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Supplemental Information

Synthetic augmentation of bilirubin metabolism in human pluripotent stem cell-derived liver organoids

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Supplementary data related to manuscript:

Synthetic Augmentation of Bilirubin Metabolism in Human Pluripotent Stem Cell-derived Liver Organoids

Reza HA *et al.*, 2023

Overview

Supplementary Figures and Legends

Figure S1. Development and characterization of HLOs from healthy iPSC. Related to Figure 1.

Figure S2. Further development and characterization of HLOs from all cell lines. Related to Figure 1.

Figure S3. Effect of Dexamethasone and Mifepristone on HLOs. Related to Figure 2-3.

Figure S4. Characterization of mGULO iPSC and eHLO. Related to Figure 4.

Figure S5. Orthotropic transplantation of HLOs improve overall liver health. Related to Figure 5.

Supplementary Tables

Table S1: List of antibodies used for immunostaining (IC), ELISA, ChIP-qPCR (ChIP) and organoid experiment. Related to all figures.

Table S2: List of TaqMan probes used for qPCR. Related to all figures.

Table S3: List of Custom primers used for ChIP-qPCR. Related to Figure 2.

Supplementary Figures and Legends

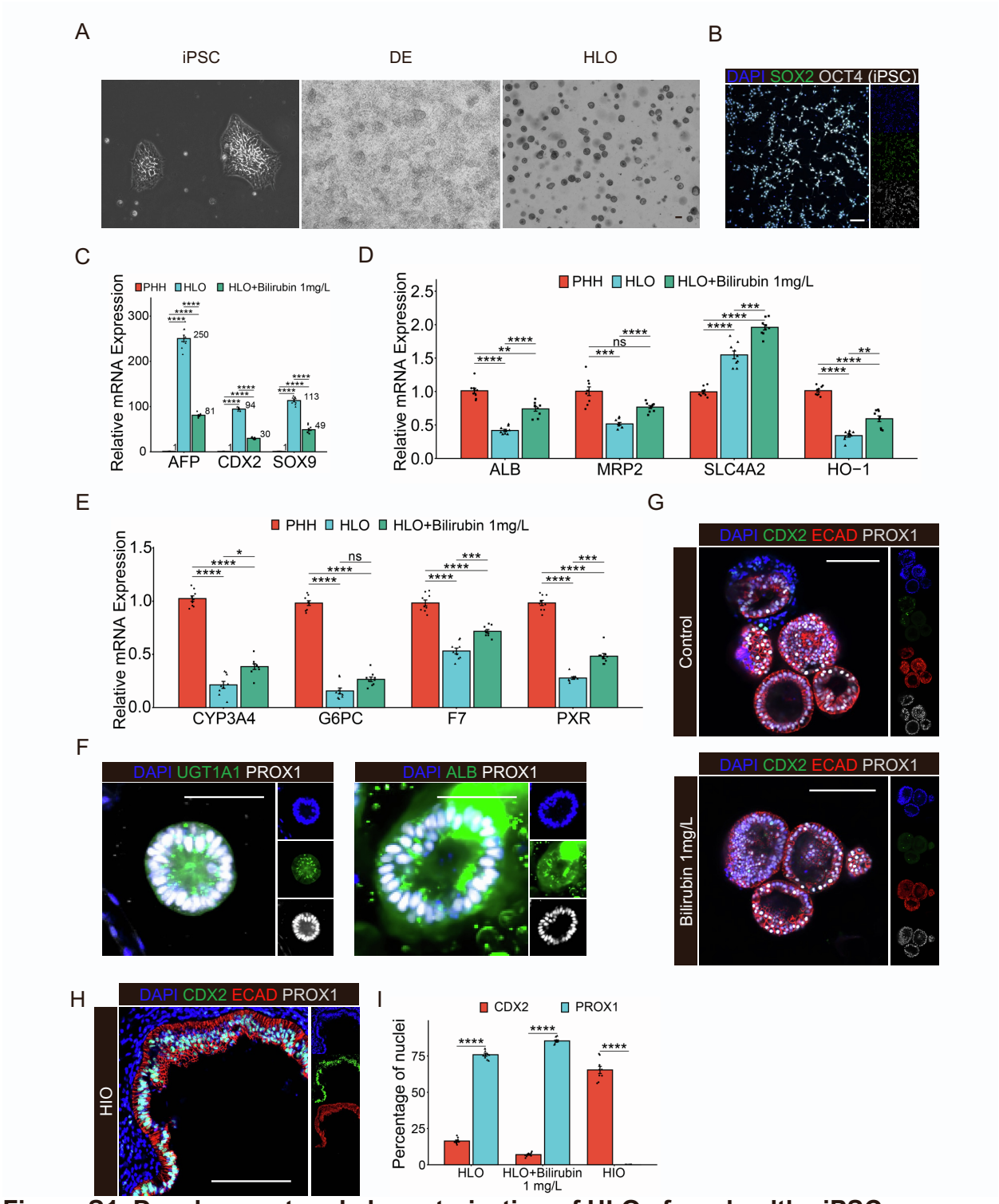


Figure S1. Development and characterization of HLOs from healthy iPSC.

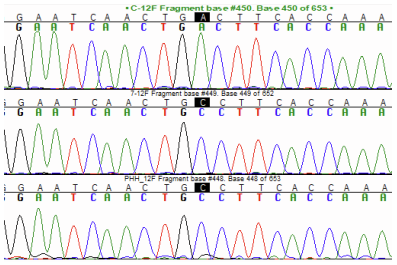
A) Brightfield image of healthy iPSCs, Definitive Endoderm, and HLOs. Scale bar indicates 200 µm.

B) Immunofluorescence images of healthy iPSCs depicting pluripotent markers such as SOX2 and OCT4. Scale bar indicates 200 μm.
C) RT-qPCR of *AFP* and *CDX2* gene for HLOs primed with bilirubin compared to control HLOs and PHH. (Data is mean ± SD, n = 9 independent experiments)
D) RT-qPCR of *ALB, NANOG, SLC4A2,* and *HO-1* gene for HLOs primed with bilirubin compared to control HLOs and PHH. (Data is mean ± SD, n = 9 independent experiments)
E) RT-qPCR of *CYP3A4, G6PC, F7,* and *PXR* gene for HLOs primed with bilirubin compared to control HLOs and PHH. (Data is mean ± SD, n = 9 independent experiments)
F) Immunofluorescence images of healthy HLOs for UGT1A1, ALB, and PROX1. Scale bar indicates 200 μm.
G) Immunofluorescence images for CDX2, PROX1, and ECAD of bilirubin primed HLOs compared to control. Scale bar indicates 200 μm.
H) Immunofluorescence images for CDX2, PROX1, and ECAD of HIOs. Scale bar indicates 200 μm.
I) Quantitation of CDX2+ and PROX1+ nuclei in bilirubin primed HLOs compared to control.

A

CNS patient profile	
Ethnicity	Japanese
Disease	CNS Type 1, Jaundice (Total Bili: 20mg/dL), growth retardation
Treatment	Non-responsive to phototherapy, phenobarbital
Mutation	UGT1A1 exon1 point mutation

B



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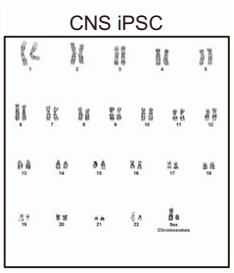

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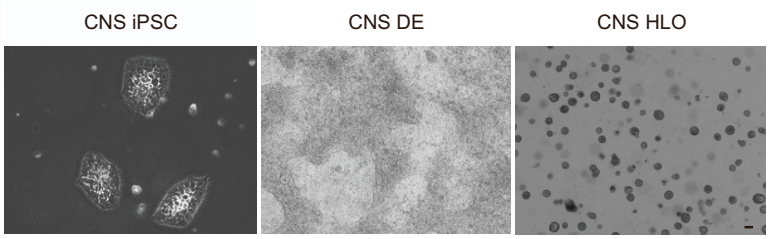

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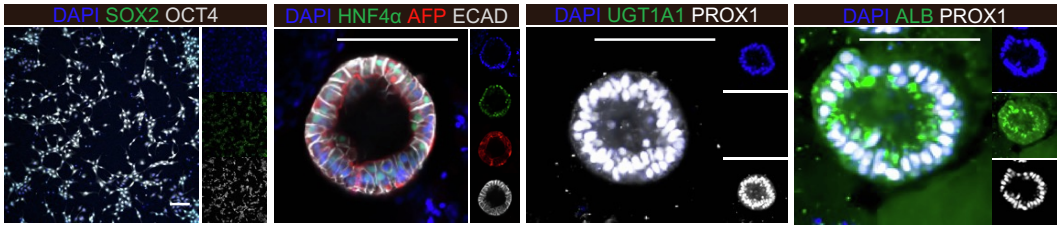

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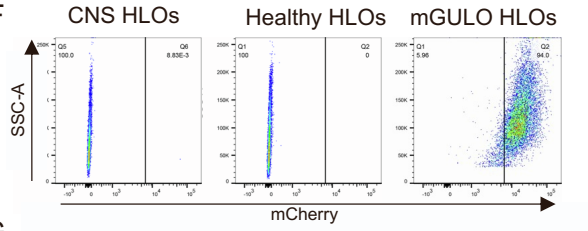

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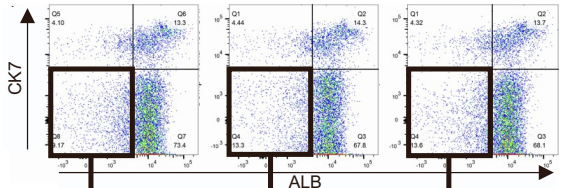

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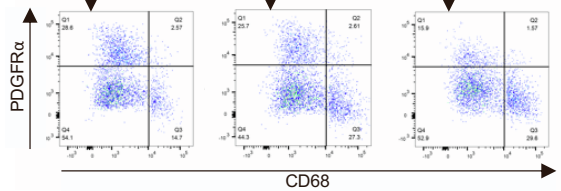

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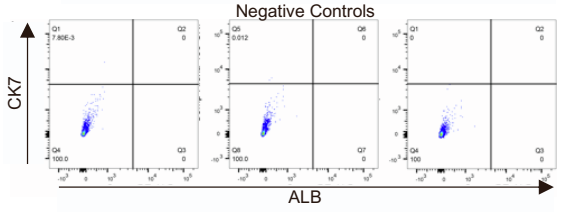

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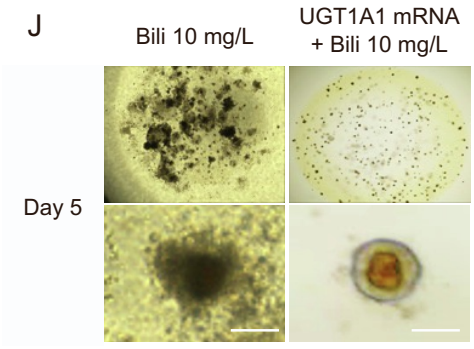

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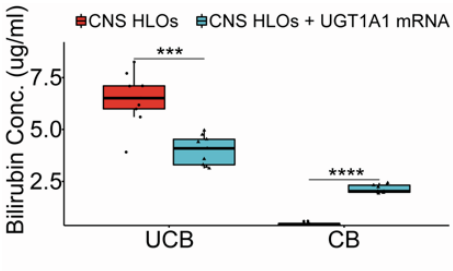

Figure S2. Further development and characterization of HLOs from all cell lines.

A) Patient details for the person from which the CNS iPSC was derived from.
B) Sanger sequencing depicting a c.858C>A (p.Cys280X) nonsense mutation in CNS iPSC compared to healthy iPSC and PHH.
C) Karyotype analysis of CNS iPSC line: standard metaphase spreads and G-banded karyotype were processed and interpreted by the CCHMC cytogenetics core.
D) Brightfield image of CNS iPSCs, Definitive Endoderm, and HLOs. Scale bar indicates 200 μm.
E) Immunofluorescence images of CNS iPSCs depicting pluripotent markers such as SOX2 and OCT4. The CNS HLOS express HNF4α, UGT1A1, ALB, PROX1, and AFP. Scale bar indicates 200 μm.
F) CNS iPSC, Healthy iPSC, and mGULO iPSC derived HLOs exhibit similar cellular diversity. Only mGULO HLOs exhibit 94% mCherry+ cells.
G) CNS HLOs, Healthy HLOs, and mGULO HLOs have 86.7, 82, and 81.7% ALB+ cells respectively indicating hepatocytes while 17.4, 18.8 and 18.1% CK7+ cells respectively were observed which were cholangiocyte like cells. Additionally, there was an ALB+ CK7+ (dual positive population) that were hepatoblast like cells in all 3 organoid lines (13.3, 14.3 and 13.7% in CNS HLOs, Healthy HLOs, and mGULO HLOs respectively).
H) Finally, 2.62, 3.42, and 2.16% (28.6, 25.7, and 15.9% of ALB- CK7-) PDGFRα+ stellate like cells and 1.35, 3.63, and 4.03% (14.7, 27.3, and 29.6% of ALB- CK7-) CD68 expressing macrophage like cells were observed in CNS HLOs, Healthy HLOs, and mGULO HLOs respectively.
I) Negative controls have almost 0% of ALB+ and CK7+ cells.
J) Brightfield image of CNS HLOs transfected with UGT1A1 mRNA compared to control after treatment with bilirubin (10 mg/L).
K) Bilirubin assay measuring unconjugated and conjugated bilirubin in CNS HLOs transfected with UGT1A1 mRNA compared to control. (n = 9 independent experiments)

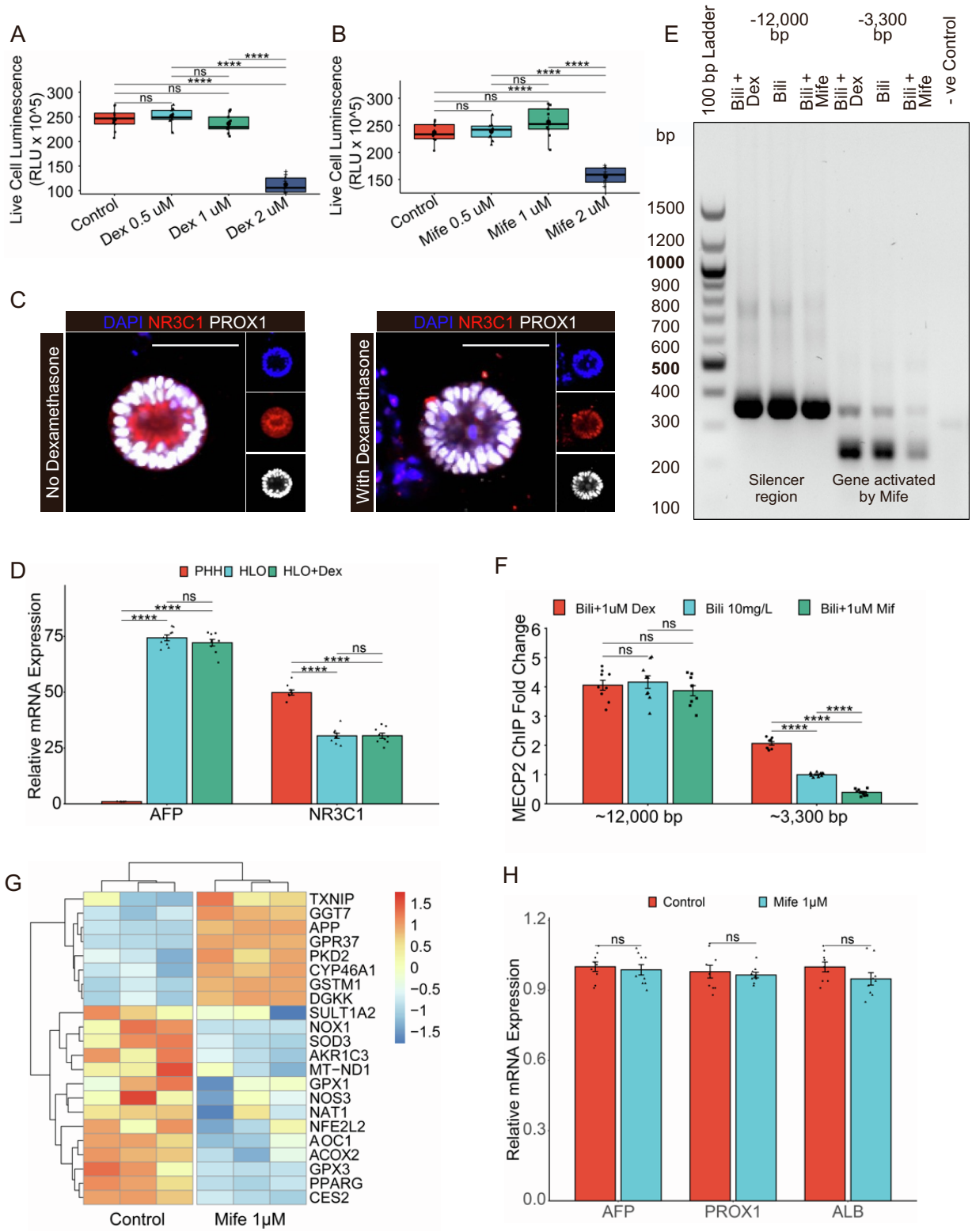

Figure S3. Effect of Dexamethasone and Mifepristone on HLOs.

A) Cell Viability Assay on HLOs treated with different concentration of Dexamethasone.

B) Cell Viability Assay on HLOs treated with different concentration of Mifepristone.

C) Immunofluorescence images for NR3C1, and PROX1of dexamethasone treated

HLOs compared to control. Scale bar indicates 200 μm.

D) RT-qPCR of *NR3C1* and *AFP* gene for HLOs treated with Dexamethasone compared to control and PHH. (Data is mean ± SD, n = 9 independent experiments)

E) MECP2 ChIP-PCR for organoids treated with 10mg/L Bilirubin and Mifepristone (1 uM) or Dexamethasone (1 uM).

F) MECP2 ChIP-qPCR for samples in (F). (Data is mean ± SD, n = 9 independent experiments)

G) Heatmap for genes important for both Xenobiotic and ROS metabolism in Mifepristone treated organoids compared to control after treatment with bilirubin.

H) RT-qPCR of *ALB, PROX1,* and *AFP* gene for Mifepristone treated organoids compared to control after treatment with bilirubin. (Data is mean ± SD, n = 9 independent experiments)

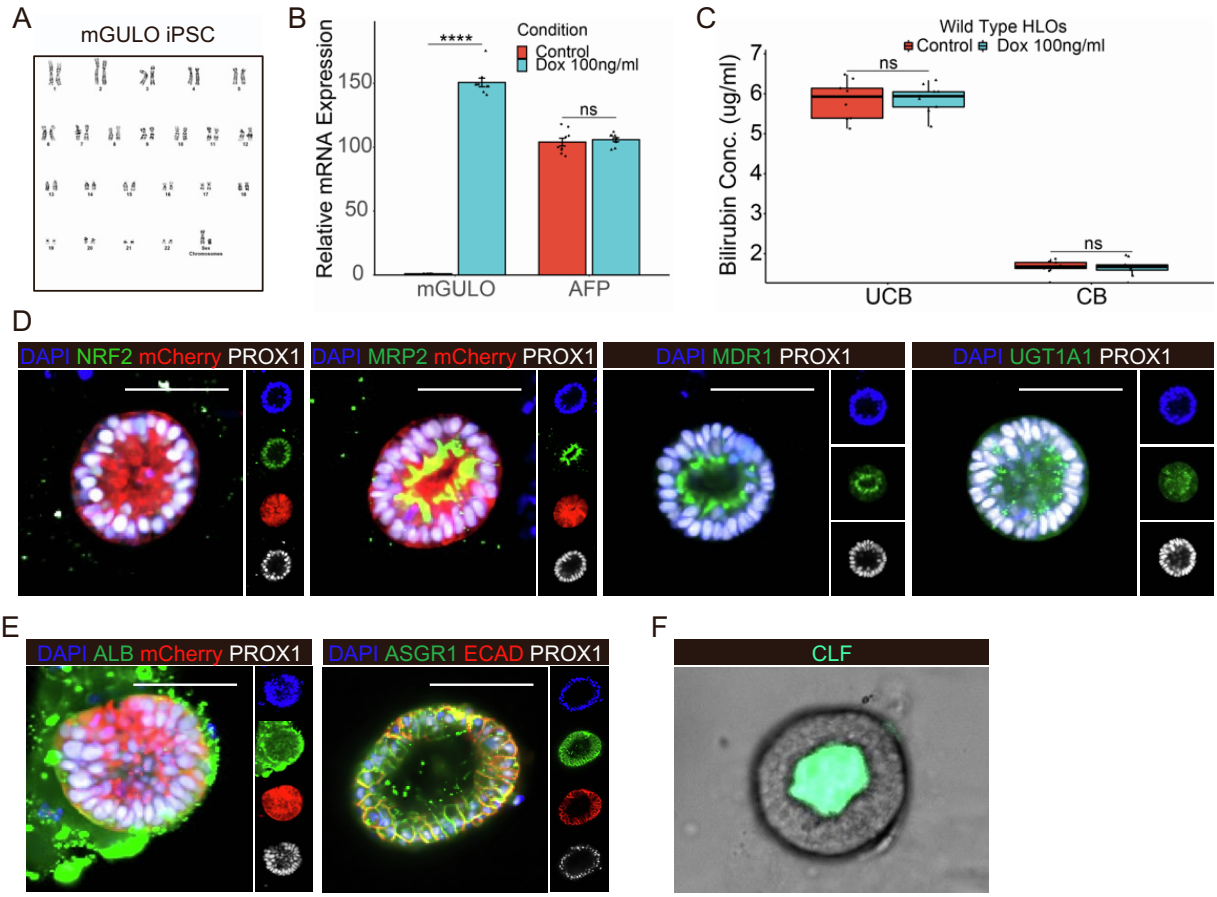

Figure S4. Characterization of mGULO iPSC and eHLO.

A) Karyotype analysis of mGULO iPSC line: standard metaphase spreads and G-banded karyotype were processed and interpreted by the CCHMC cytogenetics core.

B) RT-qPCR of *mGULO* and *AFP* gene for mGULO organoids treated with Dox (100 ng/ml) compared to control. (Data is mean ± SD, n = 9 independent experiments)

C) Bilirubin assay measuring unconjugated and conjugated bilirubin in wild type HLOs treated with bilirubin (10 mg/L) and Dox (100 ng/ml) compared to control. (n = 9 independent experiments)

D) Immunofluorescence images for NRF2, mCherry, MRP2, MDR1, UGT1A1, and PROX1 in eHLOs. Scale bar indicates 200 μm.

E) Immunofluorescence images for mCherry, ALB, PROX1, ASGR1, and ECAD in eHLOs. Scale bar indicates 200 μm.

F) Fluorescent bile acid (CLF, green) uptake assay in eHLOs. Scale bar indicates 200 μm.

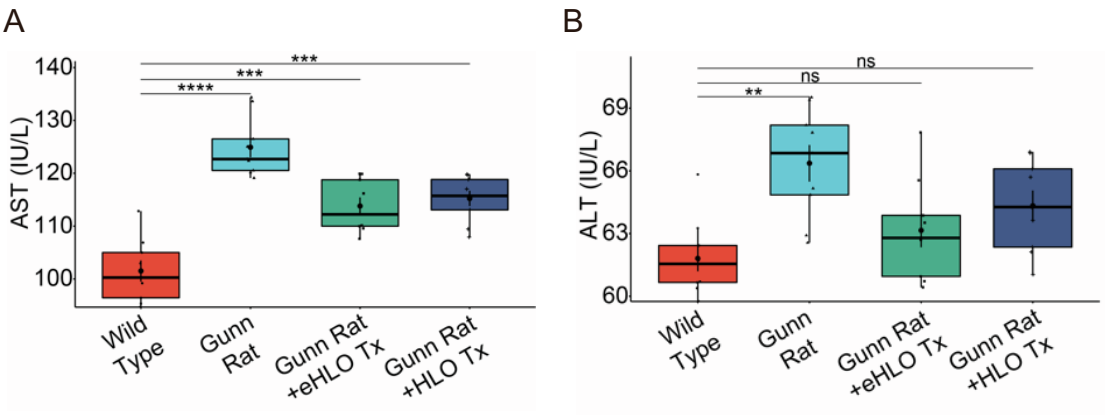

Figure S5. Orthotropic transplantation of HLOs improve overall liver health.

A) AST assay on Gunn rats after transplantation compared to sham and wild type rats. (n = 9 independent experiments)

B) ALT assay on Gunn rats after transplantation compared to sham and wild type rats. (n = 9 independent experiments)

Table S1: List of antibodies used for immunostaining (IC), ELISA, ChIP-qPCR (ChIP) and organoid experiment.

Antibody	Host	Source	Catalog #	Dilution	Method
MeCP2 ChIP Grade	Rabbit	abcam	ab2828	5μg	ChIP
HNF4α	Rabbit	abcam	ab201460	1:200	IF
AFP	Mouse	eBioscience	14-9499	1:200	IF
CDH1	Goat	R&D	AF648	1:200	IF
GCR	Rabbit	Invitrogen	PA1-511A	1:200	IF
SLCO1B1	Mouse	Novus	NB100-74482	1:200	IF
NCOR	Rabbit	abcam	ab24552	1:200	IF
MRP2	Mouse	Novus	NB110-6000	1:100	IF
SOX2	Goat	R&D	AF2018	1:200	IF
OCT4	Mouse	Santa cruz	sc-5279	1:200	IF
UGT1A1	Mouse	Santa cruz	sc-271268	1:200	IF
PROX1	Goat	R&D	AF2727	1:200	IF
CDX2	Rabbit	Bethyl	A300-691A	1:200	IF
CDH1	Mouse	Thermo	334000	1:200	IF
NR3C1	Rabbit	Abcam	ab24552	1:200	IF, ChIP
NRF2	Rabbit	Abcam	ab31163	1:200	IF
MRP2	Mouse	Novus	NB110-6000	1:200	IF
MDR1	Rabbit	Bioss	BS-0563R	1:200	IF
ALB	Mouse	R&D	MAB1455	1:200	IF
ALB	Goat	Bethyl	A80-129A	1μg	ELISA
ALB-PE	Mouse	Novus	IC1455P	1:200	Flow cytometry
CK7- Alexa Fluor 488	Rabbit	Abcam	ab185048	1:200	Flow cytometry
PDGFRα-BV711	Mouse	BD Bioscience	752901	1:200	Flow cytometry
CD68-APC	Mouse	R&D	IC20401A	1:200	Flow cytometry
normal IgG control	Rabbit	abcam	ab37415	5μg	ChIP

Table S2: List of TaqMan probes used for qPCR

Gene	Gene name	TaqMan probe (Catalog #)
UGT1A1	UDP-glucuronosyltransferase 1A1	Hs02511055_s1
NRF2	Nuclear factor erythroid 2-related factor 2	Hs00975961_g1
IL-6	Interleukin 6	Hs00174131_m1
mGULO	Murine L-Gulonolactone oxidase	Mm00626646_m1
AFP	Alpha-fetoprotein	HS01040598_m1
CDX2	Caudal type homeobox 2	Hs01078080_m1
SOX9	SRY-box transcription factor 9	Hs00165814_m1
ALB	Albumin	Hs00609411_m1
MRP2	ATP binding cassette subfamily C member 2	Hs00166123_m1
SLC4A2	Solute carrier family 4 member 2	Hs01586776_m1
HO-1	Heme oxygenase 1	Hs01110250_m1
CYP3A4	Cytochrome P450 family 3 subfamily A member 4	Hs00604506_m1
G6PC	Glucose-6-phosphatase	Hs00609178_m1
F7	Coagulation factor VII	Hs01551994_m1
PXR	Nuclear receptor subfamily 1 group I member 2	Hs01114267_m1

Table S3: List of Custom primers used for ChIP-qPCR

Orientation	Sequence
Forward *UGT1A1* XRE	AAGGTCACTCAATTCCAAGGG
Probe *UGT1A1* XRE	/56-FAM/AGGGTATTA/ZEN/GGTGTAATGAGGATGTGT/3IABkFQ/
Reverse *UGT1A1* XRE	CTCAGAAGTTTGTTCTGGTGAGA
Forward *UGT1A1* Silencer	CCTGCTGGTCTCATCATAGTG
Probe *UGT1A1* Silencer	GAAAGTGAGAGAGAGGCAAAGA
Reverse *UGT1A1* Silencer	/56-FAM/TCCCTTCAC/ZEN/TTGCAAGCTCTTCCT/3IABkFQ/

Supplementary Experimental Procedures

CNS and mGULO iPSC generation and general iPSCs maintenance

Experiments using iPSCs were approved by the Ethics Committees of Cincinnati Children's Hospital Medical Center and Kyoto University. A CNS patient from whom iPSCs were derived provided written informed consent. The iPSC lines were generated from the patient as previously reported (Okita et al., 2011; Okita et al., 2013). Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from the patient at the Kyoto University Hospital and cultured in StemSpan-ACF (STEMCELL Technologies) supplemented with 100 ng/mL IL-6, 300 ng/mL SCF, 300 ng/mL TPO, 300 ng/mL Flt3 ligand, 10 ng/mL IL-3 (PeproTech) for 6 days. The cells were reprogrammed by introducing pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, pCXLE-hUL, and pCXWB-EBNA1, which encoded OCT3/4, SOX2, KLF4, L-MYC, LIN28, and shRNA against TP53 under on feeder culture conditions. The 1383D6 and RCL-BC iPSC used in this study was kindly provided by CiRA, Kyoto University and RxCell Inc. respectively. CuSTOM1 iPSC was obtained from patient foreskin fibroblasts and reprogrammed into iPSC by Cincinnati Children’s Hospital Medical Center pluripotent stem cell core (Pitstick et al., 2022). The PCSF#117 vector with the modified GULO sequence was then inserted into the AAVS1 locus of the CuSTOM1 iPSC cell line using a lentiviral mediated CRISPR/Cas9. The correct clones were then selected using G418. The surviving clones were then verified for correct insertion, random insertion and copy number using PCR, and verified by DNA sequencing. The iPSCs were then maintained on Laminin iMatrix-511 Silk (REPROCELL USA Inc.) coated cell culture plates and maintained with StemFit Basic04 Complete Type

(Ajinomoto Company) media with Y-27632 (Stem Cell Technologies). The cells were passaged every 7 days with Accutase (Sigma-Aldrich) until passage 40 (p40).

Organoid generation

The p40 cells were plated on a 24 well plate coated with Laminin iMatrix-511 Silk at a density of 2X10^5 cells/well and maintained with Stemfit media with 10 μM Y-27632. On Day 2, the media was replaced with fresh Stemfit. The following day, the cells were treated with RPMI 1640 (Gibco) media mixed with 100 ng/ml Activin A (Shenandoah Biotechnology) and 50 ng/ml BMP4 (R&D Systems) to generate definitive endoderm. On the 4th day, the media was replaced with RPMI, 100 ng/ml Activin A and 0.2% dFBS (HyClone) which was changed to 2% dFBS on day 5. From Day 6-8, the cells were fed with 500 ng/ml FGF-4 (Shenandoah Biotechnology) and 3 μM CHIR99021 (PeproTech) in Adv. DMEM [Advanced DMEM/F-12 (Gibco) with B27 (Gibco), N2 (Gibco), 10mM HEPES (Gibco), 2 mM L-glutamine (Gibco), and GA-1000 (Lonza)] to induce posterior foregut. On Day 9, the cells were dissociated into a single cell suspension using Accutase treatment. This single cell suspension was then mixed with 50% Matrigel (Corning Cat# 356237) and 50% EP media and plated as 50 ul drops in a 6-well plate. These cells were fed with EP media every 48 hrs for 4 days to generate organoids. These organoids were then treated with Adv. DMEM and 2 μM RA (Sigma-Aldrich) every 48 hrs for 4 days to specify the hepatic lineage. The organoids were then filtered through a 160 μm mesh filter and fed with HCM (Lonza), 10 ng/ml HGF (PeproTech), 20 ng/ml Oncostatin M (PeproTech) and 0.1 μM Dexamethasone (Sigma-Aldrich) every 3-4 days to generate

HLOs and passaged as necessary by passing them through a 160 μm mesh filter to maintain consistency of HLO and mesenchyme ratio.

Flow cytometry

For flow cytometry, the organoids were dissociated from the Matrigel on Day 27 by pipetting and washed. The organoids were then dissociated into single cells using 10% 10X TrypLE Select Enzyme (Gibco, A1217701) and 90% Trypsin-EDTA (Gibco, 25200056) and centrifuged at 1500 rpm for 3 minutes. Each cell pellet was washed with 1x PBS (Gibco, 14190235), strained using a 100μm strainer (Falcon, 08-771-19) and counted using a hemocytometer before being stained using the Transcription Factor Buffer Set (BD Pharmingen, 562574) according to the manufacturer's instructions. Stained or unstained controls were included. Subsequently, the unstained or stained cells were washed at room temperature and pelleted. The pellet was then resuspended in Stain Buffer (FBS) (BD Pharmingen, 554656) and filtered into FACS tubes. Flow cytometry run was performed using BD LSR Fortessa X20 with UltraComp eBeads Compensation Beads (Invitrogen, 01-2222-41) and finally, the flow cytometry data was analyzed using FlowJo.

RNA extraction, RT-qPCR, and RNA sequencing

RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription was carried out using the High-Capacity cDNA Reverse Transcription Kit for RT-PCR (Applied Biosystems) according to manufacturer's protocol. qPCR was carried out using TaqMan gene expression master mix (Applied Biosystems) on a QuantStudio

5 Real-Time PCR System (Applied Biosystems). All the samples were amplified with TaqMan Gene Expression Assays and normalized with 18S rRNA Endogenous Control. For RNA sequencing, the service was outsourced to Novogene (USA), the extracted RNA quality was evaluated with an Agilent 2100 Bioanalyzer (Agilent). A sequence library was prepared using a TruSeq Stranded mRNA kit (Illumina) and sequenced using NovaSeq 6000 (Illumina). Reads were aligned to human genome assembly hg38 and quantified using the quasi-mapper Salmon (v1.8.0). Gene-expression analysis was performed using the R Bioconductor package DESeq2 (v1.36.0). The read count matrix was normalized by size factors, and a variance stabilizing transformation (VST) was applied to the normalized expression data. The differentially expressed genes were then extracted by applying a filter of padj > 0.05 and |log2foldchange| > 1 and mapped to org.Hs.eg.db for genome wide annotation. The data was visualized using clusterProfiler (v4.4.2) and pheatmap (v1.0.12) packages.

ChIP-PCR, and ChIP-qPCR

ChIP experiments were performed using the High Sensitivity ChiP Kit (Abcam, ab185913). Briefly, organoids were fixed with PFA and whole chromatin was prepared and then sonicated to an optimal size of 300bp which was confirmed by gel electrophoresis. Chromatin was used for immunoprecipitation (IP) with either MECP2 antibody (Abcam, ab2828) or IgG1 isotype control. DNA fragments were amplified using custom primers for PCR and qPCR, and fold enrichment data were normalized to IP from IgG controls.

ChiP-reChIP

Before the first ChIP, the NR3C1 antibody (Abcam, ab24552) was crosslinked to Protein A Dynabeads (Invitrogen, 10002D). The ChIP assay was then carried out on extracts from organoids as described above. At the end of the first ChIP, DNA was eluted with elution buffer supplemented with 10 mM DTT. The eluate was then diluted in 2 volumes of wash buffer supplemented with 1x Protease Inhibitor Cocktail and 1 mM DTT. The 2nd ChIP assay was then carried out as described above.

Metabolite assays

For UGT activity assay, the HLOs and PHH were harvested and homogenized in UGT assay buffer. The enzyme activity was then analyzed using UGT activity assay (BioVision, K692) according to the manufacturer's instructions in a time course experiment. Bilirubin levels were measured by collecting the supernatant from HLOs treated with bilirubin and serum from the rats. The supernatant and serum were assayed with Bilirubin Assay Kit (Total and Direct, Colorimetric) (abcam, ab235627) and Bilirubin Assay Kit (Sigma-Aldrich, MAK126) according to the manufacturer's instructions. Albumin ELISA was carried out on the rat serum using the Human Albumin ELISA Quantitation Set (Bethyl Laboratories) according to the manufacturer's instructions. AST and ALT assays were carried out on the rat serum using the AST Activity Assay Kit (Sigma-Aldrich, MAK055) and the ALT Activity Assay Kit (Sigma-Aldrich, MAK052) according to the manufacturer's instructions. Cell viability was tested using the CellTiter-Glo® luminescent cell viability assay (Promega, G7570) and quantified by a BioTek® Synergy H1 plate reader after 72 hrs. For the functional assay of lipid transport, the HLOs were incubated with fresh media

containing 50 nM CLF (Corning, 451041) before imaging them every 30 min for 2 days. Visualization of bilirubin conjugation was achieved with 5 μM fluorescent UnaG (a kind gift from Dr. Miyawaki) which was incubated with the HLO media and imaged for 2 days (Kumagai et al., 2013).

Supplementary References

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