1521-009X/51/1/1–7$35.00
DRUG METABOLISM AND DISPOSITION

dx.doi.org/10.1124/dmd.122.000945
Drug Metab Dispos 51:1–7, January 2023

Cytochrome P450 Transcriptional Regulation by Testis-Specific Y-Encoded-Like Protein: Identification of Novel Upstream Transcription Factors[S]

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Received May 17, 2022; accepted July 5, 2022

ABSTRACT

Cytochrome P450s (*CYP*s) display significant inter-individual variation in expression, much of which remains unexplained by known *CYP* single-nucleotide polymorphisms (SNPs). Testis-specific Y-encoded-like proteins (*TSPYL*s) are transcriptional regulators for several drug-metabolizing *CYP*s including *CYP3A4*. However, transcription factors (TFs) that might influence *CYP* expression through an effect on *TSPYL* expression are unknown. Therefore, we studied regulators of *TSPYL* expression in hepatic cell lines and their possible SNP-dependent variation. Specifically, we identified candidate TFs that might influence *TSPYL* expression using the ENCODE ChIPseq database. Subsequently, the expression of *TSPYL1/2/4* as well as that of selected CYP targets for *TSPYL* regulation were assayed in hepatic cell lines before and after knockdown of TFs that might influence *CYP* expression through TSPYL-dependent mechanisms. Those results were confirmed by studies of TF binding to *TSPYL1/2/4* gene promoter regions. In hepatic cell lines, knockdown of the REST and ZBTB7A TFs resulted in decreased *TSPYL1* and *TSPYL4* expression and increased *CYP3A4* expression, changes reversed by *TSPYL1/4* overexpression. Potential binding sites for *REST* and *ZBTB7A* on the promoters of *TSPYL1* and *TSPYL4* were confirmed by chromatin immunoprecipitation. Finally, common SNP variants in upstream binding sites on the *TSPYL1/4* promoters were identified and luciferase reporter constructs confirmed SNP-dependent modulation of *TSPYL1/4* gene transcription. In summary, we identified REST and ZBTB7A as regulators of the expression of TSPYL genes which themselves can contribute to regulation of *CYP* expression and—potentially—of drug metabolism. SNP-dependent modulation of TSPYL transcription may contribute to individual variation in both *CYP* expression and—downstream–drug response phenotypes.

SIGNIFICANCE STATEMENT

Testis-specific Y-encoded-like proteins (*TSPYL*s) are transcriptional regulators of cytochrome P450 (*CYP*) gene expression. Here, we report that variation in *TSPYL* expression as a result of the effects of genetically regulated TSPYL transcription factors is an additional factor that could result in downstream variation in *CYP* expression and potentially, as a result, variation in drug biotransformation.

Introduction

Approximately half of the population of the United States uses prescription drugs every year ((CDC), 2015–2018b). Adverse drug events and toxicity as a result of prescription drug use could potentially be decreased by enhanced understanding of variation in pharmacokinetic (PK) and/or pharmacodynamic (PD) factors that contribute to inter-individual differences in drug exposure or response ((CDC), 2015–2018a). Many studies ranging from candidate gene studies to genome-wide analyses have highlighted the contribution of genomics to individual variation in the occurrence of adverse drug events and/or inter-individual variability in drug response phenotypes (Nebert et al., 2013; Nelson, 2013; Zanger and Schwab, 2013). The cytochrome P450 (*CYP*) enzymes play an important role in Phase I drug metabolism and, as a result, have the potential to be major contributors to individual variability in PK. The *CYP* superfamily includes 18 families of protein encoding human *CYP* genes, including the *CYP1*, *CYP2*, and *CYP3* families, which include many important drug-metabolizing enzymes (Bush et al., 2016; Kozyra et al., 2017). *CYP3A4*, *CYP2C9*, and *CYP2C19* are important *CYP*s with common, functionally significant genetic polymorphisms (Evans and Relling, 1999). These three enzymes have been estimated to contribute to the metabolism of approximately 50%, 20% and 5% of drugs, respectively (Evans and Relling, 1999; Neavin et al., 2019). However, known single-nucleotide polymorphism (SNP) variants that influence the expression or function of *CYP3A4*, *CYP2C9*, and *CYP2C19* explain only a portion of inter-individual differences in drug biotransformation catalyzed by these *CYP*s (Daly, 2010; Liu et al., 2010; Zi et al., 2010; He et al., 2011; Wang et al., 2011; Motsinger-Reif et al., 2013; Wright et al., 2018). Most of the early examples of *CYP* pharmacogenomic variation involved SNPs that resulted in alterations in the amino acid sequence of the encoded protein,

This work was supported, in part, by National Institutes of Health [Grant R01GM125633-04] (LW), [Grant GM28157] (RW), and [Grant T32GM008685-24] and Grant [U01GM61388] (LW and RW).

Drs. Wang and Weinshilboum are cofounders of and stockholders in OneOme, LLC. All other authors have no conflict of interest with the contents of this article.

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dx.doi.org/10.1124/dmd.122.000945.

[S] This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: ChIP, chromatin immunoprecipitation; CYP, cytochrome P450; eQTL, expression quantitative trait loci; KD, knock-down; MAF, minor allele frequencies; OE, overexpression; PD, pharmacodynamics; PK, pharmacokinetic; RT-qPCR, reverse transcription real time polymerase chain reaction; siRNA, small interfering RNA; SNP, single-nucleotide polymorphism; TF, transcription factors; TSPYL, testis-specific Y-encoded-like protein.

alterations in gene splicing or variation in gene structure (deletions/insertions) but, increasingly, it is becoming apparent that variants which alter gene transcription represent a major source of pharmacogenomic variation—either directly or indirectly (Wang et al., 2022).

Previous studies from our group reported that testis-specific Y-encoded-like proteins (specifically *TSPYL1*, *2*, and *4*) are transcriptional regulators that can influence the expression of *CYP3A4, CYP2C9,* and *CYP2C19* (Qin et al., 2018). Elevated expression of these *TSPYLs* can suppress the expression of *CYP3A4, CYP2C9,* and *CYP2C19* (Qin et al., 2018). The *TSPYL* gene family consists of six genes, *TSPYL1* to *TSPYL6*, with *TSPYL3* being a pseudogene. The Genotype-Tissue Expression database (https://gtexportal.org/) reports that the *TSPYLs* are expressed in most human tissues, with isoform-specific variation in their tissue distribution. *TSPYLs* have multiple cellular functions (de Andrade et al., 2006; Epping et al., 2015), and genetic polymorphisms and/or variation in the methylation status of these genes have been related to disease states (Kim et al., 2006; Guo et al., 2012; Le Gallo et al., 2012). In addition, as stated above, functionally significant polymorphisms in *TSPYL* genes have been reported to alter their ability to regulate transcription and, as a result, the expression of *CYPs*, resulting in inter-individual variation in drug biotransformation (Qin et al., 2018; Qin et al., 2020). Specifically, and of importance for the studies described subsequently, we reported previously that knock-down (KD) of *TSPYL1, 2* and *4* in HepaRG cells can result in increased expression of *CYP2C9, 2C19,* and *3A4*, while overexpression (OE) of these same *TSPYL* genes can result in decreased expression of the same *CYPs*, with the most striking effects for *CYP3A4* (Qin et al., 2018; Qin et al., 2020). Given our increasing recognition of the role of the *TSPYLs* in drug metabolism, it is important to understand the possible role of upstream regulators of *TSPYL* gene expression, specifically, transcription factors (TFs) that influence *TSPYL* gene expression, to help us achieve a more comprehensive understanding of downstream variability in *CYP* expression and drug response phenotypes. In the present study, we set out systematically to identify TFs that might be involved in the regulation of *TSPYL1, 2,* and *4* expression in human hepatic cell lines as a step toward a more comprehensive understanding of the potential contribution of the *TSPYLs* to individual variation in *CYP* expression and function.

Materials and Methods

ENCODE Chromatin Immunoprecipitation (ChIP)-Seq Data. The ENCODE UCSC genome browser includes ChIP-seq data for HepG2 cells that lists TFs that bind to the promoter regions of *TSPYL1, TSPYL2,* and *TSPYL4*, 1 kbp upstream or downstream of the transcription starting site. We used that information as a starting point for this series of studies of possible transcriptional regulatory factors that might contribute to variation in the expression of human *TSPYLs*.

Hepatic Expression Quantitative Trait Loci (eQTL) Database Association Analysis. We next determined associations between TFs that bind to *TSPYL* gene promoters and *TSPYL1*, *TSPYL2,* and *TSPYL4* expression in an hepatic eQTL database (Innocenti et al., 2011) using Pearson correlation analyses and identified TFs that might bind near *TSPYL1, TSPYL2* or *TSPYL4* and, as a result, might influence the expression of genes influenced by *TSPYL* expression with P values <0.05.

Transfection of HepaRG Cells and HepG2 Cells. Specific short interfering RNAs (siRNAs) targeting the 30 candidate TFs that we had identified in the ENCODE database were then used to knock down the expression of those TFs in HepaRG and HepG2 cells using specific siRNAs, with non-targeting siRNAs as a control (see Supplemental Table 1). The cells were harvested 48 hours after transfection, and RNA was extracted for the performance of reverse transcription polymerase chain reaction (RT-PCR).

Gene Expression Quantification. Total RNA from HepG2 and HepaRG cells was extracted and was used to perform real time quantitative PCR to assay expression levels of *TSPYL1, TSPYL2, TSPYL4, CYP3A4, CYP2C9,* and *CYP2C19*, as well as after the after KD of TFs using the primers listed in Supplemental Table 2. Alterations in the expression of those genes were expressed as fold change from baseline.

ChIP for HepaRG Cells. HepaRG cells were used to perform ChIP assays to validate TF binding to promoter regions of the *TSPYL1* and *TSPYL4* genes, and the results were analyzed using real time quantitative PCR. *TSPYL1* and *TSPYL4* were selected for study because they map in relatively close proximity in the genome and because our previous experiments had demonstrated that those two *TSPYL* genes appeared to have significant impact on variation in the expression of *CYP3A4* (Qin et al., 2018).

Candidate TFs	TSPYL1	TSPYL2	TSPYL4	Candidate TFs	TSPYL1	TSPYL2	TSPYL4
SP1	Δ	Δ	Δ	FOSL2	Δ		Δ
TAF1	Δ	Δ	Δ	RCOR1	Δ		
SIN3AK20	Δ	Δ	Δ	REST	Δ		
MXI1	Δ	Δ	Δ	RAD21		Δ	
TBP	Δ	Δ	Δ	SMC3		Δ	
CEBPB	Δ	Δ	Δ	HSF1			Δ
EP300	Δ	Δ	Δ	HDAC2			Δ
CHD2	Δ	Δ	Δ	ARID3A			Δ
RFX5	Δ	Δ	Δ	TEAD4			Δ
YY1	Δ		Δ	NFIC			Δ
ZBTB7A	Δ		Δ	HNF4A			Δ
BRCA1	Δ		Δ	HNFAG			Δ
ZBTB33	Δ		Δ	Δ=Binds to promoter regions of TSPYL 1/2/4 (Chip-seq data in HepG2)			
MAFF	Δ		Δ				
MAFK	Δ		Δ				
FOXA1	Δ		Δ	Δ=Binds to promoter regions of TSPYL 1/2/4 (Chip-seq data in HepG2) + shows significant correlation with TSPYL expression in human liver tissue			
FOXA2	Δ		Δ				
MYBL2	Δ		Δ				

Fig. 1. Transcription factors that might transcriptionally regulate *TSPYL1/2/4* expression. The table lists the 30 TFs identified as binding to the promoter regions of *TSPYL 1/2/4* as well as those that also displayed significant correlations with TSPYL expression in human liver tissue (Storey et al., 2011). The three TFs that were studied in detail here, *ZBTB7A*, *REST*, and *MAFK*, are highlighted in red type in the TF gene list.

Luciferase Reporter Assay. The luciferase reporter vector, pGL4.23 (Cat#: E8411), was obtained from Promega with inserts encoding either 2 Kbp of the *TSPYL1* or 2 kbp of the *TSPYL4* promoter regions and were used to create *TSPYL1* wild-type promoter, *TSPYL1* variant promoter, *TSPYL4* wild-type promoter or *TSPYL4* variant promoter constructs (Supplemental Table 4). Those vectors were then used to transfect HepaRG cells. The cells were harvested 48 hours after transfection to assay relative luciferase and Renilla activities.

Additional methodological details have been provided as Supplemental Methods.

Results

The series of studies described subsequently was designed to pursue our previous observation of the potential importance of members of the *TSPYL* gene family in regulation of the expression of drug metabolizing *CYPs* (Qin et al., 2018; Qin et al. 2020). Specifically:

1. As a first step in the present studies, the ENCODE database was consulted to identify TFs that might bind to the promoters of the *TSPYL1/2* and *4* genes in HepG2 cells. Thirty potential candidate TFs were identified.
2. Those 30 candidate *TSPYL* TFs were then knocked down in HepaRG cells, and the effect of KD on the expression of *CYP3A4, CYP2C9,* and *CYP2C19* was determined and compared with our previous results after the KD of *TSPYL1, 2,* and *4* in this same cell line. The most striking similarities observed related to *CYP3A4* and the putative TSPYL TFs *REST* and *ZBTB7A*. Therefore, the final series of studies focused on SNPs in the promoters of *TSPYL1* and *TSPYL4*—two genes that map in close proximity to each other in the genome—as well as the effect of *REST* and *ZBTB7A* on their transcription.
3. The final series of experiments addressed the possible binding of REST and ZBTB7A to the promoters of *TSPYL1* and *TSPYL4* and the influence of SNPs in those genes on that binding and the expression of those two TSPYLs. Neither REST nor ZBTB7A appeared to bind to the promoter of *TSPYL2*, so *TSPYL2* was not included in this series of experiments.

Candidate Transcriptional Regulators of *TSPYL1*, *TSPYL2,* and *TSPYL4*. The initial list of candidate TFs that might participate in regulation of the expression of *TSPYL1*, *TSPYL2,* and *TSPYL4* was assembled based on their ability to bind to promoter regions of the genes encoding these three *TSPYLs* based on ENCODE data for HepG2 cells. Specifically, using HepG2 cell ChIP-seq data, we identified TFs that bound to 2 Kb regions extending 1 Kb on either side of the transcription start sites for *TSPYL1*, *TSPYL2,* or *TSPYL4.* As the next step, significant correlations between expression levels of these candidate TFs and the expression of *TSPYL1*, *TSPYL2,* and *TSPYL4* were determined by Pearson correlation analysis of hepatic eQTL expression data obtained from the Genotype-Tissue Expression database-with the full understanding that hepatic tissue expression might differ significantly from that for either HepG2 or HepaRG cells, the two cell lines used in our experiments. TFs with correlation coefficients >0.2 were then advanced to the next step of the analysis. By applying this step-wise narrowing-down process, we identified the 30 candidate TFs that are listed in Fig. 1. Those 30 candidate TFs were then knocked down in HepaRG cells using siRNAs with KD efficiencies as shown graphically in Supplemental Fig. 1. We used HepaRG rather than HepG2 cells in these experiments because they have been reported to better reflect the biology of hepatocytes than do HepG2 cells (Ramboer et al., 2015). The mRNA expression levels of *TSPYL1*, *TSPYL2*, and *TSPYL4* and of *CYP3A4*, *CYP2C9,* and *CYP2C19* were then assayed in HepaRG cells by qRT-PCR as shown in Fig. 2 for *CYP3A4*. Panels (a), (b), and (c) in Fig. 2 display data for the expression of *CYP3A4* versus those of *TSPYL1*, *TSPYL2,* and *TSPYL4*, respectively, after KD of the candidate TFs, with each black or red circle representing one of the 30 TFs studied and with error bars showing the impact of KD of the indicated TSPYL as a vertical line and the impact on the expression of *CYP3A4* as a horizontal line. We have highlighted points in Fig. 2 in red in which the relationship of the expression of those TFs mapped to the lower right quadrant of the four quadrant graphical representations of the data—i.e., these were TFs that were associated with increased expression of *CYP3A4* in the setting of decreased *TSPYL* expression—a relationship that we had reported previously in our KD and OE studies of TSPYL genes in HepaRG cells (Qin et al., 2018). After excluding TFs already known to be involved in the regulation of *CYP3A4* expression (Martinez-Jimenez et al., 2007; Jover et al., 2009) as well as TFs known to bind to the promoter region of *CYP3A4* based on ENCODE data, 18 TFs were found to significantly influence the mRNA expression of *CYP3A4* and at least one of the TSPYLs in HepaRG cells (fold change ≥2). However, only the KD of *REST, MAFK,* and *ZBTB7A* resulted in the downregulation of *TSPYL* expression coupled with the

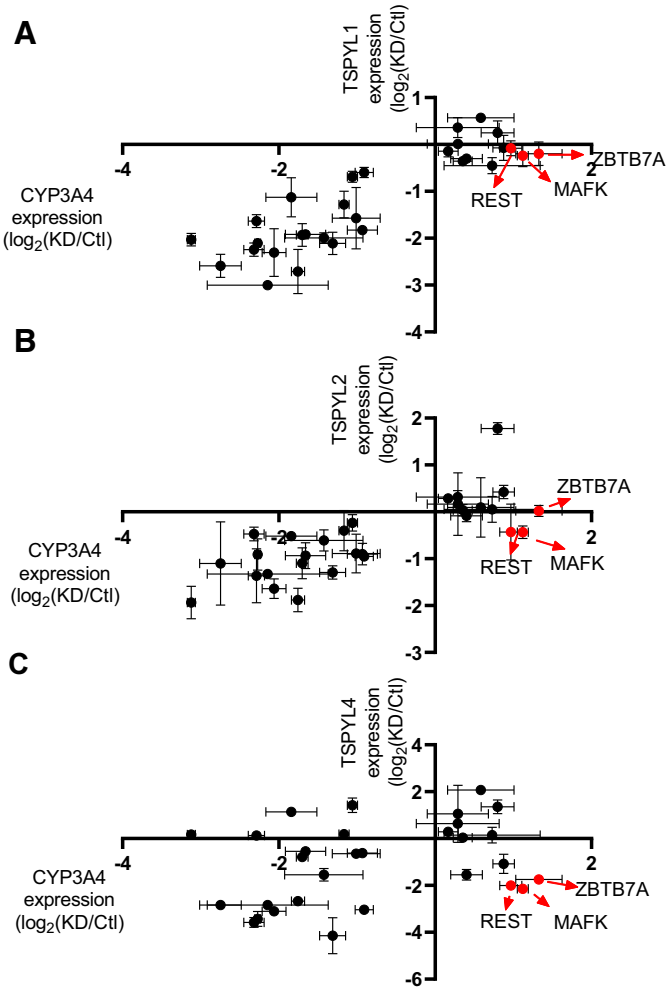

Fig. 2. RT-qPCR quantification of expression for *TSPYL* genes and *CYP3A4* in HepaRG cells after individual knockdown of 30 candidate TFs. The relative mRNA expression values for *TSPYL* genes and *CYP3A4* after KD of selected TFs, as listed in Fig. 1, were plotted on a $\log_2$ scale after being normalized to expression of the housekeeping gene *GAPDH*. Highlighted in red are data for *ZBTB7A*, *REST,* and *MAFK*. Each point represents the expression of *TSPYLs* versus *CYP3A4* after KD of one TF, measured in triplicate, and error bars indicate the S.D. for each point.

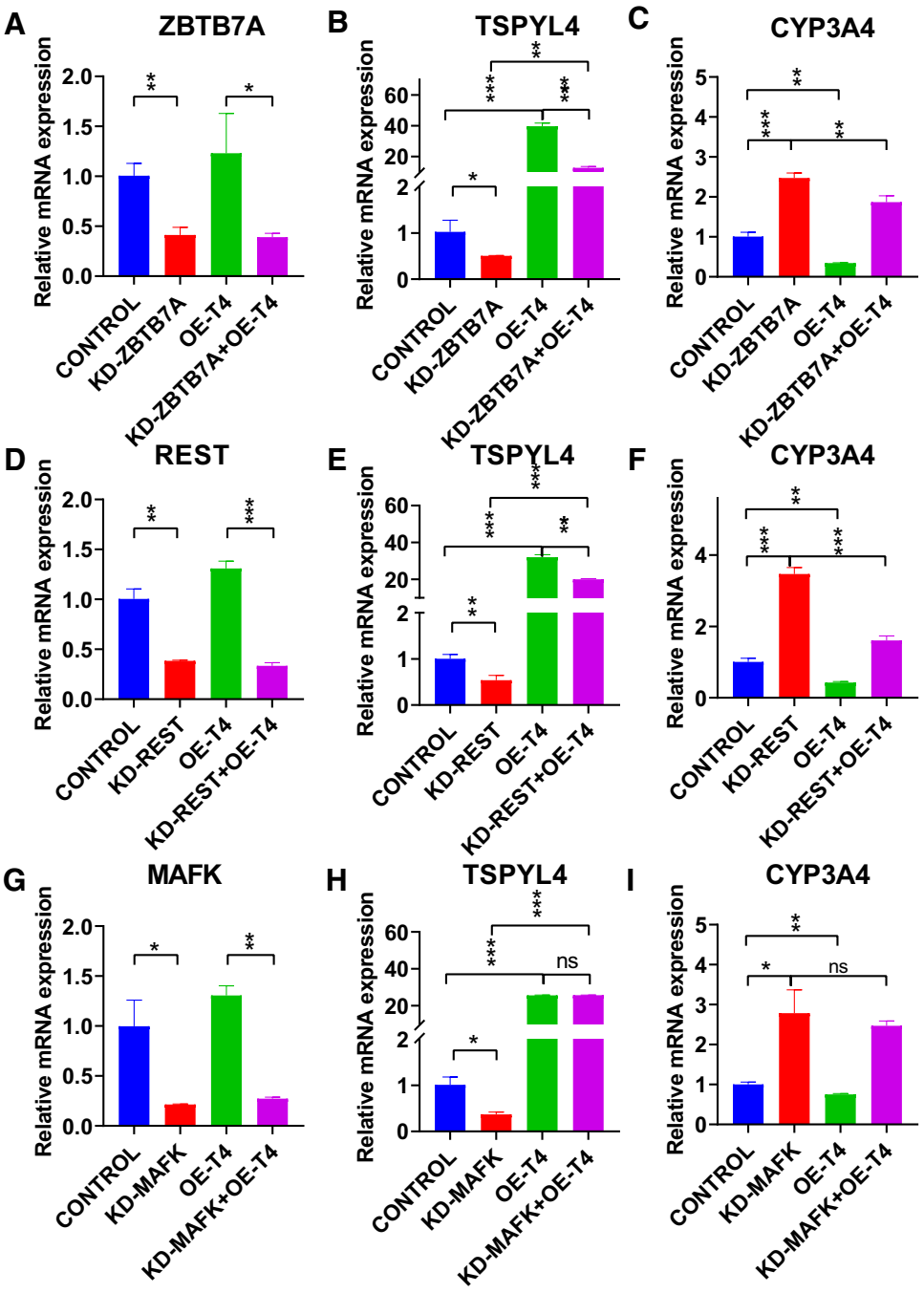

Fig. 3. Transcriptional regulation of *TSPYL4* and *CYP3A4* genes by *ZBTB7A*, *REST*, and *MAFK*. In HepaRG cells, *TSPYL4* and *CYP3A4* mRNA levels were determined after co-transfection with non-targeting siRNA (CONTROL) or siRNA targeting a–c) *ZBTB7A*, d–f) *REST*, or g–i) *MAFK*, and empty vector or plasmids overexpressing *TSPYL4*. The mRNA levels of *CYPs* in KD-only or OE-only were compared with those for cells transfected with negative siRNA and empty vector, and expression levels of KD+OE-TSPYL4 were compared with KD-only samples in three independent experiments by two-tailed student's *t* test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Error bars represent the S.D. of three replicates.

up regulation of *CYP3A4* expression (see Fig. 2 panel c), consistent with our previous findings. KD of these three TFs resulted in striking upregulation of *CYP3A4* expression coupled with the downregulation of *TSPYL4* expression. Supplemental Fig. 1A) through (f) shows similar data for *CYP2C9* and *CYP2C19*. However, for *CYP2C9* and *2C19*, as shown in the figure, there was a noticeable absence of points in the lower right quadrants of the figures. As a result, *REST, MAFK,* and *ZBTB7A* were selected for further study after a rescue experiment designed to verify our initial results and to help determine the mechanism of regulation of *CYP3A4* expression by TSPYLs—in this case focusing on *TSPYL4*.

Transcriptional Regulation of CYPs by TFs by Regulation of TSPYL Expression. As the next step in this series of experiments, a candidate TF KD and *TSPYL4* OE rescue study was performed for *REST*, *MAFK,* and *ZBTB7A*, the three TFs that displayed the most striking upregulation of *CYP3A4* expression after the downregulation of *TSPYL4* (see Fig. 2C). The results of that experiment, as shown in Fig. 3, demonstrated that KD of *ZBTB7A, REST,* and MAFK consistently resulted in increased expression of *CYP3A4*, while OE of *TSPYL4* decreased *CYP3A4* expression. However, *TSPYL4* OE was able to reverse the upregulation of *CYP3A4* expression (Fig. 3, B and E) only after *ZBTB7A* or *REST* KD, but not after *MAFK* KD (Fig. 3H).

Based on the results of the rescue experiment, we concluded that *ZBTB7A* and *REST* clearly enhanced *TSPYL4* expression which, in turn, downregulated the expression of *CYP3A4*. Similar results were observed when we used NCI-H2405 human lung adenocarcinoma cells to perform similar studies (see Supplemental Fig 2, B and C). Therefore,

REST and *ZBTB7A* appeared to be negative regulators of CYP3A4 expression as a result, at least in part, of an effect on *TSPYL4* expression. As the next step in the analysis, ChIP-qPCR experiments were performed using HepaRG cells to validate specific TF binding sites in the promoter regions of the *TSPYL1* and *TSPYL4* genes (Fig. 4). Specifically, primers were designed to target the promoter regions of *TSPYL4* and *TSPYL1* based on *ZBTB7A* ChIPseq performed with HepG2 cells (ENCODE experiment ENCSR000BQA) and REST ChIPseq performed with hepatic tissue (ENCODE experiments ENCSR867WPH and ENCSR893QWP) (see Supplemental Table 3). We chose to study *TSPYL1* and *TSPYL4* together because those two genes map only 20 kbp away from each other. The results of ChIP-qPCR, as shown in Fig. 4, demonstrated significant enrichment of the binding of ZBTB7A or REST antibody to the promoter regions of both *TSPYL1* and *TSPYL4* as compared with IgG, indicating that both *ZBTB7A* and *REST* could bind directly to the promoter regions of these two *TSPYLs*.

SNP-Dependent Modulation of Transcriptional Regulatory Activity for TSPYL Expression by REST and ZBTB7A.

We next identified six common SNPs, rs9400898(G/C), rs3828743(G/A), rs3749895 (C/G), rs910391(T/G), rs17524614 (G/T), and rs2232470 (C/A) that mapped within the ChIPseq peaks for *REST* (ENCODE experiments ENCSR867WPH and ENCSR893QWP) and *ZBTB7A* (ENCODE experiment ENCSR000BQA) on the *TSPYL1* and *TSPYL4* gene promoter regions. Specifically, the first three SNPs mapped to the *TSPYL1* promoter region and were in tight linkage disequilibrium, with minor allele frequencies (MAFs) that ranged from 0.26 to 0.30 for the group near the transcription start site for *TSPYL1* based on 1000 Genomes Project data (https://www.genome.gov/27528684/1000-genomes-project) (Supplemental Table 5), while the latter three SNPs mapped to the *TSPYL4* promoter region with MAF values that ranged from 0.16 to 0.33, as depicted graphically in Fig. 5A and Supplemental Table 6.Luciferase reporter constructs were then designed that incorporated wild-type and variant SNP *TSPYL* promoter region SNPs, as shown schematically in Fig. 5, B and C to study possible SNP-dependent modulation of regulatory activity. Specifically, after transfecting HepaRG cells with a series of luciferase reporter constructs, we found that the *TSPYL1* variant promoter displayed reduced luciferase activity (Fig. 5B) as compared with the *TSPYL1* wild-type promoter, while the *TSPYL4* variant promoter resulted in increased luciferase activity (see Fig. 5C) as compared with the *TSPYL4* wild-type promoter, thus revealing SNP-dependent regulatory differences in their effect on *TSPYL* gene expression. Very similar results were observed when we transfected luciferase reporter constructs into Caco2 and HepG2 cells, using the same constructs that had been employed in a previous *TSPYL* study (Qin et al., 2020) (See Supplemental Fig. 4, A–D).

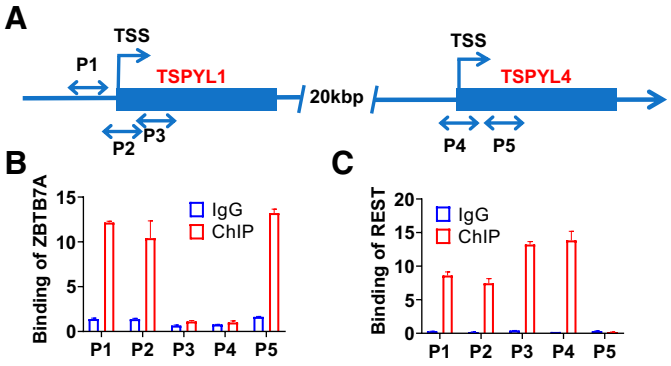

Fig. 4. ChIP assays for *ZBTB7A* and *REST* in HepaRG cells. a) Primer sets targeting *TSPYL1* and *TSPYL4* promoter regions are indicated as follows: for *TSPYL1* P1: −280 bp to −30 bp; P2: −50 bp to +114 bp; P3: +92 bp to +264 bp) and for *TSPYL4* (P4: −86 bp to +93 bp; P5: +262 bp to +433 bp). Nucleotides have been designated as negative or positive numbers if they are downstream or upstream from the transcription start site for *TSPYL1* or *TSPYL4*. Bindings of the transcription factors b) *ZBTB7A* or c) *REST* to promoter regions of *TSPYL1* and *TSPYL4* were determined by qRT-PCR and are shown as fold enrichment over input. Error bars represent the S.D. of two replicates.

Discussion

Our previous studies showed that *TSPYLs* are transcriptional regulators of several *CYPs* and that downregulation of the expression of *TSPYLs* can result in the upregulation of *CYP3A4, CYP2C9,* and *CYP2C19* gene expression (Qin et al., 2018; Qin et al., 2020). Furthermore, genetic polymorphisms present in both the *TSPYLs* and *CYPs* [27,28,29] are known to be associated with variation in drug response phenotypes, but those polymorphisms only explain a portion of the population variability that has been observed in *CYP* expression. The present study of upstream regulators of *TSPYL* expression has revealed additional factors that could contribute to individual variation in *CYP*-dependent drug metabolism pathways. The results of the series of experiments described here may help us better understand molecular factors that contribute to that variation.

We used the HepaRG cell line for most of our studies since those cells have been reported to be more similar biologically to human hepatocytes than are many other widely used hepatic cell model systems such as HepG2, Huh7 or the Hep3B (Zeilinger et al., 2016), especially in terms of basal *CYP* expression and/or induction (Ramboer et al., 2015). Our mRNA expression results for *TSPYLs* and *CYP3A4* after the silencing of *REST* and *ZBTB7A* reflect known eQTL relationships that have been reported between *TSPYLs* and *CYPs* in HepaRG cells (Qin et al., 2018; Qin et al., 2020), and they made it possible for us to identify REST and ZBTB7A as upstream regulators of *TSPYL4* expression. Our studies of the expression of *TSPYLs* and *CYPs* after silencing these TFs as well as rescue experiments performed with *TSPYL* overexpression vectors further supported the apparent regulation of *TSPYL* gene expression by *REST* and *ZBTB7A*. Future proteomic analysis (Graves and Haystead, 2002) of HepaRG cells might provide insight into additional transcriptional cofactors involved in the sequential, stepwise regulation of the expression of *TSPYLs* and, downstream, of *CYPs*.

In one of our previous reports, 4 missense variants in *TSPYL* genes were studied, with the identification of one missense SNP, rs3828743 (G/A) (Pro62Ser), in the *TSPYL1* open reading frame that abolished the suppression of *CYP3A4* expression by *TSPYL1* due to loss of the ability of *TSPYL* variant protein to bind to the *CYP* promoter region (Qin et al., 2018). In the present study, we observed that the rs3828743 variant genotype results in SNP-dependent modulation of transcriptional regulatory effects on the expression of *TSPYL1*. That SNP, rs3828743, resides in the binding region for *REST* and *ZBTB7A*, as shown by published ChIPseq data and by our ChIP-qPCR data. As a result, variation of *TSPYL1* expression based on rs3828743genotype may contribute, at least in part, to variation in the impact of *REST* and *ZBTB7A* on transcriptional activity at this locus.

Based on our previous studies, we know that the *TSPYL4* rs910391 SNP, an SNP that is in tight linkage disequilibrium with the *TSPYL1* rs10223646 SNP, is associated with baseline depression severity in major depressive disorder patients (Qin et al., 2020). In the present study, we showed that rs910391 maps to the binding region for *REST* and *ZBTB7A*, as demonstrated by published ChIPseq data and by our own ChIP-qPCR data. In addition, we observed that the rs910391 genotype variants contribute to SNP-dependent modulation of *TSPYL4* expression, which is at least partially responsible for variation in the transcription activity of *TSPYL4*. Further study of cis eQTL SNPs for additional upstream transcription factors like those identified in the present study might provide mechanistic insight into regulation of the expression of drug metabolizing

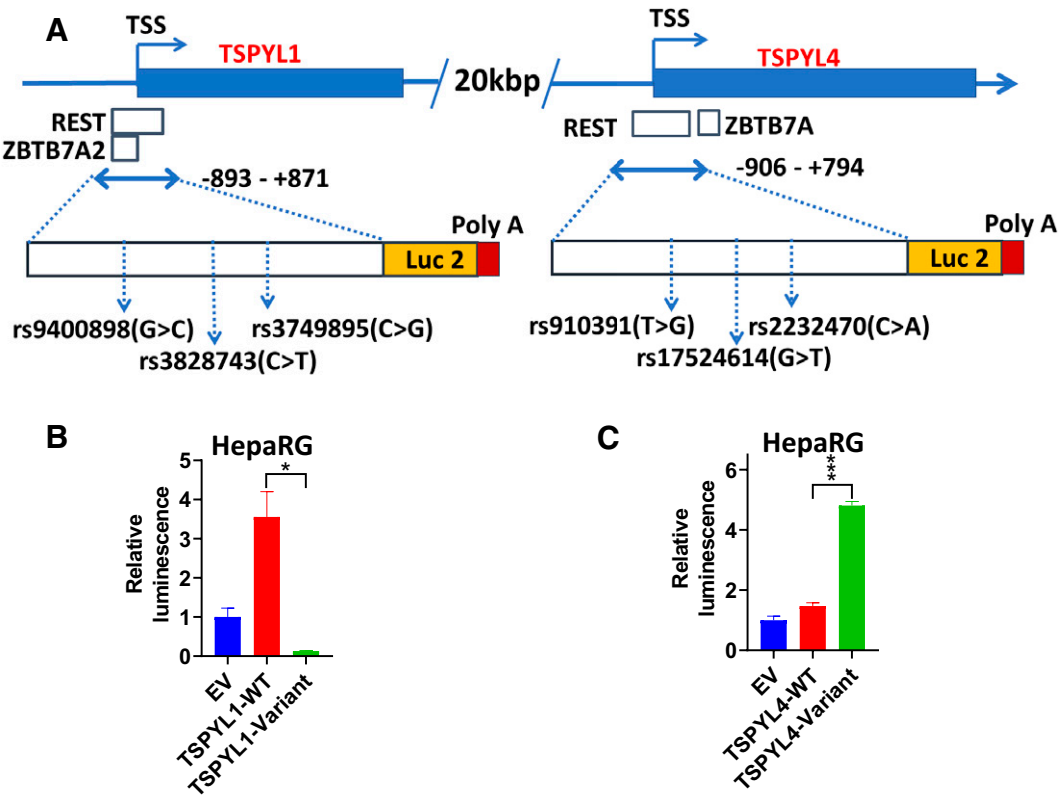

Fig. 5. SNP-dependent modulation of transcriptional activity for *TSPYL1* and *TSPYL4* promoter regions. a) Genome line diagrams of the *TSPYL1* and *TSPYL4* promoter regions showing the locations of common SNPs in Caucasians with MAF values >1% (arrows). Binding regions for *ZBTB7A* and *REST* based on published ENCODE ChIPseq data are also indicated. b) Luciferase reporter constructs were created for TSPYL promoter regions either with wild-type or variant genotypes for the indicated SNPs to study SNP-dependent transcriptional activity of *ZBTB7A* and *REST* binding loci. b) and c) Luciferase assays using HepaRG cells co-transfected for b) *TSPYL4* or c) *TSPYL1* promoter firefly luciferase reporters and Renilla luciferase constructs. Transfection efficiencies have been normalized based on Renilla luciferase reporter signals. Differences in normalized luciferase activity between WT and variant TSPYL promoter constructs were then compared in three independent experiments by two-tailed student's *t* test, $*P < 0.05$, $***P < 0.001$. Error bars represent the S.D. for three replicates.

enzymes and of genetic polymorphisms associated with variability in drug response.

In summary, the novel transcription factors *REST* and *ZBTB7A* appear to be transcriptional regulators of *TSPYL* gene expression resulting in variation in expression which then plays a role downstream in CYP expression and CYP-mediated variation in drug metabolism. This series of events represents a novel upstream source of variation in downstream *CYP* expression that may mechanistically help us to better understand variation in *CYP* expression. Ultimately, this novel SNP-dependent modulation of transcription regulating *TSPYL* expression and activity may contribute to variability in both *CYP* expression and, as a result, variation in drug response phenotypes.

Authorship Contributions

Participated in research design: Shivaram, Gao, Qin, Liu, Weinshilboum, Wang.

Conducted experiments: Shivaram, Qin.

Performed data analysis: Shivaram, Gao, Qin, Liu.

Wrote or contributed to the writing of the manuscript: Shivaram, Gao, Liu, Weinshilboum, Wang.

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Drug Metabolism and Disposition

Supplementary Materials

Cytochrome P450 Transcriptional Regulation by TSPYLs: Identification of Novel Upstream Transcription Factors

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Article number: DMD-AR-2022-000945

Supplementary Table 1: SiRNAs from Dharmacon/Horizon Discovery

Gene	Dharmacon Catalog number
SP1	M-026959-00
TAF1	M-005041-01
SIN3A	M-012990-00
MXI1	M-009947-02
TBP	M-011790-01
CEBPB	M-006423-03
EP300	M-003486-04
CHD2	M-008948-01
RFX5	M-011103-01
YY1	M-011796-02
ZBTB7A	M-020818-00
BRCA1	M-003461-02
ZBTB33	M-019982-01
MAFF	M-003903-00
MAFK	M-008580-01
FOXA1	M-010319-01
FOXA2	M-010089-01
MYBL2	M-010444-01
FOSL2	M-004110-00
RCOR1	M-014076-01
REST	M-006466-02
RAD21	M-006832-01

SMC3	M-006834-01
HSF1	M-012109-01
HDAC2	M-003495-02
ARID3A	M-012032-01
TEAD4	M-019570-03
NFIC	M-008362-00
HNF4A	M-003406-02
HNF4G	M-003407-02

Supplementary Table 2: Prime time primers for qRT-PCR

Gene	Forward	Reverse
TSPYL1	CTAGAAATCCAAGTGACAGCAAC	AGCCAAGCAAATGATACTGTAGA
TSPYL2	ATGTCTTCATCACGTCGGTT	GTCTCAAGCGCAAGTTCATC
TSPYL4	CTTCCTCAATGCCCCAACTC	ACTCCTAGCCAACAAACCATC
CYP3A4	ATCATGTCAGGATCTGTGATAGC	GGGAAATATTTTGTCCTACCATAAGG
CYP2C19	GGTAATCACTGCAGCTGACT	TCAATCTCTTCCTGGACTTTAGC
CYP2C9	CTGCAGTTGACTTGTTTGGAG	GTTTCTGCCAATCACACGTTC
ARID3A	GACTTGTTCAGCTTCATGCAG	CTTCTCCGTCACCAGCAC
BRCA1	AATGGAAGGAGAGTGCTTGG	ATACCTGCCTCAGAATTTCCTC
CEBPB	AGAAACGTCTATGTGTACAGATGA	GATTGCATCAACTTCGAAACCG
CHD2	ATCCGAAGGTTCATCAAGGC	TGCCACCGACTTATCTACCA
EP300	GCGGCCTAAACTCTCATCTC	GTAAGTCGTGCTCCAAGTCA
FOSL2	GATCAAGACCATTGGCACCA	CAGCCAGCTTGTTCCTCT
FOXA1	CCAGGATGTTAGGAACTGTGAA	CTGAGTTCATGTTGCTGACC
FOXA2	GGAGCGGTGAAGATGGAAG	TGTTCATGCCGTTCATCCC
HNF4A	GCCATCATCTTCTTTGACCCA	GATGTAGTCCTCCAAGCTCAC
HNF4G	GAGCAACAGGAAAACACTATGG	CAACACATTGCCGACTGAAC
HSF1	CCTCCACCCCTGAAAAGTG	GGAGTCCATAGCATCCAAGTG
MAFF	GCTAGGAGTGAGGGATGTGA	CTCAGCTCTCGCTTGATCT
MAFK	GACGCCAGCTACGAGTTC	GACACCAGCTCATCATCGC
MXI1	AGCACCCAAGTCTAAGTCAAC	CCGCTGCTGTGTTTCTGT
MYBL2	GATTCCTGTAACAGCCTCACG	CTCTCCAGCTCCAATGTGTC
NFIC	GGCGGCGATTACTACACTTC	CTTGTCCATCTCTGTCTTCTTCA
RAD21	GGAAAGAGACAGGAGGAGTAGA	GTGTAAGACAGCGTGTAAAGAG
RCOR1	CCCAGATAATTCCATTAAGATGCC	TAACACAGTAGTCCACACCAAG
REST	ACTAGACATATGCGTACTCATTCAG	CCATTGTGAACCTGTCTTGC
RFX5	ACCTACCACCCTTCTTCAGA	GTAGAGATACAGCTTGTCATTGTC
SIN3A	AGATGTTCACCATTCATGCCT	CACACAGATCTCATCACTCACG
SMC3	TGAGTTTAGTCATCTTCGTCCAG	TCCACAAAAGCAGAAATAACACG
SP1	GGTACTTCAGGAATCCAGGTG	GCTGTGTCATCATGTATTCCATC
TAF1	ATGGTTTGGAGGATAGCAACA	CTCCTCATCTTCTTCCTCCTCT
TBP	GATAAGAGAGCCACGAACCAC	CAAGAACTTAGCTGGAAAACCC
TEAD4	GACTCCTTGGAACTGGCTTAG	GATGTGGCTGGAGACCTG
YY1	CAGAATTTGCTAGAATGAAGCCA	CCGAGTTATCCCTGAACATCT
ZBTB33	TGGAGCGACGTTTAAAGAAGG	GCCAAGTGAAGTCAACAGACA
ZBTB7A	GAAGCCCTACGAGTGCAAC	GGTTCTTCAGGTCGTAGTTGTG
GAPDH	ACATCGCTCAGACACCATG	TGTAGTTGAGGTCAATGAAGGG

Supplementary Table 3: ChIP-qPCR primer sets for promoter regions of TSPYL1 and TSPYL4

Gene	Sequences
TSPYL1 **- P1**	**Forward: TTCCAGACTCAGCACAATCG** **Reverse: TTTCCTCAGAGGCCGAACT**
TSPYL1 **- P2**	**Forward: AGTTCGGCCTCTGAGGAAA** **Reverse: AGCCTCAGGTACTGGTGTGC**
TSPYL1 **- P3**	**Forward: GACGCACACCAGTACCTGAG** **Reverse: CAACTCGGATCTGGGGAGTA**
TSPYL4 **- P4**	**Forward: AGTAAAGGAGGGGTGGTGCT** **Reverse: GGATCTCCTGAGGCATGGT**
TSPYL4 **- P5**	**Forward: GGATGCTCCACCTTCTACGA** **Reverse: TTCTTCCCCGGTATCATCTG**

Supplementary Table 4: Primer sets for TSPYL1 and TSPYL4 promoter region amplification

Name	Restriction Site	Primer sequence
TSPYL1 - forward	Kpn1	aatgGGTACCAGCCTTTCCTCACAAAATGG
TSPYL1 - reverse	HindIII	actgAAGCTTGATGTAGTTCCTCCGCTCCA
TSPYL4 - forward	Kpn1	aatgGGTACCTCCACAGGCATTATGAAGCA
TSPYL4 - reverse	HindIII	actgAAGCTTCTCCTCCACCTCCAAATTGA

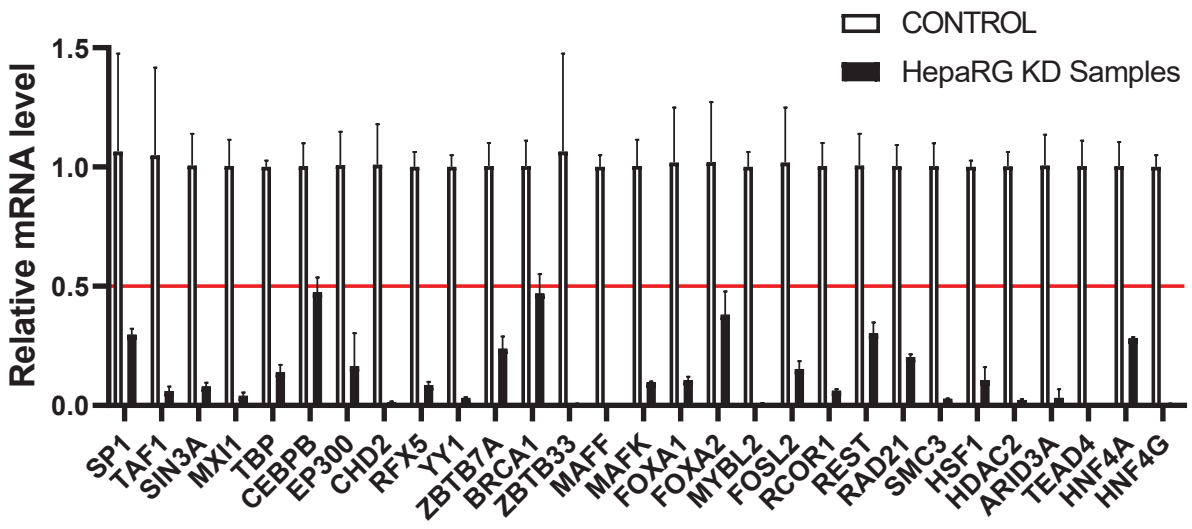
Supplementary Table 5: Linkage disequilibrium (LD) and minor allele frequencies (MAFs) for selected SNPs for the *TSPYL1* promoter regions

Promoter Region	SNPid	LD (r^2)			Ref	Alt	MAF (Eur)
		rs3749895	rs3828743	rs9400898			
TSPYL1	rs3749895		0.81	0.81	C	G	0.3
TSPYL1	rs3828743			1	C	T	0.26
TSPYL1	rs9400898				G	C	0.26

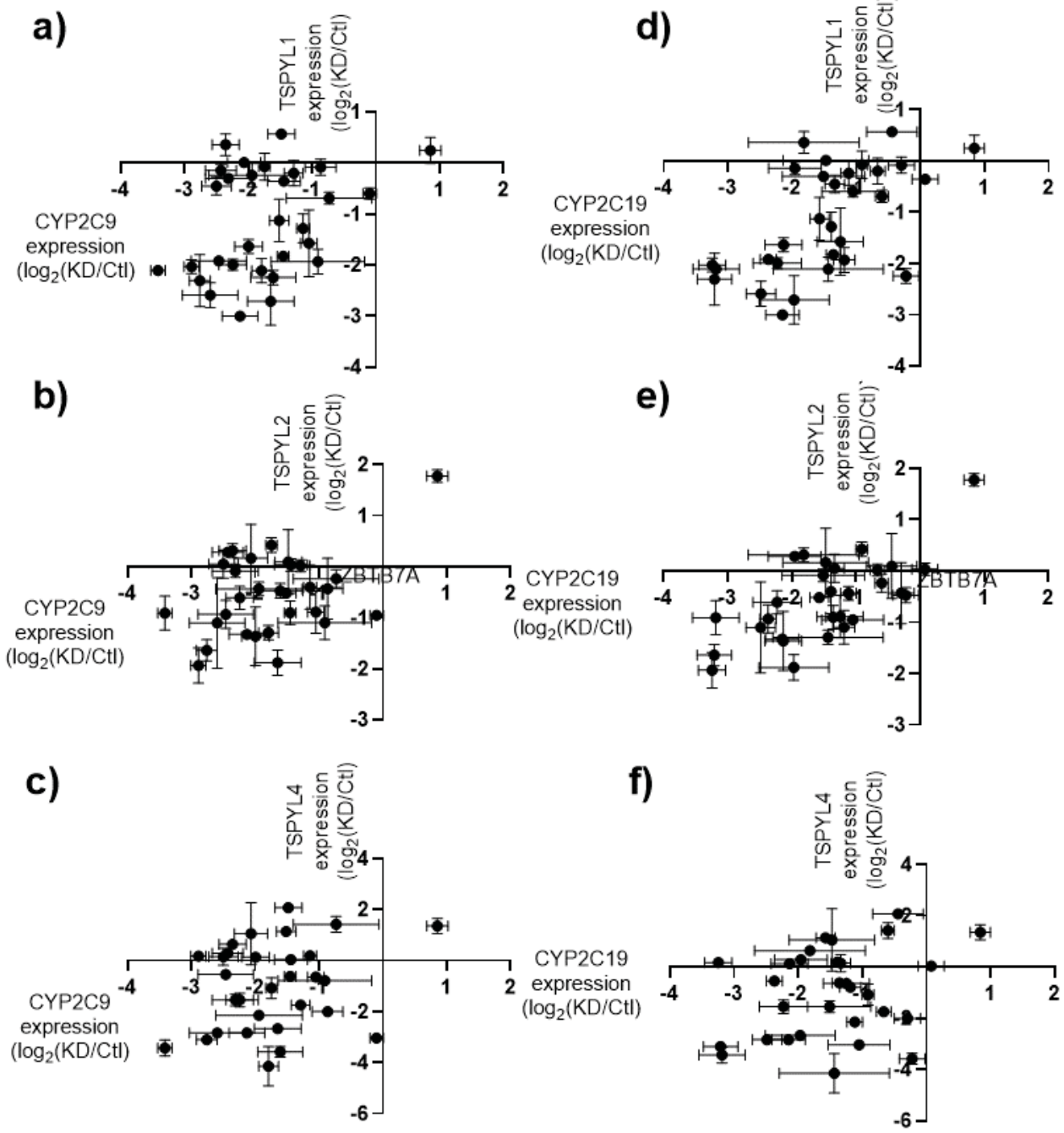
Supplementary Table 6: LD and MAFs for selected SNPs for the *TSPYL4* promoter regions

Promoter Region	SNPid	LD(r^2)			Ref	Alt	MAF (Eur)
		rs2232470	rs910391	rs17524614			
TSPYL4	rs2232470		0.96	<0.2	C	A	0.67
TSPYL4	rs910391			<0.2	T	G	0.67
TSPYL4	rs17524614				G	T	0.16

Supplementary Figures

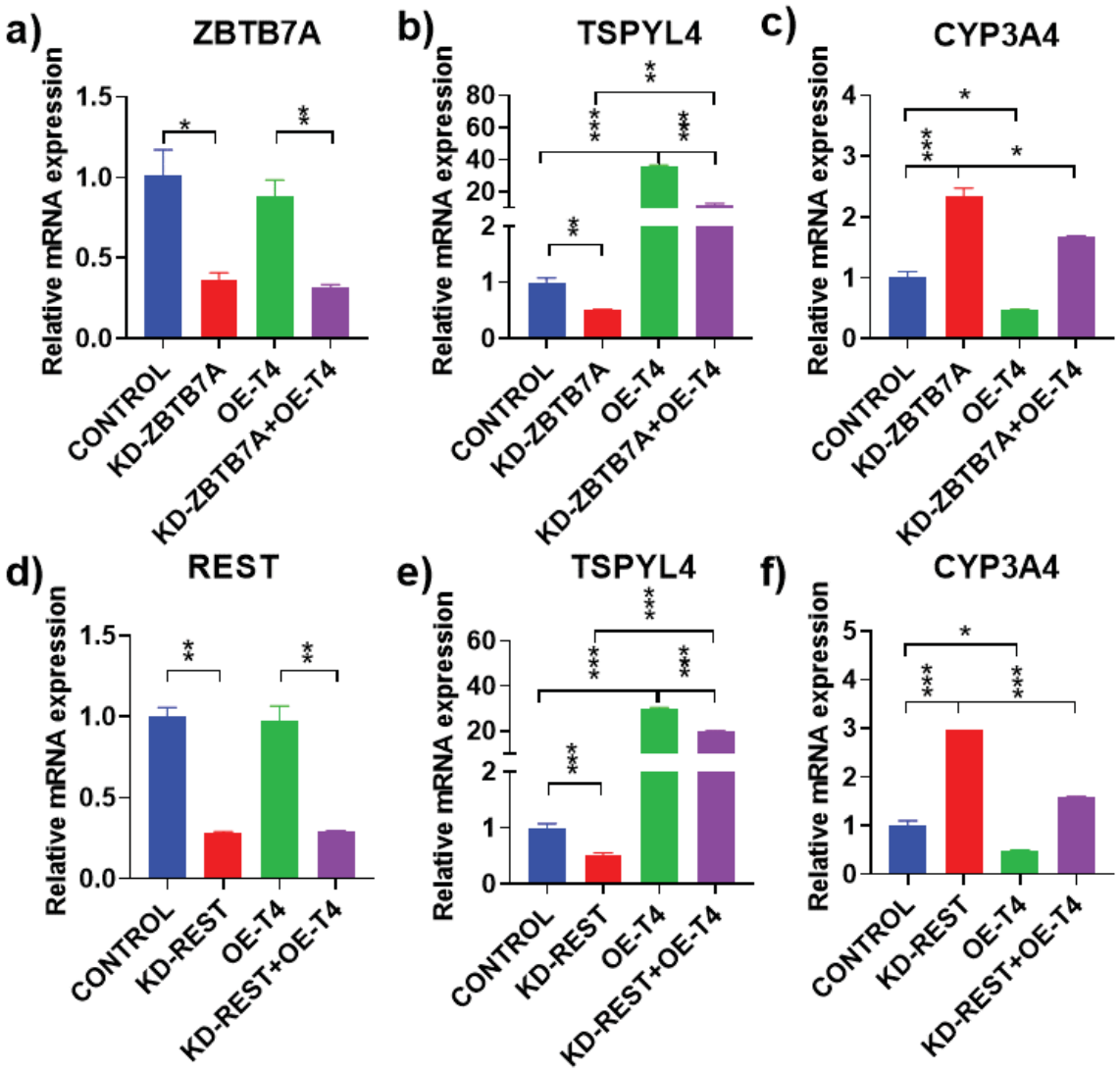


Supplementary Figure 1: Knockdown efficiency achieved in HepaRG cells. The mRNA levels of candidate TFs were compared to those in cells transfected with negative SiRNA in three independent experiments. Error bars represent SDs of three replicates.



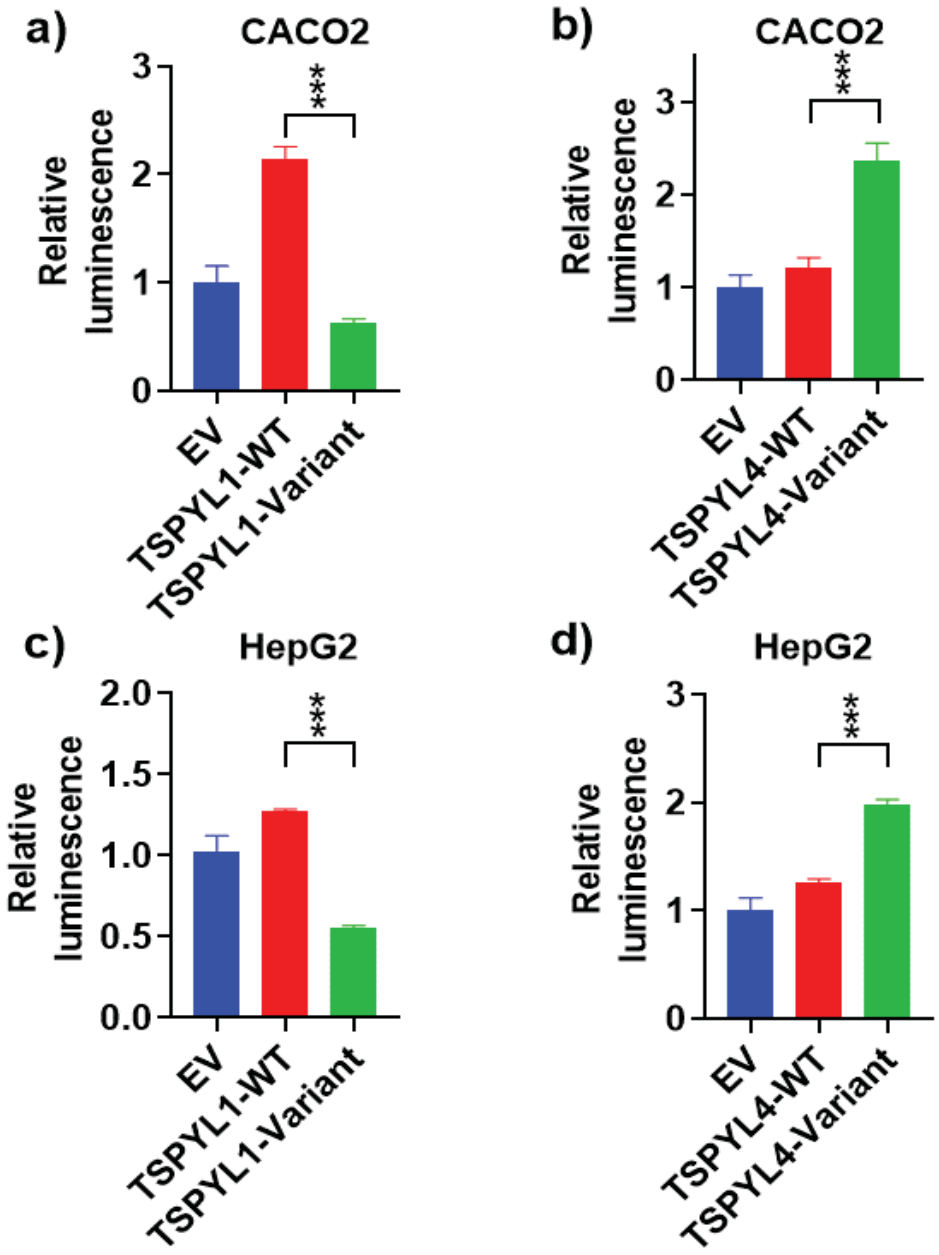
Supplementary Figure 2: RT-qPCR quantification of expression for TSPYL genes and *CYP2C9* and *CYP2C19* in HepaRG cells after selected TF knockdown. The relative mRNA expression values for TSPYL genes and CYP genes after selected TF Knock Down were plotted on log scales after being normalized to the expression of the housekeeping gene *GAPDH*. a) CYP2C9 vs TSPYL1; b) CYP2C9 vs

TSPYL2; c) CYP2C9 vs TSPYL4; d) CYP2C19 vs TSPYL1; e) CYP2C19 vs TSPYL2; f) CYP2C19 vs TSPYL4. Error bars represent SDs for three independent replicates.



Supplementary Figure 3: Transcriptional regulation of the expression of *TSPYL4* and *CYP3A4* by ZBTB7A and REST. NCI-H2405 cells were co-transfected with non-targeting siRNA (CONTROL) or siRNA targeting **a-c**) ZBTB7A or d-f) REST, and empty vector or a plasmid overexpressing *TSPYL4*. mRNA levels for **a**) *ZBTB7A* **b, e**) *TSPYL4*, **c, f**) *CYP3A4* and **d)** REST were analyzed by RT-qPCR. The mRNA levels after KD-only or OE-only samples were compared to those in cells transfected with

negative siRNA and empty vector, and mRNA levels of KD+OE-TSPYL4 were compared with KD-only samples in three independent experiments with significance tested by two-tailed student's t-test, *p <0.05, **p < 0.01, ***p <0.001. Error bars represent the SDs of three independent replicates.



Supplementary Figure 4: SNP-dependent modulation of the transcriptional regulation of TSPYLs at *REST* and *ZBTB7A* binding sites. a) and **b**) Luciferase Assays for **a**) *TSPYL4* and **b)** *TSPYL1* promoter reporter constructs in *CACO2* cells and for luciferase Assays for **c)** *TSPYL4* and **d)** *TSPYL1* promoter reporter constructs in HepG2 cells. Transfection efficiencies were normalized using Renilla luciferase reporter genes. The differences in normalized luciferase activity between WT and variant TSPYL promoter constructs were compared in three independent experiments by two-tailed student's t-test, ***p <0.001. Error bars represent SDs of three independent replicates.

Supplementary Methods and Materials:

ENCODE ChIP Seq data:

The ENCODE UCSC genome browser includes ChIP-seq data for HepG2 cells that shows TF binding to the promoter regions of *TSPYL1,* TSPYL2 and *TSPYL4*.

LIVER eQTL data-based association analysis:

We determined associations between expression of these TFs and *TSPYL1, TSPYL2* or *TSPYL4* in liver eQTL databases using Pearson correlations and identified TFs that were significantly associated with *TSPYL1, TSPYL2* or *TSPYL4* expression with p- values < 0.05.

Cell Culture:

Undifferentiated HepaRG ™ cells (HPR101) were purchased from Biopredic Internationals, Saint-Grégoire, France. Undifferentiated HepaRG cells are human hepatic stem cell lines with the ability to express the full array of cytochrome P450s with function that mimicks that of primary human hepatocytes. The HepaRG cells were cultured in base Williams' Medium E (12551032) with growth medium supplementation (ADD711C) (Biopredic internationals, Saint-Grégoire, France) for 2 weeks. After obtaining a sufficient number of cells, they were cultured in base Williams' Medium E (12551032) with differentiation medium supplementation

(ADD721C) (Biopredic internationals, Saint-Grégoire, France) for 2 weeks to transform them into mature human hepatocytes.

HepG2 cells (ATCC® HB-8065™), NCI H2405 cells (ATCC® CRL-5944™) and Caco2 cells (ATCC® HTB-37™) were purchased from American Type Culture Collection (ATCC, Manassas, VA). HepG2 cells are a human hepatoma cell line which was cultured in base medium Eagle's minimum essential medium EMEM (ATCC 30-2003) with 10% Fetal Bovine Serum FBS (ATCC 30-2020).

NCI H2405 cells are human lung adenocarcinoma cells which were cultured in RPMI-1640 Medium (ATCC 30-2001) with 5% Fetal Bovine Serum (FBS; ATCC 30-2020).

Caco2 cells are a colorectal adenocarcinoma cell line. These cells were cultured in base medium Eagle's minimum essential medium EMEM (ATCC 30-2003) with 10% Fetal Bovine Serum FBS (ATCC 30-2020).

KD screening of HepaRG cells:

Specific targeting short interfering RNAs (siRNAs) for 30 potential TFs and nontargeting siRNA were ordered from Dharmacon (Lafayette, CO). Differentiated HepaRG cells were seeded in 6-well plates at a density of 2×10^5 cells/well with appropriate medium overnight. The next day, cells were transfected with either nontargeting siRNA or siRNAs targeting one of the 30 TFs with lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific, Waltham, MA) in OptiMEM media following the manufacturer's instructions. Cells were harvested 48 hours after transfection and RNA was extracted.

Co-transfection of siRNA and *TSPYL4* overexpression plasmids:

Specific targeting short interfering RNAs (siRNAs) for ZBTB7A, REST and nontargeting siRNAs were ordered from Dharmacon (Lafayette, CO). *TSPYL4* OE plasmid construct and

respective empty vector pCMV6-XL4 were purchased from Origene Technologies (Rockville, MD). Differentiated HepaRG, HepG2, or NCI H2405 cells were seeded in 6-well plates at a density of 4×10^5 cells/well overnight. The next day, the cells were co-transfected using nontargeting siRNA or siRNAs targeting *REST* or *ZBTB7A, as well as* empty vector or TSPYL4 OE plasmids using lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) in OptiMEM media following the manufacturer's instructions. Cells were harvested after 48 hours and RNA was extracted.

Gene expression quantification:

Total RNA was extracted from HepG2 and HepaRG cells with Zymo research the Quick RNA prep kit (Irvine, CA). The concentration of RNA was measured using a Nano drop 8000. PCR reactions were performed with100 ng of total RNA, 5 µl of 2X SYBR green qPCR master mix (Life technologies, CA USA), 1 µl of gene specific primer and distilled water up to 10 µl final volume per reaction. Primer sets for real time PCR are listed in **Supplementary Table 2**. Real time PCR reactions were performed in triplicate using the Applied Biosystems Vii A 7™ real-Time PCR System (Life Technologies, Carlsbad, CA, USA). RNA concentrations across all KD samples and negative controls were normalized to 100ng/µl. Expression levels for TSPYL1, TSPYL 2, TSPYL4, CYP3A4, CYP2C9, and CYP2C19 were tested in all TF KD samples and in negative controls by using the ΔΔCT method in qRT-PCR. GAPDH was used as an internal control for quantification. Alteration of gene expression levels for TSPYL1, TSPYL 2, TSPYL4, CYP3A4, CYP2C9 and CYP2C19 were noted as fold changes as well as statistically significant p-values using student's t- test with < 0.05 being significant.

Chromatin Immunoprecipitation (ChIP) in HepaRG:

HepaRG cells were used to perform ChIP assays to validate TF binding to the promoter regions of the *TSPYL1* and *TSPYL4* genes. Primer sets (see **Supplemental Table 3**) covered 500bp upstream and downstream of exon 1 of the *TSPYL1* and *TSPYL4* genes. ChIP assay was performed using the SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell Signaling Technology®, Boston, MA) followed by quantitative PCR using Takara SYBR Green PCR Master Mix reagent (Takara Bio, Saint-Germain-en-Laye, France). The real time Quantitative PCR assays were performed with 2µl of template, 1 µl of primers, 5 µl of PCR Master Mix reagent and nuclease free water to a 10 µl reaction volume. Real time PCR reactions were performed in triplicate using the Applied Biosystems Vii A 7™ real-Time PCR System (Life Technologies, Carlsbad, CA, USA). IP efficiency was calculated using the Percent Input method in which signals obtained from each immunoprecipitation (IP) were expressed as a percent of the total input chromatin using the following formula: Percent Input = 2% x 2 **(CT of 2% input sample – CT of IP samples)**. Input enrichment values for IP samples were compared with IgG control samples using student's t-test with a significant p-value threshold < 0.05.

TSPYL Promoter Constructs:

Lymphoblastoid cell lines (LCL) with known *TSPYL1* and *TSPYL4* promoter SNP genotypes were used as PCR templates. The primers were designed to amplify 2Kbp promoter regions of the *TSPYL1* and *TSPYL4* genes with KPNI and HINDIII restriction sites at the ends of the primers using the primer3 tool. The primer sets are reported in **Supplementary Table 4**. The PCR amplification was performed with Kapa HIFI hot start ready-mix reagents (KR0370) (Kapa Biosystems, Wilmington, MA) and with primers designed to obtain inserts for ligation with vector. The PCR reactions were set up with 1 ng of genomic DNA, 25µl of Kapa HIFI hot start ready-mix reagent, 1µl of forward and reverse primers and distilled water up to 50 µl. PCR

products were purified with the QIAquick PCR Purification Kit (28104) (QIAGEN, Germantown, MD). KPNI and HINDIII Restriction sites for both pGL4.23 [luc2/minP] vector (Promega, Madison, WI) and purified PCR products were digested at 37°C with restriction enzymes in smart cut buffer (New England Biolabs, Ipswich, MA). Digested PCR products and digested luciferase vectors were separated in 1% agarose gel. Appropriate bands were cut, and gel purified with the QIAquick Gel Extraction Kit (28704) (QIAGEN, Germantown, MD). The ligation reaction included digested 100ng luciferase vector DNA, 48ng digested insert DNA, 1µl Ligase 10X Buffer, 0.1–1unit of T4 DNA Ligase and Nuclease-Free Water to a final volume of 10µl at 4°C overnight (New England Biolabs, Ipswich, MA). The sequences of luciferase constructs (*TSPYL4* promoter Wild Type (WT) and *TSPYL4* promoter Variant (VT)) were confirmed by Sanger sequencing.

Luciferase Reporter Assay:

The firefly luciferase reporter vector, pGL4.23 (Cat#: E8411) was obtained from Promega with inserts encoding either 2Kbp of *TSPYL1* or 2Kbp of *TSPYL4* promoter regions and were used to create *TSPYL1* Wild Type promoter, *TSPYL1* variant promoter, *TSPYL4* wild type promoter or *TSPYL4* variant promoter constructs. HepaRG cells, HepG2 cells and Caco2 cells were seeded in 6 well plates at a density of 4×10^5 for overnight. The next day, the cells were co-transfected with firefly luciferase constructs and Renilla luciferase vector pRL-TK (Promega, Madison, WI) at a ratio of 20:1 using lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) in OptiMEM media following manufacturer's instructions. 48 hrs after transfection, luciferase assays were performed on these cells with the Dual-Luciferase Reporter assay system (Promega) following the manufacturer's instructions. Luciferase activities were normalized to Renilla

luciferase activities. The relative luciferase activity of variant samples was compared with WT samples using student's t-test with a significant p-value threshold < 0.05.