Transcriptional regulation of carboxylesterase 1 (CES1) in human liver: role of the nuclear receptor NR1H3 (LXRα) and its splice isoforms

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Regulation of CES1 expression by NR1H3

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Abbreviations:

ACE: angiotensin-converting enzyme

AHR: aryl hydrocarbon receptor

CAR: constitutive androstane receptor

CES1: Carboxylesterase 1

ChIP-Seq: chromatin immunoprecipitation followed by sequencing

CRISPR: clustered regularly interspaced short palindromic repeats

gRNA: guide RNA
GTEx: Genotype-Tissue Expression

HNF4α: hepatocyte nuclear factor 4α

iPSCs: induced pluripotent stem cells

KD: knock-down

LXR: Liver X Receptor

NR1H3: Nuclear Receptor Subfamily 1 Group H Member 3

NRF2: nuclear factor erythroid related factor 2

PXR: pregnane X receptor

siRNA: small interfering RNA

SSI: Sobol’s Sensitivity Indices

TA: transcriptional activation

TFs: Transcription Factors
Abstract:

Carboxylesterase 1 (CES1) is the predominant carboxylesterase in the human liver, involved in metabolism of both xenobiotics and endogenous substrates. Genetic or epigenetic factors that alter CES1 activity or expression are associated with changes in drug response, lipid, and glucose homeostasis. However, the transcriptional regulation of CES1 in the human liver remains uncertain. By applying both the random forest and Sobol’s Sensitivity Indices (SSI) to analyze existing liver RNA expression microarray data (GSE9588), we identified NR1H3 (LXRα) as a key factor regulating constitutive CES1 expression. This model prediction was validated using siRNA knockdown and CRISPR-mediated transcriptional activation of NR1H3 in Huh7 and HepG2 cells. We found that NR1H3’s activation of CES1 is splice isoform-specific, namely that increased expression of the NR1H3-211 isoform increased CES1 expression while NR1H3-201 did not. Also, in human liver samples, expression of NR1H3-211 and CES1 are correlated, while NR1H3-201 and CES1 are not. This trend also occurs during differentiation of induced pluripotent stem cells (iPSCs) to hepatocytes, where only expression of the NR1H3-211 isoform parallels expression of CES1. Moreover, we found that treatment with the NR1H3 agonist T0901317 in HepG2 cells had no effect on CES1 expression. Overall, our results demonstrate a key role of NR1H3 in maintaining the constitutive expression of CES1 in the human liver. Furthermore, our results support that the effect of NR1H3 is splice isoform-specific and appears to be ligand independent.

Significance statement:
Despite the central role of CES1 in metabolism of numerous medications, little is known about its transcriptional regulation. Here we identify NR1H3 as a key regulator of constitutive CES1 expression, and therefore is a potential target for future studies to understand inter-person variabilities in CES1 activity and drug metabolism.

**Introduction:**

Carboxylesterase 1 (CES1) is the predominant carboxylesterase in the human liver and intestine. CES1 catalyzes the ester cleavage of a large number of structurally diverse ester- or amide-containing substrates and is involved in the metabolism of both xenobiotics and endogenous compounds. CES activity is also a major determinant for the bioconversion of prodrugs to the active parent drugs (Imai and Hosokawa, 2010). Common drugs metabolized by CES1 include the anti-platelet prodrug clopidogrel (Lins et al., 1999), angiotensin-converting enzyme (ACE) inhibitors (imidapril, enalapril, trandolapril, ramipril) (Song and White, 2002; Thomsen et al., 2014), chemotherapeutic agents (irinotecan) (Humerickhouse et al., 2000), ADHD medications (methylphenidate) (Sun et al., 2004) and others. CES1 is also known to metabolize endogenous esters including cholesteryl esters, triacylglycerols, and other endogenous lipids that have vital physiological functions in lipid homeostasis (Lian et al., 2018). For example, reduced DNA methylation of the CES1 gene is associated with childhood obesity (Li et al., 2018), and CES1 knockout mice are more susceptible to high cholesterol diet-induced liver injury (Li et al., 2017).

There exists large inter-person variability in CES1 expression and activity, which affects drug response. Nonsynonymous loss of function genetic polymorphisms in CES1 have been associated with prodrug (e.g. dabigatran, etexilate, and oseltamivir)
activation, pharmacokinetics, and efficacy (Shi et al., 2016a; Shi et al., 2016b; Mu et al., 2020), and some variants have been proposed to serve as biomarkers for predicting clopidogrel efficacy (Lewis et al., 2013). However, the allele frequencies of these coding region variants are low, and therefore cannot explain the large variability in CES1 activity between individuals. Several potential CES1 regulatory polymorphisms have also been identified (Geshi et al., 2005; Bruxel et al., 2013; Johnson et al., 2013), including structural variants arising from genomic translocation of the 5’ region from the poorly expressed pseudogene CES1P to CES1 (Sanford et al., 2016). However, the functional consequences of these regulatory variants are uncertain.

Little is known about transcriptional regulation of CES1 in the human liver. Chemical induction experiments in mice showed that the transcription factors (TFs) aryl hydrocarbon receptor (AHR), constitutive androstane receptor (CAR, NR1I3), pregnane X receptor (PXR, NR1I2), and the nuclear factor erythroid related factor 2 (NRF2, NFE2L2) were involved in expression of the CES genes (Zhang et al., 2012). In patients with alcoholic steatohepatitis, the mRNA of both the hepatocyte nuclear factor 4α (HNF4A) and CES1 were markedly reduced (Xu et al., 2016), implying a potential regulatory role of HNF4A on CES1 expression during alcoholic steatohepatitis. In HepG2 cells, PXR is involved in insulin- (Yang et al., 2019) and fluoxetine-mediated (Shang et al., 2016) CES1 transcriptional regulation and a variety of stimuli that alter signaling pathways have been shown to change CES1 expression, including: the steroid hormone (17β-estradiol) (Wu et al., 2018), antioxidants (Chen et al., 2012), and disease states (e.g. type 2 diabetics) (Chen et al., 2015). However, the primary TFs controlling constitutive CES1 expression remains largely unknown.
The purpose of this study was to identify TFs regulating constitutive CES1 expression in the human liver. We applied both random forest and Sobol’s Sensitivity Indices (SSI) (Lu et al., 2017) on existing microarray liver gene expression data (GSE9588) (Yang et al., 2010), as described previously for CYP3A4 (Wang et al., 2019). Of the 44 liver enriched TFs (Yang et al., 2010) analyzed, we identified NR1H3 (LXRα) and several others as the top TFs associated with CES1 expression. siRNA-mediated knock down (KD), CRISPR-mediated transcriptional activation (TA), and quantitative liver gene expression validated the regulatory role of NR1H3 in constitutive CES1 expression and demonstrate that this role of NR1H3 is splice isoform specific.

Material and Methods:

Human liver samples. Human liver samples were obtained from the Cooperative Human Tissue Network (CHTN, Bethesda, MD). Demographics of liver samples are mean age-60 ± 13 years, female-52%, all samples were from Caucasian American donors (n=140). The University of Florida internal review board approved the human tissue study.

Random forest and SSI analysis of TF interactions with CES1. The mRNA dataset used is published microarray data (GSE9588) from 427 liver samples (Yang et al., 2010). We selected 44 liver-enriched TFs (Yang et al., 2010), represented by 78 probes in microarray data (some TFs were measured by multiple probes) (Supplemental Table 1). We estimated the mean decreases in Gini by fitting a random forest classifier of CES1 and estimated the main effect Sobol’s indices by using the empirical variance of the best-fitting polynomial expression (Lu et al., 2017; Wang et al., 2019). The most influential TF was identified by the largest mean decreases in Gini, the
largest Sobol’s indices, and the shortest distance between CES1 and TF in network analysis, which represents the strength of the interaction between CES1 and TF (the shorter the distance, the stronger the interaction).

**Cell culture and iPSC differentiation.** Huh7 and HepG2 cells were cultured at 37°C in a humidified incubator at 5% CO\textsubscript{2} in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/100 μg). Human iPSCs (ASE-9203) were purchased from Applied StemCell (Milpitas, CA, USA) and were cultured at 37°C in a humidified incubator at 5% CO\textsubscript{2} in DEF-CS medium (Takara Bio, Mountain View, CA, USA). iPSC to hepatocyte differentiation was performed using the Cellartis iPSC to hepatocyte differentiation system (Takara Bio, Mountain View, CA, USA) according to manufacturer’s instructions. The system progresses through directed differentiation of iPSCs into definitive endoderm (DE) (completed on day 14), which are then differentiated into hepatocytes (completed on day 32).

**Gene knockdown using siRNA.** Silencer siRNA targeting NR1H3 (#138007), NR1I3 (#5535), HNF4A (#290203) and NR1I2 (#6638) and the negative control #1 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). siRNA was introduced into cells using the lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA, USA). After incubation for 72 hours, the cells were harvested for total RNA preparation, reverse transcription, and quantitative real-time polymerase chain reaction (qRTPCR).

**CRISPR-mediated NR1H3 transcriptional activation.** By fusing VP64 (the universal transcriptional activator) with an inactive mutant Cas9 protein, dCas9, the dCas9-VP64
fusion protein can specifically activate transcription when directed by a guide RNA (gRNA) to a target gene promoter (Konermann et al., 2015). We used the lentiviral-based vectors lentiviral-VP64-dCas9 (#61429; Addgene) and LentisgRNA vector (#61427; Addgene), for VP64-dCas9 fusion protein and gRNA delivery. We used previously reported gRNA sequences to target NR1H3 (Konermann et al., 2015). We targeted three separate promoters of NR1H3 corresponding to three different isoforms: NR1H3-235 (NM_001251934), NR1H3-211 (NM_001130101), and NR1H3-201 (NM_001130102) (see Supplemental Figure 1 for liver NR1H3 splice isoforms). Each promoter was targeted with three gRNAs, and a gRNA without a human genome target served as a negative control (Supplemental Table 2 contains the gRNA sequences).

Lentiviral particles containing the expression vectors for VP64-dCas9 and a mix of the three gRNAs targeting a specific gene promoter or the negative control gRNA were incubated with Huh7 or HepG2 cells in the presence of 8 µg/ml SureEntry transduction reagent (Qiagan, Valencia, CA) for 24 hours. Cells were harvested 72 hours after transduction.

**RNA preparation and gene expression analysis.** Total RNA was prepared using RNA mini prep kits from Zymo Research (Irvine, CA). RNA was reverse transcribed into cDNA using the RTIV reverse transcriptase (Life Technologies, Carlsbad, CA). Gene expression levels were measured using qRTPCR with gene-specific primers (Supplemental Table 2) and the SYBR Green PCR master mix (Life Technologies, Carlsbad, CA), using GAPDH as an internal control as described (Collins and Wang, 2021). The Quantabio Q real-time PCR instrument (VWR, PA, USA) was used to measure the signal. The relative expression of each gene was calculated using the
following formula: expression level of tested gene = antilog2(mean Ct value of GAPDH – mean Ct value of tested gene)*10^6. After Log10 transformation, the expression level of NR1H3 and CES1 in liver samples followed a normal distribution.

**CES1 protein quantification in human liver tissues.** Relative CES1 protein expression in 46 individual human liver tissues was determined using a western blot assay that we described in a previous publication (Sanford et al., 2016).

**Results**

SSI and random forest analysis identified NR1H3 as a main regulator of CES1 expression. By applying SSI analysis to published microarray mRNA expression data (GSE9588) from 427 liver samples (Yang et al., 2010), we identified PGRMC1, NR1I3, NHF4A, NR1H3 and ARNT1 as the top five TFs with largest Sobol’s indices for CES1 expression among the 44 liver-enriched TFs tested (Supplemental Table 3). Similarly, these five TFs also showed the largest mean decreases in Gini using random forest classification (Supplemental Figure 1). We also employed SSI network analysis to help determine which TF may be directly regulating CES1 expression in the liver. Compared to the other four TFs, NR1H3 has the shortest distance to CES1 (Figure 1), and therefore became the primary focus for experimental validation.

siRNA knockdown (KD) of NR1H3 and other TFs. To validate the in-silico predictions, we used siRNA KD in HepG2 and Huh7 cells to determine the effect of decreased NR1H3 on CES1 expression. NR1H3 siRNA reduced NR1H3 expression 83% in Huh7 and 43% in HepG2 cells and significantly decreased CES1 mRNA in both cell lines. CES1 was decreased to a greater extent in HepG2 cells compared to Huh7 cells (Figure 2), which may be due to different expression of CES1 in these two cells lines, as
Huh7 has much lower CES1 levels (313-fold) compared to HepG2 (Huh7: 8.6 ± 1.1 and HepG2: 2691 ± 712, arbitrary units resulting from comparison to an internal control GAPDH). We also tested four other TFs based on their SSI values (Supplemental Table 1) and network distance to CES1 (Figure 1). These included (as compared to NR1H3): NR1I3 (larger SSI value and similar distance), PGRMC1 and HNF4A (larger SSI value and more distal), and NR1I2 (smaller SSI value and closer distance). siRNA KD of all four of these TFs did not affect CES1 expression in Huh7 cells (Supplemental Figure 2), indicating that they do not directly control the constitutive expression of CES1. Instead, their associations from the models may have resulted from indirect regulation or inducible expression of CES1, consistent with previous studies showing involvement of NR1I3, NR1I2 and HNF4A in chemical- and lipid-mediated CES1 induction (Zhang et al., 2012; Shang et al., 2016; Xu et al., 2016; Yang et al., 2019).

**CRISPR-mediated transcriptional activation (TA) of NR1H3 and the effects on CES1 expression.** To further validate the impact of NR1H3 on CES1 expression, we used CRISPR-mediated TA to increase the expression of NR1H3 in the same two cell lines, as reported (Wang et al., 2019). Based on Genotype-Tissue Expression (GTEx) data (Carithers et al., 2015), transcription of NR1H3 can be initiated from at least three different promoters, producing three main splice isoforms, NR1H3-211, NR1H3-201 and NR1H3-235, all of which are expressed in human livers (Supplemental Figure 3). We designed gRNA targeting all three promoters, using three gRNAs per promoter (see Supplemental Table 2 for gRNA sequences). Transcription of NR1H3-201 and NR1H3-211 was significantly increased (1.4-2-fold) by CRISPR-mediated TA in both Huh7 and HepG2 cells, while NR1H3-235 was not (Figure 3). NR1H3-235 is expressed at a low
level in the liver (Supplemental Figure 3), implying that additional regulatory mechanisms are controlling its expression. Increased expression of NR1H3-211 enhanced CES1 mRNA levels in both Huh7 and HepG2 cells, in agreement with the KD results. In contrast, enhanced expression of NR1H3-201 failed to increase CES1 in either cell line (Figure 3). Overall, these results agree with the SSI prediction indicating NR1H3 as a key TF controlling constitutive CES1 expression. The results also indicate that the regulation of CES1 by NR1H3 is splice isoform-specific.

Changes in NR1H3 splice isoforms and CES1 expression during iPSC to hepatocyte differentiation. We next leveraged an iPSC to hepatocyte differentiation model to determine changes in the expression of CES1, NR1H3, and its splice isoforms during development. We measured developmental markers during the different cell stages to confirm proper differentiation: POU5F1 (Oct4) for the iPSC stage (day 0), CER1 for the definitive endoderm (DE) stage (day 14), and CYP3A4 for hepatocyte-like cells (day 32) (Ghosheh et al., 2017). The markers followed an expression pattern in agreement with previous report (Ghosheh et al., 2017): Oct4 expression was high in iPSCs and declined throughout differentiation, CER1 peaked at day 14, and CYP3A4 progressed from being undetectable in the iPS and DE cells to a marked increase in expression in the hepatocytes at day 32 (Supplemental Figure 4). These results indicated that our cell differentiation was successful.

Expression of CES1 increased with differentiation of the iPSCs: its relative expression was low in iPSCs, increased roughly two-fold at day 14 in the DE cells, and drastically increased (15-fold, compared to iPSCs) at day 32 in the hepatocyte cells (Figure 4a). NR1H3 followed a very similar pattern to CES1, while a different type of
LXR, NR1H2 (LXRβ), did not (Figure 4a). Furthermore, only the expression of splice isoform NR1H3-211 peaked at day 32, while the other isoforms, NR1H3-201 and NR1H3-235 peaked at day 14, and then declined by day 32 (Figure 4b). Thus, at day 32 in the hepatocyte cells, NR1H3-211 is the primary isoform (Figure 4c) and thereby coincides with the highest level of CES1 expression (Figure 4a). These co-expression results support that NR1H3 (particularly NR1H3-211) may have a regulatory role in controlling CES1 expression during the transition from iPSCs to hepatocytes, and thereby contribute to CES1 expression in the human liver.

Correlation between expression of CES1 and NR1H3 splice isoforms in cell lines and liver samples.

We compared the expression levels of CES1 to the overall expression of NR1H3 and its three splice isoforms in Huh7 cells, HepG2 cells, and liver samples. CES1 expression varies in the different cell lines; compared to its lowest expression in Huh7 cells, CES1 is 313-fold higher in HepG2 cells and 4308-fold higher in the liver (average of 140 liver samples). In contrast, when considering total NR1H3 mRNA levels, NR1H3 expression is relatively similar across all three cell types and is only 1.2-fold higher in HepG2 cells and 3.8-fold higher in liver tissues (compared to Huh7 cells). However, analysis of the individual NR1H3 splice isoforms shows large differences between the liver and the two cell lines. Over 90% of the total NR1H3 transcripts in liver tissues are the NR1H3-211 isoform, while NR1H3-211 only comprises ~50% of the total NR1H3 transcript pool in the Huh7 and HepG2 cells (Supplemental Figure 5). Furthermore, in 140 liver samples, we observed a strong positive correlation between levels of CES1 and NR1H3-211 (P<0.0001) (Figure 5a), while there was no correlation between
expression of CES1 and NR1H3-201 (P=0.367) (Figure 5b). We also measured CES1 protein levels in 46 samples using a western blot approach. Similar to the mRNA expression results, CES1 protein levels are positively correlated with NR1H3-211 (P<0.0001) (Figure 5c) but not with NR1H3-201 (r=-0.115, P=0.451) (Figure 5d). These results indicate that NR1H3-211 is the predominate splice isoform regulating expression of CES1.

NR1H3 agonists do not activate CES1 expression. NR1H3 encodes LXRα, a transcriptional regulator that has previously been shown to be strongly activated by T0901317 (Hoang et al., 2012). We therefore tested whether this agonist would also cause a concomitant increased in CES1 expression. For positive controls, we also tested expression of two genes ABCG1 and FAS that are known to be induced by T0901317 (Hoang et al., 2012). While T0901317 treatment (0.3 or 1 µM, 24h) drastically increased the expression of ABCG1 (~100-fold) and FAS (~4-fold) (Figure 6b), it did not alter CES1 expression (Figure 6a). Similar results were observed in Huh7 cells (data not shown).

Discussion:

We have identified NR1H3 as a key regulator for constitutive CES1 expression in the human liver using SSI and random forest analyses. siRNA mediated KD or CRISPR-mediated gene TA of NR1H3 in HepG2 and Huh7 cells also caused a corresponding change in CES1 expression. To our knowledge, this is the first study showing the regulation of CES1 by NR1H3. Moreover, our results demonstrate that the NR1H3-211 splicing isoform is the key NR1H3 splice isoform controlling constitutive CES1 expression.
NR1H3 (LXRα) is a ligand-activated TF of the nuclear receptor superfamily, playing important roles in lipid and carbohydrate metabolism (Baranowski, 2008). The role of ligand-activated NR1H3 in gene expression regulation is well studied; for example, NR1H3 agonists are known to increase the expression of many genes related to lipid and glucose homeostasis, and display potent antiatherogenic and antidiabetic effects (Baranowski, 2008). NR1H3 agonists also induce the expression of several phase I and phase II drug metabolizing enzymes, for example, CYP1A1 (Shibahara et al., 2011), CYP3A4, CYP2B6 (Duniec-Dmuchowski et al., 2007) and UGT1s (Hansmann et al., 2020), in cells and mouse models. However, a role of unliganded NR1H3 has yet to be reported. Our results, for the first time, demonstrate the critical role of NR1H3 on maintaining basal CES1 expression and showed correlation between the expression of NR1H3 and CES1 in human liver. No NR1H3 agonists were added during our siRNA or CRISPR-mediated transcription activation experiments, where we saw corresponding changes in expression of both NR1H3 and CES1 (Figures 2 & 3), supporting a role of unliganded NR1H3 in controlling CES1 expression in hepatic cells. Furthermore, activation of NR1H3 by agonist T0901317 did not induce the expression of CES1 in HepG2 and Huh7 cells, suggesting potential different signaling pathways of ligand-free and ligand-bound NR1H3. This result is consistent with our recent findings regarding a different nuclear receptor, the estrogen receptor α (ESR1). Although canonically ESR1 is considered a ligand-activated nuclear receptor, we demonstrated the different roles of ligand-free and ligand-bound ESR1 in regulating the expression of cytochrome P450s (Wang et al., 2019) and our ChIP-Seq experiments showed distinct binding motifs and binding sites for these two forms of ESR1 (Collins et al., 2021).
These results suggest that having different chromatin binding and signaling pathways in the presence or absence of ligands may be a general phenomenon of the nuclear receptors. However, we cannot rule out that endogenous NR1H3 ligands may have different effects on NR1H3-mediated regulation than synthetic ligands, and thus, the contribution of endogenous NR1H3 ligands on regulation of CES1 remains unclear and will require further investigation.

NR1H3 has numerous splice isoforms exist, with 35 transcripts listed in the Ensembl database (Howe et al., 2021). According to the GTEx portal (Carithers et al., 2015), thirteen of these transcripts are expressed in the liver, with five of them (NR1H3-211, -201, -217, -235 and -221) being predicted as protein coding (Supplemental Figure 3). These five transcripts are initiated from three different promoters, and we chose a major isoform from each promoter for this study (NR1H3-211, NR1H3-201 and NR1H3-235, Supplemental Figure 3). NR1H3-221 is initiated from the same promoter as NR1H3-201 but is not (or nearly not) expressed in the liver, while NR1H3-217 shares the same promoter with NR1H3-211 and has low liver expression (Supplemental Figure 3). NR1H3-211 and NR1H3-217 encode the same protein but differ at the 5'UTR due to retention of an intron in NR1H3-217. The qPCR primers used in this study cannot differentiate NR1H3-211 from -217, and thus, may represent the sum of these two isoforms. The expression level of NR1H3-235 is low in hepatic cells and in the liver (<1% of total) and therefore may not play major regulatory role. Conversely, the expression levels of the other two isoforms, NR1H3-211 and NR1H3-201, are dynamic depending on the cell type (Figures 3 & 4). Although the expression of both NR1H3-211 and NR1H3-201 were activated by CRISPR-mediated TA in HepG2 and Huh7 cells,
only NR1H3-211 enhanced CES1 expression, indicating different regulatory roles of these two splice isoforms. In further support of this, expression of CES1 only paralleled the NR1H3-211 isoform during iPSC to hepatocyte differentiation, and only NR1H3-211 is correlated with CES1 expression in human liver samples. These results indicate that the regulation of NR1H3 on CES1 transcription is mediated by NR1H3-211 but not the NR1H3-201 isoform. These two isoforms differ at their 5'UTR and have different translation start sites (Supplemental Figure 3). Compared to NR1H3-211, the NR1H3-201 protein is shorter and lacks 45 amino acids at the N-terminal. A previous study showed that the N-terminal truncated NR1H3-201 isoform has lower basal and agonist-induced transcriptional activity than the full-length isoform, indicating that the N-terminal 50 amino acids are critical for full NR1H3 transcriptional function (Chen et al., 2005). Indeed, further studies are needed to elucidate the mechanisms underlying NR1H3 regulation of basal CES1 expression in human liver.

In conclusion, our results demonstrate the regulation of CES1 by the nuclear receptor NR1H3 in a ligand-independent and splice isoform-specific manner. Therefore, genetic, or epigenetic factors affecting the expression of NR1H3 will have the potential to alter CES1 expression, opening new research directions for understanding variable expression of CES1 in the human liver.

Authorship Contributions:

Participated in Research design: D. Wang and Collins

Conducted experiments: D. Wang, Collins, X. Wang, and Zhu

Performed data analysis: Lu, Collins and D. Wang
Wrote or contributed to the writing of the manuscript: D. Wang, Collins, Lu, X. Wang and Zhu

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Footnotes

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The authors declare no conflicts of interest.
Figure legends

Figure 1. Transcription factors identified using SSI analysis and their predicted interactions affecting CES1 expression. Dot sizes represent the effect of each TF on CES1 expression: the larger the dot, the greater the predicted regulatory effect of that TF on CES1 expression. The connecting lines illustrate predicted interactions occurring between the TFs and/or CES1, with the length of the line indicating the overall impact of each interaction (the shorter the distance, the higher the SSI value of the interaction). TFs measured by more than one microarray probe (for example, NR1I3 and NR1I3.1; HNF4A.2 and HNF4A.4) yielded similar results.

Figure 2. NR1H3 knockdown by siRNA and the effect on expression of CES1 in a) Huh7 and b) HepG2 cells. Mean ± SD, n=4. Compared to negative control (NC), * p<0.05; ** p<0.01; *** p<0.001, ANOVA with Bonferroni: compared selected pairs post hoc test.

Figure 3. Effect of NR1H3 transcriptional activation (TA) on the expression of CES1 in a) Huh7 and b) HepG2 cells. Mean ± SD, n=4. Compared to negative control (NC), *p<0.05; ** p<0.01; *** p<0.001, ANOVA with Bonferroni: compared selected pairs post hoc test. Note: The measured NR1H3-211 level is the sum of NR1H3-211 and NR1H3-217 due to the lack of qPCR primer specificity.

Figure 4. Gene expression changes during iPSC to hepatocyte differentiation. (a & b) Expression levels of genes were measured at day 0, day 14, and day 32 using qRT-PCR with GAPDH as an internal control. (c) Relative expression level of each NR1H3 splicing isoform at the different stages of differentiation. Data expressed as % of the
total \textit{NR1H3} level. Note: \textit{NR1H3}-235 expression is too low (<1\%) to be visible in the graph.

Figure 5. Correlation between the levels of mRNA (a & b) and protein (c and d) of \textit{CES1} and two \textit{NR1H3} isoforms. (a & c) \textit{NR1H3}-211 and (b & d) \textit{NR1H3}-201.

Figure 6. Effect of the \textit{NR1H3} agonist T0901317 on gene expression in HepG2 cells. (a) \textit{CES1} (24hrs & 48hrs) (b) ABCG1 and FAS (24hrs). Panel b is in log10 scale.

Mean ± SD, n=4. *** compared to DMSO, p<0.0001, ANOVA with Bonferroni: compared selected pairs post hoc test.
Figure 2.
Figure 3.
Figure 4
Figure 5

- Panel a: Scatter plot showing a positive correlation between Log10(CES1 mRNA) and Log10(NR1H3-211 mRNA), with $r = 0.767$ and $P < 0.0001$.

- Panel b: Scatter plot showing a weak positive correlation between Log10(CES1 mRNA) and Log10(NR1H3-201 mRNA), with $r = 0.100$ and $P = 0.241$.

- Panel c: Scatter plot showing a positive correlation between Log10(CES1 protein) and Log10(NR1H3-211 mRNA), with $r = 0.461$ and $P = 0.001$.

- Panel d: Scatter plot showing a negative correlation between Log10(CES1 protein) and Log10(NR1H3-201 mRNA), with $r = -0.115$ and $P = 0.451$. 

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Figure 6.