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The role of retinoic acid in hepatic lipid homeostasis defined by genomic binding and transcriptome profiling

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Abstract

Background: The eyes and skin are obvious retinoid target organs. Vitamin A deficiency causes night blindness and retinoids are widely used to treat acne and psoriasis. However, more than 90% of total body retinol is stored in liver stellate cells. In addition, hepatocytes produce the largest amount of retinol binding protein and cellular retinoic acid binding protein to mobilize retinol from the hepatic storage pool and deliver retinol to its receptors, respectively. Furthermore, hepatocytes express the highest amount of retinoid x receptor alpha (RXR α) among all the cell types. Surprisingly, the function of endogenous retinoids in the liver has received very little attention.

Results: Based on the data generated from chromatin immunoprecipitation followed by sequencing, the global DNA binding of transcription factors including retinoid x receptor α (RXR α) along with its partners i.e. retinoic acid receptor α (RAR α), pregnane x receptor (PXR), liver x receptor (LXR), farnesoid x receptor (FXR), and peroxisome proliferator-activated receptor α (PPAR α) has been established. Based on the binding, functional annotation illustrated the role of those receptors in regulating hepatic lipid homeostasis. To correlate the DNA binding data with gene expression data, the expression patterns of 576 genes that regulate lipid homeostasis were studied in wild type and liver RXR α -null mice treated with and without RA. The data showed that RA treatment and RXR α -deficiency had opposite effects in regulating lipid homeostasis. A subset of genes (114), which could clearly differentiate the effect of ligand treatment and receptor deficiency, were selected for further functional analysis. The expression data suggested that RA treatment could produce unsaturated fatty acids and induce triglyceride breakdown, bile acid secretion, lipolysis, and retinoids elimination. In contrast, RXR α deficiency might induce the synthesis of saturated fatty acids, triglyceride, cholesterol, bile acids, and retinoids. In addition, DNA binding data indicated extensive cross-talk among RAR α , PXR, LXR, FXR, and PPAR α in regulating those RA/RXR α -dependent gene expression levels. Moreover, RA reduced serum cholesterol, triglyceride, and bile acid levels in mice.

Conclusions: We have characterized the role of hepatic RA for the first time. Hepatic RA mediated through RXR α and its partners regulates lipid homeostasis.

Keywords: Nuclear receptor, Retinoids x receptor, Retinoic acid receptor, Farnesoid x receptor, Peroxisomal proliferator-activated receptor α , Liver x receptor, Pregnane x receptor, Chromatin immunoprecipitation, Sequencing, Microarray

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Background

The eyes and skin are obvious retinoid target organs. Vitamin A deficiency causes night blindness and retinoids are widely used to treat acne and psoriasis. However, more than 90% of total body retinol (retinylpalmitate, the storage form) is stored in liver stellate cells [1]. In addition, hepatocytes produce the largest amount of retinol binding protein and cellular retinoic acid binding protein to mobilize retinol from the hepatic storage pool and deliver retinol to its receptors, respectively [2]. Furthermore, hepatocytes express the highest amount of retinoid x receptor alpha (RXR α) among all the cell types. Surprisingly, the function of endogenous retinoids in the liver has received very little attention. Thus, the current study aims to identify the bona fide RXR α and RAR α targets in the liver.

The broad and complicated roles of retinoids can be explained by the presence of multiple receptors for retinoic acid (RA), the biological active form of retinol. The receptors for RA are retinoic acid receptor (RAR) as well as retinoid x receptor (RXR) [2]. In addition, RXR is essential for many other receptors to function. These receptors for RA belong to a nuclear receptor family whose members are transcriptional factors. Thus, RA exerts its biological effects by regulating gene expression. RXR is unique in that it not only forms homodimers, but also dimerizes with other nuclear receptors, which include receptors for fatty acids (peroxisomal proliferator-activated receptors, PPARs), bile acids (farnesoid x receptor, FXR), oxysterols (liver x receptor, LXR), xenobiotics (pregnane x receptor, PXR, and constitutive androstane receptor, CAR), vitamin D (vitamin D receptor, VDR), and RA (RAR). Hence, most RXR partners participate in regulating lipid homeostasis. Within these heterodimers, RXR can be either a permissive or a silent partner. When RXR serves as a silent partner, the heterodimer does not respond to RA. When it is a permissive (active) partner, RA and the ligand for the heterodimeric partner can both activate the heterodimer. For example, RXR is a permissive partner for PPAR [3]. Similarly, heterodimeric complexes of RXR with LXR [4] or FXR [5] also retain RA responsiveness. Furthermore, retinoids also activate PXR, VDR, and CAR thus are able to thus regulate xenobiotic metabolism and potentially their own oxidation [6-8]. Since most of these receptors are abundantly expressed in the liver, the endogenous RA may regulate many hepatic nuclear receptor-mediated pathways. Therefore, the role of RA in the liver is unpredictable. In order to understand the endogenous function of RA and its receptors, it is crucial to identify RA receptor targets (genes and pathways) genome-wide.

RXR α is highly expressed in the liver [9]. Liver specific RXR α -deficient mice have increased serum triglyceride and cholesterol levels [10,11]. In addition, lack of hepatic RXR α increases sensitivity to alcohol- and non-alcohol-induced steatosis and steatohepatitis [12,13]. Besides regulating

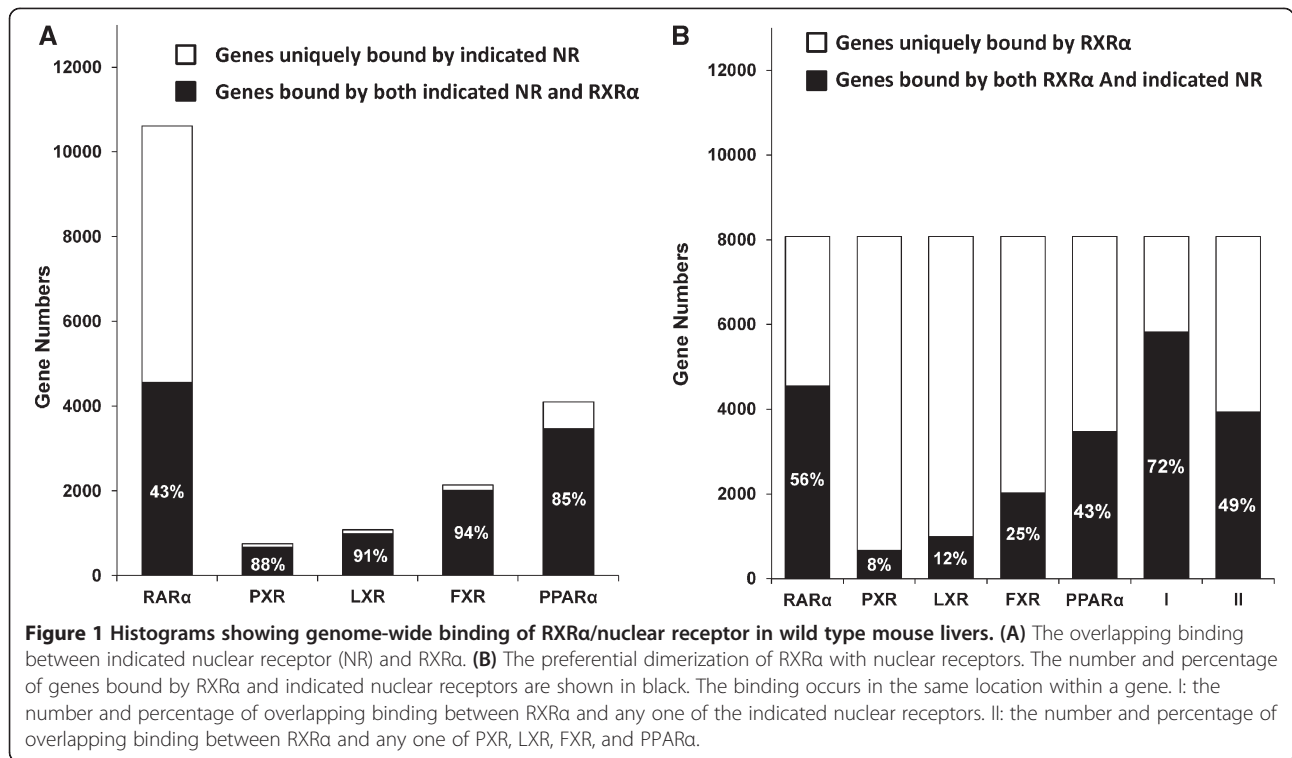
lipid metabolism, hepatocyte RXR α also controls xenobiotic [14-16], carbohydrate [17], and amino acid metabolism [17]. These findings indicate that RXR α -mediated signaling has a huge impact on maintaining liver health and in regulating many disease processes.

To understand the global roles of RXR α and RAR α at the genomic level, chromatin immunoprecipitation using anti-RXR α and -RAR α antibodies followed by sequencing (ChIP-seq) was performed. Since RXR α is an essential partner for other nuclear receptors, we compared ChIP-seq data to RXR α binding locations with locations from previous studies for PXR [18], LXR [19], FXR [20], and PPAR α [19]. Meanwhile, the expression levels of the genes responsible for lipid homeostasis were studied in wild type and hepatic RXR α -deficient mouse livers. Both genome-wide DNA-binding and hepatic gene expression data were used to define the role of RA in the liver. Our data uncovered the unknown function of retinoic acid and RXR vs. RAR in the liver. Using different approaches, we showed for the first time that retinoic acid-activated RXR α and RAR α have distinct effects. Moreover, the action of retinoic acid in the liver is to regulate lipid homeostasis specifically by reducing serum cholesterol, triglyceride and bile acid levels. The data provided may lead to future development of synthetic retinoid that can target metabolic syndrome or other types of lipid-associated health issues.

Results

Genome-wide binding of RXR α , RAR α , PXR, LXR, FXR, and PPAR α in mouse livers

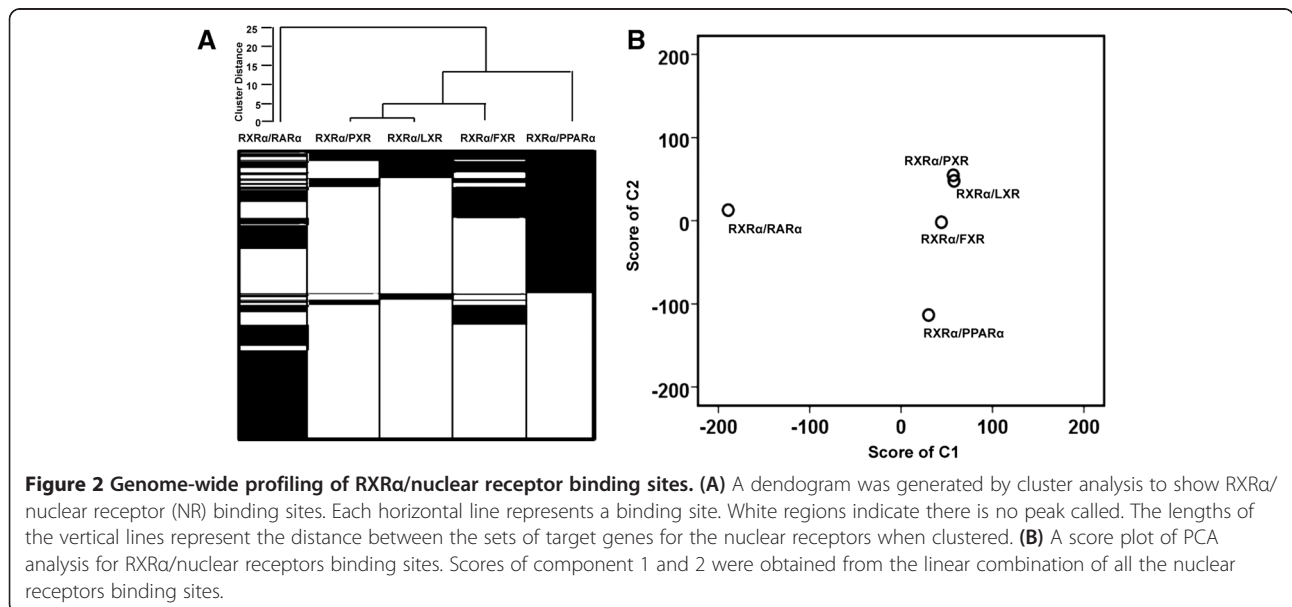
To understand the global roles of RXR α and RAR α at the hepatic genome level, ChIP-seq was performed using anti-RXR α and -RAR α antibodies. Single read sequencing yielded 18 and 32 million uniquely mapped reads for RXR α and RAR α , respectively. After filtering by including peak scores that were greater than 20 and distance within 10 kb from the transcriptional start site, 17,973 peaks were detected for RXR α and 18,697 peaks for RAR α . Since RXR α is an essential partner for other nuclear receptors, we compared ChIP-seq data of RXR α with those of PXR [18], LXR [19], FXR [20], and PPAR α [19]. Our data showed that the numbers of peaks, which were commonly bound by RXR α and other nuclear receptors, were 6,577 for RAR α , followed by 5,154 for PPAR α , 2,846 for FXR, 1,190 for LXR, and 868 for PXR in the mouse liver genome. RAR α had less than 50% overlapping bindings with RXR α while PPAR α , FXR, LXR, and PXR had over 85% overlapping bindings with RXR α (Figure 1A). These findings suggest that RXR α is indispensable for the function of PPAR α , FXR, LXR and PXR in the mouse liver. In contrast, RAR α may work in the absence of RXR α . More than 4000 of RAR α binding genes were bound by RXR α , and occupied 56% of total RXR α bindings, followed by PPAR α (43%), FXR (25%),



LXR (12%), and PXR (8%). The well-known lipid regulators including PPARα, FXR, LXR, and PXR bound to almost 50% of RXRα-bound regions suggesting the extensive role of RXRα in regulating lipids.

Cluster and principal component analysis (PCA) were performed using the binding data. Overlapping bindings between RXRα and RARα, PPARα, FXR, LXR, and PXR were analyzed. According to the length of the vertical branches (Figure 2A), LXR and PXR were clustered into one basic

group. FXR was clustered into a subgroup with LXR and PXR. PPARα, PXR, LXR, and FXR were classified into another group. However, RARα was distant from the others. The same datasets were subjected to PCA analysis. After dimension deduction, two components were picked to describe the global properties of the samples. The two-dimension score plot showed that LXR and PXR had the shortest distance, implying their properties on the genome-wide binding level were most



similar (Figure 2B). The distance between FXR and LXR/PXR was shorter than the distances between other receptors and LXR/PXR. RAR α was far from all other nuclear receptors. Thus, the information generated from the PCA and cluster analysis was consistent. The difference among the binding profiles of PXR, LXR, FXR, and PPAR α were described by component 2 while the difference between the binding profile of RAR α and those four nuclear receptors were described by component 1 in the PCA.

Function analysis of the genes that have overlapping binding sites between RXR α and each of RAR α , PXR, LXR, FXR, and PPAR α

To understand the potential biological role of genes likely to be targeted by RXR α -heterodimers, DAVID functional annotation was performed. The top ten processes for each of the heterodimers formed by RXR α and other nuclear

receptors were investigated in this study (Table 1). Although common pathways among these heterodimers, such as carboxylic acid catabolic process, organic acid catabolic process, and oxidation reduction, appeared in the top 10 pathways, the heterodimers still have their unique biological functions. RXR α -RAR α uniquely regulated protein transport, localization, and RNA processing. The heterodimers of RXR α and PXR, LXR, FXR, and PPAR α have a great impact on various lipid processing pathways. For example, RXR α -PPAR α and RXR α -LXR tend to bind to the genes involved in acylglycerol, glycerol ether, and neutral lipid metabolic processes, which are fatty acid derived. In addition, RXR α -PPAR α distinctly binds to the genes that are involved in fatty acid metabolic processes, and RXR α -LXR is more prone to bind to the genes that participate in steroid metabolic process. In addition to steroid metabolic process, RXR α -FXR also tends to bind to genes that dictate lipid

Table 1 Common and unique biological function of genes bound by RXR α coupled with RAR α , PXR, LXR, FXR and PPAR α

Pathways	RAR α	PXR	LXR	FXR	PPAR α
Acylglycerol metabolic process			1.9E-07		3.8E-10
Neutral lipid metabolic process			2.7E-07		9.4E-10
Glycerol ether metabolic process			2.7E-07		1E-10
Organic ether metabolic process					4.5E-11
Carboxylic acid catabolic process	6.1E-11	7.3E-07	2.8E-08	1.9E-09	6.1E-11
Organic acid catabolic process	6.1E-11	7.3E-07	2.8E-08	1.9E-09	6.1E-11
Coenzyme metabolic process	9.4E-13			2E-07	1.7E-10
Cofactor metabolic process	1.6E-17	3.0E-06		2.1E-07	2E-11
Fatty acid metabolic process					1.8E-12
Oxidation reduction	1.7E-23	1E-11	1.4E-16	6.1E-14	3.4E-28
Sterol homeostasis			7.2E-08		
Cholesterol homeostasis			7.2E-08		
Lipid homeostasis				4.8E-07	
Lipid transport				2E-07	
Hexose metabolic process				2.1E-07	
Steroid metabolic process			2.6E-11	2E-08	
Monosaccharide metabolic process				1.4E-07	
Pyruvate metabolic process		1.3E-05			
Acute-phase response		3.2E-05			
Cellular carbohydrate biosynthetic process		5.9E-06			
Acute inflammatory response		7.3E-07			
Glucose metabolic process		0.00001			
Response to wounding		3.5E-07			
Electron transport chain	2.7E-10				
ncRNA metabolic process	4.2E-15		1.8E-09		
mRNA processing	6.7E-16				
Translation	7E-16				
RNA processing	8.8E-16				

Numbers in the table represent the *p* value given by DAVID.

transport and regulate steroid and monosaccharide homeostasis. RXR α -PXR targets specific biological processes like regulating pyruvate and carbohydrate biotransformation, but it also has a role in acute-phase response, acute inflammatory response, and response to wounding. Taken together, RXR α -RAR α targeted protein and RNA processes while RXR α -PXR/LXR/FXR/PPAR α targeted homeostasis regulation of small molecules, which include monosaccharide and lipids.

Global profiling of the expression of lipid homeostasis genes in wild type and RXR α KO mice treated with and without RA

Hepatic RXR α KO mice have elevated serum cholesterol and triglyceride levels [21], and the majority of PXR, LXR, FXR, PPAR α -bound genes were also bound by RXR α . We next tested a hypothesis that RA and hepatic RXR α could regulate lipid homeostasis in the mouse liver via RXR α and its heterodimeric partners. The expression levels of the lipid homeostasis genes (579) in the KEGG pathway database were studied in wild type and hepatic RXR α KO mice treated with and without RA. The PCA score plot showed that RA treatment of wild type mice caused a downward shift in C2 from the untreated control group (Figure 3A). In contrast, an upward shift was found due to hepatic RXR α deficiency in comparison to the untreated wild type mice. Thus, RA treatment and RXR α deficiency had opposite effects. In addition, no significant change was noted when RA was used to treat hepatic RXR α KO mice. These findings unequivocally prove that the effects

of RA on regulating those lipid homeostasis genes were RXR α dependent. Score plot (Figure 3A) indicates component 2 made a contribution to distinguish groups of control, RA-treated, and RXR α -deficient mice. Thus, 114 out of 579 genes with high loading values (>0.5 or <-0.5) in component 2 were selected for further analysis (Figure 3B). Among them, 55 genes were induced by RA and had decreased expression levels due to RXR α deficiency. The other 59 genes, whose expression levels were suppressed by RA, had increased expression levels due to a lack of RXR α . Thus, the expression levels of those 114 lipid-related genes are ligand (RA)-responsive and receptor (RXR α)-dependent. Based on the known function of those genes described in KEGG and PubMed, the role of those 114 genes was assigned and summarized in Table 2. Remarkably, RA regulated many genes involved in certain pathways. For example, RA decreased the expression of 10 genes in the cholesterol biosynthesis pathway, but did not increase the expression of any other genes in the same pathway. Thus, it is very likely that RA inhibited the biosynthesis of cholesterol in an RXR α -dependent manner. RA also induced the expression of 13 genes in the RA elimination process and yet did not reduce the expression of any gene in the same process. Thus, RA can self-regulate its own level. Furthermore, RA also induced the expression of 9 genes in the biosynthesis of unsaturated fatty acids responsible for anti-inflammation. Since there was no inhibition of gene expression in the same pathway, it is very likely that RA up-regulates the synthesis of unsaturated fatty acids and has an anti-inflammatory role.

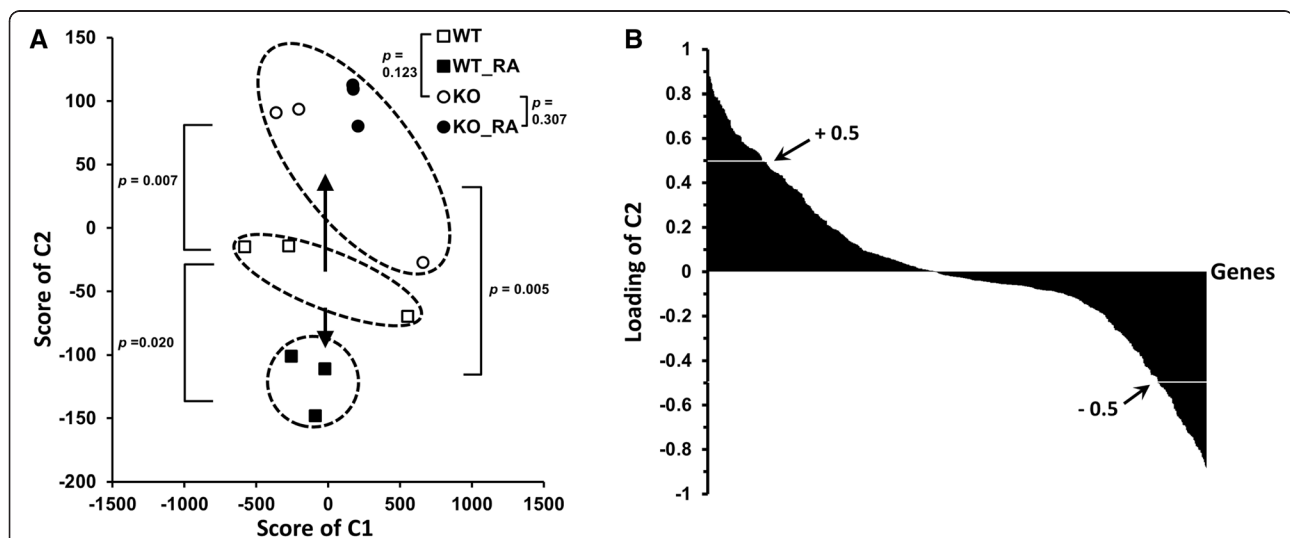


Figure 3 PCA of the expression level of 579 lipid genes in wild type and hepatic RXR α -KO mice treated with and without RA. Wild type and hepatic RXR α KO mice were treated with and without RA for 7 days (150 mg/kg diet, n = 3). The expression of 579 genes involved lipid homeostasis was studied. **(A)** Score plot of PCA showing the difference among the groups. Spots within an ellipse belong to the same group. Arrows represent the direction separating groups from the wild type (WT) mice (open square \square). There is no significance in the C1 component observed across all groups. **(B)** A histogram that shows the loading value of genes on C2. Genes with high loading value (≥ 0.5) on C2 had higher mRNA levels in RXR α KO than wild type livers. In contrast, genes with low loading value (≤ -0.5) on C2 had increased mRNA levels due to RA treatment.

Table 2 Biological functions responding to RA treatment and RXR α knockout in wild type mice

Biological functions	Gene number (with RXR α bindings)	
	RA induced & RXR α KO repressed	RA repressed & RXR α KO induced
Lipid droplet growth	1 (0)	2 (2)
Transportation of bile acids for bile excretion	1 (0)	0
Dehydrogenation of saturated fatty acids to unsaturated fatty acids	3 (2)	0
Biosynthesis of glycerol phospholipids	4 (3)	1 (1)
Tryglyceride degradation	4 (3)	0
Biosynthesis of unsaturated fatty acids responsible for anti-inflammation	9 (7)	0
Elimination of retinoic acids	13 (11)	0
Biosynthesis of bile acids	4 (4)	4 (4)
Biosynthesis of retinoic acids	1 (1)	2 (2)
Biosynthesis of saturated fatty acids	5 (5)	10 (10)
Degradation of glycerol phospholipids	1 (1)	4 (4)
Degradation of saturated fatty acids	10 (10)	5 (5)
Elimination of steroid hormones	3 (3)	5 (5)
Fat digestion and absorption	3 (3)	7 (7)
Lipid droplet breakdown (fat mobilization)	1 (1)	1 (1)
Recycle of bile acids via hepatic-intestine	1 (1)	3 (3)
S1P degradation	1 (1)	1 (1)
Transportation of bile acids for kidney excretion	2 (2)	3 (3)
DHS1P degradation	1 (1)	0
Elimination of unsaturated fatty acids (PGE2) responsible for lipolysis inhibition	1 (1)	0
phosphatidylcholine to phosphatidylethanolamine	1 (1)	0
Sphingolipid biosynthesis	1 (1)	0
SPH (SM) degradation	0	1 (0)
Biosynthesis of cholesterol	0	10 (10)
Biosynthesis of steroid hormone	0	1 (1)
Biosynthesis of tryglycerides	0	1 (1)
Biosynthesis of unsaturated fatty acids responsible for pro-inflammation	0	1 (1)
Breakdown of phospholipid to form unsaturated fatty acids	0	1 (1)
Elimination of cholesterol (from circulation back to liver for catabolism)	0	1 (1)
Elimination of cholesterol via steroid hormone pathway	0	1 (1)
Phosphatidylethanolamine to phosphatidylcholine	0	1 (1)

ChIP-Seq data indicated that most of the genes (87 out of 114) had RXR α binding implying direct gene regulation.

Taken together, ligand (RA) treatment and hepatic RXR α deficiency resulted in opposite effects. Figure 4 summarizes the effect of RA and hepatic RXR α deficiency on lipid homeostasis. RXR α deficiency tends to favor saturated fatty acids, triglyceride, cholesterol, and bile acids synthesis. In contrast, RA treatment leads to unsaturated fatty acids and phospholipid synthesis and lipolysis as well as triglyceride breakdown.

Binding of RA/RXR α responsive genes by other nuclear receptors

Additional analysis was done to understand which other nuclear receptors may be involved in regulating the expression of these 114 RA/RXR α target genes, which have a role in lipid homeostasis. The binding data generated in the current study (RXR α and RAR α) were compared with the binding data of PXR, LXR, FXR, and PPAR α . Figure 5 shows overlapping genes with RXR α -heterodimers, as assessed by overlapping binding of RXR α and other nuclear receptors. The data were organized by the number of different nuclear receptors binding the genes. For example, motifs located in the *Abca1*, *Abhd5*, *Acs1*, and *Aldh3a2* genes could be bound by RXR α and all five nuclear receptors. Peaks located in the *Apoa4*, *Cyp51*, *Cyp7b1*, and *Elovl1* could be bound by RXR α and any 4 out of the 5 studied nuclear receptors (Figure 5). Some of the commonly regulated genes have nuclear receptor binding site at the same location. The data indicated extensive crosstalk among nuclear receptors in regulating the expression of those genes.

Quantification of serum cholesterol, triglyceride, and bile acid levels

ChIP-Seq and RNA expression profiling indicate the role of RA in controlling lipid homeostasis in the liver. Serum cholesterol, triglyceride, and bile acid levels were quantified to test the genetic findings. The data showed that RA reduced serum cholesterol, triglyceride as well as bile acid levels in wild type mice (Figure 6). However, such effects were not found in hepatic RXR α KO mice. In addition, serum cholesterol and triglyceride, but not bile acid, levels were elevated due to RXR α deficiency. These biochemical findings confirm the role of RA in regulating lipid homeostasis in the liver.

Discussion

This study establishes the role of nuclear receptors and RA in regulating lipid homeostasis in the liver. In addition, the mechanisms by which nuclear receptors and RA regulate lipid homeostasis were illustrated at the gene, pathway, and systemic levels. Furthermore, relationships between RXR α and PXR, LXR, FXR, as well as PPAR α in regulating lipid homeostasis were analyzed. These nuclear receptors depend

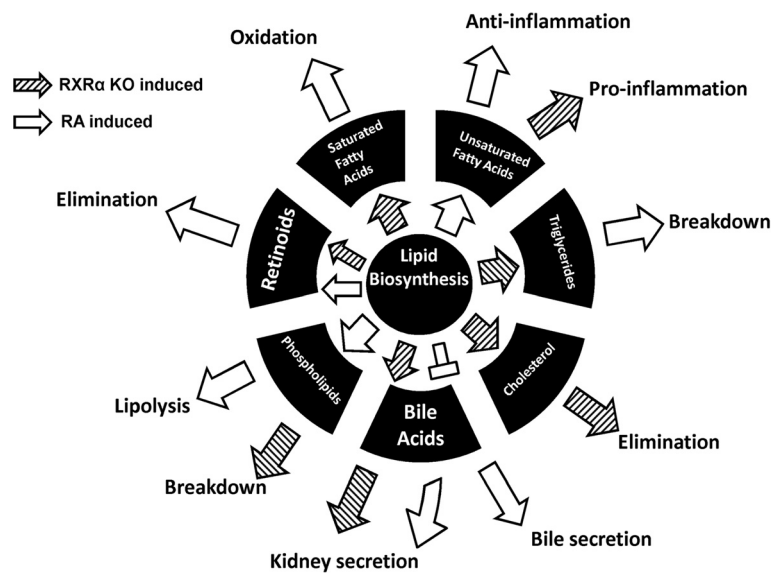


Figure 4 Lipid synthesis and metabolism pathways regulated by RA treatment and RXR α deficiency. The diagram represents the effect of RA treatment and RXR α deficiency on the expression of 114 lipid genes based on PCA analysis. Fifty-five genes were induced by RA and inhibited due to the lack of RXR α , and fifty-nine of them were induced because of RXR α deficiency and RA suppressing. Gene functions were obtained from the KEGG database and confirmed in PubMed gene database. The arrows inside the circle represent the lipid synthesis processes, and the outer arrows represent the lipid elimination processes. The "T" sign represents inhibition and all arrows represent inductions.

on RXR α to execute their functions and more than 85% of their bound genes overlap with those bound by RXR α . The nuclear receptor binding data were strengthened by profiling the expression patterns of 576 lipid genes, which showed that RA treatment and RXR α -deficiency had an opposite effect in regulating lipid homeostasis. Nuclear receptor binding data also illustrated extensive cross talk among the studied nuclear receptors. Thus, our novel *in vivo* data provided extensive evidence showing the role of RA in dictating lipid homeostasis in the liver.

It is intriguing that more than 85% of the PXR, LXR, FXR, and PPAR α binding sites overlapped with those of RXR α . In contrast, only 43% of RAR α binding sites overlapped with those of RXR α . It has been shown that RAR α can form homodimers [22]. It is also possible that RAR α may dimerize with RXR β and RXR γ to exert its function. Although the percentage of overlapping bindings between RAR α and RXR α is not as high as others, the number of genes that could be bound by RXR α /RAR α (4554) is the highest, followed by RXR α /PPAR α (3468), RXR α /FXR (2019), RXR α /LXR (988), and RXR α /PXR (666), implying the relatively extensive role of these nuclear receptors in regulating hepatic gene expression. It is important to further study the role of RAR α and other RARs in the liver. By forming partners with other nuclear receptors, RXR α is a master regulator. Our data showed that more than 8000 hepatic genes were bound by RXR α , and 72% of them overlapped with the genes bound by RAR α , PXR, LXR, FXR, or PPAR α . The remaining 28% of RXR α binding sites might be bound by RXR α homodimer or the heterodimer of

RXR α and VDR or CAR. Thus, the five nuclear receptors (RAR α , PXR, LXR, FXR, or PPAR α) analyzed in the current study account for almost three quarters of RXR α binding genes in the liver. Furthermore, nearly 50% of RXR α bindings overlapped with the bindings of PXR, LXR, FXR, and PPAR α (Figure 1B). Hence, lipid regulation should be one of the major functions of RXR α .

Clustering and PCA showed that the genome-wide binding pattern of RAR α is not similar to that of PPAR α , LXR, PXR, and FXR. Biological function annotation also showed that RAR α has some unique features including protein processing, protein localization, and RNA processing. However, the five studied nuclear receptors also demonstrated functional redundancy. For example, there are four pathways, including oxidation reduction, carboxylic acid catabolic process, organic acid catabolic process, and cofactor metabolic process, that can be regulated by more than four nuclear receptors. This finding suggests the importance of these four pathways in the liver, and the role of RAR α in them.

Although PPAR α , LXR, FXR, and PXR have extensive roles in regulating lipids, they also have specific roles in regulating different types of lipids. RXR α /PPAR α prefers to bind to genes that participate in neutral lipids, glycerol ether, and organic ether as well as fatty acid metabolism processes. All of which are either fatty acid-derived products or precursors for the biosynthesis of fatty acids. Another pathway bound by RXR α /PPAR α is the acylglycerol metabolic process, which is involved in triglyceride homeostasis. RXR α /LXR tends to regulate genes involved in sterol

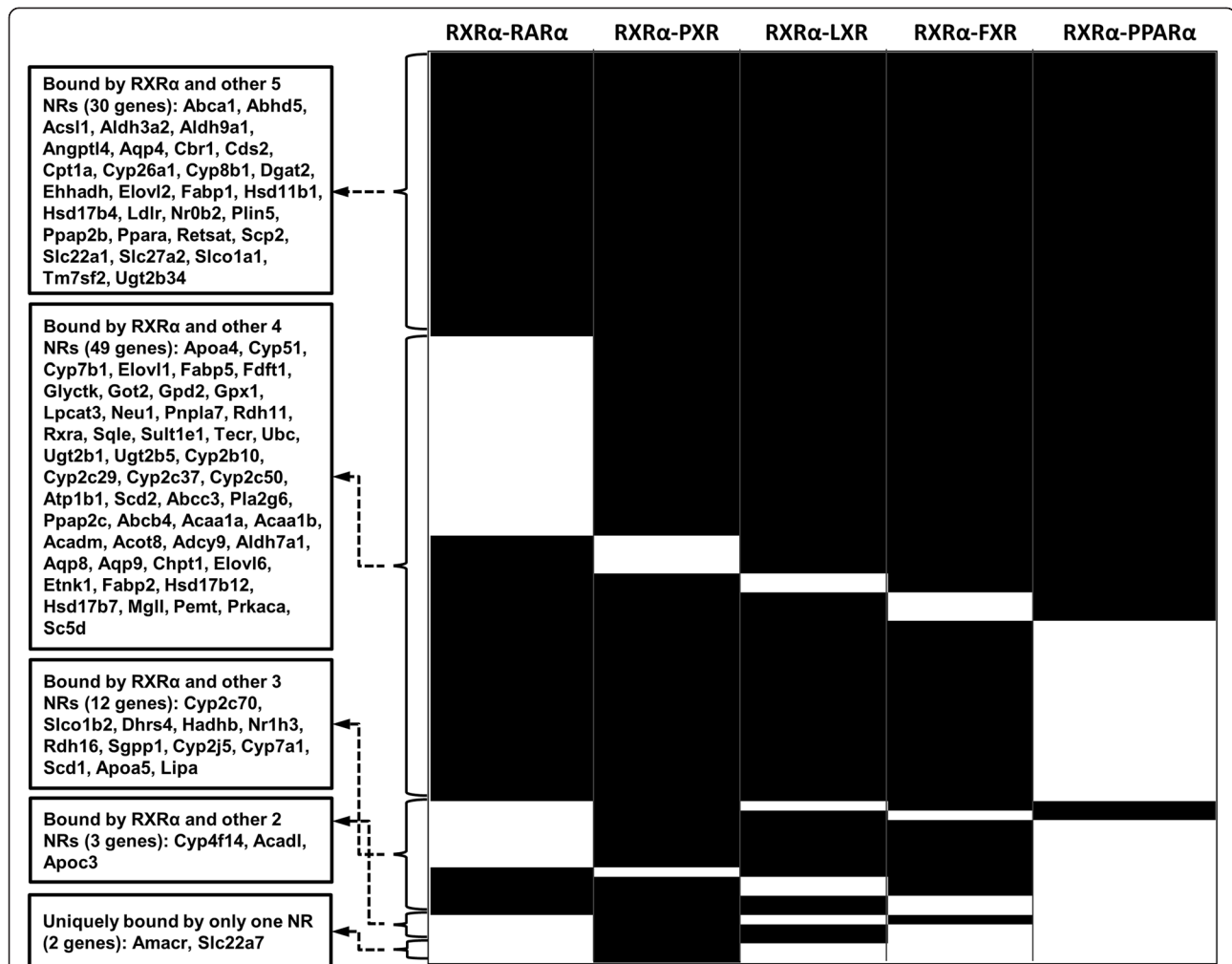


Figure 5 Heat maps showing the binding profiles of RXRα/nuclear receptor on 96 RA-responsive and RXRα-dependent lipid genes. RA responsive and RXRα-dependent genes (114) were analyzed by nuclear receptor binding. Among those 114 genes, 96 genes shown in black lines can be commonly regulated by RXRα and its partner. Some of the commonly regulated genes have nuclear receptor binding site at the same location.

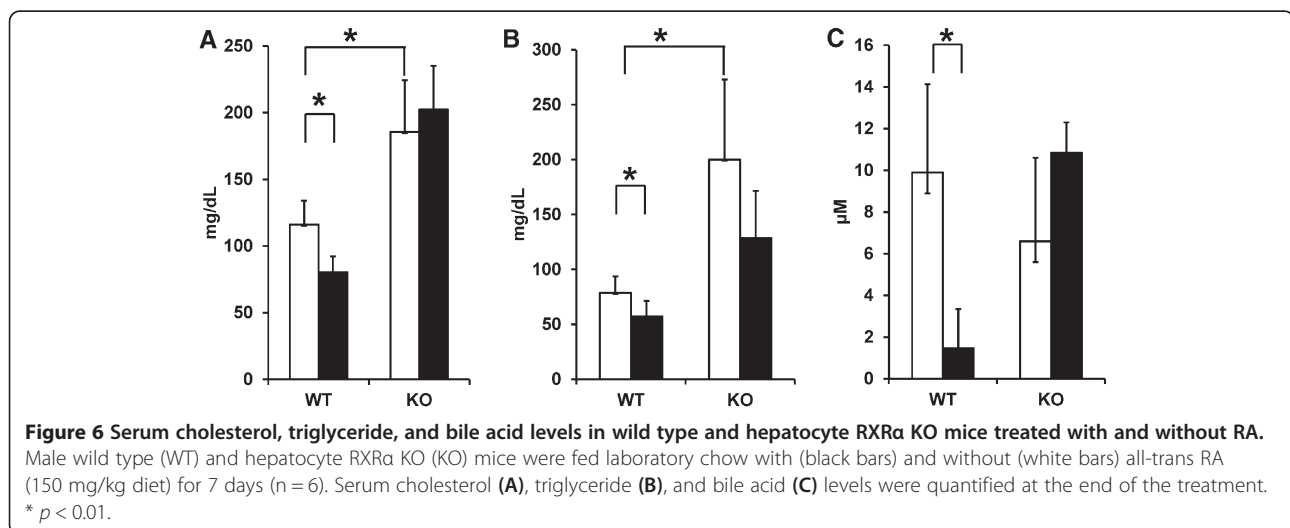


Figure 6 Serum cholesterol, triglyceride, and bile acid levels in wild type and hepatocyte RXRα KO mice treated with and without RA. Male wild type (WT) and hepatocyte RXRα KO (KO) mice were fed laboratory chow with (black bars) and without (white bars) all-trans RA (150 mg/kg diet) for 7 days (n = 6). Serum cholesterol (A), triglyceride (B), and bile acid (C) levels were quantified at the end of the treatment. * $p < 0.01$.

metabolism, which is consistent with its known role [23]. RXR α /FXR not only binds to the genes participating in steroid metabolism process, but also those involved in lipid transport and carbohydrate metabolism processes. RXR α /PXR binds to the genes involved in regulating the pyruvate metabolic process at the DNA binding level. Pyruvate is a key intersection for fatty acid, carbohydrate, and protein metabolisms. In addition, RXR α /PXR also regulates response to acute phase, inflammatory, and wounding, implying that PXR can be an excellent target for metabolism and inflammation-related health issues. Lastly, FXR binds to the genes involved in monosaccharide metabolism, which shows the intimate relationship between bile acid and glucose homeostasis [24].

All of the 114 genes that showed differential effects of RA treatment and RXR α deficiency are bounded by RXR α and RAR α , PXR, LXR, FXR, and PPAR α heterodimers. These findings indicate that those studied nuclear receptors retain RA response in vivo and the effect of RA is dependent upon those nuclear receptors. RA has a broad spectrum of effects including biosynthesis of retinoids, phospholipids, and unsaturated fatty acids. It also has a role in eliminating retinoids, oxidizing saturated fatty acids, and breaking down triglycerides. It seems that RA has extensive beneficial effects in maintaining the health of the liver. Specifically, RA induced the expression of *Cyp2c37/38/50/54/70* and *Cyp2j5*. These genes encode enzymes involved in the generation of epoxyeicosatrienoic acids [25], which have anti-inflammatory effects [26]. In contrast, RXR α deficiency induces the gene expression of *Cyp4f* that is responsible for the generation of 20-hydroxyeicosatetraenoic acid, a pro-inflammation molecule [25]. In addition, RA increases mRNA levels of *cbr1* (carbonyl reductase 1), which is responsible for transforming prostaglandin E2 to prostaglandin F2 α . Prostaglandin E2 and F2 α have different effects in regulating lipid breakdown. Prostaglandin E2 is a lipolysis inhibitor [27]; whereas, prostaglandin F2 α has not been shown to have the same effect. Thus, the induction of *cbr1* gene expression could be a mechanism by which RA induces lipolysis. RA also induces expression levels of gene encoding proteins for phospholipid biosynthesis, but RXR α deficiency increases the expression of the genes that have a role in the degradation of phospholipids. This finding suggests the potential role of RA in maintaining the normal structure of the cell membrane. Formation of the monolayer of lipoprotein or lipid droplet is one of the major ways that phospholipids regulate lipid metabolism [28]. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are two important phospholipids that show different effects on lipid metabolism in humans and rodents. Lower PC/PE ratio induces steatosis or even steatohepatitis in humans

[29], however, PE has a greater effect than PC in reducing the cholesterol level in rodents [30]. Our data showed that RA induced the gene expression of *ptdss1* (phosphatidylserine synthase 1), which converts PC to PE. Consistently, the *ptdss1* gene expression is reduced due to hepatic RXR α deficiency. These gene expression levels and DNA binding data not only showed the underlying mechanism for RA in regulating liver gene expression, but also suggested the biochemical outcome.

Conclusions

Taken together, the differentiation and morphogenetic effect of RA is well known. However, the current study provides a comprehensive analysis of the role of RA in lipid homeostasis. All-trans RA is the most abundant retinoid that can be easily detected in the liver. Thus, RA is likely to act as a regulator to control hepatic lipid metabolism. Since the effect of RA is broad, it is important to develop specific retinoids in order to target specific pathways. Such efforts may allow us to identify compounds that can be used to treat or prevent metabolic syndromes and other lipid-related health issues.

Methods

Materials and animal models

Male wild type mice (12 weeks old) and hepatocyte RXR α -deficient mice (KO) [10,11], which have the same genetic background of C57BL/6, were used. The RXR α KO mice were produced and characterized previously [10,11]. The LoxP sites were inserted into introns flanking the fourth exon of the RXR α gene covering the DNA binding domain, which is deleted after crossing the floxed RXR α allele against a transgenic line in which cre recombinase is expressed under the control of the albumin promoter. The mutant mice express a truncated protein that has the intact ligand binding domain, but lacks the DNA binding domain. Animal protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kansas Medical Center and the University of California, Davis.

All ChIP-grade antibodies except anti-RNA Pol II (Millipore, MA) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). DNA purification kit was purchased from Qiagen Co. (Valencia, CA). All other ChIP-related reagents were obtained from Invitrogen Co. (Carlsbad, CA).

Animal treatment

Retinoic acid (Sigma-Aldrich, MO) was given to wild type and hepatic RXR α -deficient mice at a dosage of 150 mg/kg diet for 7 days. As controls, mice were fed with normal diet.

Chromatin immunoprecipitation (ChIP)

ChIP was performed according to our previously published study [31]. After fixation, the mouse livers were subjected to lysis with cell and nuclear lysis buffer. Sonication was used to fragment the chromatin, followed by precipitation with specified antibodies. The target DNA fragments were obtained by reverse crosslinking and purification. Antibodies against IgG and RNA Pol II were used as negative and positive controls, respectively.

DNA library preparation and sequencing

By using the End-It DNA End Repair Kit (Illumina, Madison, WI), DNA fragments prepared from ChIP were ligated with specified adaptors and amplified, then size-selected (175-225 bp) on an agarose gel followed by sequencing (High-Seq 2000, Illumina, Madison, WI).

Alignment, call peak, and annotation of ChIP-seq data

The target sequences were aligned to the mouse genome (<http://hgdownload.cse.ucsc.edu/goldenPath/mm10/bigZips/>) by Bowtie 0.12.7 [32] followed by peak-calling using MACS (version 1.4.1) [33]. The peaks were annotated using the database (NCBI37/mm9) by Peak Analyzer [34]. The background cut off standard was set to be 20 fold of the input signals [18]. The cut off distance from the transcription start site (TSS) was set to be 10 kb. Co-localization is defined as having at least 25% overlap in their peak widths.

Microarray

Affymetrix 430 A_2 Chip (Santa Clara, CA) was used to determine the genome-wide mRNA expression levels. Microarray data were annotated using Affymetrix Expression Console (MAS5). The probe signal with *p* values less than 0.05 were used for further analysis.

ChIP-seq data analysis

All data were treated with the same cut off criteria. The generated RXR α binding data were compared with the data for RAR α , PXR, LXR, FXR, and PPAR α . The principle component analysis (PCA) and cluster analysis package in SPSS program was used to analyze the global binding data. For both PCA and cluster analysis, called peaks were assigned the value of 1. Not called peaks were assigned the value of 0. Genes with overlapping binding sites of RXR α and each of RAR α , PXR, LXR, FXR, and PPAR α at the same location were functionally analyzed by the DAVID (<http://david.abcc.ncifcrf.gov/>) [35].

Lipid homeostasis analysis based on mRNA expression

Genes (579) involved in regulating lipid homeostasis were extracted from the KEGG database (Kyoto Encyclopedia of Genes and Genomes, <http://www.genome.jp/kegg/>). The expression of those 579 genes were determined in wild type and liver RXR α KO mice treated with and without

RA (*n* = 3) for 7 days. After multiple comparisons, only 30 and 36 out of all 579 lipid homeostasis genes showed significant change at the corrected *p*-value of 0.05 after RA treatment and RXR α knockout, respectively. Therefore, IBM SPSS PCA package was used to differentiate groups based on the global expression pattern of all 579 lipid homeostasis genes.

Serum lipid assays

Triglyceride, cholesterol, and bile acids in the serum were assayed using a commercially available kit (Pointe Detroit, Michigan) that was modified to a 96-well format. Spectrophotometric analysis was conducted with a Bio-Tek microtiter plate reader (Bio-Tek, VT).

Availability of supporting data

The microarray and ChIP-Seq data supporting the results of this article are available with accession numbers of GSE50028 and GSE46762, respectively, in the GEO repository (<http://www.ncbi.nlm.nih.gov/geo/>).

Abbreviation

RA: Retinoic acid; ChIP: Chromatin immunoprecipitation; seq: Sequencing; RXR α : Retinoid x receptor alpha; RAR α : Retinoic acid receptor alpha; PXR: Pregnane x receptor; LXR: Liver x receptor; FXR: Farnesoid x receptor; PPAR α : Peroxisome proliferator-activated receptor alpha; DAVID: Database for annotation, visualization and integrated discovery; PCA: Principal component analysis; WT: Wild type; KO: Knockout; NR: Nuclear receptors; KEGG: Kyoto encyclopedia of Genes and genomes; PE: Phosphatidylethanolamine; PC: Phosphatidylcholine.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YH: Performed experiments, analyzed data, generated figures and tables as well as prepared manuscript. LG: Microarray experiments and analysis. YF: Sequence alignment and call peaks. QZ: Functional annotation. H-XL: Animal experiments. YL: Statistics analysis. GLG: FXR ChIP Seq data generation. LL-M: Microarray data generation. JF: Sequence alignment and call peaks. Y-JYW: Generated idea and supervised the all overall performance of the project. All authors read and approved the final manuscript.

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