (from white, no expression to red, highest expression), numbers are the averaged RPKM.

798

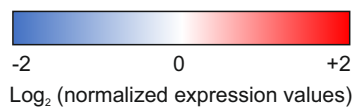
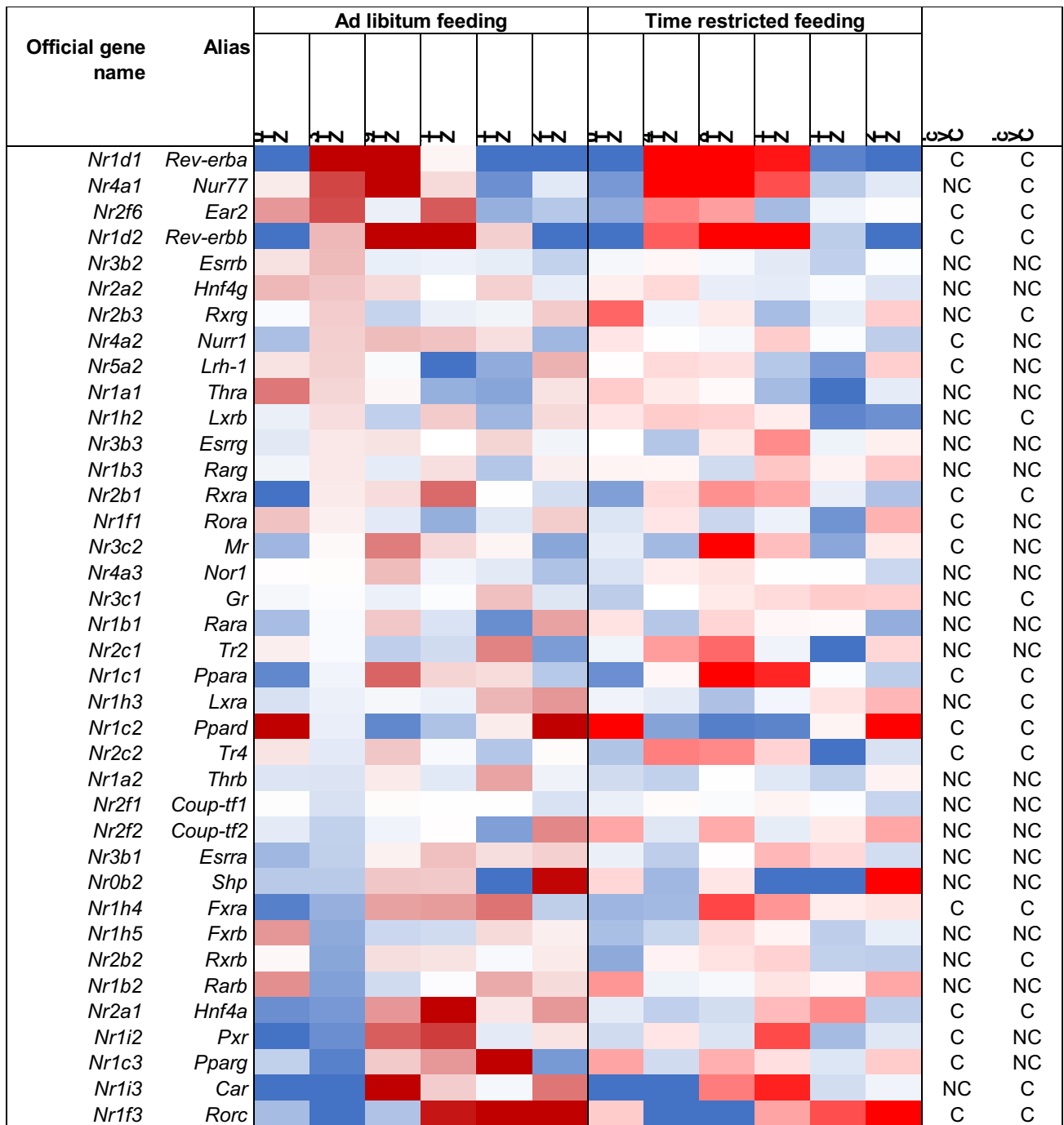
799



Zummo et al., Figure 1

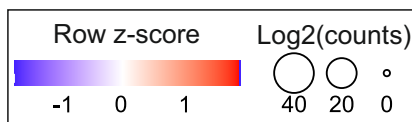
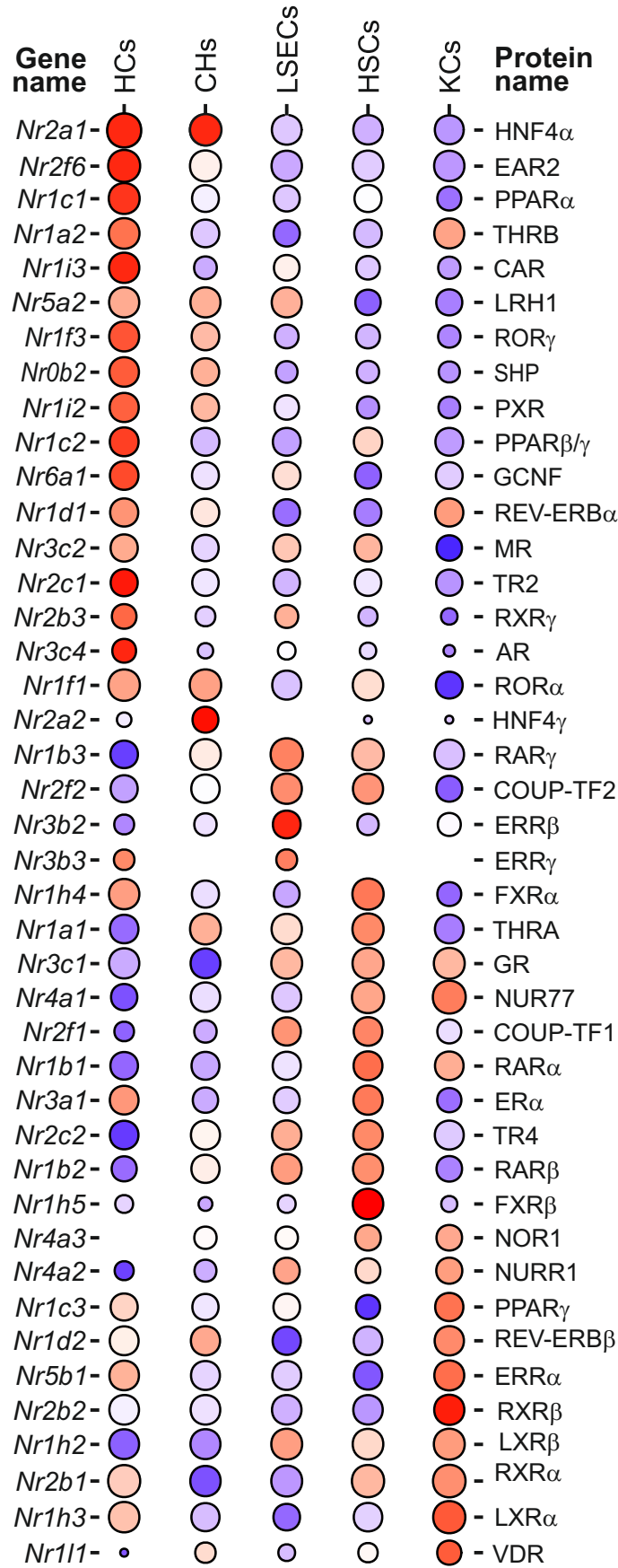


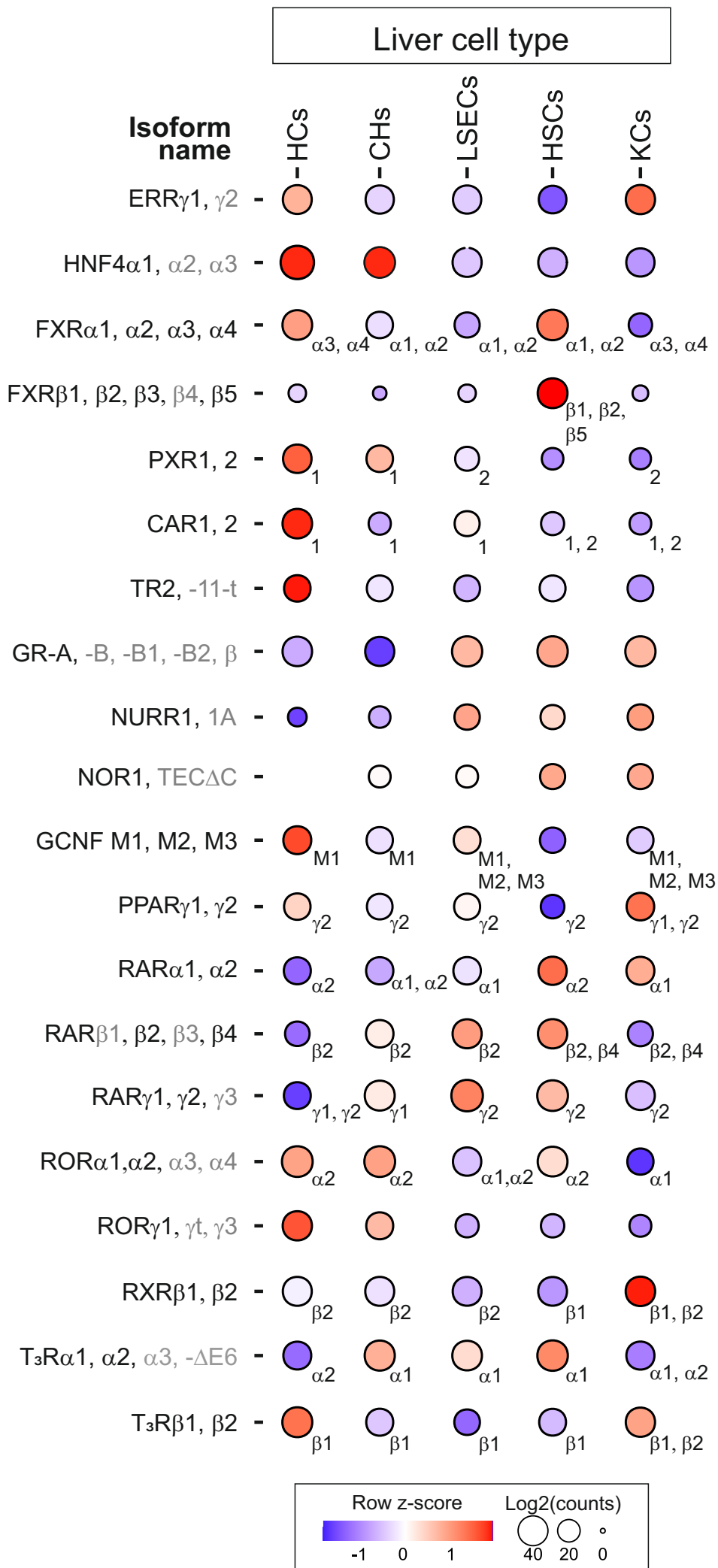
Zummo et al., Figure 2



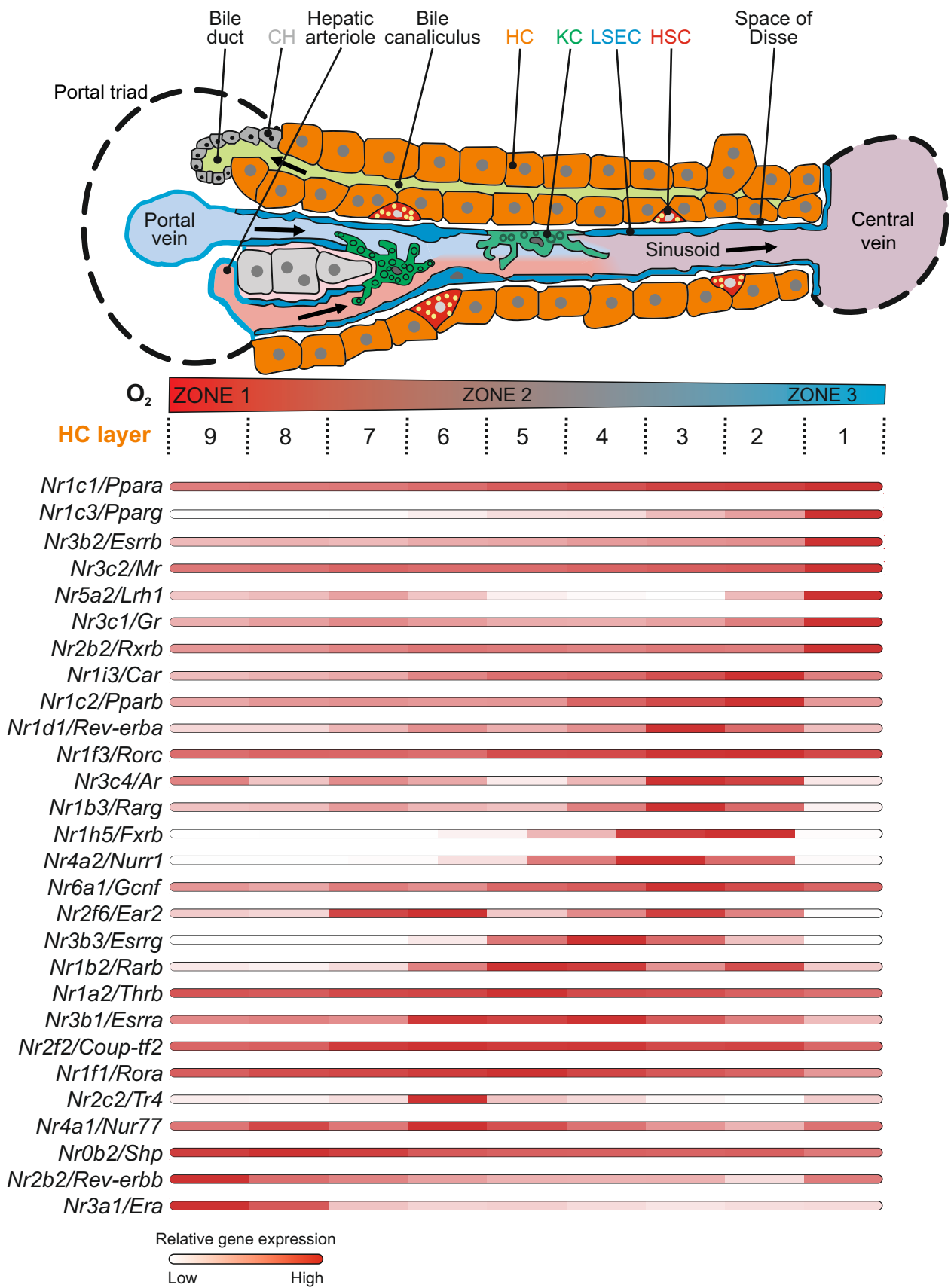
Zummo et al., Figure 3

Liver cell type



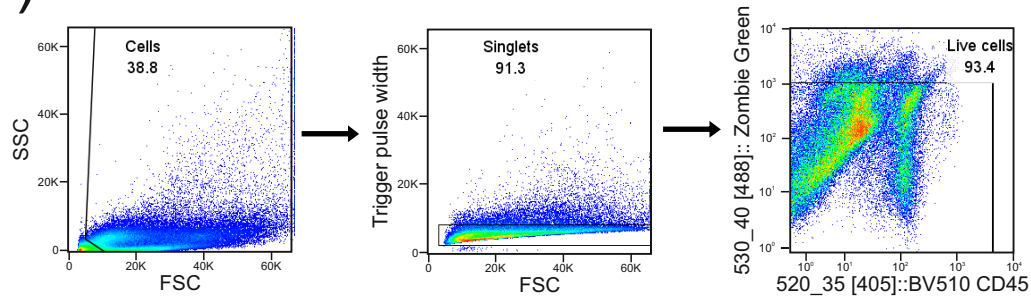


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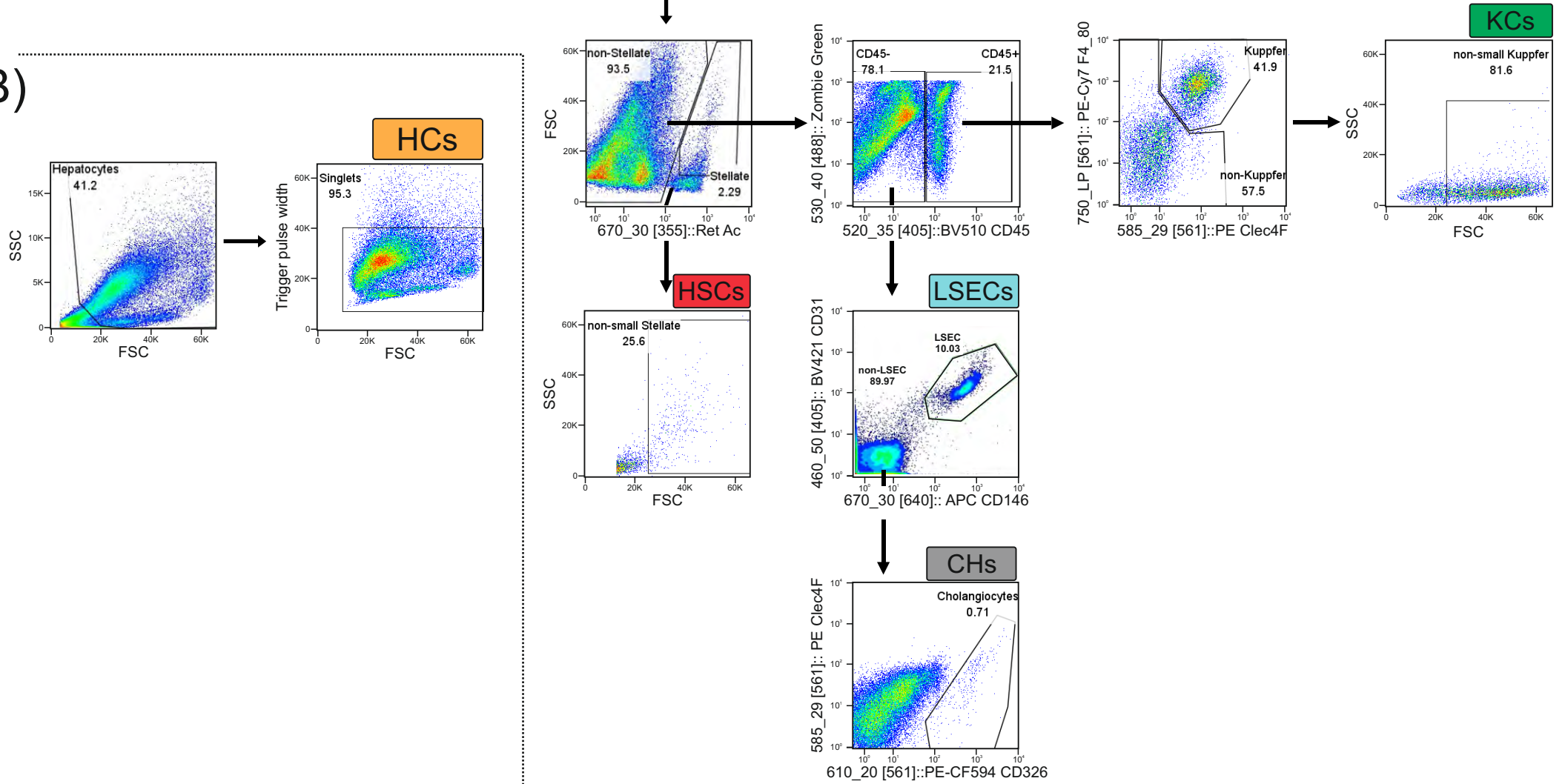


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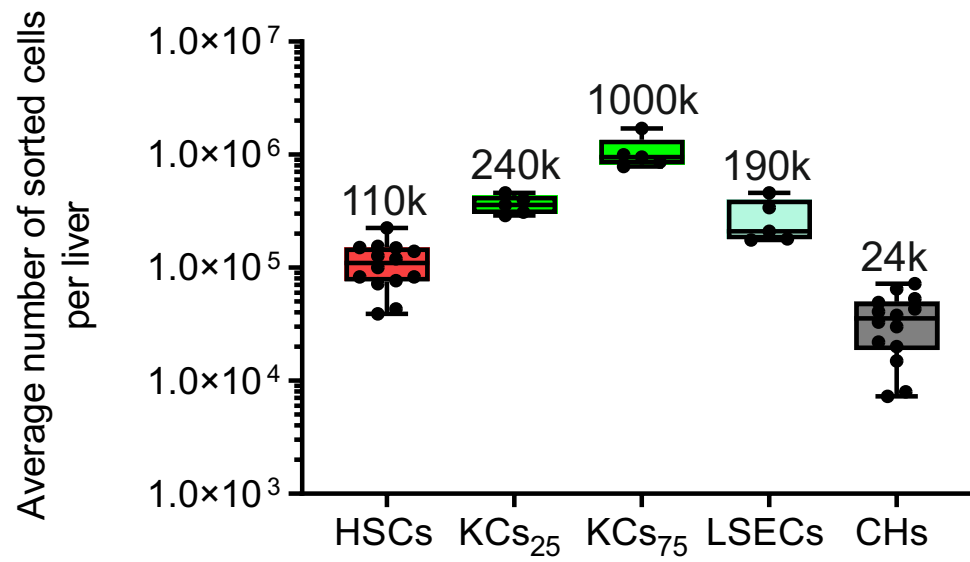
A)



B)

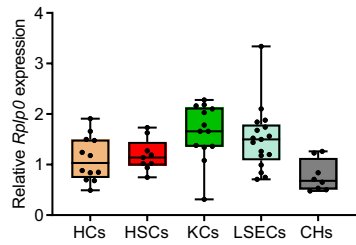


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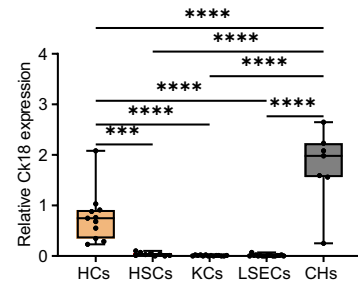


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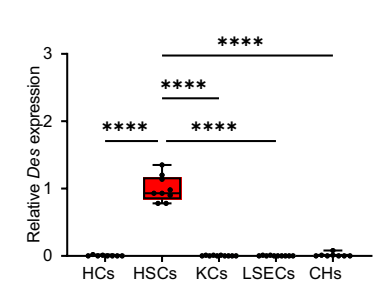
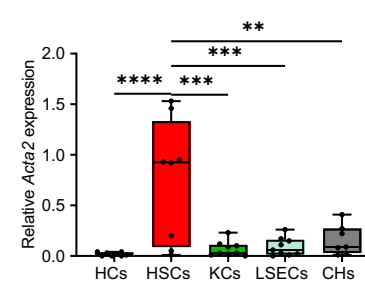
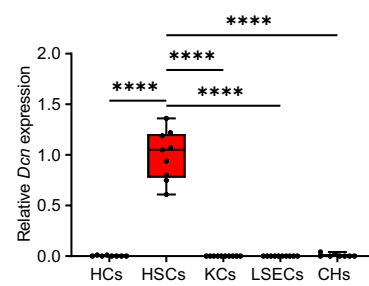
Ubiquitous



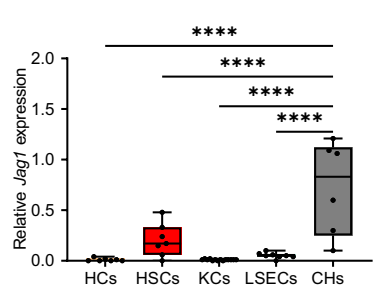
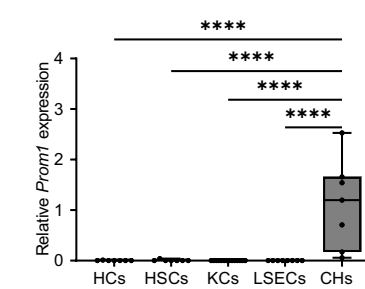
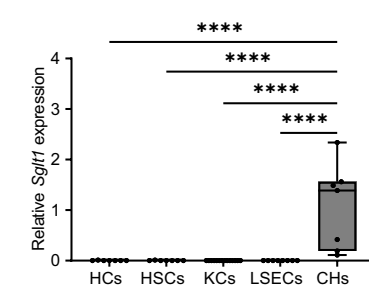
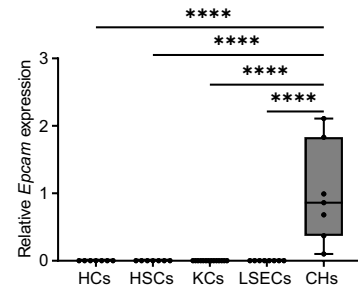
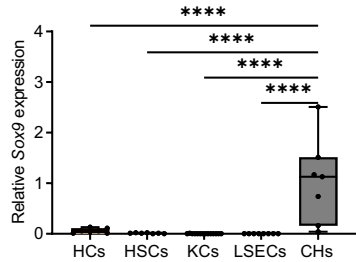
HCs & CHs



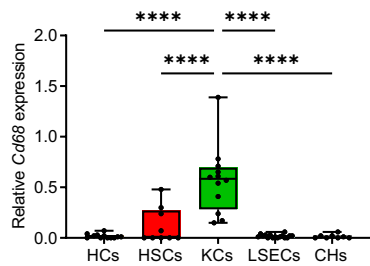
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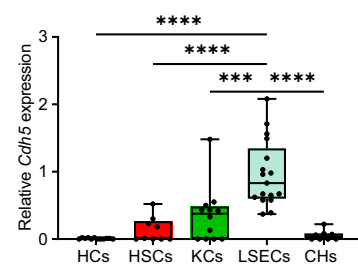
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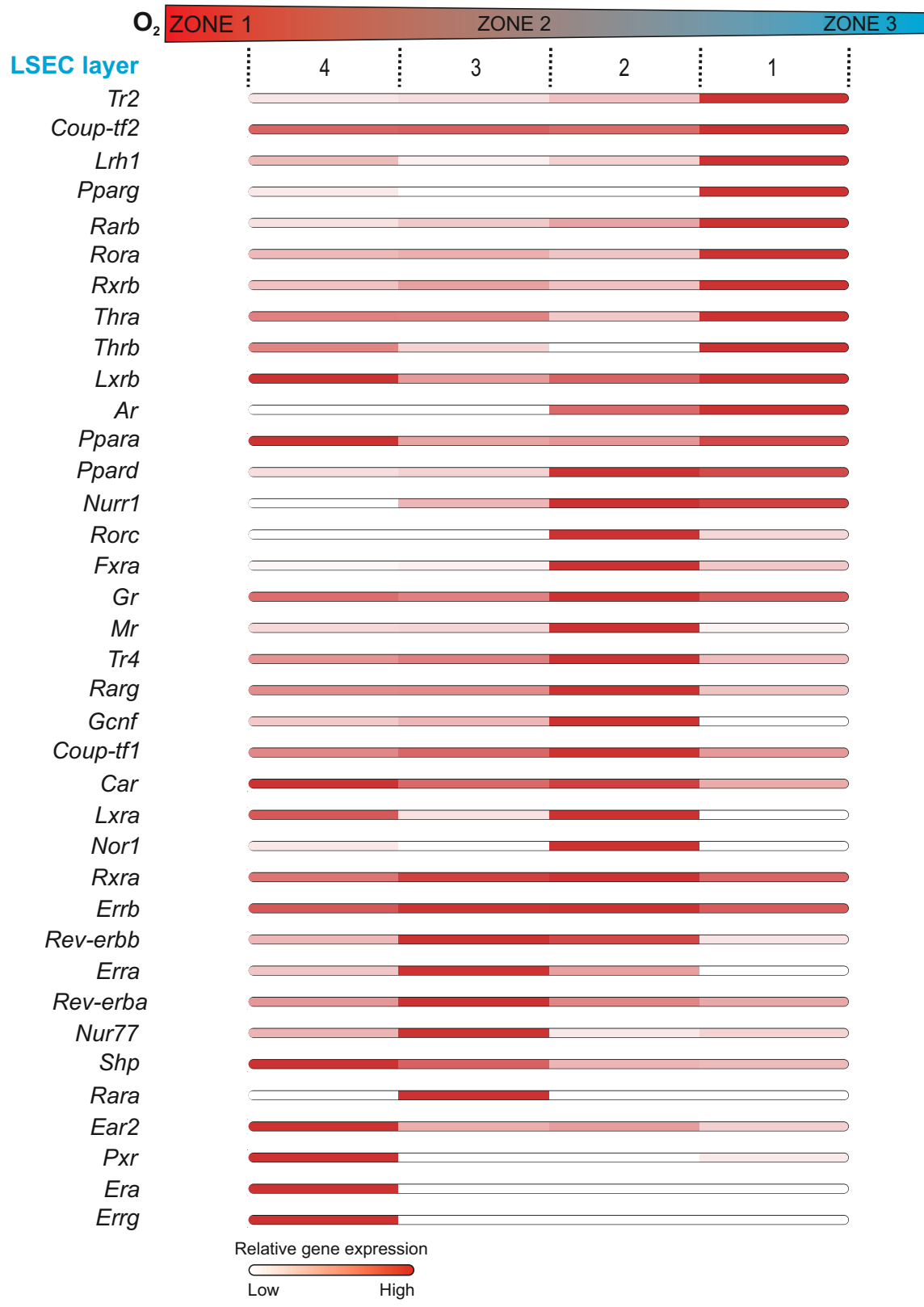
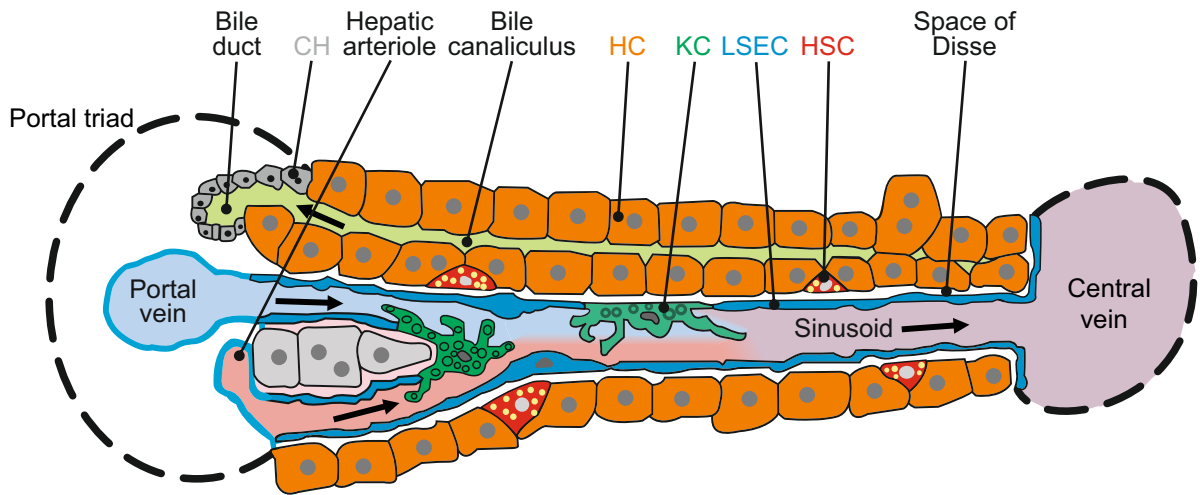


KCs

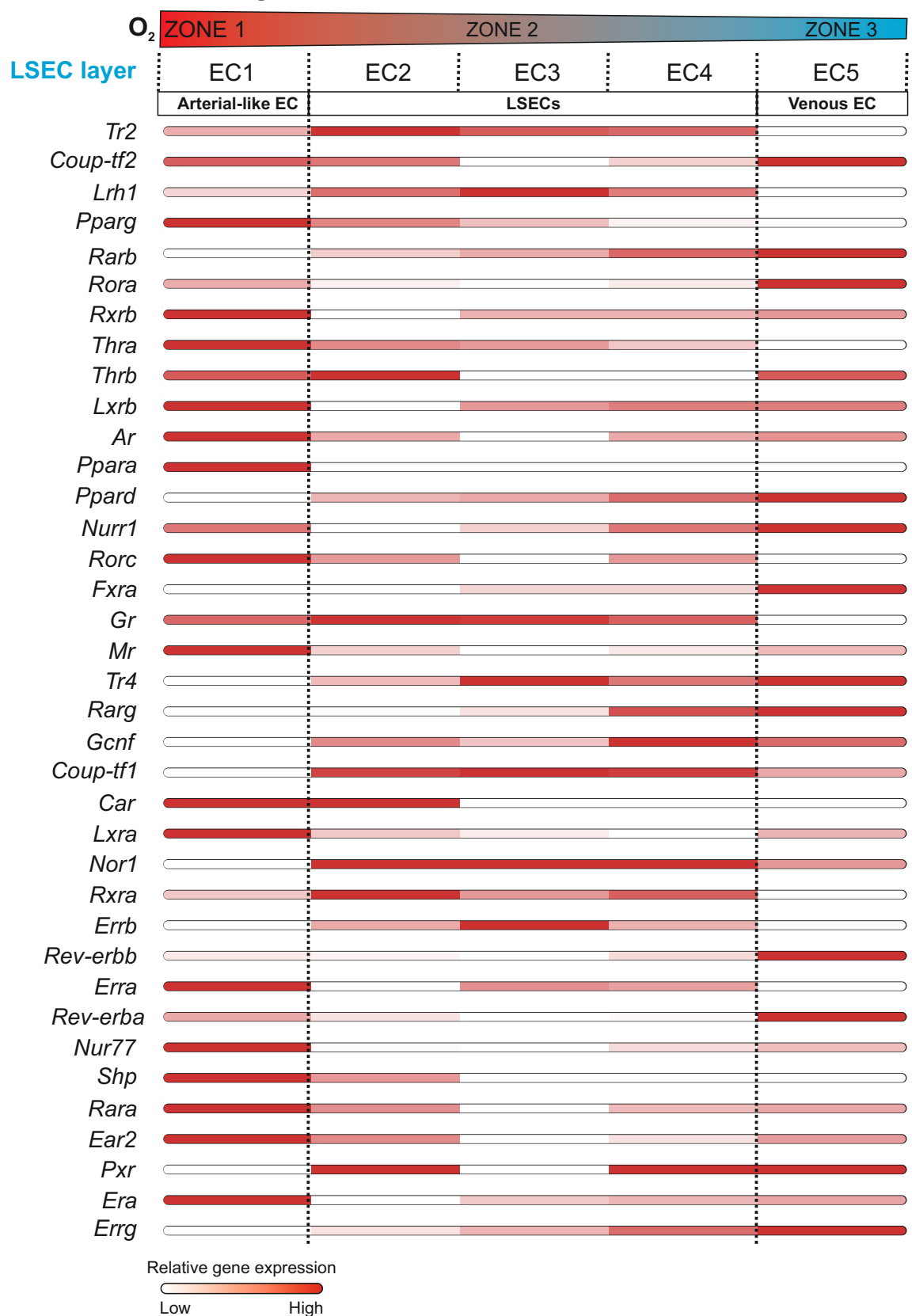
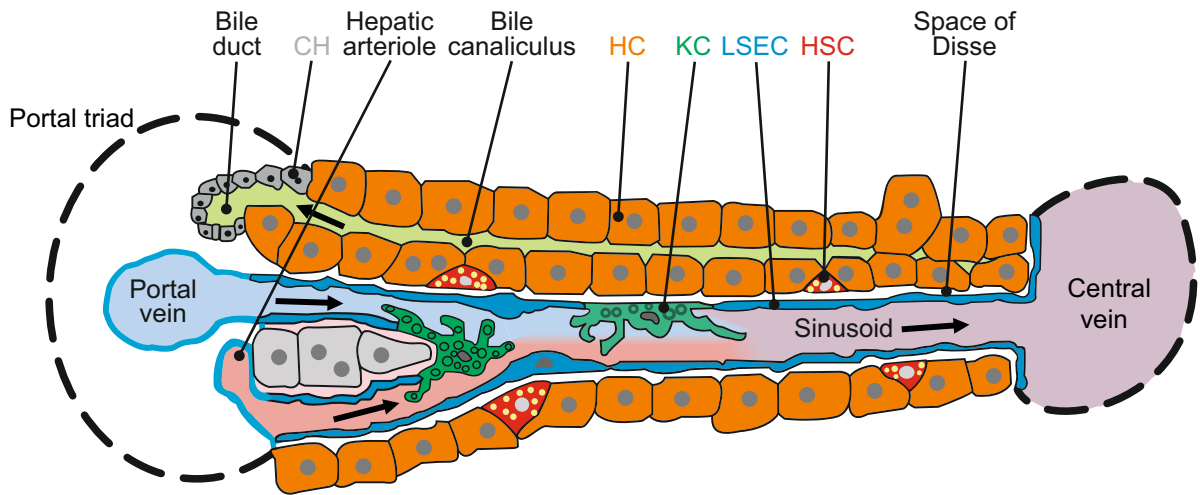


LSECs

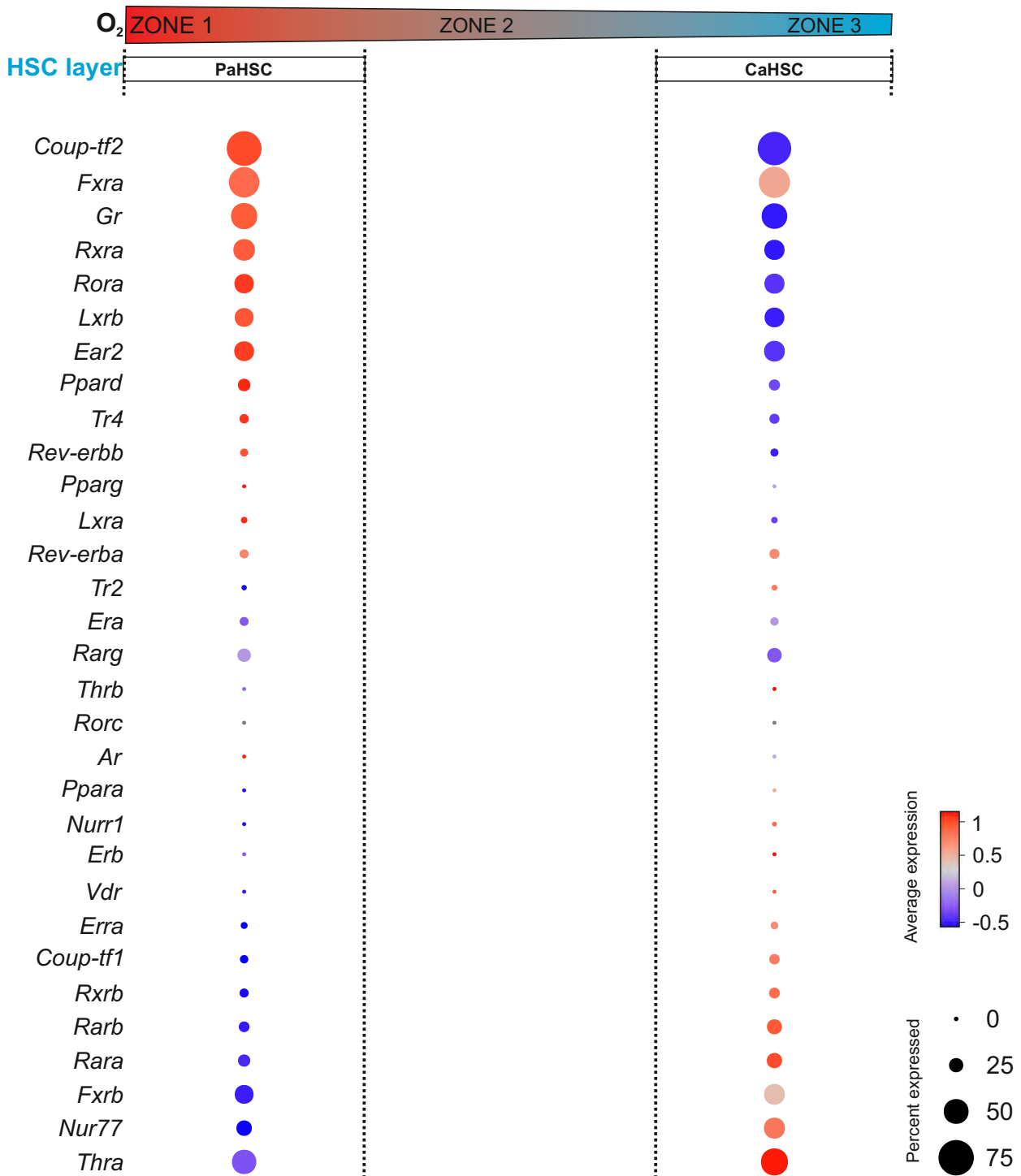
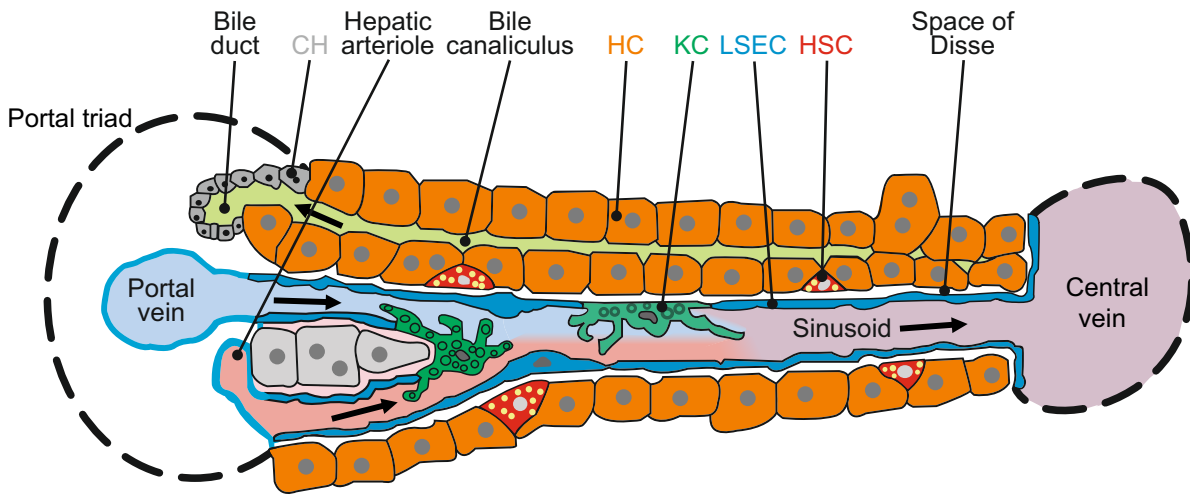




Zummo et al., Supp. Figure 4



Zummo et al., Supp. Figure 5



Zummo et al., Supp. Figure 6