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TRANSCRIPTION FACTOR BINDING SITE ENRICHMENT ANALYSIS PREDICTS DRIVERS OF ALTERED GENE EXPRESSION IN NONALCOHOLIC STEATOHEPATITIS

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Abstract

The molecular mechanisms behind the transition from simple steatosis to nonalcoholic steatohepatitis (NASH) in nonalcoholic fatty liver disease (NAFLD) are not clearly understood. This hinders development of effective therapies for treatment and prevention of NASH. In this study expression profiling data from normal, steatosis, and NASH human livers was used to predict transcription factors that are misregulated as mechanistic features of NAFLD progression. Previously-published human NAFLD gene expression profiling data from normal, steatosis, and NASH livers was subjected to transcription factor binding site enrichment analysis. Selected transcription factors that bind enriched transcription factor binding sites were analyzed for changes in expression. Distinct transcription factor binding sites were enriched in genes significantly up- or down-regulated in NASH livers. Those enriched in up-regulated genes were bound by transcription factors such as FOXA, CEBP, and HNF1 family members, while those enriched in down-regulated genes were bound by nuclear receptors involved in xenobiotic sensing and lipid metabolism. Levels of mRNA and protein for selected transcription factors were significantly changed during disease progression. The study indicates that NAFLD progression involves changes in activity or expression of transcription factors that regulate genes involved in hepatic processes known to be altered in NASH. Transcription factors such as PPAR receptors, FoxA family members, and HNF4A might be targeted therapeutically to prevent NAFLD progression.

Graphical abstract

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Keywords

transcription factor; liver; nonalcoholic steatohepatitis; gene expression; metabolism; bioinformatics

1. Introduction

Approximately 30–40% of the U.S. population has nonalcoholic fatty liver disease (NAFLD) [1] and 5–17% are estimated to have progressed to nonalcoholic steatohepatitis (NASH) [2]. NAFLD occurs when altered processing of fatty acids and triglycerides leads to abnormal hepatic lipid accumulation resulting in steatosis. Increased inflammation and oxidative stress can cause progression to NASH, which is characterized by fibrosis and higher risk of cirrhosis and hepatocellular carcinoma [2]. While the pathological progression and characteristics of NAFLD are defined, a clear understanding of the underlying mechanisms is lacking.

Gene expression profiling (GEP) has been used to identify changes in regulatory mechanisms and signaling that drive disease progression [3;4]. However, this remains challenging due to the complexity of the resulting data and the poorly-understood functions of many genes. Cell signaling pathways ultimately target transcription factors (TFs) to alter gene expression. Thus, identifying TFs that drive changes in gene expression during disease progression may reveal key signaling pathways that are misregulated and could be targeted therapeutically. To regulate transcription TFs bind specific DNA sequences, generally referred to as transcription factor binding sites (TFBS). TFBS enrichment analysis has gained utility in elucidating the mechanistic basis for altered gene expression [4]. In this method, potential TFBS within regulatory regions of genes that show significant changes in expression between distinct conditions are identified. Statistical analysis is then performed to identify TFBS enriched around genes that are either up-or down-regulated. The enrichment of particular TFBS in the promoters of co-expressed genes can predict which TFs drive changes in their expression and ultimately reveal underlying regulatory mechanisms.

Previously, a GEP data set representing individual microarrays for multiple normal, steatosis, and NASH liver samples was generated to study mechanisms underlying NAFLD progression [5]. In the current study, TFBS enrichment analysis was performed to identify those that are over-represented in the promoter regions of genes either significantly up-regulated or down-regulated in NASH livers compared to normal or steatosis livers. The results revealed enriched TFBS bound by TFs that regulate key liver functions. In some cases their expression was found to be altered during NAFLD progression. Altogether the results indicate changes in the activity and/or expression of specific TF families during

NAFLD progression that could be targeted therapeutically to prevent NASH or limit its severity.

2. Materials and Methods

2.1 Human Liver Samples

Human liver samples representing the complete spectrum of NAFLD were acquired from the Liver Tissue Cell Distribution System. Post mortem and liver biopsy samples were scored and diagnosed using the Kleiner NAFLD activity scoring method [6], as previously described [5]. Demographics and other information have been previously published [7].

2.2 Human NAFLD Microarray Data

GEP data from individual Affymetrix Human GeneChip 1.0 ST arrays of normal (n=19), steatosis (n=10), NASH (n=16) liver samples were utilized in the analyses of TFBS enrichment and TF mRNA levels. A total of 33,252 genes were analyzed and are accessible at the ArrayExpress public repository under the accession number E-MEXP-3291 (<http://www.webcitation.org/5zyojNu7T>). All microarray data, archiving, and analysis were generated as previously described [5]. Analysis of gene expression was done as follows. In most cases, expression data for each gene examined was derived from GEP for each biopsy sample. These data were then converted to Log2 transformed values (+/- the standard deviation) normalized to the median of the normal or steatosis groups (see Fig. 2 as an example). In some cases Log2 transformed values for a gene were derived through normalization to the median expression of a related gene in the normal or NASH groups (see Fig. 1 for an example) Statistical analysis of the Log2 transformed values was performed using 1-way ANOVA with post hoc Tukey testing.

2.3 Transcription Factor Binding Site Analysis

Genes that were significantly up-or down-regulated between normal and NASH microarray data sets were subjected to TFBS enrichment analysis using the TRANSFAC (Biobase) TFBS database and software package. Predicted TFBS were identified in gene regions 5000 bp upstream and downstream of the transcription start site (TSS) for all genes contained in the microarrays. Statistical analysis was then performed to identify TFBS enriched in the significantly up-or down-regulated genes relative to all genes in the expression profiling set. This yielded two significance scores for each TFBS: the Fisher score, which measured its occurrence in each gene, and the Z score, which measured the number of its occurrences in the promoter of each gene. In the TRANSFAC analysis Z scores and Fisher of 0.05 were considered significant. An additional analysis was done using genes that were significantly up-or down-regulated between steatosis and NASH microarray data sets. In this case, the JASPAR TFBS database and the oPOSSOM platform were used for enrichment analysis. Z scores and Fisher scores were generated but, in this case, Z scores were expressed differently and scores >1.6 were considered significant.

2.4 Immunoblot Analysis

Protein from human liver whole cell lysates (80 µg/sample) was separated in 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membrane blocking

and antibody application was accomplished using 5% non-fat dry milk dissolved in phosphate-buffered saline with 0.1% Tween-20. Primary antibodies for HNF3A/B (sc-377033) and HNF1A (sc-10791) were acquired from Santa Cruz Biotechnology (Santa Cruz, CA) while those for LXR α/β (ab24532) and Pan-Cadherin (ab16505) were purchased from Abcam (Cambridge, MA). Immunoblots were imaged using Supersignal West Femto chemiluminescent substrate (Fisher Scientific, Rockford, IL). Densitometry was performed using ImageJ software (NIH, Bethesda, MD).

3. Results

3.1 TFBS Enrichment Analysis of Human NAFLD GEP Data

Previously-obtained GEP data were used to identify TFBS significantly-enriched in the extended promoter regions ($\pm$ 5000 bp relative to the TSS) of genes up-regulated or down-regulated in NASH. Significantly-enriched TFBS from the normal versus NASH comparison are listed in Tables 1 (up-regulated genes) and 2 (down-regulated genes) while those from the steatosis versus NASH comparison are listed in Table 3 (upregulated genes, columns 1–3 and down-regulated genes, columns 4–6). For both comparisons, the TFBS enriched in the up-regulated genes were mostly distinct from those enriched in the down-regulated genes. While the two comparisons did not yield identical results, many enriched TFBS were common (Table 4). The liver-specific actions of multiple TFs listed, such as NKX and PAX families, are poorly understood, so further analysis was focused on those with known hepatic functions. HNF4A, and members of the C/EBP, HNF1, HNF3 (*FOX4*), PPAR, LXR, and COUP-TF families regulate lipid metabolism, xenobiotic sensing/metabolism, and lipotoxic stress, all of which are impacted in NASH [8–11]. Changes in TF activity or expression during NAFLD progression may alter their ability to regulate target genes and thereby contribute to NAFLD progression and NASH pathology.

3.2 TFs that bind TFBS enriched in genes up-regulated in human NASH livers

TFs that bind TFBS enriched in NASH up-regulated genes include members of the C/EBP, FOXA, and HNF1 families. Their enrichment in up-regulated genes implies that their transcription-promoting activity is enhanced or that their transcription-repressing activity is diminished.

C/EBP family members bind similar DNA sequences as homo- or heterodimers [12]. Further analysis was focused on those with known hepatic functions, namely C/EBP α (*CEBPA*), C/EBP β (*CEBPB*), C/EBP δ (*CEBPD*), and C/EBP γ (*CEBPG*). These TFs regulate hepatic energy metabolism, liver regeneration, and the inflammatory acute phase response [8]. The GEP data set was analyzed to reveal that *CEBPA*, *CEBPB*, and *CEBPD* mRNAs were expressed at similar levels in normal (not shown) and NASH livers while *CEBPG* mRNA levels were significantly lower (Fig. 1A). Furthermore, C/EBP mRNA levels do not significantly change between normal, steatosis, and NASH livers (Fig. 2A–D), indicating that potentially-increased transcriptional activity is not due to elevated expression.

HNF3 TFs are essential regulators of liver-specific gene expression [9] and include three isoforms, now referred to as FOXA1, FOXA2, and FOXA3. FOXA1 and FOXA2 mRNAs

were expressed at significantly higher levels than FOXA3 mRNA in normal and NASH livers (Fig. 1B,C), but all FOXA mRNAs were significantly down-regulated in NASH (Fig. 2E–G). These TFs act primarily as transcriptional activators, so down-regulated mRNA expression is not consistent with enrichment of their binding sites in NASH up-regulated genes. Western blotting shows that, in contrast to mRNA, the combined level of the FOXA1 and FOXA2 proteins was significantly elevated in NASH livers (Fig. 3A,B), which is consistent with increased expression of their target genes.

The HNF1 family consists of HNF1A and HNF1B, which can form homo- or heterodimers to bind DNA [9]. Only HNF1A is robustly expressed in adult liver [13;14], consistent with results from the GEP data, which shows that HNF1B mRNA levels are significantly lower than those of HNF1A in normal liver (Fig. 1D). GEP data analysis shows that HNF1A mRNA is significantly down-regulated upon progression to NASH (Fig. 2H). Because HNF1A functions predominantly as a transcriptional activator and its binding site was enriched in NASH-upregulated genes, HNF1A protein levels were measured by Western blot (Fig. 3A,C). The results showed a significant increase of HNF1A protein in NASH livers (Fig. 3B). HNF1B mRNA levels are significantly up-regulated in NASH (Fig. 2I) to the extent that they become significantly higher than those of HNF1A (Fig. 1D). Due to the very limited amount of liver extracts, measurement of HNF1B protein was not feasible. However, the results suggest that combined HNF1 expression is elevated in NASH livers and drives increased expression of target genes.

3.3 TFs that bind TFBS enriched in genes down-regulated in human NASH livers

TFBS enriched in NASH-downregulated genes include those bound by members of the nuclear receptor superfamily that have hepatic functions perturbed in NASH, including xenobiotic sensing/metabolism, lipid metabolism, and inflammation. Their enrichment in down-regulated genes implies that their transcription-repressing activity is enhanced or that their transcription-promoting activity is diminished.

As heterodimers with RXR, both LXR isoforms bind the same DNA element [15]. LXRalpha (*NR2H3*) is the primary regulator of cholesterol and bile acid metabolism in adult liver [16]. Accordingly, LXRalpha mRNA is expressed at higher levels relative to LXRbeta mRNA (*NR2H2*) in normal livers (Fig. 4A). However, progression to NASH results in a significant drop in both mRNAs (Fig. 4B,C). Western blot analysis of liver extracts with an antibody recognizing both LXR isoforms shows that, in contrast to the decline in mRNA, LXR protein levels trend upward in NASH livers (Fig. 4D). The increase did not reach significance but the median level of LXR protein is clearly higher in NASH relative to normal and steatosis.

HNF4A is a nuclear receptor that regulates many liver-specific genes. The GEP data shows that its mRNA is significantly decreased in NASH livers (Fig. 5A). The same was observed for GABPA (Fig. 5B) which has multiple interactions with HNF4A. HNF4A binds to the *GABPA* promoter, suggesting a positive role in its expression [17] and consistent with the coordinate down-regulation of both mRNAs in NASH. A recent study showed significant co-occupancy of GABPA and HNF4A near the TSS of selected HNF4A target genes and established that HNF4A and GABPA interact by co-immunoprecipitation [18]. Thus,

decreased expression of HNF4A and GABPA may cause down-regulation of their target genes in NASH.

The PPAR family of nuclear receptors has three isoforms: PPARalpha (*PPARA*), PPARdelta (*PPARD*), and PPARgamma (*PPARG*). Various fatty acid derivatives bind PPARs and increase or inhibit transcription at target genes [19]. PPARs can also repress transcription in the unliganded state [20;21]. In normal livers, *PPARA* mRNA is expressed at significantly higher levels than mRNAs for *PPARD* or *PPARG* (Fig. 5C). During NAFLD progression, *PPARA* and *PPARG* mRNA levels do not change significantly (Fig. 5D,F) while *PPARD* mRNA is significantly up-regulated in NASH livers (Fig. 5E).

The nuclear receptors COUP-TFI (*NR2F1*) and COUP-TFII (*NR2F2*) are transcriptional repressors [22]. GEP data analysis shows that COUP-TFII is expressed at significantly higher levels in normal and NASH livers (Fig. 7A). mRNAs for both COUP-TFs increase significantly in NASH relative to steatosis (Fig. 7B,C) but the magnitude of the change is greater for COUP-TFII. Thus, the increased expression of COUP-TFs may cause down-regulation of genes in NASH.

4. Discussion

The mechanisms underlying NAFLD progression to NASH are not well-understood resulting in a dearth of effective therapeutic options. The current study utilizes bioinformatics to predict TFs that drive changes in gene expression which occur upon progression to NASH. These TFs and the signaling pathways that regulate their activity and/or expression provide potential targets for therapeutic intervention. TFBS enrichment analyses performed revealed a potential role for hepatic nuclear factors and nuclear receptors in the altered expression profile that characterizes NASH.

Distinct groups of TFBS were enriched in the genes repressed or activated upon progression to NASH. The activated genes were enriched in sequences bound by members of the C/EBP, FOXA, and HNF1 families, suggesting that the transcription-promoting function of these proteins is increased. Levels of C/EBP proteins were not measured in this study but their activity can be regulated by other mechanisms. C/EBPalpha and C/EBPbeta are expressed as multiple isoforms through alternative translation start sites [8;12]. C/EBPbeta isoforms include LAP and LAP*, that activate transcription, and LIP, that represses transcription when hetero-dimerized with LAP or LAP*. The relative expression of these isoforms may alter transcription of target genes. In addition, post-translational modification regulates C/EBP DNA binding and transcriptional activity [12]. C/EBP proteins are targets of cytokine signaling, which is activated in NASH due to chronic inflammation. Expression of C/EBP isoforms and their post-translational modifications in NAFLD have not been investigated.

FoxA proteins are critical for proper hepatic function. FoxA1 and A2 mRNAs were significantly decreased upon progression to NASH. In contrast, combined expression of these proteins is significantly increased in NASH livers. Consistent with the current findings, FOXA2 overexpression in transgenic mouse liver caused significant down-regulation of

mRNAs from the endogenous FOXA genes [23;24], indicating the existence of a feedback mechanism for regulation of FOXA expression that is perturbed in NASH. Elevated FOXA1 and A2 levels could increase expression of their target genes.

Hepatic FOXA2 overexpression was also associated with increased lipid-containing vesicles, elevated serum bile acids, decreased expression of sinusoidal bile uptake transporters, mitochondrial membrane damage/swelling, influx of immune cells, and fibrosis [23;24]. Since many of these features are also observed in NASH, dysregulated FOXA2 expression and activity may contribute to NAFLD progression, and thus highlight its potential as a therapeutic target. Deacetylation by SIRT1 leads to reduced levels of FOXA2 protein through increased degradation [25]. Small molecule sirtuin activators are under development, and their potential use in NAFLD treatment has been suggested [26].

While HNF1A mRNA levels decline upon NAFLD progression, protein levels increase in NASH livers along with levels of HNF1B mRNA. While HNF1B protein levels were not measured, the combined results raise the possibility that total HNF1 expression is elevated in NASH. Because normal hepatocytes express only HNF1A [13;14], it is not clear whether HNF1B up-regulation occurs in hepatocytes or in another cellular compartment that expands in NASH, such as stellate or inflammatory immune cells. Because HNF1A regulates genes involved with carbohydrate and lipid metabolism, drug metabolism and transport, co-expression of both HNF1 proteins in NASH hepatocytes could have a significant impact. It is unclear whether target gene specificity would be altered upon heterodimer formation or whether heterodimers would be cooperative or antagonistic at target genes.

Genes down-regulated in NASH were enriched in sequences bound by several nuclear receptors. While both LXR mRNAs were down-regulated upon progression to NASH, combined protein levels trend upward. In support, Ahn et al. demonstrated that LXRalpha staining was more intense in NASH livers and correlated with the extent of hepatic inflammation and fibrosis [27]. The enrichment of LXR binding sites in NASH-downregulated genes could be explained in several ways. First, LXR agonist treatment in mouse liver caused both activation and repression of genes [15]. Interestingly, LXR-repressed genes are enriched in those associated with PPAR signaling [15]; PPAR binding sites were also enriched in NASH down-regulated genes. The gene repressive activity of LXR may predominate in NASH. Second, the endogenous ligands for LXR nuclear receptors are oxysterol cholesterol derivatives [28]. Because cholesterol and bile acid metabolism is altered during NAFLD progression [29–31], the mix of endogenous LXR ligands, both agonists and antagonists, could influence the balance of transcription-activating versus –repressing LXR activity.

Consistent with the enrichment of its binding site in NASH down-regulated genes, HNF4A mRNA was significantly decreased in NASH livers. Metabolomic analysis of NAFLD liver samples revealed a shift from classical bile acid synthesis toward the alternative pathway in NASH [31]. Chenodeoxycholic acid, a product of the alternative pathway, negatively affects HNF4A expression and activity [32]. Down-regulation of HNF4A mRNA in NASH is also consistent with elevated IL-1beta signaling, which occurs in human NASH livers [33]. Treatment of mice with IL-1beta downregulated HNF4A mRNA and protein [34].

While HNF4A protein expression was not measured in the current study, several lines of evidence suggest reduced expression or activity. First, HNF4A binds and activates the HNF1A [17;35] and CYP8B1 [36] promoters; mRNA levels for both are significantly decreased in NASH (Fig. 2H and [31]). Second, the effects of hepatic HNF4A knockout in mice share key characteristics with human NAFLD, including hepatic lipid accumulation and increased serum bile acids [37]. The latter was associated with downregulation of mRNAs for the NTCP and Oatp1a1 bile acid uptake transporters. While NTCP mRNA was unchanged during human NAFLD progression, *SLCO1B3* (OATP1B3) mRNA is significantly down-regulated [5]. HNF4A binds linoleic acid [38], and one synthetic ligand has been reported [39]. Accumulated evidence suggests that impaired expression or activity of HNF4A contributes to NAFLD progression or severity, indicating its potential as a therapeutic target.

While levels of PPAR mRNAs were not dramatically altered during NAFLD progression, the enrichment of PPAR binding sites in NASH down-regulated genes suggests that PPAR activity is impaired. In support, multiple PPAR agonists have been shown to slow NAFLD progression and/or ameliorate its severity in animal models [40–42]. While fibrates, which are weak PPARalpha agonists, have variable effects on NAFLD in humans, newer, more potent agonists such as the dual PPARalpha/delta agonist, GFT505, and the PPARalpha-selective agonist, K-877, have shown promise in clinical trials [40–42].

COUP-TFI and COUP-TFII, generally considered to be transcriptional repressors, can antagonize the activating function of nuclear receptors such as PPARs and HNF4A [43]. Although COUP-TF protein levels were not measured, their mRNAs were significantly up-regulated in NASH suggesting the possibility they down-regulate gene expression in NASH through increased expression. COUP-TF expression has not been measured in NAFLD biopsies but other evidence suggests a potential role for COUP-TFII in NAFLD progression. Mice heterozygous for the COUP-TFII gene are lean and protected from the effects of high fat diet, including excess hepatic lipid storage [44]. COUP-TFII mRNA levels are low in human hepatocytes but high in hepatic endothelial and Kupffer cells [45]. The latter are activated by lipotoxic stress and secrete IL-1 β and TNF- α [46], which activate stellate cells to produce collagen that contributes to fibrosis. Thus, activated Kupffer cells may contribute to increased COUP-TFII expression in NASH.

This study combined TFBS enrichment with analysis of TF expression to reveal potential drivers of altered gene expression in NASH. Among hepatic TFs, the current study indicates that FOXA2, PPARs, LXRs, and HNF4 are misregulated in NASH and represent promising therapeutic targets. Finally, this study revealed a disconnect between the pattern of mRNA and protein expression for multiple TFs during NAFLD progression. This is consistent with several studies that measured the correlation between protein and RNA abundance in cell populations. The results showed a limited correlation, implying that only about 40% of variation in protein abundance can be explained by abundance of the corresponding mRNA and that post-transcriptional mechanisms generally make a strong contribution to protein expression levels [47–50]. Altogether these observations suggest that quantitative proteomic analysis of patient biopsies would add significantly to knowledge of NAFLD pathology by

providing a more accurate readout of protein expression than can be predicted from GEP analysis.

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ABBREVIATIONS

TF	Transcription factor
TFBS	Transcription factor binding sites
GEP	Gene Expression Profiling
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
LXR	Liver X receptor
PPAR	Peroxisome proliferator-activated receptor
HNF	Hepatocyte nuclear factor

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Figure 1. Relative Expression of CEBP, HNF1, and FOXA TF Families in human livers
Log2 transformed values $\pm$ the standard deviation (from GEP data) were normalized to the median of the samples indicated for each panel. A.) Expression of *CEBPB*, *CEBPD*, and *CEBPG* mRNAs relative to *CEBPA* mRNA in NASH livers. Asterisks indicate significantly different expression from *CEBPA*. B,C.) Expression of *FOXA2* and *FOXA3* mRNAs relative to *FOXA1* mRNA in normal (B) and NASH (C) livers. Asterisks indicate significantly different expression from *FOXA1*. D.) Expression of HNF1 mRNAs relative to *HNF1A* mRNA in normal livers. Asterisks indicate significantly different expression of *HNF1B* relative to *HNF1A*.

Figure 2. Relative mRNA Expression of TFs that Bind TFBS enriched in genes upregulated in NASH

Relative mRNA expression of TFs in normal, steatosis, and NASH livers are shown. Log2 transformed values $\pm$ the standard deviation are normalized to the median of the normal samples. Significance from normal or from steatosis is represented by asterisk (*) or pound sign (#), respectively. Significance was set at p values ≤ 0.05 .

Figure 3. Expression of FOXA1/FOXA2 and HNF1A proteins in human livers

(A) Representative Western blot with whole cell lysates from liver biopsies using antibodies against HNF1A or both FOXA1 and FOXA2. Pan-Cadherin was utilized as a loading control. B., C.) Western blotting was performed using antibodies against FOXA1/FOXA2 (B) or HNF1A (C) on whole cell lysates from normal (4 samples), steatosis (6 samples) and NASH (13 samples) livers. The band observed for HNF1A migrates around 80 kD consistent with the expected MW of 79 kD. The major band observed for FOXA1/A2 migrated around 50 kD, the expected MW of both proteins. Protein levels were quantitated by densitometry. Results are expressed graphically as fold change relative to levels in normal livers. Significance is denoted as described in the legend to Fig. 2.

Figure 4. Relative Expression of LXR α and LXR β mRNA and protein

A.) Relative expression of LXR α and LXR β mRNAs in normal and NASH livers relative to LXR α mRNA in normal livers. Asterisks indicate significantly different expression from LXR α mRNA. B,C.) Relative mRNA expression levels of LXR α (B) and LXR β (C) in normal, steatosis, and NASH livers are shown. Log2 transformed values and significance are denoted as described in the legend to Fig. 2. D.) Western blotting was performed using an antibody against both LXR α and LXR β on whole cell lysates from normal (4 samples), steatosis (6 samples), and NASH (13 samples) livers. The predicted MW of LXR α and LXR β is 50 kD, consistent with the size of the observed band. Protein levels were analyzed by densitometry. Results are expressed graphically as fold change relative to levels in normal livers. Inset -Representative Western blot. Pan-Cadherin was utilized as a loading control.

Figure 5. Relative mRNA Expression of HNF4A, GABPA, and PPAR Isoforms
 (A,B) Relative mRNA expression levels of *HNF4A* (A), and *GABPA* (B) in normal, steatosis, and NASH livers are shown. (C) Expression of *PPARD* and *PPARG* mRNAs relative to *PPARA* mRNA in normal livers. Asterisks indicate significantly different expression relative to *PPARA*. (D–F) Relative mRNA expression levels of the *PPARA* (D), *PPARD* (E), and *PPARG* (F) genes in normal, steatosis, and NASH livers are shown. Log2 transformed values and significance are denoted as described in the legend to Fig. 2.

Figure 6. Relative mRNA Expression of COUP-TF Isoforms

A.) Expression of *NR2F1* and *NR2F2* mRNAs in normal and NASH livers relative to the level of *NR2F1* mRNA expressed in normal livers. Asterisks indicate significantly different expression relative to *NR2F1* mRNA in normal or NASH livers. (B,C) Relative mRNA expression levels of the *NR2F1* (B) and *NR2F2* (C) genes in normal, steatosis, and NASH livers. Log2 transformed values and significance are denoted as described in the legend to Fig. 2.

Table 1

Enriched Transcription Factor Binding Sites from TRANSFAC Analysis: Up-regulated genes, normal versus NASH.

TFBS	Z Scores	Fisher Scores	TFBS	Z Scores	Fisher Scores
CDXA	0	1.00E-21	RUSH1A_Q2	0.001	4.00E-04
FOXJ2	0	1.20E-21	GATA_C	0.003	2.70E-04
OCT4_Q2	0	1.60E-16	GATA4_Q3	0.001	9.60E-04
OCT1_Q3	0	1.60E-16	PAX4_Q2	0.132	1.50E-05
TST1_Q1	0	1.50E-12	RSRFC4_Q2	0	2.80E-03
OG2_Q1	0	8.40E-12	HNF3ALPHA_Q6	0.003	9.10E-04
CEBPG_Q6	0	1.60E-11	SRF_C	0.002	1.30E-03
OCT_Q6	0	3.60E-11	CDPCR3_Q1	0	4.70E-03
PLZF_Q2	0	9.50E-11	KROX_Q6	0.01	4.70E-04
SRY_Q2	0	2.00E-10	BRCA_Q1	0	5.90E-03
XVENT1_Q1	0	1.30E-09	PAX6_Q1	0	5.90E-03
TBP_Q6	0	2.20E-09	ISRE_Q1	0.005	1.50E-03
SOX9_B1	0	2.50E-09	POU3F2_Q1	0.268	3.80E-05
POU3F2_Q2	0	3.90E-09	AHRHF_Q6	0.01	1.20E-03
POU6F1_Q1	0	6.60E-09	PAX2_Q2	0.003	3.90E-03
HNF1_Q6	0	3.40E-08	CHOP_Q1	0.021	1.00E-03
OCT4_Q1	0	3.70E-08	TCF11_Q1	0.001	1.50E-02
NKX25_Q2	0	4.20E-08	ZF5_B	0	5.20E-02
ETF_Q6	0	8.10E-08	MEF2_Q3	0.008	5.90E-03
POU1F1_Q6	0	1.00E-07	DBP_Q6	0.002	2.80E-02
CEBP_Q3	0	3.80E-07	MRF2_Q1	0.014	6.10E-03
HFH1_Q1	0	4.40E-07	HSF1_Q1	0.019	5.50E-03
NKX3A_Q1	0	1.20E-06	SRF_Q6	0.005	2.40E-02
MEIS1BHOXA9_Q2	0	2.30E-06	HNF6_Q6	0.054	2.90E-03
PBX1_Q3	0	4.10E-06	CDPCR1_Q1	0.133	6.20E-03
OCT1_Q2	0	4.10E-06	IRF2_Q1	0.034	2.60E-02
CDP_Q2	0	1.10E-05	AR_Q2	0.031	3.20E-02

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TFBS	Z Scores	Fisher Scores	TFBS	Z Scores	Fisher Scores
HMG1Y_Q6	0	1.20E-05	AIRE_Q2	0.038	2.90E-02
CEBPA_Q1	0	2.30E-05	GFI1_Q6	0.062	2.10E-02
HNF3B_Q1	0	2.60E-05	S8_Q1	0.023	6.00E-02
OCT1_Q5_Q1	0	3.50E-05	PR_Q1	0.035	4.70E-02
IPF1_Q4	0	6.40E-05	AP2_Q6	0.06	2.80E-02
NCX_Q1	0	6.80E-05	AP2ALPHA_Q1	0.141	1.30E-02
FAC1_Q1	0.001	1.00E-04	SRF_Q4	0.039	5.90E-02
NKX22_Q1	0	2.10E-04	IRF_Q6	0.05	7.20E-02
CART1_Q1	0.001	1.10E-04	HLF_Q1	0.05	7.40E-02
OCT1_Q7	0	2.60E-04	TFE_Q6	0.037	1.30E-01

Table 2

Enriched Transcription Factor Binding Sites from TRANSFAC Analysis; Down-regulated genes, normal versus NASH.

TFBS	Z Scores	Fisher Scores	TFBS	Z Scores	Fisher Scores
DR4_Q2	0	2.70E-09	GABP_B	0	1.50E-02
EBOX_Q6_01	0	3.20E-08	HNF4_Q6_01	0.006	2.90E-03
CACD_01	0	2.40E-07	HIF1_Q3	0.002	7.10E-03
P300_01	0	1.20E-06	COUP_DR1_Q6	0	2.70E-02
AP2_Q6	0.001	7.80E-07	PPAR_DR1_Q2	0.002	1.40E-02
AHRARNT_01	0	1.60E-06	ATF6_01	0.004	8.70E-03
PAX6_Q2	0	5.00E-06	MYOGNF1_01	0	4.30E-02
SREBP1_01	0	6.30E-06	ZIC2_01	0.001	2.50E-02
ZF5_B	0.018	6.70E-07	VMYB_02	0.021	3.50E-03
PPARA_02	0	1.50E-05	NFY_01	0.006	1.10E-02
CETS1P54_03	0	1.90E-05	DR3_Q4	0	8.20E-02
SREBP_Q6	0	2.70E-05	HIC1_02	0.008	1.20E-02
AHR_Q5	0	4.40E-05	SF1_Q6_01	0.008	1.20E-02
MYOD_Q6_01	0	6.20E-05	TBX5_01	0	1.10E-01
CP2_02	0	7.20E-05	STAF_02	0.011	1.10E-02
MTF1_Q4	0	9.30E-05	CRX_Q4	0.016	8.00E-03
LRF_Q2	0.001	5.20E-05	VDR_Q3	0.002	6.00E-02
PAX3_B	0	1.40E-04	PAX8_01	0.012	2.10E-02
PPARG_01	0	2.30E-04	PAX4_03	0	2.80E-01
TAXCREB_01	0	3.60E-04	E2A_Q2	0.02	2.00E-02
ARNT_01	0.001	1.90E-04	MAZ_Q6	0.032	1.30E-02
SP1_Q2_01	0.011	3.60E-05	LXRA_RXRA_Q3	0	4.90E-01
MINI19_B	0.001	2.90E-04	USF_Q6_01	0.072	8.50E-03
PAX5_02	0	6.60E-04	GEN_INT3_B	0	6.30E-01
PAX4_01	0	8.20E-04	NF1_Q6_01	0.015	3.90E-02
TTF1_Q6	0	8.40E-04	AP2ALPHA_01	0.082	1.00E-02
SP3_Q3	0.002	2.80E-04	CREB_02	0.041	2.20E-02

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TFBS	Z Scores	Fisher Scores	TFBS	Z Scores	Fisher Scores
DR1_Q3	0	1.40E-03	KID3_01	0	1.00E+00
AP2_Q6_01	0.01	1.40E-04	PPARA_01	0.022	4.50E-02
AP4_01	0.002	5.20E-04	E2F_03	0.025	6.80E-02
ER_Q6	0	1.90E-03	E2F_Q6_01	0.03	8.90E-02
PAX5_01	0	2.60E-03	NFKB_Q6_01	0.082	3.40E-02
PAX4_04	0	2.70E-03	CEBPD_Q6	0.043	9.90E-02
LMO2COM_01	0.001	1.50E-03	MYCMAX_03	0.046	1.30E-01
TEL2_Q6	0	5.70E-03	MEIS1_01	0.08	8.00E-02
SZF11_01	0.001	3.00E-03	PAX_Q6	0.013	5.50E-01
ETF_Q6	0.908	7.00E-06			
SREBP_Q3	0	9.60E-03			

Table 3
Enriched Transcription Factor Binding Sites from JASPAR Data Analysis: Differentially-expressed genes, Steatosis versus NASH.

Up-regulated Genes	Z.score	Fisher score	Down-regulated Genes	Z.score	Fisher score
Nkx2-5	39.65	1.35E-016	ZNF42_1-4	14.85	0.7719
SRY	39.13	6.78E-022	NR2F1	14.79	0.01691
Sox5	34.8	8.98E-024	deltaEF1	13.65	0.5039
Foxa2	32.46	9.79E-026	RXR- VDR	12.72	0.009483
Prrx2	32.09	6.32E-019	Staf	12.45	0.09576
Foxd3	31.13	1.34E-024	MYC-MAX	11.87	0.2704
FOXI1	28.93	6.24E-032	NR1H2-RXR	10.24	0.04824
cEBP	22.51	1.45E-029	HNf4	10.07	0.405
MEF2A	20.64	4.52E-031	MAX	10.07	0.5477
Gfi	20.53	1.98E-021	USF1	9.334	0.7279
FOXDI	20.23	5.61E-029	ELK4	9.265	0.1091
SOX9	19.68	1.97E-025	YY1	8.743	0.8619
Foxq1	19.17	4.96E-033	GABPA	8.466	0.4904
FOXP2	18.81	3.31E-029	HAND1-TCF3	7.101	0.423
SRF	17.95	1.49E-013	Myf	6.985	0.9879
IRF1	17.29	4.29E-028	ELK1	6.843	0.8577
TCF1	16.62	3.20E-020	Arnt	6.814	0.8456
Sox17	13.61	1.95E-019	Mycn	6.71	0.8492
NR3C1	12	9.19E-018	Pax5	5.65	0.4501
HLF	11.84	3.02E-022	Bapx1	5.497	0.7584
TEAD	10.49	8.88E-021	CREB1	4.324	0.8145
NFIL3	10.38	1.91E-014	PPARG	4.323	0.3129
Pbx	10.3	2.37E-016	PPARG-RXRA	4.32	0.3482
Evil	9.705	1.48E-010	Roaz	3.52	0.9587
Fos	8.122	1.07E-025	SP1	2.935	0.5094
RELA	5.911	7.62E-028	T	2.438	0.479
IRF2	5.755	1.91E-005	NHLH1	2.412	0.9081

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Up-regulated Genes	Z-score	Fisher score	Down-regulated Genes	Z-score	Fisher score
Pax6	5.061	8.87E-009	ESR1	2.285	0.6298
Chop-cEBP	3.372	2.71E-016	ZNF42_5-13	2.227	0.9535
Pax4	2.884	0.008918	Amt-Ahr	2.049	0.5808
REL	2.312	2.183E-025	NF-kappaB	1.610	0.8767
RORA1	2.257	1.827E-012			
Bapx1	1.830	1.108E-026			
HAND1-TCF3	1.820	3.083E-021			

TABLE 4

Transcription Factors that Bind Enriched TFBS Common to Analysis with TRANSFAC and JASPAR Databases.

Up-regulated Genes	Down-regulated Genes
CEBP family	AHR/ARNT
SOX9	PAX5
HNF1	PPAR family
NKX3-1/NKX3-2	ETS family
NKX2-5	ER
HNF3 (FOXA family)	GABP
SRF	HNF4
MEF2A	COUP-TF Family (NR2F1/2)
IRF1/IRF2	STAF (ZNF143)
PAX4	LXR family (NR1H1/2)
PAX6	USF1
HLF	MYC/MAX
GFI1	CREB
PBX1	
AR/PR/GR	