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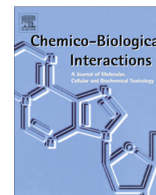

A map of the PPARα transcription regulatory network for primary human hepatocytes

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ABSTRACT

Nuclear receptor activation in liver leads to coordinated alteration of the expression of multiple gene products with attendant phenotypic changes of hepatocytes. Peroxisome proliferators including endogenous fatty acids, environmental chemicals, and drugs induce a multi-enzyme metabolic response that affects lipid and fatty acid processing. We studied the signaling network for the peroxisome proliferator-associated receptor alpha (PPARα) in primary human hepatocytes using the selective PPARα ligand, GW7647. We measured gene expression over multiple concentrations and times and conducted ChIP-seq studies at 2 and 24 h to assess genomic binding of PPARα. Over all treatments there were 192 genes differentially expressed. Of these only 51% showed evidence of PPARα binding–either directly at PPARα response elements or via alternative mechanisms. Almost half of regulated genes had no PPARα binding. We then developed two novel bioinformatics methods to visualize the dose-dependent activation of both the transcription factor circuitry for PPARα and the downstream metabolic network in relation to functional annotation categories. Available databases identified several key transcription factors involved with the non-genomic targets after GW7647 treatment, including SP1, STAT1, ETS1, ERα, and HNF4α. The linkage from PPARα binding through gene expression likely requires intermediate protein kinases to activate these transcription factors. We found enrichment of functional annotation categories for organic acid metabolism and cell lipid metabolism among the differentially expressed genes. Lipid transport processes showed enrichment at the highest concentration of GW7647 (10 μM). While our strategy for mapping transcriptional networks is evolving, these approaches are necessary in moving from toxicogenomic methods that derive signatures of activity to methods that establish pathway structure, showing the coordination of the activated nuclear receptor with other signaling pathways.

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1. Introduction

Much of what we understand about responses to toxic compounds comes from decades of experimentation in animal models. This knowledge underwrites the safety regulations concerning acceptable exposures to hazardous compounds in commercial, industrial, and environmental applications. However, differences between human biology and animal models make it difficult to definitively assess the safety of a compound from such studies. Additionally, extrapolating from high-dose conditions typically used for in-life animal testing to low-dose chronic exposures relevant to human safety is complicated by nonlinear dose–response relationships. A transition away from in-life toxicity testing to mode of action-based testing is now underway to improve the basis for inferring likely risks to humans from various chemicals [1–3].

Perturbations induced by environmental chemicals often lead to different physiological outcomes in humans and rodents, further complicating extrapolation of animal test results for human risk assessment [3]. For example, peroxisome proliferators including endogenous fatty acids [4] and various synthetic ligands induce lipid metabolism enzymes in humans [5,6] and have been used successfully as therapeutic strategies for various dyslipidemias and

Abbreviations: PPARα, peroxisome proliferator-associated receptor alpha; ChIP-seq, chromatin immunoprecipitation and next-generation sequencing.

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diabetes. In rodents, however, peroxisome proliferators also influence peroxisome assembly, inflammatory responses [7–9], and cellular proliferation in addition to their role in regulating fatty acid metabolism. Many peroxisome proliferators are potent carcinogens in rodents but do not appear to be carcinogenic in humans [10,11].

Peroxisome proliferators act in both human and rodent cells through the peroxisome proliferator-activated receptor (PPAR) family of proteins. The three members of the PPAR family (α, γ, and δ) are ligand-activated nuclear receptors. They mediate highly context-specific gene transcription in response to environmental or endogenous stimuli through a succession of steps (Fig. 1). These steps include: (i) ligand-induced phosphorylation of receptor in the cytosol; (ii) translocation of the receptor–ligand complex to the nucleus; (iii) heterodimerization with binding partners; (iv) binding of the heterodimer at DNA-response-elements in the promoters of target genes; and (v) recruitment of co-activators and co-repressors, leading to altered gene expression. The three PPAR subtypes have tissue-specific expression, and vary in the responses they mediate. PPARα is primarily expressed in the liver–where peroxisome proliferators induce cancer in rodents.

The canonical mechanism of PPARα-mediated response involves the direct binding of activated PPARα to a well-established consensus binding site, the peroxisome proliferator response element (PPRE) [12,13]. However, changes in PPAR binding upon treatment with exogenous ligands do not accurately predict changes in gene expression [14]. Also, the majority of peroxisome proliferator induced transcriptional changes are dampened or eliminated in PPARα-deficient cells for both mice and humans [15,16]. These facts indicate that mechanisms other than transactivation by direct binding of PPARα to its cognate site must contribute to the effects of peroxisome proliferators, but that these mechanisms depend on PPARα activity.

As a first step in extending the canonical model of hormone receptor regulation for PPARα, we describe here the response of isolated human primary hepatocytes to the PPARα-specific exogenous ligand GW7647 [17], and infer a transcriptional network underlying the response. Recognizing the importance of the dynamics of nuclear receptor biology, we collected dense dose and time response gene expression data. We also functionally characterize the genes differentially induced in the network at various doses of the ligand by their biological process annotation. Our work has also produced several tools for visualizing network structure and gene-ontology dose response that should be widely useful in establishing and characterizing signaling networks with other nuclear receptors.

2. Methods

2.1. Cell culture and treatment

Primary human hepatocytes were obtained from Invitrogen (Carlsbad, CA). Cells were plated on Rat Collagen I-coated plates and Geltrex was added for a final concentration of 0.25 mg/ml approximately 6 h after plating. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere in William E medium supplemented with cell maintenance cocktail B (Invitrogen). GW7647 was obtained from Tocris (Bristol, UK).

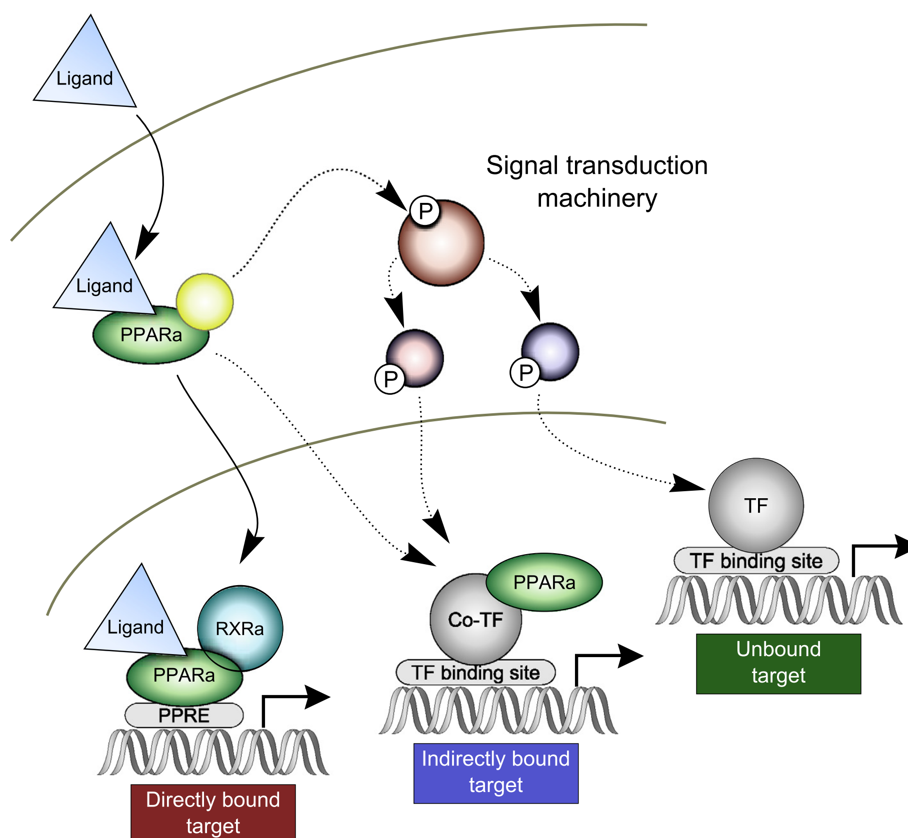

Fig. 1. Identification of the PPARα regulatory network. Three distinct mechanisms for regulating the targets of PPARα: (i) direct genomic binding, (ii) indirect genomic binding, and (iii) unbound non-genomic interactions.

2.2. Preparation of RNA

Total RNA was isolated using mirVANA kit by Ambion (Austin, TX) according to manufacturer's instructions. The RNA was diluted in RNase-free H_2O and quantified by Nanodrop (Thermo, Wilmington, DE) at 260 nm. The quality of RNA samples was confirmed using RNA Nano Chips with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbron, Germany). RNA samples were stored at −80 °C until use.

2.3. Microarray experiments and data analysis

From 5 μg total RNA, cDNA was synthesized using a one-cycle cDNA synthesis kit (Affymetrix Corp., Santa Clara, CA). cDNA was transcribed to cRNA which was then biotin-labeled using GeneChip IVT labeling kit (Affymetrix). 15 μg labeled cRNA were then hybridized to an Affymetrix Human Genome U133 Plus PM Array at 45 °C for 16 h. Biological cRNA replicates ($n = 4$) were each hybridized to an individual array. After being washed using the GeneChip Fluidics Station 450, arrays were scanned using a GeneTitan and intensity values were extracted from the CEL file using Partek software (St. Louis, MO). All gene expression data have been made publicly available (GEO: GSE53399).

2.4. Statistical analysis

Prior to performing data analysis, intensities were normalized using robust multi-array average (RMA) method [18]. Evaluation of the time- and dose-dependent effects of PPARα activation were performed using two-way ANOVA with the Benjamini–Hochberg false discovery rate controlling procedure [19]. Data with a false discovery rate-corrected $p \leqslant 0.05$ were considered significant.

2.5. Chromatin immunoprecipitation

The human hepatocytes used for chromatin immunoprecipitation (ChIP) of PPARα were cultured from different donors than those used for the gene expression microarray experiments. Hepatocytes incubated with GW7647 or DMSO were fixed with 1% formaldehyde at room temperature for 15 min and quenched with 0.125 M glycine and shipped to Active Motif (Carlsbad, CA, USA) for ChIP-seq analysis. Chromatin was isolated by adding lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated using a Misonix Sonicator 3000 equipped with a microtip in order to shear the DNA to an average length of 300–500 bp. Lysates were cleared by centrifugation and stored at −80 °C.

Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by phenol/chloroform extraction and ethanol precipitation. Purified DNA was quantified on a Nanodrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantification of the total chromatin yield.

For each ChIP reaction, 30 μg of chromatin was precleared with protein A agarose beads (Invitrogen). ChIP reactions were set up using precleared chromatin and antibody PPARα (sc-9000, Lot# K2911) and incubated overnight at 4 °C. Protein A agarose beads were added and incubation at 4 °C was continued for another 3 h. Immune complexes were washed two times each with a series of buffers consisting of the deoxycholate sonication buffer, high salt buffer, LiCl buffer, and TE buffer. Immune complexes were eluted from the beads with SDS buffer, and subjected to RNase treatment and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65 °C, and ChIP DNA was purified by phenol–chloroform extraction and ethanol precipitation.

2.6. ChIP sequencing

ChIP and input DNAs were prepared for amplification by converting overhangs into phosphorylated blunt ends and adding an adenine to the 3′-ends. Illumina genomic adapters were ligated and the sample was size-fractionated (200–250 bp) on a 2% agarose gel. After a final PCR amplification step (18 cycles), the resulting DNA libraries were quantified and sequenced on HiSeq 2000. Sequences (50 nucleotide reads, single end) were aligned to the human genome (hg19) using the BWA algorithm [20]. Aligns were extended *in silico* at their 3′-ends to a length of 150 bp, which is the average genomic fragment length in the size-selected library, and assigned to 32-nt bins along the genome. Peak locations were determined using the MACS algorithm (v1.4.2) with a cutoff of $p = 10^{-7}$ [21]. Signal maps and peak locations were used as input data to Active Motif's proprietary analysis program, which creates excel tables containing detailed information on sample comparison, peak metrics, peak locations and gene annotations. Peaks were assigned to genes with a transcription start site within 50 kb up- or downstream (see Supplemental Text).

2.7. De novo binding motif detection

MEME-ChIP was used for ab initio identification of sequences enriched in the sequenced ChIP fragments [22]. Binding sites were compared to known binding sites in the Jaspar and UniPROBE databases using TOMTOM [23]. Motifs with expectation values greater than 0.01 were considered significantly enriched. The TRANSFAC ExPlain module was used to identify ChIP intervals matching the PPARα motif, controlling the false discovery rate at 0.05.

3. Results

3.1. Transcriptional response by PPARα activation in primary hepatocytes

We used microarray-based transcriptome profiling experiments to identify gene targets whose expression changed as a consequence of GW7647 treatment. mRNA from primary human hepatocytes isolated from four independent donors was collected at five time points (2, 6, 12, 24, and 72 h) from cells treated with five concentrations of GW7647 between 0.001 and 10 μm. Over 54,000 probe sets representing more than 14,000 genes were evaluated for this sampling of 25 different concentration and time conditions.

Only 192 genes were statistically significantly up- or down-regulated upon treatment of GW7647 at any of the dose or time points, with more than 80% showing up-regulation (Fig. 2B). These up-regulated genes encompassed many lipid metabolism pathway genes that are known targets of PPARα, including ACOX1 [24], CPT1A [25,26], and APOA4 [27]. The differentially expressed genes we identified correlate strongly with the set of genes regulated by another PPARα-selective agonist, Wy14643 (Figure S1) [28]. The relatively small number of differentially expressed genes was surprising, but it is unlikely that higher doses or longer exposure time would induce a substantial number of additional genes, as the conditions we investigated were sufficient to produce saturated dose and time response and were well above the EC_{50} for PPARα activation by GW7647 (0.006 μM; [17]). The small number of differentially expressed genes helped us develop our visualization tools on a more manageable number of features before moving onto larger data sets, for instance with PPARα activation in rat hepatocytes, where one expects many more genes are altered by treatment.

Transactivation of several of the differentially expressed target genes by peroxisome proliferators results from binding of activated PPARα at its cognate consensus promoter element, the peroxisome

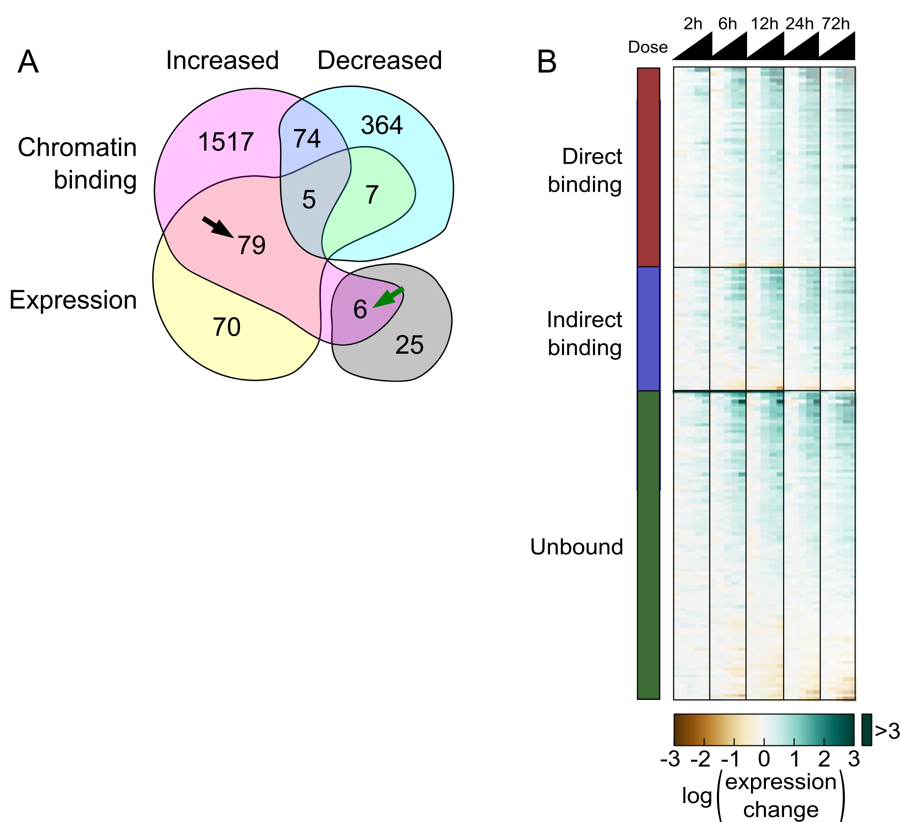

Fig. 2. Genomic response to GW7647. (A) Venn diagram summarizing ChIP and gene expression experiments. Regions not shown denote empty sets. Black arrow indicates the set of genes that are up-regulated and associated with increased PPARα binding. Most peroxisome proliferator-induced expression changes are not associated with increased binding of PPARα. Green arrow indicates genes that are associated with increased PPARα binding and decreased expression. (B) Matrix of 192 differentially regulated genes. Rows are individual genes, organized according to mechanism of regulation. Columns are treatment conditions, increasing in dose from left to right.

proliferator response element (PPRE). We investigated the extent to which this canonical mechanism explains the gene expression in primary human hepatocytes. Because no genome-wide study of PPARα binding had previously been reported in primary human hepatocytes, we used ChIP-seq to identify PPARα binding sites that were not previously characterized.

We considered PPARα binding with and without activation by GW7647 at 2 and 24 h of exposure. Long-range chromatin interactions are important for nuclear receptors [29,30], complicating the assignment of binding sites to their transcriptional targets. We developed a novel statistical strategy for determining a reasonable threshold for promoter length (see Supplemental Text). Here, we define the promoter as the sequence bound within 50,000 bases up and downstream of any transcription start site for the gene.

We identified 1766 binding sites mapping to the promoters of 1769 genes associated with increased PPARα binding, and 340 binding sites mapping to the promoters of 475 genes associated with decreased binding (Fig. 2A). Of the 192 genes we identified as differentially expressed, 98 were associated with a site with altered PPARα binding. Interestingly, while there were 31 genes down-regulated by GW7647, none of these were associated with a decrease in PPARα binding.

We compared the results of our ChIP-seq study with results from a ChIP-on-chip experiment studying the effect of the same ligand on PPARα binding in HepG2 human hepatoma cells [14] (Figure S2). Of the 3670 genes with enriched PPARα binding in HepG2 cells, only 46 (1.3%) mapped to our differentially expressed targets. While some of this discrepancy may be due to differences in biology between primary hepatocytes and immortalized hepatoma cells, the authors of the previous study noticed a similar difference between PPARα-bound and differentially expressed genes in the HepG2 line. The small number of differentially expressed genes with direct or indirect association with PPARα suggests additional layers of regulation beyond the traditional ligand-activated hormone receptor model. This finding indicates that a substantial fraction of genes affected by peroxisome proliferators are regulated by mechanisms other than the classical model of nuclear receptor biology.

In addition to direct binding of ligand-bound PPARα to its response element, PPARα may influence the expression of target genes through the formation of complexes with other transcription factors – e.g. by "tethering" to other transcription factors that bind directly to their cognate binding sites [31]. This group is the indirectly bound targets (Fig. 1). Indeed, PPARα interacts with a large number of other transcription factors and accessory proteins, including Src, CBP/p300, and PGC1 [32]. Such complexes, when they associate with DNA, are detectable by chromatin immunoprecipitation. To differentiate direct binding of PPARα to the promoter from indirect binding through a coactivator, we performed a computational search for PPREs within the enriched ChIP fragments. 61 of 98 transcriptional targets bound by PPARα have direct binding to a PPRE (Fig. 3A). These direct targets are on average more strongly up-regulated than indirect targets, which are not regulated by PPREs (Fig. 3B).

To validate these direct targets, we compiled a list of 11 of our differentially expressed genes that have been shown in the literature to be direct targets of PPARα via low-throughput methods (Table 1). For each of these targets, the location of a PPRE has been experimentally identified via gel shift, low-throughput chromatin immunoprecipitation (ChIP), fluorescent reporter construct, or

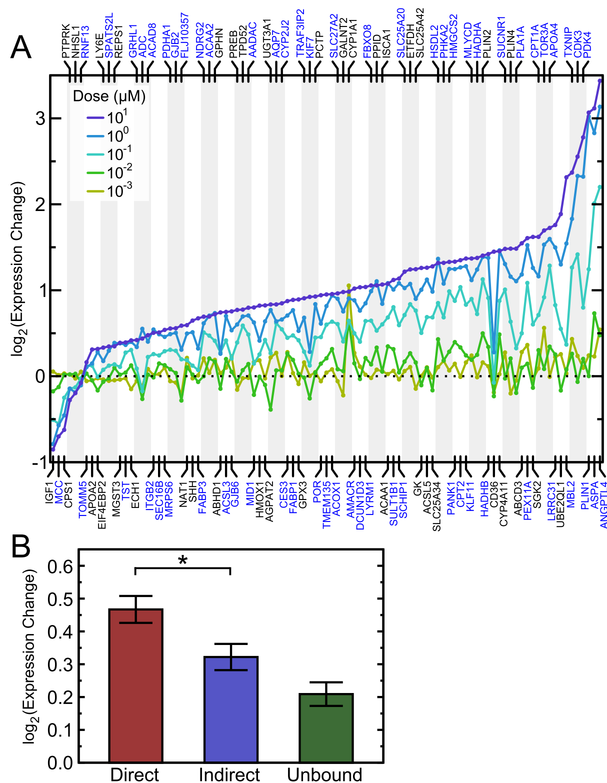

Fig. 3. Expression changes are more pronounced for direct and indirect genomic targets. (A) Expression changes of the PPARα-bound targets as a function of dose in cells treated for 24 h suggest a monotonic dose response. Genes are ordered by expression change at the highest dose tested (10 μM). Directly bound targets (blue text) have on average higher expression changes than do indirectly bound targets (black text). (B) The expression changes of directly regulated targets is higher than indirectly regulated targets (*$p < 0.05$, permutation test). Also, indirectly regulated targets are more likely to have higher expression changes than unbound targets.

similar evidence in human cells. These genes are not intended to be a comprehensive list of direct targets; there are certainly many other sites with PPREs that have not been characterized to the degree that these have. Of these targets, 8 were bound by PPARα at a PPRE in response to GW7647 (Table 1). APOA2 and PLIN2 were bound by PPARα at previously described sites [33,34], but these sites were insufficiently similar to the PPREs we identified in other binding sites to be classified as such. CYP1A1 had no differential binding in response to GW7647 at the characterized site [35]–the nearest binding site was 500,000 bases downstream of the gene.

To further characterize PPARα regulation, we performed a de novo motif search using the chromatin sequences bound by PPARα (Figure S3). We compared these motifs to two databases of transcription factor binding sites, Jaspar and UniPROBE. As expected, the most highly enriched motif corresponds well to the consensus PPRE. We identified a long, high-fidelity motif with no established consensus (Figure S3, line 2). This putative site was found in 9 PPARα binding segments. We did not identify sites of known PPARα coactivators within the segments.

3.2. Construction of a putative PPARα regulatory network

Nearly half of the differentially expressed genes we identified neither contain a functional PPRE nor are bound by PPARα upon treatment with a PPARa-selective agonist. We next turned to a database of transcription factor-target associations from literature-curated high-throughput ChIP studies to identify possible alternative pathways of regulation [36]. We screened the transcription factors in the database to identify those that have appreciable expression in liver tissue (see Table S1). We used a constrained definition for promoter size to minimize false positives: transcription factors were mapped to target genes if a ChIP fragment for the transcription factor was within 10 kb upstream or 1 kb downstream of the gene's transcription start site. Together, the ensemble of the differentially expressed genes and these transcriptional regulators form a putative regulatory network (Fig. 4). Because these data come from a diverse set of experiments in a number of cell types, some of the interactions may not be present in the context of PPARα biology in human hepatocytes. However, this provisional network suggests the scope of the regulatory landscape of the peroxisome proliferator response.

The associations between PPARα–as well as other transcription factors–and the target genes constitute a map of the regulatory landscape of PPARα activation (Fig. 4). Here, nodes represent differentially expressed target genes (circles; a target is represented if it was identified as differentially expressed under any of the 25 dose/time conditions we considered) and transcription factors (rectangles). Three distinct types of edges are represented in the network (Figs. 1 and 4, Figure S4):

(i) *Direct bound* interactions: PPARα binds to its consensus response elements (PPREs) at the promoters of target genes (shown in blue lines extending from PPARα)
(ii) *Indirect bound* interactions: PPARα is bound to promoter regions of target genes—but not to PPREs—presumably through tethering with other transcription factors that directly bind to the promoters of target genes (shown in black lines extending from PPARα)

Table 1
Confirmed direct targets of PPARα that are differentially expressed in human primary hepatocytes upon treatment of GW7647. APOA2 and PLIN2 are bound by PPARα at the reported site; however our computational search did not identify a PPRE at either of these locations. CYP1A1 do not bind PPARα at the reported sites in response to GW7647.

Symbol	Name	Reference
ACOX1	Acyl-Coenzyme A oxidase 1, palmitoyl	[24]
ANGPTL4	Angiopoietin-like 4	[67]
APOA2	Apolipoprotein A-II	[33]
APOA4	Apolipoprotein A-IV	[27]
CPT1A	Carnitine palmitoyltransferase 1a, liver	[25]
CPT1B	Carnitine palmitoyltransferase 1b, muscle	[68]
CYP1A1	Cytochrome P450, family 1, subfamily a, polypeptide 1	[35]
FABP1	Fatty acid-binding protein 1	[69]
HMGCS2	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 2	[70]
PLIN2	Perilipin 2	[34]
SLC25A20	Carnitine/acylcarnitine translocase	[71]

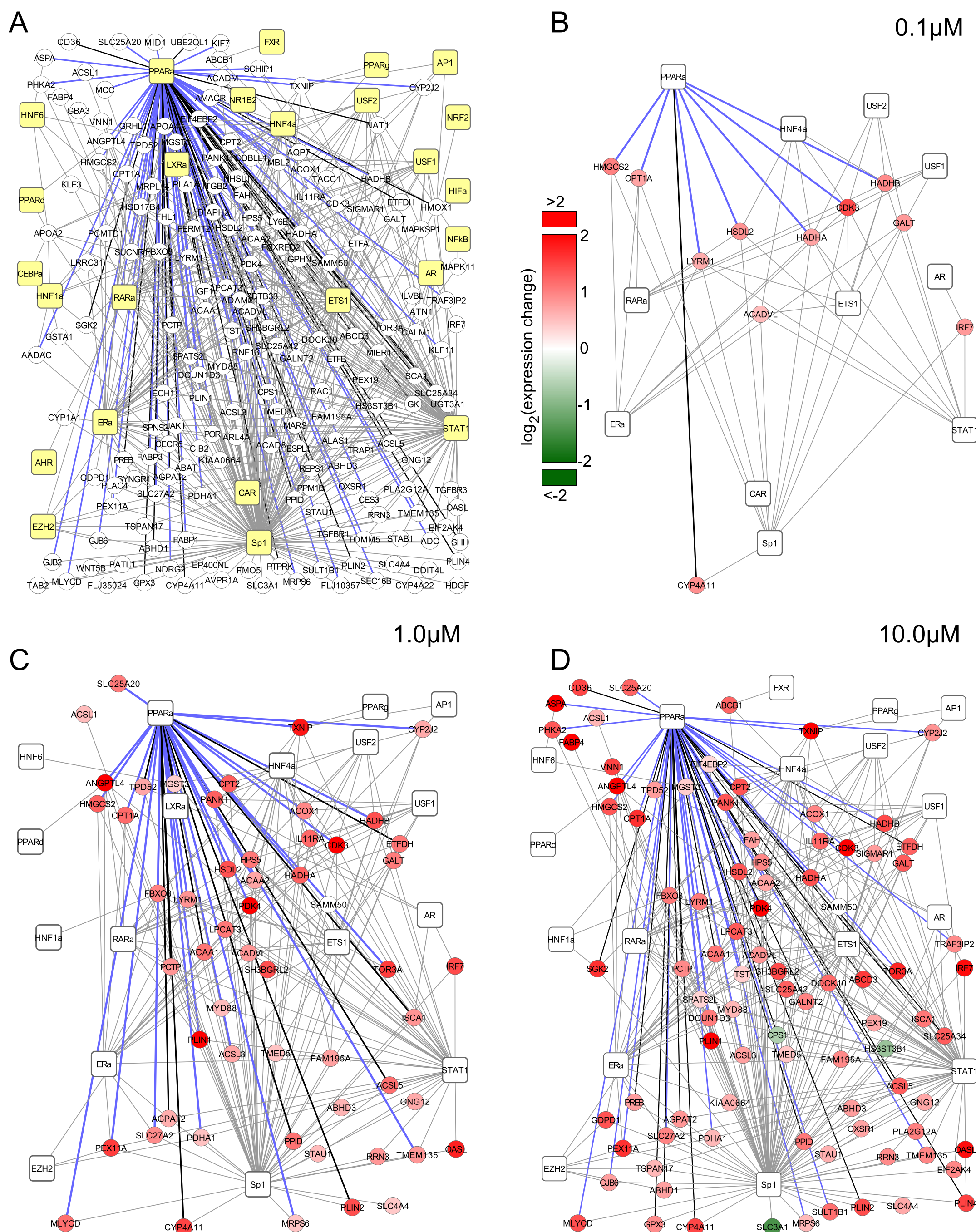

Fig. 4. The PPARα regulatory landscape. (A) We inferred the transcriptional regulatory network of PPARα using an online database of promoter-transcription factor interactions [36]. A target is featured if it was differentially expressed at one or more time or dose point. Circular nodes represent target genes; rectangular nodes are inferred transcription factors. (B–D) Dose response of the peroxisome proliferator regulatory network at 24 h exposure. For each panel, genes are only shown if they are differentially expressed at the indicated dose. Transcription factors are displayed if they are associated with at least one gene at the indicated dose. No genes were differentially expressed at 0.001 or 0.01 μM at 24 h.

(iii) *Indirect unbound* interactions: PPARα is not bound to the promoter region of a differentially-expressed gene, which is presumably activated or repressed by other TFs activated by phosphorylation or crosstalk with alternative signaling pathways (all remaining interactions).

3.3. Dose response of the PPARα regulatory network

The gene targets in Fig. 4A represent the union of all the differentially expressed genes observed over all experimental conditions (5 doses; 5 time points). However, whether these targets are differentially expressed at a particular condition (and to what extent) is a function of both time and ligand dose. To visualize how these factors affect the PPARα regulatory network, we visualized the evolution of the network in response to dose after 24 h treatment (Figs. 4B–D). To further demonstrate the dynamics of the network, we have created an interactive web applet as a tool for exploring the changes in the PPARα regulatory network in response to exposure time and dose (http://www.thehamner.org/ppara_network). We observed little gene expression change at any time point at

0.001 and 0.01 μM exposures. Genes regulated by PPARα binding (either direct or indirect) were more likely to be differentially expressed at lower doses (Fig. 4B); thus PPARα seems to be activated at lower doses compared to other transcription factors in the network. We were unable to identify any major differences in the dose response of the genes regulated by any transcription factor other than PPARα.

3.4. Functional characterization of peroxisome proliferator response

The gene ontology (GO) is a collection of terms that ascribe biological context to cellular species. Its descriptions fall under three domains: biological processes, cellular components, and molecular functions. GO categories have proven instrumental in summarizing the character of collections of genes, and are commonly used in the interpretation of microarray and other high-throughput experiments. The genes of an organism are annotated with GO terms based on what is known of their biology.

To better understand the transcriptional program initiated upon GW7647 treatment, we searched for commonalities among the functions of the differentially expressed genes. We identified 22 GO biological process terms that are overrepresented in the differentially expressed genes, relative to their occurrence in the genome at large [37] (Fig. 5 and S5). Interestingly, none of the GO terms we identified are differentially enriched in the subset of genes that are down-regulated upon treatment. This difference is likely because the set of down-regulated genes is too small to afford the statistical power necessary to establish a gene ontology category as enriched.

Because GO is a collection of biological concepts that form a hierarchical directed acyclic graph, terms that are near each other in semantic meaning are also close to each other in the ontology. As gene ontology annotations are curated from the literature and from automated searches, the specific set of terms associated with a gene is somewhat subjective. GO terms for two related genes may not perfectly coincide, but will typically be nearby in the ontological space. As such, we compiled the minimum network of gene ontology terms that is required to connect the 22 GO terms overrepresented among the target genes (Fig. 5).

This process revealed that there are two semantic groups of gene ontology terms that are enriched upon PPARα activation in primary human hepatocytes: amino acid processing and fatty acid processing (Fig. 5). This observation is consistent with what is known about the role of PPARα in these processes [38]. These categories remain relatively consistent between dose and time factors; the principal difference being that higher doses and longer exposure times yield a larger set of differentially expressed genes, which make enriched GO categories easier to identify (hence, lower *p*-values). However, it was interesting to observe the enrichment of "carboxylic acid transport" and "lipid localization" at high doses of GW7647 (Fig. 5), suggesting the activation of dose-specific mechanisms for coping with high concentrations of metabolites produced after near-maximal activation of these various metabolizing enzymes and lipid transport proteins.

3.5. The metabolic response to PPARα activation

Because of the important role of PPARα in sensing and responding to the lipid balance in the liver, we are also attempting to use gene expression experiments to develop a better understanding of the role of PPARα-responsive genes in fatty acid metabolism. Though the set of genes regulated by PPARα is small, it consists of nearly every major enzyme involved in lipid transport and β-oxidation in both the mitochondria and the peroxisome (Fig. 6).

Interestingly, the regulation of the individual components of β-oxidation depends upon their function and position in the β-oxidation cascade. Proteins involved in transport of fatty acids into the mitochondria (CPT1A, CPT2, and SLC25A20) and fatty acid activation (ACADM and ACOX1) are among the direct bound targets. Also, these proteins are up-regulated to a higher degree and up-regulated at earlier exposure times compared to the other proteins in the cascade (Figs. 2A and 6). This correlation indicates that direct binding of PPARα governs aspects of the temporal and dose-dependent organization of the metabolic response.

4. Discussion

4.1. Identifying network structure

The nuclear receptor superfamily coordinates the genomic response to a wide array of xenobiotic compounds by binding exogenous ligands and directly interacting with chromatin to promote transcription. Our results suggest that this mechanism only accounts for a small fraction of the response to peroxisome proliferators.

The canonical model of PPARα-mediated response involves the direct binding of activated PPARα to a well-established consensus binding site. Indeed, many of the genes we identified as differentially expressed upon GW7647 treatment participate in fatty acid metabolism. However, we found that a substantial fraction of the up-regulated genes were not bound by PPARα in primary hepatocytes or in HepG2 cells [14], indicating gaps in our understanding of how peroxisome proliferators induce their genomic response.

4.2. Implications of indirect PPARα binding

Transcription factor tethering is a likely mechanism by which PPARα could indirectly influence gene expression without binding to a canonical PPRE [31,39–42]. Tethering has been implicated in the regulation of about 25% of estrogen receptor-induced genes; and 30% of glucocorticoid receptor binding to chromatin appears to be indirect, likely by tethering to co-localized transcription factors like RUNX1 and AP1 [39–42]. Also, while there are a vast number of potential nuclear receptor binding sites across the genome, only a small fraction of these sites is occupied, with an even smaller number of sites likely contributing to functional gene regulation *in vivo* [29,43,30]. This latter observation supports approaches like those shown in the present paper to combine gene expression data from transcriptome profiling with genome-wide location analysis to provide a more accurate picture of nuclear receptor-mediated gene regulation.

Gene transcription via PPARα binding to a PPRE requires the assembly of a coactivator complex [32]. The assembly of this complex—rather than the translocation of PPARα to its cognate site—may control gene expression [44]. This hypothesis is consistent with the discordance between previously reported enhancement of PPARα binding [14] and the ligand-activated gene expression identified here. One of the genes down-regulated in our study was TACC1. TACC1 modulates transcription associated with other RXR-heterodimeric nuclear receptors (e.g., thyroid receptor and retinoic acid receptor) and appears to be a scaffold protein providing the structure to build transcriptional complexes for these nuclear receptors. In HepG2 cells [22], PPARα indirectly bound this gene (Figure S2). Our ChIP-seq study in primary hepatocytes did not identify binding of PPARα to TACC1 and there is no evidence of a functional PPRE in its promoter.

4.3. Understanding the nongenomic PPARα response

The relatively small fraction of genes bound by activated PPARα suggests that non-genomic mechanisms control a major

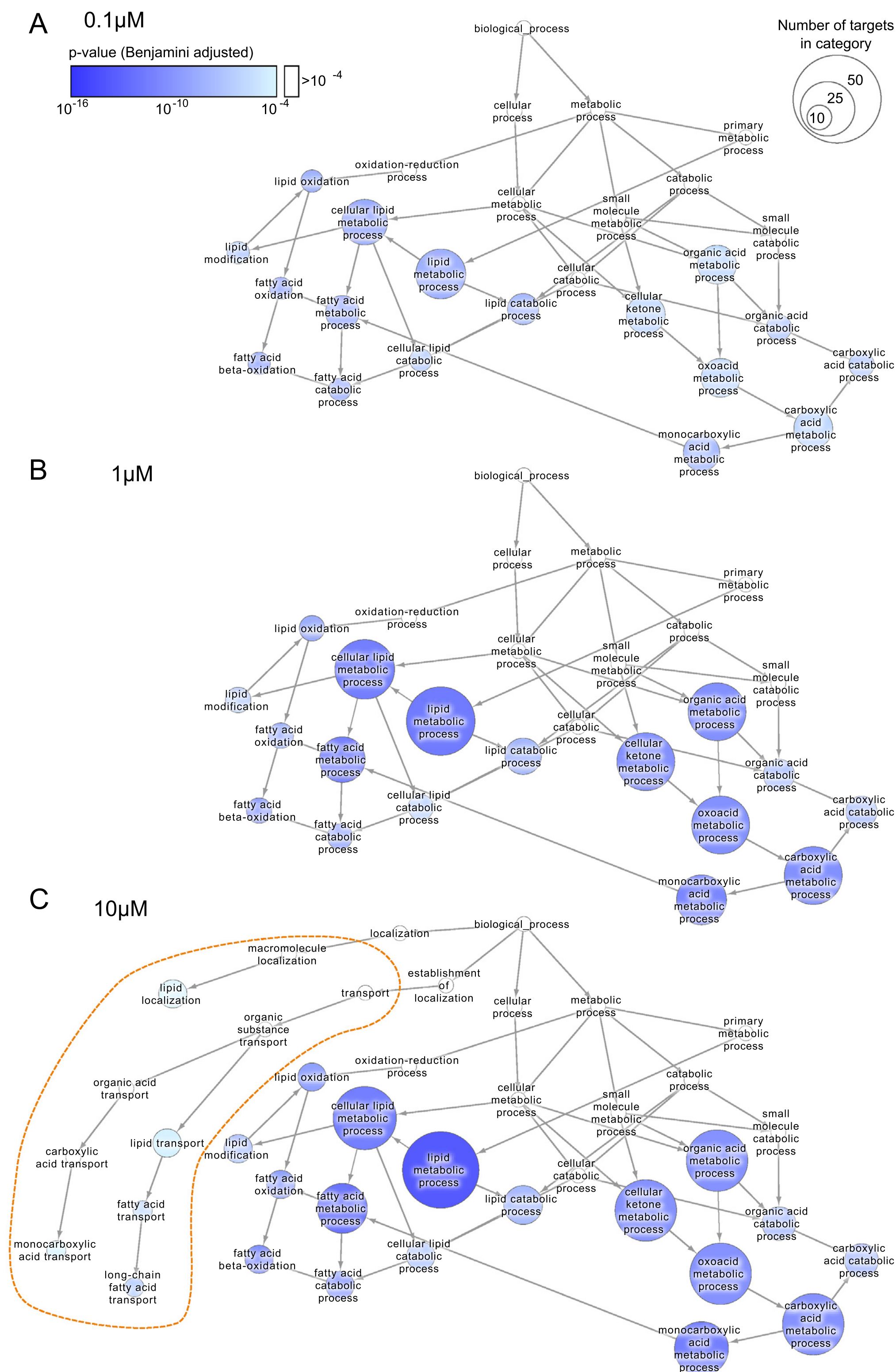

Fig. 5. Functional characterization of the peroxisome proliferator response. The network of gene ontology (GO) biological processes enriched for the union of genes showing differential expression at least one dose or time point. The number of genes annotated with each term is reflected by node size and the significance of the enrichment by node color (hypergeometric test, GO terms with Benjamini-adjusted *p*-value $<10^{-4}$ shown). Non-significant nodes were included (white) as needed to complete the minimal spanning tree. As expected, peroxisome proliferators have a profound effect on lipid and carboxylic acid metabolism processes. With increasing dose, the number of genes in, and consequently, the significance of, each functional annotation category increases (exposure time is 24 h). At high doses, fatty acid transport processes are activated (orange trace).

component of the peroxisome proliferator response. Peroxisome proliferators activate protein kinase cascades, leading to phosphorylation of kinases related to the extracellular signal-regulated kinase pathway, including Src [45], p38 [46], ERK1/2 [47–49], MEK1 [48], Ras [45], and EGFR [50]. PPARα agonists may affect ERK phosphorylation via a mechanism that depends on phosphati-

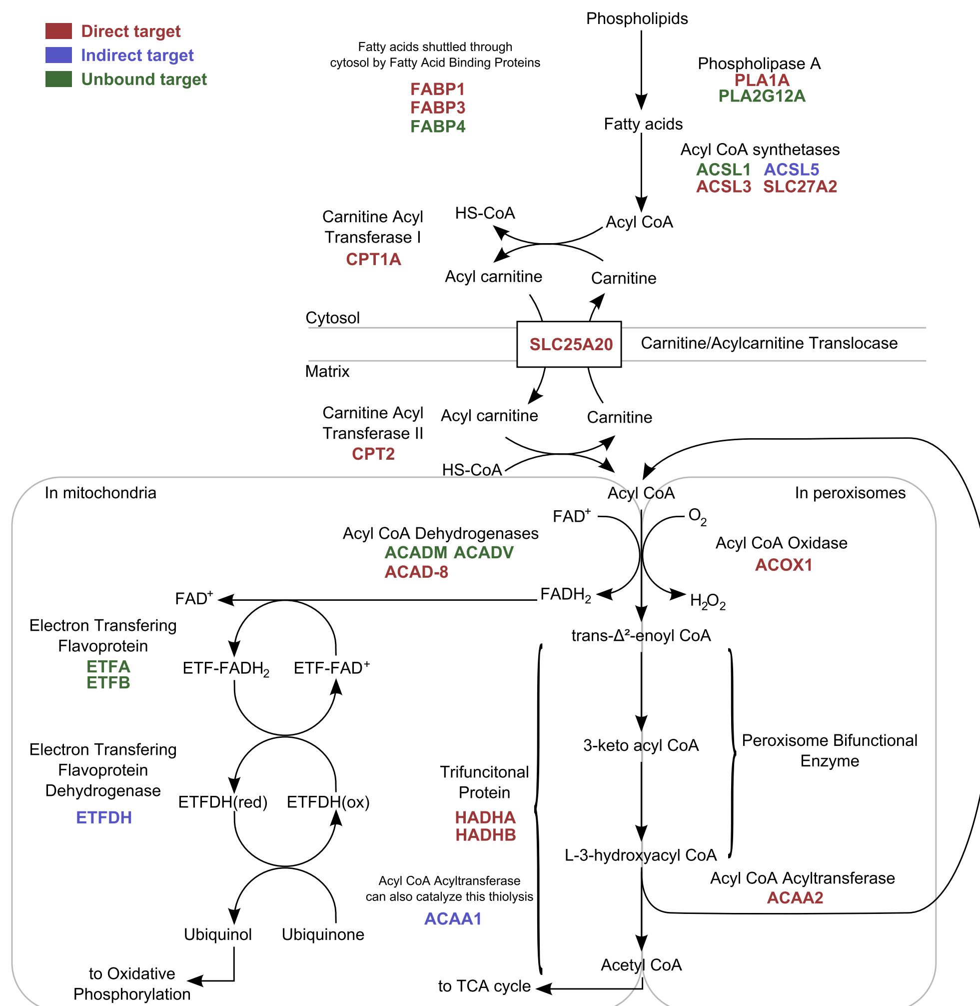

Fig. 6. The core machinery of mitochondrial and peroxisomal β-oxidation. Human genes with increased expression in response to the PPARα activator GW7647 are labeled in red, blue, or green according to their regulatory mechanism. With few exceptions (most notably, the peroxisome bifunctional enzyme), every major enzyme involved in lipid transport, oxidation, and electron capture is up-regulated in our study.

dylinositol signaling [50]. Separately, several PPAR agonists have been demonstrated to lead to the transient activation of p38 [45]. GW7647 and other PPARα agonists affect calcium signaling in various cell types, including pancreatic β-cells [51] and eosinophils [52]. These various mechanisms contribute to activation of transcription factors and induction of their target genes. While the specific mechanisms by which non-genomic processes are initiated by peroxisome proliferators are not yet clear, they might explain expression changes in genes responsive to peroxisome proliferators that have no demonstrated PPARα binding in their promoter regions. We made preliminary attempts to determine the role of kinase pathways in the PPARα network by examining the effects of inhibition on gene expression in primary hepatocytes. An alternative strategy with the human hepatocytes might be to examine the kinase-mediated activation of PPARα itself to see if the active kinase also phosphorylates key transcription factors identified in the pathway (Fig. 4).

4.4. Network components other than regulators of fatty acid metabolism

PPARα and the associated peroxisome proliferator response are a normal part of hepatic biology. Persistent activation of this pathway in rats leads to hepatic responses, including hypertrophy, cell proliferation and hepatocellular cancer [53]. These responses are considered unlikely to occur in humans based on various studies of peroxisome proliferating compounds in human hepatocytes that provide little evidence for enhancement of cell proliferation [54,55]. In our gene expression analysis in human primary hepatocytes we saw no alteration in genes that would induce proliferation at any of these times or concentrations. However, here were changes in genes that might have a negative consequence for proliferation. Of the down-regulated genes that are bound by PPARα (Fig. 2A, Table 2), the two with the greatest change are IGF1 and MCC. MCC (mutated in colorectal cancer) negatively regulates cell cycle progression. IGF1 (Insulin like growth factor 1) protein is

Table 2
Down-regulated genes with direct or indirect binding of PPARα.

Symbol	Name
IGF1	Insulin-like growth factor 1
MCC	Mutated in colorectal cancer
CPS1	Carbamoyl-phosphate synthase 1, mitochondrial
PTPRK	Protein tyrosine phosphatase, receptor type K
NHSL1	NHS-like 1
RNF13	Ring finger protein 13

similar to insulin in structure and function. Down-regulation of these genes and others may partially explain failure to cause proliferation in human hepatocytes. CPS1 (carbamoyl-phosphate synthase 1), which controls entry of nitrogen into the urea cycle, is also down-regulated. This implies that PPARα mediates a negative interplay between beta-oxidation and urea metabolism. Other down-regulated genes also appear to have roles in growth and differentiation. PTPRK (protein tyrosine phosphatase, receptor type K) is a signal transducer that modulates epidermal growth factor receptor activity in human keratinocytes [56].

Among the up-regulated genes that bind PPARα, there is a mix of metabolism related genes, including PLIN1, PDK4, PEX11A, PLA1A, CYP4A11, CYP1A1, HADHA, HADHB, PANK1, FABP1, and genes with more diverse function. Some of these other genes are involved in organelle formation and cell adhesion/integration—LYRM1, MID1, and ITGB2. In addition, the network includes transcription factors and cellular signaling proteins. CDK3 (cyclin-dependent kinase 3) is a serine/threonine kinase involved in cell cycle control. TXNIP (thioredoxin interacting protein), in addition to modulating thioredoxin activity, is a transcriptional co-repressor and cell cycle modulator [57]. We are now completing a similar pathway analysis with rat primary hepatocytes. A comparison of the regulatory networks between the two species with respect to genes that indirectly bind PPARα should help identify aspects of the network that either activate proliferative responses in the rat or serve to limit proliferative responses in humans.

4.5. Dynamic properties of network activation

Our study moves away from providing a list of genes and their connectivity to examining the transcriptional network associated with nuclear receptor function. With several nuclear receptors that are now known to be active as RXR heterodimers, responses appear to include bimodal induction in rodent cells (cells appear either in a basal or fully activated state) [58,59]. There is also hepatocyte proliferation in the intact rat after treatment with agonists for these nuclear receptors [60]. Both single-cell bimodal induction and proliferation likely require ultrasensitive signaling motifs leading to bistable states for the cell transcriptional programs [61]. Candidate motifs for driving these bistable decision-making behaviors are MAP kinase cascades linked to coordinated activation of groups of transcription factors. At this time, investigations are ongoing to characterize the role of kinases, the phosphoproteomic state of PPARα and implicated kinases, and the metabolic state of hepatocytes in response to PPARα activation. With proliferation driven by nuclear receptors, Src pathways associated with membrane-tethered nuclear receptors are a common network structure [62–66]. The change in gene expression profiles may be associated with kinase pathways linked to receptor phosphorylation after ligand binding.

4.6. Human risk/safety assessments and toxicity pathways

A 2007 National Research Council (NRC) publication, Toxicity Testing in the 21st Century; A Vision and A Strategy, proposed a new safety assessment approach based on *in vitro* assays in human cells relying on knowledge of modern biology and modes of action of compounds in humans. The assays would examine specific pathways or networks (such as with PPARα signaling in liver) without regard to specific apical responses seen in toxicity tests in animals or from human disease or epidemiology. The NRC report referred to testing responses of toxicity pathways, which are simply normal biochemical pathways that could lead to adverse consequences if activated or suppressed at a sufficient level over time. The larger suite of nuclear receptor pathways provides important targets for toxicity and good case studies for looking at the approaches needed to move to *in vitro* platforms for risk assessment. Our approach here is a first step to outline the network for PPARα and we expect to extend it to other nuclear receptor pathways following further progress in establishing pathway dynamics for bimodal patterns of the hepatocyte gene expression profiles in the presence and absence of ligand. With PPARα, an interesting modality for pathway identification and dose response would be metabolomics applied with an active biological ligand whose concentration would be reduced by the PPARα pathway.

For some nuclear receptor pathways, the endogenous ligand or class of ligands is well understood. PPARα binds a number of representative endogenous fatty acids and cannabinoids. For many other nuclear receptor pathways—CAR, AhR, PXR, etc.—that increase Phase I, II, and III enzymatic pathways, endogenous ligand are not as well established. Presumably, these pathways map the signal from an endogenous ligand to a response that is related to the quantity of that ligand, much like fatty acids activate PPARα to induce β-oxidation. Applied to other nuclear receptor toxicity pathways, we expect our approach to reveal the endogenous role of the pathway and shed light on the native ligand of the receptor system.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2013.11.006.

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