

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

Francesco Paolo Zummo, Alexandre Berthier, Céline Gheeraert, Manjula Vinod, Marie Bobowski-Gérard, Olivier Molendi-Coste, Laurent Pineau, Matthieu Jung, Loic Guille, Julie Chevalier-Dubois, et al.

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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 C57BI6/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 (Fxra) 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.

#### INTRODUCTION

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 and centrifugal bile circulation (Nagy et al., 2020). 61

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 signals regulating transcriptional networks in hepatic cells (Soccio, 2020). NR structure allows them to 64 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 functions have been mostly defined in a hepatocyte background but their expression territory is more 80 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

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

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

#### 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, ZTO 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)["T(ime)-R(estricted) F(eeding) condition]. Liver samples were collected every 3 hours at ZT0, ZT3, ZT6, ZT9, ZT12, ZT15, ZT18 and ZT21 for ad libitum fed mice. Livers were collected every 4 hours 104 105 at ZTO, ZT4, ZT8, ZT12, ZT16, and ZT20 for the time restricted feeding study.

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#### 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 by cervical dislocation at ZT3 and liver was perfused through the vena cava. After a first perfusion with 113 114 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 CaCl<sub>2</sub> 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  $\mu$ m filtration), centrifuged for 5 min at 580 x G and incubated with collagenase (100 U/mL), and 0.5

mg/mL pronase (Sigma-Aldrich, #10165921001) and 10  $\mu$ g/mL DNAseI (DNase I grade II, from bovine pancreas, Sigma-Aldrich, # 10104159001) for 20 min at 37°C. The remaining 25% (NPC25) was further digested with collagenase alone for 10 min at 37°C. Cellular preparations were filtered through a 70  $\mu$ m filter and centrifuged for 5 min at 580 x G in Wash buffer at 37°C. Both NPC fractions are 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 NH<sub>4</sub>Cl, 10mM NaHCO<sub>3</sub>, 0.127mM EDTA, pH7.4) for 4 min before adding 1 ml PBS + 0.5% BSA 134 to stop lysis. Two to  $3x10^6$  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 Sortware. Compensation particles were from Becton-Dickinson (BD<sup>™</sup> 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 visualized on FSC/SSC and FSC-W/FSC-A dotplots, respectively and live cells were then selected as 154 155 "Zombie Green low" events. HSCs were selected as UV<sup>+</sup> granular cells out of the NPC75 fraction, taking advantage of UV light excitation of retinol and retinoic acid contained in HSCs granules (Mederacke et 156 al., 2015). A "non-small HSCs" gating was applied in order to avoid sorting of degranulated or damaged 157 HSCs. LSECs were selected as UV<sup>-</sup> CD45<sup>-</sup> CD146<sup>hi</sup> events of the NPC25 fraction as pronase digestion was 158

not compatible with CD31 detection. CHs were selected as UV<sup>-</sup> CD45<sup>-</sup> CD146<sup>low</sup> CD326<sup>+</sup> events of the
 NPC75 fraction. KCs were selected as UV<sup>-</sup> CD45<sup>+</sup> F4/80<sup>+</sup> CLEC4F<sup>+</sup> events of the NPC75 fraction, and a
 "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).

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#### 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.

Primary antibodies used were: Anti-KRT18 (C-04, Abcam, #ab668)(dilution 1:100), anti-DESMIN (Y66, Abcam, #ab32362)(dilution 1:50), anti-CLEC4F (ThermoFisher, # MA5-24113)(dilution 1:100), anti-VECAD (ThermoFisher, # 36-1900)(dilution 1:50), anti-KRT19 (EP1580Y, Abcam, # ab52625)(dilution 1:100). Secondary antibodies (ThermoFisher Scientific) were used at 1:100 dilution and were goat anti-mouse AF568 (A-11004), donkey anti-rabbit AF488 (A-21206), donkey anti-mouse 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<sup>™</sup> Mini kit Nucleospin<sup>™</sup> (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 reversetranscribed using random primers and the High Capacity cDNA Reverse Transcription Kit 187 188 (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 RplpO (acidic ribosomal phosphoprotein PO) mRNA levels and were used to calculate fold changes using the cycle threshold  $(2^{-\Delta\Delta Ct})$  method (Schmittgen and Livak, 2008). Primer sequences are 193 194 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<sup>™</sup> WT PLUS Reagent Kit (Thermo Fisher
203 Scientific, # 902280), retrotranscribed to single-stranded complementary (ssc) DNA and labeled using
204 GeneChip<sup>™</sup> 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<sub>2</sub> transformation. Then 213 normalized expressions were averaged per Gene Symbol (NetAffx Annotation Release 36, July, 2016) and transcripts within the 10<sup>th</sup> lowest percentile were considered as technically unreliable and 214 215 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.

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

3'end adenylation, adapter ligation and PCR amplification were performed using the TruSeq Stranded
 total RNA sample preparation kit (Illumina). DNAs were sequenced using an Illumina HiSeq 4000
 sequencer in 50 bp Single-Read following Illumina's instructions. Image analysis and base calling were
 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 0.6.1p1 (Anders et al., 2015), with annotations from Ensembl version 94 and "union" mode. Read 231 232 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).

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

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#### 240 Single cell data extraction.

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

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

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

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266

#### 267 Statistical analysis.

268 Statistical analysis was performed using GraphPad Prism (v. 9). Data are plotted as the mean 269 ± SEM. At least 3 independent experimental replicates were obtained. Data were determined to have equal variances using the F test. For 2-group comparisons, an unpaired 2-tailed t-test with Welch 270 271 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.

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

285

### 286 Data availability.

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

291

#### RESULTS

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 x10<sup>6</sup> cells per liver. Further digestion by collagenase and pronase yielded the total nonparenchymal cell (NPC) fraction which was sorted using the indicated combination of antibodies 302 303 (Figure 1 and Supplemental Figure 1). This yielded per liver variable amounts of HSCs, KCs, LSECs and 304 CHs with numbers routinely exceeding 10<sup>5</sup> 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

libitum-fed: Nr4a2(Nurr1), Nr5a2(Lrh1), Nr1f1(Rora), Nr3c2(Mr), Nr1i2(Pxr), Nr1c3(Pparg); timerestricted feeding: Nr2b3(Rxrg), Nr1h2(Lxrb), Nr3c1(Gr), Nr1h3(Lxra), Nr2b2(Rxrb), Nr1i3(Car)].
Irrespective of the functional consequences of such oscillations, these differential expression levels
should be considered when comparing expression levels of NRs in different conditions. We thus
selected ZT3 (3 hours after light-on) as a convenient reference time point to initiate liver cell type
isolation from ad-libitum fed mice (Figure 1). In these conditions, Nr1d1(Rev-erba) and Nr1d2(Reverbb) 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 Nr1b1(Rara). Finally, Nr2b2(Rxrb), Nr4a1(Nur77), Nr1c3(Pparg) and Nr1h3(Lxra) elevated levels were 339 340 a feature of KCs. On the opposite, hepatocytes were characterized by undetectable levels in 341 *Nr4a3(Nor1)* and LSECs by the total absence of *Hnf4q*. 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)]. Twenty out of the 42 detected NRs-encoding transcripts can be potentially expressed as distinct 351 352 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 $\alpha$ , PXR, CAR, GCNF, PPARy,

RARα, β and γ, RORα, RXRβ, T3Rα and β, whose expression levels were qualitatively assessed as 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 $\alpha$  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 $\beta$ ) 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(Esrrb) 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 Figure 5). Zonation patterns matched for 50% (18 out 37) NRs, showed minimal discrepancies for 15 380 381 and were strikingly different for Nr1c1(Ppara), Nr3b1(Esrra), Nr3b3(Errq) 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 Figure 4). Along this axis, Nr3a1(Era), Nr3b1(Esrra) and Nr3b3(Esrrg) displayed an opposite gradient of 384 385 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 2 studies, calling for additional strictly comparative studies to reach a consensual cartography of NRs
 in LSECs. Of note, our LSEC purification procedure relies on a CD31/CD146 double-positive labeling to
 obtain highly pure cell preparations, which may have nevertheless selected a particular subpopulation.

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

DISCUSSION

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/BI6J mice at ZTO (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 C57BI/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 any liver cell types [Nr0b1(Dax1), Nr1f2(Ror $\beta$ ), Nr2e1(Tlx), Nr3e3(Pr) and Sf1(Nr5a1)]. Sexual 418 419 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 transcript431 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, *Nr4a1*(*Nur77*)- and *Nr2c2*(*Tr4*)-encoded transcripts gained strong cyclicity in time-restricted fed mice. 437 438 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 Rarq2 displayed highest levels of expression, while Era and Exra1 and a2 expression were hallmarks of HSCs. Finally, KCs displayed highest levels in Nr3b1(Esrra), Nr1h3(Lxra) 443 444 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 zonated 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 zonated expression of 451 PPARs in HCs (Berthier et al., 2021). PPAR $\alpha$  appears to be preferentially pericentral, together with 452 PPARα-driven lipogenic enzymes. However, fatty acid oxidation, which is also controlled by PPARα, 453 mostly occurs in the oxygen-rich periportal area where a decreased, but not absent, expression of 454 *Ppara* is observed. In contrast, *Ppary* is uniquely expressed in pericentral HCs, in line with its pro-455 lipogenic activities and the metabolic zonation of HCs. We note that Rxr8 is also preferentially 456 expressed in the pericentral area. As a PPARy heterodimerization partner, RXRB confers increased 457 transcriptional activity to PPARγ when compared to RXRα (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

area. We note that NR agonists efficiently blocking NASH and fibrosis progression, at least in rodent
 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 functional repertoire of NRs, as described for "metabolic NRs" (Mukha et al., 2021). While our study 467 468 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 Pparq2; Rara1 vs Rara2; Rarg1 vs RARq2; 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, PPARy), 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 $\alpha$ 1 and  $\alpha$ 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 $\alpha$ 3 and 488  $\alpha$ 4, whereas HSCs express mostly FXR $\alpha$ 1 and  $\alpha$ 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.

Taken as a whole, this study provides a compendium of NR expression in parenchymal and non-parenchymal liver cells which calls for an in-depth investigation of NR functions in liver cell populations. In addition to the multiple layers of NR activity regulation, that ranges from ligand availability, dimerization, transcriptional comodulator interaction and post-translational 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.

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708

#### FIGURE LEGENDS

## 710 711

Figure 1 – Liver cell type isolation protocol. The sequential steps of liver cell type purification by 712 713 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

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

726

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

733

Figure 4. NR expression in purified liver cell types. RNA extracted from each cell type preparation (n=3) was analyzed by single-end 50b RNAseq. After (pre)processing, mapping and normalization by the median-of-ratios method to make counts comparable between samples, log<sub>2</sub> expression values were used to generate a bubble plot in which row Z-score of RPKM (p adj < 0.05) is indicated (genes in red are up-regulated, genes in blue are down-regulated) on a row-by-row basis. The size of the bubble is proportional to the expression level (empty spaces indicate no significant expression).

740

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

744

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

752

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

758

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

762

Supplemental Figure 3. Cell purity assessment by gene expression profiling. RNAs extracted from
 each purified cell type was analyzed by RT-qPCR (n=9-16) and probed for the expression of ubiquitous
 (*Rplp0/36b4*), common (HCs and CHs, *Ck18*) or specific (HSCs: *Dcn, Acta2, Des*; CHs: *Sox9, Epcam, Sglt1, Prom1, Jaq1*; KCs: *Cd68*; LSECs: *Cdh5*) cell markers.

767

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

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

774

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

781

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

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

794

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

798





			Ad	libitu	m fee	ding			Time	e restri	cted f	eeding	3		
Official gene	Alias														
name															
		3-N	7-N	л <del>н</del> л	ΗN	ΗN	<b>7</b> -7	-N	3H-N	d-N	ΗN	ΗN	<b>1</b> -1	.u>U	.ഗ>ഗ
Nr1d1	Rev-erba													С	С
Nr4a1	Nur77													NC	С
Nr2f6	Ear2													С	С
Nr1d2	Rev-erbb													С	С
Nr3b2	Esrrb													NC	NC
Nr2a2	Hnf4g													NC	NC
Nr2b3	Rxrg													NC	С
Nr4a2	Nurr1													С	NC
Nr5a2	Lrh-1													С	NC
Nr1a1	Thra													NC	NC
Nr1h2	Lxrb													NC	С
Nr3b3	Esrrg													NC	NC
Nr1b3	Rarg													NC	NC
Nr2b1	Rxra													С	С
Nr1f1	Rora													С	NC
Nr3c2	Mr													С	NC
Nr4a3	Nor1													NC	NC
Nr3c1	Gr													NC	С
Nr1b1	Rara													NC	NC
Nr2c1	Tr2													NC	NC
Nr1c1	Ppara													С	С
Nr1h3	Lxra													NC	С
Nr1c2	Ppard													С	С
Nr2c2	Tr4													С	С
Nr1a2	Thrb													NC	NC
Nr2f1	Coup-tf1													NC	NC
Nr2f2	Coup-tf2													NC	NC
Nr3b1	Esrra													NC	NC
Nr0b2	Shp													NC	NC
Nr1h4	Fxra													С	С
Nr1h5	Fxrb													NC	NC
Nr2b2	Rxrb													NC	С
Nr1b2	Rarb													NC	NC
Nr2a1	Hnf4a													С	С
Nr1i2	Pxr													С	NC
Nr1c3	Pparg													С	NC
Nr1i3	Car													NC	С
Nr1f3	Rorc													С	С

-2 0 +2 Log<sub>2</sub> (normalized expression values)









Zummo et al., Supp. Figure 1

KCs

non-small Kuppfer 81.6

40K

20K FSC 60K

Kuppfer

41.9

non-Kuppfei

57.5

10° 10' 102 103 104 585\_29 [561]::PE Clec4F

60K

20K

SSC



Zummo et al., Supp. Figure 2



CHs





Zummo et al., Supp. Figure 3



<b>O</b> <sub>2</sub>	ZONE 1			ZONE	E 2				ZONE 3
LSEC layer		4		3		2		1	
Tr2			:		:		:		
Coup-tf2									
Lrh1									
Pparg									
Rarb									
Rora									
Rxrb	Ĩ								
Thra									
Thrb									
Lxrb									
Ar	0								
Ppara									
Ppard									
Nurr1	C								
Rorc	0								
Fxra									
Gr									
Mr									
Tr4									
Rarg									
Gcnf	Ĩ								$\square$
Coup-tf1									
Car									
Lxra									$\square$
Nor1									$\square$
Rxra									
Errb									
Rev-erbb	Ē								
Erra									$\square$
Rev-erba									
Nur77									
Shp									
Rara	0								$\square$
Ear2									
Pxr									
Era									
Errg									$\square$
	Re	lative gene e	expression						
	L	ow	High						

# Zummo et al., Supp. Figure 4





	NE 1	ZONE 2	ZONE 3	
HSC layer	PaHSC		CaHSC	
Coup-tf2				
Fxra				
Gr	<b></b>			
Rxra	ē			
Rora	•			
Lxrb	•			
Ear2	•			
Ppard	•		•	
Tr4	•		•	
Rev-erbb	•		•	
Pparg	•		•	
Lxra	•		•	
Rev-erba	•		•	
Tr2	•		•	
Era	•		•	
Rarg	•		•	
Thrb	•		•	
Rorc				
Ar	•		•	
Ppara	•		•	Ę
Nurr1	•		•	essic
Erb	•		•	expre
Vdr	•		•	age i
Erra	•		•	Avera
Coup-tf1	•		•	
Rxrb	•		•	
Rarb	•		•	sed
Rara	•		•	ress
Fxrb	•			t exp
Nur77	•			rcen
Thra				Ре
	•		• •	-

Zummo et al., Supp. Figure 6