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Regulation of Bile Acid Synthesis by Fat-soluble Vitamins A and D*[S]

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Bile acids are required for proper absorption of dietary lipids, including fat-soluble vitamins. Here, we show that the dietary vitamins A and D inhibit bile acid synthesis by repressing hepatic expression of the rate-limiting enzyme CYP7A1. Receptors for vitamin A and D induced expression of *Fgf15*, an intestine-derived hormone that acts on liver to inhibit *Cyp7a1*. These effects were mediated through distinct *cis*-acting response elements in the promoter and intron of *Fgf15*. Interestingly, transactivation of both response elements appears to be required to maintain basal *Fgf15* expression levels *in vivo*. Furthermore, whereas induction of *Fgf15* by vitamin D is mediated through its receptor, the induction of *Fgf15* by vitamin A is mediated through the retinoid X receptor/farnesoid X receptor heterodimer and is independent of bile acids, suggesting that this heterodimer functions as a distinct dietary vitamin A sensor. Notably, vitamin A treatment reversed the effects of the bile acid sequestrant cholestyramine on *Fgf15*, *Shp*, and *Cyp7a1* expression, suggesting a potential therapeutic benefit of vitamin A under conditions of bile acid malabsorption. These results reveal an unexpected link between the intake of fat-soluble vitamins A and D and bile acid metabolism, which may have evolved as a means for these dietary vitamins to regulate their own absorption.

Lipid-soluble vitamins are essential for human health and must be obtained from the environment. They exist in four major groups (A, D, E, and K), each of which comprises a series of structurally related compounds with multiple biological functions. Members of the nuclear receptor family of transcription factors are central to the mechanism of action of the lipid-soluble vitamins A and D. Metabolites of vitamin A regulate gene transcription by binding to and activating the retinoic acid receptors (RARs[4]; NR1B) and the retinoid X receptors (RXRs; NR2B) (1). Similarly, the genomic actions of vitamin D are mediated by the vitamin D receptor (VDR; NR1I1) (2).

Retinol, the basic form of vitamin A, is obtained from foods of animal origin, where it is present in the form of retinyl esters. Vitamin A can also be synthesized in the liver from β-carotene, which is present in foods of plant origin. In the body, retinol is converted to several metabolites that function as signaling molecules in various biological processes, including vision, development, growth, metabolism, and cell differentiation (3–5). Most of the transcriptional actions of vitamin A *in vivo* have been shown to require the RXR/RAR heterodimer (3).

Vitamin D is synthesized in the skin from 7-dehydrocholesterol through a process that requires sunlight, or it can be obtained directly from the diet. Dietary sources include cholecalciferol (vitamin D_3, animal origin) and ergocalciferol (vitamin D_2, plant origin). Whether obtained from the diet or photoactivation in the skin, vitamin D must be converted to its bioactive form, 1α,25-dihydroxyvitamin D_3, by the action of cytochrome P450 enzymes in the liver and kidney (6). Vitamin D is best known for its essential role in regulating calcium and phosphate homeostasis (7). Recently, the repertoire of physiological systems regulated by vitamin D and its receptor has been expanded to include both innate and adaptive immunity and bile acid detoxification (8–10).

The absorption of lipid-soluble vitamins from the diet requires the detergent actions of bile acids. Bile acids are amphipathic sterols synthesized from cholesterol in the liver and secreted into the intestine, where, when present at high concentrations, they function to emulsify dietary lipids (11). Cholesterol 7α-hydroxylase (CYP7A1), which catalyzes the rate-limiting step in bile acid biosynthesis, is tightly regulated at the transcriptional level by bile acids and other signaling molecules (12). Positive transcriptional regulators of *Cyp7a1* include orphan nuclear receptors, LRH-1 (liver-related homologue-1; NR5A2) and hepatocyte nuclear factor 4α (NR2A1) (13, 14). Negative feedback regulation of *Cyp7a1 in vivo* involves two complementary mechanisms. First, bile acids activate the nuclear bile acid receptor (farnesoid X receptor (FXR); NR1H4) in the intestine to induce expression of *Fgf15* (fibroblast growth factor 15; *FGF19* in humans), which signals from the intestine

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[4] The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; VDR, vitamin D receptor; FXR, farnesoid X receptor; RT, reverse transcription; ChIP, chromatin immunoprecipitation; TTNPB, 4-((E)-2(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzoic acid.

to repress hepatic *Cyp7a1* through a mechanism that involves the atypical nuclear receptor SHP (small heterodimer partner) and the membrane receptor FGFR4 (fibroblast growth factor receptor 4) (15). Second, bile acids activate FXR in the liver to induce transcription of SHP, which subsequently binds to LRH-1 and hepatocyte nuclear factor 4α, resulting in the repression of *Cyp7a1* (16–18). How these two FXR-regulated pathways interact to control *Cyp7a1* expression is not clear, albeit both are required for the FXR-mediated feedback repression of bile acid biosynthesis (19).

Although it is well established that bile acids are essential for the absorption of lipid-soluble vitamins, it is not known whether lipid-soluble vitamins affect bile acid biosynthesis. In this study, we demonstrate that vitamins A and D regulate bile acid synthesis by overlapping but distinct mechanisms. As expected, the action of vitamin D on bile acid homeostasis occurs through activation of VDR. Interestingly, the action of vitamin A is mediated through the RXR/FXR heterodimer. This latter finding supports a novel role for the RXR/FXR heterodimer as a dietary sensor for vitamin A.

EXPERIMENTAL PROCEDURES

Animals and Animal Husbandry—Male C57BL/6 mice were purchased from Charles River Laboratories and used for all experiments involving only wild-type animals. $VDR^{+/+}$ and $VDR^{-/-}$ mice were obtained from heterozygous breeders on a pure 129T2 background. $FXR^{+/+}$ and $FXR^{-/-}$ mice were obtained from heterozygous breeders on a pure 129S background. $FGF15^{+/+}$ and $FGF15^{-/-}$ mice were obtained from homozygous breeders on a mixed C57BL/6;129S background. $SHP^{+/+}$ and $SHP^{-/-}$ mice were obtained from homozygous breeders on a pure 129S background. All animals were housed in the same specific pathogen-free facility. Animals were maintained under a temperature-controlled environment with 12-h light/dark cycles with *ad libitum* access to water and irradiated rodent chow (TD.2916, Harlan-Teklad). The expression of metabolic genes analyzed in this study is affected by circadian and feeding cycles; therefore, the following steps were taken to ensure a synchronous feeding cycle. On the day prior to death, chow was removed 10 h before the dark cycle, replaced at the onset of the dark cycle, and removed 4 h after the onset of the dark cycle. The change in body weight between the beginning and end of the dark cycle was <5% for all animals. All experiments were done with 3–6-month-old (age-matched) male mice killed between 4 and 6 h after the beginning of the light cycle. Mice were killed by isoflurane inhalation and exanguinated via the descending vena cava prior to tissue collection.

Animal Treatments—Vitamin D (1α,25-dihydroxycholecalciferol; Sigma) in sterile saline was administered by intraperitoneal injection at the end of the dark cycle and 4 h before death. Vitamin A (retinyl palmitate, ~1800 USP units/mg; Sigma) and vitamin E (*d*-α-tocopherol acetate, ~1360 IU/g; Sigma) in 1% Tween 80, 1% methylcellulose, and 25 mM HEPES were administered by oral gavage at the beginning and end of the dark cycle (16 and 4 h before death). Cholestyramine was admixed in the diet (custom diet TD.07658, Harlan-Teklad). GW4064 (GlaxoSmithKline) and TTNPB (Sigma) were admixed in the diet (500 and 0.25 mg/kg diet, respectively) and provided *ad libitum* for 12 h before death. LG268 in 0.25% Tween 80 and 1% methylcellulose was administered by oral gavage 12 h before death at a dose of 30 mg/kg. In all cases, animals in the vehicle group received the appropriate vehicle solutions and diets in a manner identical to the treatment groups.

Mouse Ileum Explant Culture—Following death, 5 cm of the terminal ileum was collected and flushed with phosphate-buffered saline to remove the contents. Segments (1 × 3 mm) of the ileum were cultured at 37 °C and 95% oxygen for 6 h in Dulbecco's modified Eagle's medium (containing 4 g/liter glucose and L-glutamine; Invitrogen) supplemented with 10% charcoal-stripped heat-inactivated fetal bovine serum, 25 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and either vitamin D or 0.1% ethanol (vehicle). Each condition contained four 1 × 3-mm segments of ileum, from which total RNA was extracted at the end of the 6-h culture period.

Bile Acid Extraction and Analysis by Liquid Chromatography/Mass Spectrometry—Bile acid pool size measurements were performed as described previously with minor modifications (20). The following is a brief description of the protocol, including modified parameters. Liver, gallbladder, intestines, and attached mesentery were removed *en bloc* and homogenized in ethanol. Homogenates were heating to boiling, cooled, and then filtered. This step was repeated twice. Filtrates were combined and adjusted to a constant volume. Bile acids were resolved by reverse-phase liquid chromatography (C_8 precolumn, C_{18} analytical column) and quantified by mass spectrometry with electrospray ionization in negative ion mode. The following unconjugated and taurine-conjugated bile acids were used as calibration standards: cholic acid (5β-cholanic acid-3α,7α,12α-triol), α-muricholic acid (5β-cholanic acid-3α,6β,7α-triol), β-muricholic acid (5β-cholanic acid-3α,6β,7β-triol), ω-muricholic acid (5β-cholanic acid-3α,6α,7β-triol), hyocholic acid (5β-cholanic acid-3α,6α,7α-triol), chenodeoxycholic acid (5β-cholanic acid-3α,7α-diol), deoxycholic acid (5β-cholanic acid-3α,12α-diol), hyodeoxycholic acid (5β-cholanic acid-3α,6α-diol), ursodeoxycholic acid (5β-cholanic acid-3α,7β-diol), murocholic acid (5β-cholanic acid-3α,6β-diol), lithocholic acid (5β-cholanic acid-3α-ol), taurocholic acid (5β-cholanic acid-3α,7α,12α-triol-*N*-(2-sulfoethyl)amide), tauro-α-muricholic acid (5β-cholanic acid-3α,6β,7α-triol-*N*-(2-sulfoethyl)amide), tauro-β-muricholic acid (5β-cholanic acid-3α,6β,7β-triol-*N*-(2-sulfoethyl)amide), tauro-ω-muricholic acid (5β-cholanic acid-3α,6α,7β-triol-*N*-(2-sulfoethyl)amide), taurohyocholic acid (5β-cholanic acid-3α,6α,7α-triol-*N*-(2-sulfoethyl)amide), taurochenodeoxycholic acid (5β-cholanic acid-3α,7α-diol-*N*-(2-sulfoethyl)-amide), taurodeoxycholic acid (5β-cholanic acid-3α,12α-diol-*N*-(2-sulfoethyl)amide), tauroursodeoxycholic acid (5β-cholanic acid-3α,7β-diol-*N*-(2-sulfoethyl)amide), taurohyodeoxycholic acid (5β-cholanic acid-3α,6α-diol-*N*-(2-sulfoethyl)amide), taurolithocholic acid (5β-cholanic acid-3α-ol-*N*-(2-sulfoethyl)amide), and taurolithocholic acid 3-sulfate (5β-cholanic acid-3α-ol-*N*-(2-sulfoethyl)amide 3-sulfate). Deuterium-labeled cholic acid (5β-cholanic acid-3α,7α,12α-triol-2,2,4,4-*d*4) and chenodeoxycholic acid (5β-cholanic acid-3α,7α-diol-2,2,4,4-*d*4) were used as recovery controls. Norcholic acid (23-

nor-5β-cholanic acid-3α,7α,12α-triol) was used as the sample loading control.

RNA Extraction and Quantitative Reverse Transcription (RT)-PCR—Following death, the left lateral lobe of the liver and distal ileum (5 cm proximal to the ileocecal junction, flushed with phosphate-buffered saline) was collected and frozen immediately in liquid nitrogen. Total RNA was extracted using RNA STAT-60™ (IsoTex Diagnostics). RNA (4 μg) from each sample was DNase-treated and reverse-transcribed using random hexamers. The resulting cDNA was analyzed by quantitative RT-PCR using a protocol described previously (21). Briefly, quantitative PCRs containing 25 ng of cDNA, 150 nmol of each primer, and SYBR® GreenER™ PCR Master Mix (Invitrogen) were carried out in triplicate in 384-well format using an ABI PRISM® 7900HT instrument (Applied Biosystems). Relative mRNA levels were calculated using the comparative C_T method normalized to *U36b4*. The primer sequences used for gene expression analyses are listed in supplemental Table 1. They were designed using Primer Express® software (Applied Biosystems) and validated as described previously (21).

Chromatin Immunoprecipitation (ChIP) Analysis—Following death, the ileum was collected, flushed with phosphate-buffered saline, and frozen immediately in liquid nitrogen. Samples from four to five mice were pulverized and pooled. 300 mg from each pool was fixed in phosphate-buffered saline with 1% formaldehyde for 10 min and quenched with glycine for 5 min. Samples were then Dounce-homogenized in hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2% Nonidet P-40, 1 mM EDTA, and 5% sucrose) and layered over cushion buffer (10 mM Tris-HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 1 mM EDTA, and 10% sucrose), followed by centrifugation at 200 × *g* to collect the crude nuclear pellet. Subsequent ChIP steps were done using the ChIP EZ kit (Upstate) materials and methods. 3 μg of protein from sonicated chromatin was used for pulldown assay with rabbit IgG (Santa Cruz Biotechnology), anti-VDR (Santa Cruz Biotechnology C-20x), or anti-RXR (Santa Cruz Biotechnology ΔN-197x). Primers scanning −1500 to +3000 of the *Fgf15* locus were designed and used for analyzing VDR and RXR binding by quantitative PCR as described (21). Primer sequences are shown in supplemental Table 2.

Expression Plasmids—pCMX-hFXR, pCMX-hVDR, and pCMX-hRXRα expressing full-length nuclear receptors under the control of the constitutive cytomegalovirus promoter have been described previously (18, 22, 23).

Fgf15 Promoter and Intron 2 Cloning—Total DNA was isolated from mouse colon using the DNeasy Blood & Tissue kit (Qiagen) and protocol. A 2338-nucleotide region of the *Fgf15* promoter including six nucleotides downstream of the transcriptional start site was amplified using the following primers containing restriction endonuclease sites (underlined): CCTAAACCAAGCTTCTGGCCATCTG (forward) and AGAGTTACTGCGTCGACAGTGG (reverse). This product was inserted upstream of the thymidine kinase promoter sequence in the pTk-LUC plasmid (24) to generate p2300Fgf15+TATA_TkLUC. This plasmid was used to amplify shorter regions of the *Fgf15* promoter, which were likewise inserted into pTk-LUC. Promoter regions were amplified using distinct forward primers (CTGCGAAAGCTTGCTAAAGGAGAG for p400Fgf15+TATA_TkLUC, CCACCAAGCTTCTGTGCATTGAAC for p230Fgf15+TATA_TkLUC, and CCTGTCGACGCATCAAGTCTCC for p100Fgf15+TATA_TkLUC) and a common reverse primer (GGATCCTCTAGAGTCGACAGTGG). p230Fgf15+TATA_TkLUC was amplified using the following overlapping primers containing two altered nucleotides (underlined): GGCCTGGGCGGGACCACTGGGTTGGGG (forward) and CCCAACCCAGTGGTCCCGCCCAGGCC (reverse). The *Fgf15* promoter region containing the two-nucleotide mutation was reinserted into pTk-LUC to generate p230(mut-145)Fgf15+TATA_TkLUC. A 2460-nucleotide region encompassing the second intron of *Fgf15* was amplified using the following primers containing restriction endonuclease sites (underlined): GAGCGCGGTCGACAAGATATACG (forward) and AAGGTACAGTCGACCTCCGAGTAG (reverse). The product was inserted in reverse orientation upstream of the thymidine kinase promoter sequence in pTk-LUC to generate pFgf15intron2rev_TkLUC. The modified regions of all plasmids were verified by DNA sequencing at the McDermott Sequencing Core of the University of Texas Southwestern Medical Center.

Cotransfection and Luciferase Assay—HEK293 cells were grown at 37 °C and 5% CO_2 in 96-well plates in Dulbecco's modified Eagle's medium (containing 4 g/liter glucose and L-glutamine) supplemented with 10% charcoal-stripped heat-inactivated fetal bovine serum and transfected by calcium phosphate co-precipitation as described previously (23). Following a 16-h treatment with GW4064 and vitamin D, luciferase and β-galactosidase activities were measured as described previously (18). Luciferase activity was normalized for transfection efficiency using β-galactosidase activity and expressed as relative luciferase units. For each experiment, all conditions were tested in triplicate, and all experiments were repeated three times. Data shown are the mean ± S.D. of triplicate assays from one representative experiment.

Statistical Analysis—Data are presented as the mean ± S.E. and were analyzed by two-tailed unpaired Student's *t* test. *p* values <0.05 were considered significant.

RESULTS

Bile Acid Metabolism Is Dysregulated in VDR$^{-/-}$ Mice—Activation of VDR in the intestine by 1α,25-dihydroxyvitamin D_3 or lithocholic acid results in the induction of bile acid-detoxifying genes such as *CYP3A4* (22, 25, 26). To determine the extent of VDR contribution to bile acid metabolism *in vivo*, we measured the bile acid pool size and composition in VDR$^{-/-}$ mice. Surprisingly, we found that VDR$^{-/-}$ mice had an ~30% larger bile acid pool size at 3 months of age and more than twice the amount of bile acids at 6 months of age as their wild-type littermates (Fig. 1*A*). The loss of VDR also increased the hydrophobicity of the bile acid pool due to a greater increase in taurocholic acid relative to tauromuricholic acids (Fig. 1*A*).

To determine the mechanism underlying the perturbation of bile acid homeostasis caused by the loss of VDR, we analyzed the expression of genes in the liver known to play a role in bile acid metabolism. In humans and rodents, bile acids are synthesized from cholesterol in the liver via two pathways (27). CYP7A1 catalyzes the first and rate-limiting step in the classic

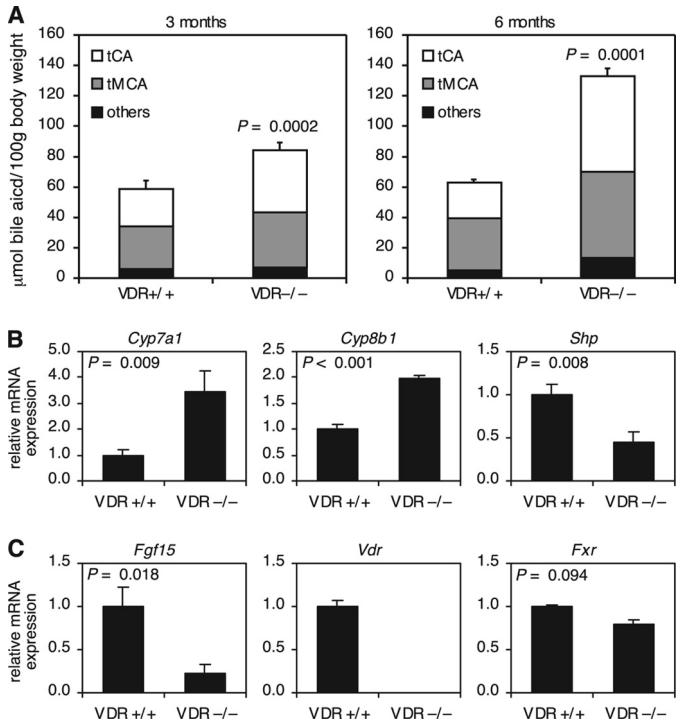


FIGURE 1. **Bile acid metabolism is dysregulated in VDR$^{-/-}$ mice.** *A*, total bile acids were extracted from the gallbladder, liver, intestine, and portal blood and quantitated by liquid chromatography/mass spectrometry. The results shown are bile acid pool size and composition in 3-month-old ($n = 7$) and 6-month-old ($n = 4$) male mice of the indicated genotype. Taurocholate (*tCA*) and tauromuricholates (*tMCA*; includes α-, β-, and ω-muricholate) compose the majority of bile acids in the mouse. See "Experimental Procedures" for a complete list of bile acids grouped as "*others*." Data represent the mean ± S.E. *B* and *C*, total RNA was extracted from the liver (*B*) and ileum (*C*) and analyzed by quantitative RT-PCR. The level of mRNA expression was normalized to *U36b4* and graphed relative to the wild-type control. Data represent the mean ± S.E. of seven animals/group. For all panels, the *p* value was determined by Student's *t* test.

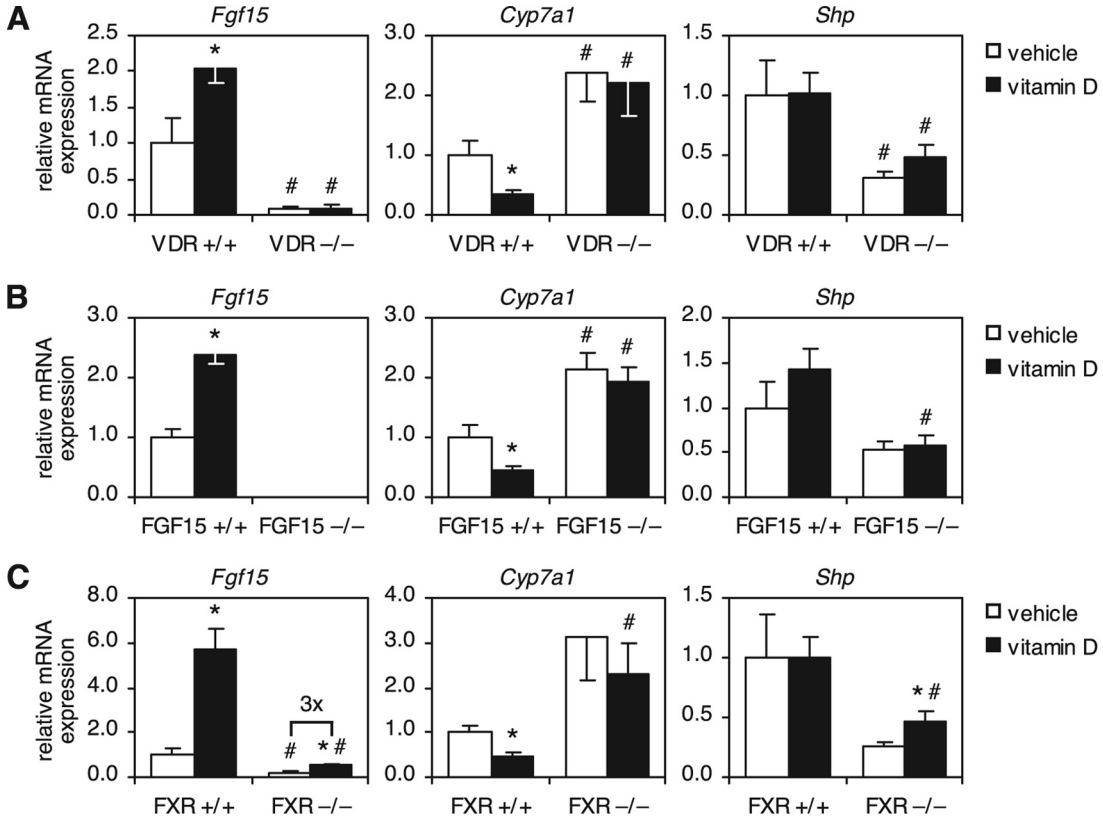
(neutral) pathway of bile acid synthesis, whereas CYP27A1 catalyzes the first step in the alternative (acidic) pathway. Under normal physiological conditions, the majority of bile acids are produced via the classic pathway. Consistent with an increased rate of bile acid synthesis, we found that VDR$^{-/-}$ mice had 3-fold higher expression of *Cyp7a1* (Fig. 1*B*). In addition, the enzyme that catalyzes the final step in the synthesis of cholic acid, *Cyp8b1*, was increased (Fig. 1*B*). These changes were accompanied by a 2-fold decrease in *Shp* mRNA and more modest changes in FXR, hepatocyte nuclear factor 4α, and *Lrh-1* expression (Fig. 1*B* and supplemental Fig. 1*A*). Expression analysis of the major bile acid transporters revealed decreased expression of the canalicular bile acid export pump *Abcb11*, a small increase in the phospholipid exporter *Abcb4*, and no change in the expression of the sinusoidal sodium taurocholate import pump *Slc10a1* (supplemental Fig. 1*B*). In contrast to enzymes of the classic bile acid synthesis pathway, no change was seen in expression of genes encoding enzymes of the alternative bile acid synthesis pathway (supplemental Fig. 1*C*). These alterations in gene expression in the livers of VDR$^{-/-}$ mice were somewhat surprising given that VDR is not expressed in hepatocytes and suggested that the changes either were secondary to alterations in the bile acid pool size and composition or were caused by perturbation of vitamin D signaling outside the hepatocyte.

We next examined gene expression in the terminal small intestine (ileum), which is the major site of bile acid reabsorption and thus plays an integral role in maintaining bile acid homeostasis. Here, bile acid activation of FXR induces expression of bile acid transporters and *Fgf15* (15, 28). As described previously, FGF15 is essential for FXR-mediated feedback repression of *Cyp7a1* and bile acid synthesis. Surprisingly, despite normal levels of FXR and bile acid transporters, the expression of *Fgf15* was decreased markedly in VDR$^{-/-}$ mice (Fig. 1*C* and supplemental Fig. 1*D*).

Vitamin D Induces Fgf15 to Suppress Cyp7a1—Because VDR is expressed in enterocytes and *Fgf15* expression was lower in VDR$^{-/-}$ mice, we hypothesized that VDR might regulate *Fgf15* at the transcriptional level. Indeed, treatment of wild-type but not VDR$^{-/-}$ mice with 1α,25-dihydroxyvitamin D_3 (hereafter referred to as vitamin D) for 4 h increased *Fgf15* expression in the intestine and decreased *Cyp7a1* in the liver (Fig. 2*A*). Intestinal bile acid transporters were also decreased, whereas the gene encoding the ileal bile acid-binding protein (an FXR target gene also known as *Fabp6*) was not changed (supplemental Fig. 2). Importantly, the repression of *Cyp7a1* by vitamin D was absent in FGF15-null (FGF15$^{-/-}$) mice, demonstrating that FGF15 is required for vitamin D-dependent suppression of *Cyp7a1* (Fig. 2*B*).

To determine whether physiological levels of vitamin D contribute to the regulation of *Fgf15*, we compared the induction of *Fgf15* with that of the gene encoding Calbindin-D9k (a known VDR target gene) in ileum explants. Notably, both genes were induced by vitamin D in a comparable dose-dependent manner (Fig. 2*D*). Taken together with the finding that VDR is required for normal expression of *Fgf15*, these data provide evidence that the transcriptional regulation of *Fgf15* by vitamin D is physiologically relevant.

Previous work has shown that FXR directly regulates *Fgf15* expression by binding to an FXR/RXR response element in the second intron of the gene (15). Given the rapid effect of vitamin D treatment on *Fgf15* expression, it seemed likely that the regulation of *Fgf15* by VDR was also direct. ChIP analysis was performed to identify VDR/RXR-binding sites in the regulatory regions of *Fgf15*. As expected, a strong RXR-binding site was detected in the second intron, and an additional binding site was found in the proximal promoter near the transcriptional start site (Fig. 2*E*). VDR binding was also detected at both sites (Fig. 2*E*). Interestingly, analysis of the *Fgf15* regulatory regions by reporter gene assay showed that VDR transactivation occurred exclusively through the proximal promoter site, whereas FXR transactivation occurred exclusively at the second intron (Fig. 2*F*). Promoter truncations localized the vitamin D-responsive *cis*-acting element to a region between 100 and 200 nucleotides upstream of the transcriptional start site (Fig. 2*F*). Analysis of this region revealed a DR3 site with similarity to known VDR response elements, and transactivation of the *Fgf15* promoter by VDR was eliminated by mutating two nucleotides within this site (Fig. 2*F*). These results demonstrate that VDR binds directly to the *Fgf15* promoter and regulates *Fgf15* expression through a direct transcriptional mechanism.


A
Fgf15
Cyp7a1
Shp
relative mRNA expression
vehicle
vitamin D
VDR +/+
VDR –/–
B
FGF15 +/+
FGF15 –/–
C
FXR +/+
FXR –/–
3x
D
CalbindinD9k
% mRNA expression relative to maximum dose
vitamin D, [nM]
E
RXR binding
VDR binding
RXR
IgG
VDR, VDR+/+
VDR, VDR–/–
IgG, VDR+/+
relative binding (% input)
Fgf15 locus
IR1
DR3
+1964 AGTTCAaTGACCT
-148 GGGTCActgGGTTGG
F
RLU (fold-induction)
Tk luciferase
FXR + RXR
VDR + RXR
+3133
+673
-2332
+6
-396
-228
-102
DR3 mutation
vehicle
GW4064
vitamin D

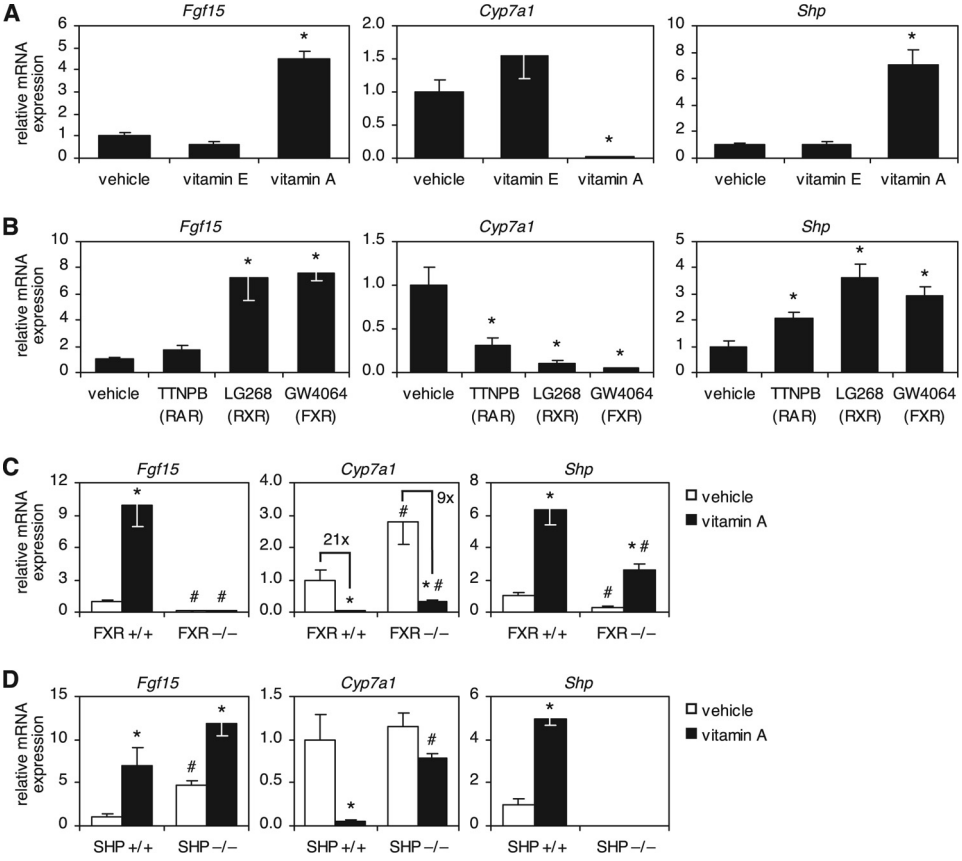


FIGURE 3. **Vitamin A induces *Fgf15* and *Shp* to suppress *Cyp7a1*.** *A*, wild-type mice were treated for 16 h by oral gavage with 500 mg/kg *d*-α-tocopherol (vitamin E) or 100 mg/kg retinyl palmitate (vitamin A). mRNA expression in the ileum (*Fgf15*) and liver (*Cyp7a1* and *Shp*) was determined by quantitative RT-PCR, normalized to *U36b4*, and graphed relative to the vehicle-treated control. *B*, wild-type mice were treated for 1 day with diets containing the indicated synthetic ligands. The target nuclear receptor is shown in *parentheses*. Data analysis was as described for *A*. *C* and *D*, mice of the indicated genotype were treated for 16 h by oral gavage with 100 mg/kg retinyl palmitate (vitamin A). mRNA expression in the ileum (*Fgf15*) and liver (*Cyp7a1* and *Shp*) was determined by quantitative RT-PCR, normalized to *U36b4*, and plotted relative to the wild-type vehicle-treated control. For all panels, data represent the mean ± S.E. of five animals/group. *, $p < 0.05$ compared with the vehicle of the same genotype; #, $p < 0.05$ compared with the wild type of the same treatment group.

Given that both VDR and FXR induce *Fgf15*, we sought to determine whether VDR could substitute for FXR in mediating repression of bile acid synthesis. Although the -fold induction of *Fgf15* by vitamin D was similar in wild-type and $FXR^{-/-}$ mice, the absolute levels of *Fgf15* in vitamin D-treated $FXR^{-/-}$ mice remained below those in vehicle-treated wild-type mice (Fig. 2*C*). Notably, the repression of *Cyp7a1* by vitamin D was absent in $FXR^{-/-}$ mice (Fig. 2*C*). These data indicate that the transcriptional regulation of *Fgf15* by VDR is not dependent on FXR; however, FXR is required for the induction of *Fgf15* to levels that suppress *Cyp7a1*.

Vitamin A Induces Fgf15 and Shp to Suppress Cyp7a1—Given the effects of vitamin D on *Fgf15* and *Cyp7a1* regulation, we sought to determine whether other lipid-soluble vitamins also cause repression of bile acid synthesis. Although *d*-α-tocopherol (*i.e.* vitamin E) had no effect on *Cyp7a1* or any other genes analyzed, retinyl palmitate (*i.e.* vitamin A) dramatically increased *Fgf15* expression in the intestine and suppressed *Cyp7a1* in the liver (Fig. 3*A*). Furthermore, vitamin A increased expression of *Shp* in the liver (Fig. 3*A*).

In vivo, vitamin A may be metabolized to all-*trans*-retinoic acid, which activates RAR, and 9-*cis*-retinoic acid, which activates both RAR and RXR. To determine which nuclear receptor was mediating the transcriptional effects of vitamin A on *Fgf15*, *Cyp7a1*, and *Shp*, we treated wild-type mice with the synthetic ligands TTNPB (selective for RAR) and LG268 (selective for RXR). Both RAR and RXR induced *Shp* and suppressed *Cyp7a1*; however, *Fgf15* was induced only by RXR (Fig. 3*B*). Because the VDR/RXR heterodimer is not activated by RXR ligands (29), we hypothesized that the induction of *Fgf15* by RXR ligand occurred through activation of the RXR/FXR heterodimer. To test this idea, we treated $FXR^{-/-}$ mice with vitamin A and found that, in the absence of FXR, vitamin A had no effect on *Fgf15* expression (Fig. 3*C*). Taken together, these data suggest that *Fgf15* is induced by vitamin A through the RXR partner of the RXR/FXR heterodimer complex.

Interestingly, despite the absence of *Fgf15* induction, vitamin A still efficiently repressed *Cyp7a1* in $FXR^{-/-}$ mice, indicating

FIGURE 2. **VDR induces *Fgf15* to suppress *Cyp7a1*.** *A–C*, mice of the indicated genotype were treated for 4 h by intraperitoneal injection of 75 μg/kg (*A*) or 50 μg/kg (*B* and *C*) 1α,25-dihydroxyvitamin D_3. mRNA expression in the ileum (*Fgf15*) and liver (*Cyp7a1* and *Shp*) was determined by quantitative RT-PCR, normalized to *U36b4*, and graphed relative to the wild-type vehicle-treated control. Data represent the mean ± S.E. of five to six animals/group. *, $p < 0.05$ compared with the vehicle of the same genotype; #, $p < 0.05$ compared with the wild type of the same treatment group. *D*, ileum explants were treated for 6 h with the indicated concentrations of 1α,25-dihydroxyvitamin D_3. Total RNA was extracted and analyzed by quantitative RT-PCR. The level of mRNA expression was normalized to *U36b4* and graphed relative to the highest treatment dose. Data represent the mean ± S.D. of two independent experiments. *E*, pooled ilea from three to four $VDR^{+/+}$ and $VDR^{-/-}$ mice treated for 2 h with 50 μg/kg 1α,25-dihydroxyvitamin D_3 were analyzed by ChIP using specific antibodies for VDR and RXR and an isotype-matched IgG control antibody. Bound DNA was quantitated by quantitative PCR and normalized to the input. Data represent the mean ± S.E. of four independent experiments. *Numbers* indicate nucleotide position relative to the transcriptional start site. The location and sequence of the IR1 FXR/RXR response element and the putative DR3 VDR/RXR response element are shown below the graph. Half-sites are in *boldface*, and nucleotides mutated in the promoter analysis shown in *F* are *underlined*. *F*, the promoter (−2332 to +6; *white boxes*) and intron 2 (+673 to +3133; *gray boxes*) of FGF15 were cloned upstream of the thymidine kinase minimal promoter (*Tk*) and luciferase gene as shown. HEK293 cells were cotransfected with the indicated reporter constructs, β-galactosidase, human RXR, and either human FXR or human VDR. Following treatment with 1 μM GW4064 (FXR agonist), 100 nM 1α,25-dihydroxyvitamin D_3, or vehicle, luciferase activity was quantitated and normalized to β-galactosidase activity. Data are graphed relative to the first data point and represent the mean ± S.D. of three replicates. *X* indicates the mutated DR3 site. *RLU*, relative luciferase units.

that additional vitamin A-dependent mechanisms exist to suppress *Cyp7a1* (Fig. 3*C*). Further analysis of hepatic gene expression demonstrated that, despite lower basal levels of *Shp* in $FXR^{-/-}$ mice, FXR was not required for induction of *Shp* by vitamin A (Fig. 3*C*), suggesting that *Shp* induction contributed to *Cyp7a1* repression by vitamin A. In support of this idea, we found that repression of *Cyp7a1* by vitamin A was greatly reduced in the absence of SHP (Fig. 3*D*).

Vitamin A Rescues Fgf15 and Shp Expression and Suppresses Cyp7a1 under Conditions of Impaired Bile Acid Feedback Repression—Our finding that vitamin A could repress *Cyp7a1* expression by inducing bile acid feedback regulatory genes in both the liver and intestine suggested that vitamin A analogs might be useful therapeutically in pathological conditions in which feedback repression of bile acid synthesis is interrupted. One such condition is bile acid malabsorption, which is characterized by decreased intestinal bile acid reabsorption, resulting in low FGF19 levels (human ortholog of FGF15) and excessive bile acid synthesis by the liver (30). The effects of bile acid malabsorption on expression of bile acid feedback regulatory genes can be mimicked by administering the bile acid-binding resin, cholestyramine. As expected, cholestyramine treatment of wild-type mice dramatically decreased *Fgf15* and *Shp* expression and increased *Cyp7a1* expression (Fig. 4). Amazingly, vitamin A completely rescued *Fgf15* and *Shp* expression and reversed the derepression of *Cyp7a1* caused by interrupted bile acid reabsorption in cholestyramine-treated animals (Fig. 4). Consistent with our previous results, in the absence of bile acids, vitamin D modestly induced *Fgf15* but did not change *Shp* or *Cyp7a1* expression (Fig. 4). These data highlight the potential of vitamin A analogs to correct disrupted feedback repression of bile acid biosynthesis.

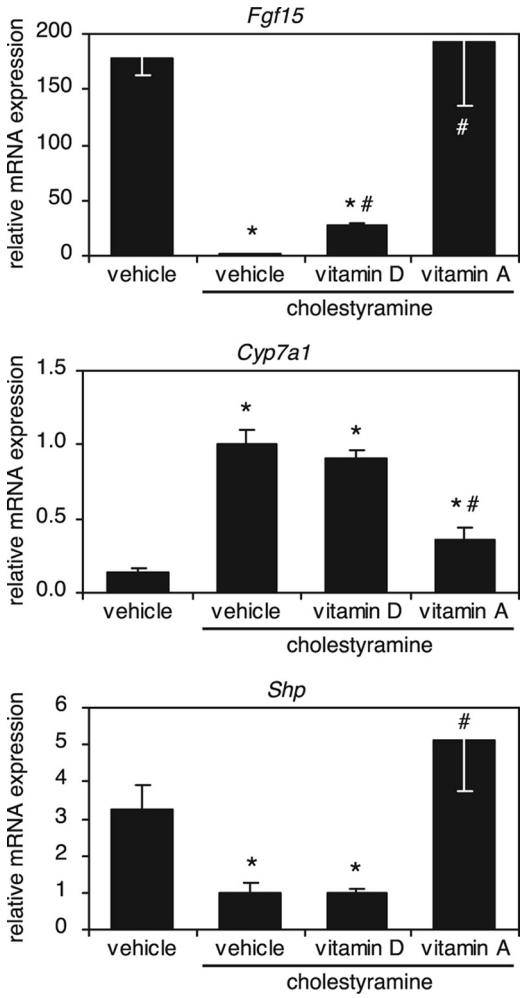


FIGURE 4. **Vitamin A rescues *Fgf15* and *Shp* expression and suppresses *Cyp7a1* under conditions of impaired bile acid feedback repression.** Wild-type mice were treated for 2 days with a 2% (w/w) cholestyramine diet and either 50 μg/kg 1α,25-dihydroxyvitamin D_3 (vitamin D) or 100 mg/kg retinyl palmitate (vitamin A). Vitamin D and A treatments were administered as described in the legends to Figs. 2 and 3, respectively. The control group received standard chow and the appropriate vehicle treatments. mRNA expression in the ileum (*Fgf15*) and liver (*Cyp7a1* and *Shp*) was determined by quantitative RT-PCR, normalized to *U36b4*, and plotted relative to vehicle-treated control. Data represent the mean ± S.E. of three animals/group. *, $p < 0.05$ compared with the control; #, $p < 0.05$ for vitamin treatment compared with cholestyramine alone.

DISCUSSION

Because of their unique physicochemical properties, bile acids are essential structural components of lipid micelles (11). In this capacity, bile acids promote the intestinal absorption of lipids and lipid-soluble vitamins. Here, we have revealed an unexpected link between lipid-soluble vitamins and bile acid biosynthesis. Surprisingly, vitamins A and D exert negative feedback on bile acid synthesis *in vivo* by decreasing *Cyp7a1* expression. FGF15 and SHP play a central role in the feedback regulation of bile acid synthesis by bile acids and FXR (31). Our study shows that FGF15 is integral to the mechanism of *Cyp7a1* regulation by vitamin D and that both FGF15 and SHP are important for the regulation of bile acid synthesis by vitamin A.

We found that VDR transcriptionally regulated *Fgf15* in the intestine and that this pathway was essential for the repression of bile acid synthesis by vitamin D. Surprisingly, VDR was required to maintain normal *Fgf15* expression and bile acids levels *in vivo*. Together, these results demonstrate that vitamin D and its receptor contribute to the feedback regulation of bile acid synthesis by controlling expression of the endocrine hormone FGF15. We note that VDR expression has been reported in non-parenchymal cells in the liver (32), leaving open the possibility that paracrine signals might also contribute to VDR-dependent regulation of bile acid synthesis.

Interestingly, *Cyp7a1* repression by vitamin D required an intact FXR signaling pathway, indicating that VDR activation alone is not sufficient to suppress bile acid synthesis. There are at least two possible explanations for this finding. First, activation of both VDR and FXR may be required to induce *Fgf15* to the level required for *Cyp7a1* suppression. Second, as has recently been shown for FXR (19), feedback repression of bile acid synthesis may require corresponding signals in the intestine and liver, the latter of which does not occur upon activation of VDR alone.

Vitamin A repressed *Cyp7a1* through both FXR-dependent and FXR-independent mechanisms. Ligands for RXR, but not RAR, induced *Fgf15*, and FXR was required for the induction of *Fgf15* by vitamin A. These results indicate that induction of *Fgf15* expression by vitamin A occurs through RXR as the obligate heterodimeric partner of the RXR/FXR heterodimer com-

plex. This finding provides evidence that RXR functions as a vitamin A receptor *in vivo* and demonstrates that the RXR/FXR heterodimer can serve as a sensor for dietary vitamin A. In contrast to its effects on FGF15 expression, vitamin A-dependent induction of *Shp* expression did not require FXR. Interestingly, we found that the RAR ligand TTNPB induced *Shp* and suppressed *Cyp7a1*, suggesting that *Shp* induction by RAR may be an FXR-independent mechanism whereby vitamin A suppresses *Cyp7a1*. Taken together, these results point to two distinct nuclear receptor-mediated mechanisms by which vitamin A regulates bile acid synthesis.

Under normal physiological conditions, bile acids are efficiently reabsorbed in the ileum (11). Bile acid malabsorption is a pathological condition often seen in patients with Crohn disease or ileal resection and is characterized by reduced ileal bile acid reabsorption and delivery of large quantities of bile acids to the colon (30). Increased luminal concentrations of bile acids in the colon induce fluid secretion, resulting in cholerheic enteropathy and the characteristic symptom of watery diarrhea. Bile acid sequestrants are currently the primary therapy and provide symptomatic relief but do not correct bile acid overproduction and hypersecretion by the liver. The exciting finding that vitamin A induces *Fgf15* and suppresses bile acid synthesis under conditions of interrupted bile acid reabsorption suggests that vitamin A analogs may provide therapeutic benefit to patients with bile acid malabsorption and increased hepatic bile acid synthesis. Interestingly, a subset of patients with bile acid malabsorption have increased hepatic bile acid synthesis despite normal ileal bile acid transport. This condition, termed idiopathic bile acid malabsorption, has recently been shown to be associated with decreased plasma levels of FGF19 (33). It is not known why FGF19 levels are abnormally low in these patients. Our data suggest that vitamins A and D may be useful tools to examine the underlying cause of low FGF19 levels in these patients.

Elevated levels of bile acid in the colon may promote colon cancer, whereas vitamin D is associated with reduced risk of colon cancer (34–37). We showed previously that VDR is activated by lithocholic acid and induces enzymes that detoxify bile acids in the colon (22). The present study suggests that vitamin D-dependent regulation of bile acid synthesis may be an additional mechanism by which vitamin D protects against the tumor-promoting effects of toxic bile acids.

With regard to the mechanism underlying the hormonal effects of vitamin D, an interesting parallel emerges between the regulation of bile acid synthesis by FGF15 and the role of FGF23 in renal phosphate metabolism. Previous studies have shown that vitamin D induces *Fgf23* in bone and that FGF23 signals in a bone-kidney axis to control phosphate absorption and vitamin D metabolism in the kidney (38). In this study, we have shown that vitamin D induces *Fgf15* in the intestine, which signals in an intestine-liver axis to regulate bile acid synthesis in the liver. Thus, a paradigm emerges in which endocrine fibroblast growth factors function as downstream messengers to mediate the homeostatic effects of vitamin D and coordinate vitamin D signaling between organ systems.

In summary, our findings highlight the importance of nuclear receptors in the regulation of bile acid metabolism and provide mechanistic insight into the elegant signaling pathways involving FGF15 and SHP that govern feedback repression of bile acid biosynthesis. We speculate that the mechanisms allowing vitamins A and D to control feedback repression of bile acid synthesis may have evolved to protect the organism from exposure to potentially toxic levels of lipid-soluble vitamins in the diet.

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REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) *Cell* **83,** 835–839
2. Haussler, M. R., Haussler, C. A., Jurutka, P. W., Thompson, P. D., Hsieh, J. C., Remus, L. S., Selznick, S. H., and Whitfield, G. K. (1997) *J. Endocrinol.* **154,** S57–S73
3. Mark, M., Ghyselinck, N. B., and Chambon, P. (2009) *Nucl. Recept. Signal.* **7,** e002
4. Ross, A. C. (1993) *J. Nutr.* **123,** 346–350
5. Zile, M. H. (1998) *J. Nutr.* **128,** 455S–458S
6. Prosser, D. E., and Jones, G. (2004) *Trends Biochem. Sci.* **29,** 664–673
7. DeLuca, H. F. (2004) *Am. J. Clin. Nutr.* **80,** 1689S–1696S
8. Liu, P. T., Stenger, S., Li, H., Wenzel, L., Tan, B. H., Krutzik, S. R., Ochoa, M. T., Schauber, J., Wu, K., Meinken, C., Kamen, D. L., Wagner, M., Bals, R., Steinmeyer, A., Zügel, U., Gallo, R. L., Eisenberg, D., Hewison, M., Hollis, B. W., Adams, J. S., Bloom, B. R., and Modlin, R. L. (2006) *Science* **311,** 1770–1773
9. Mangelsdorf, D. J., and Motola, D. L. (2005) in *Vitamin D* (Feldman, D., ed) p. 863, Elsevier Academic Press, London
10. Moro, J. R., Iwata, M., and von Andriano, U. H. (2008) *Nat. Rev. Immunol.* **8,** 685–698
11. Hofmann, A. F. (1999) *News Physiol. Sci.* **14,** 24–29
12. Gilardi, F., Mitro, N., Godio, C., Scotti, E., Caruso, D., Crestani, M., and De Fabiani, E. (2007) *Pharmacol. Ther.* **116,** 449–472
13. Crestani, M., Sadeghpour, A., Stroup, D., Galli, G., and Chiang, J. Y. (1998) *J. Lipid Res.* **39,** 2192–2200
14. Nitta, M., Ku, S., Brown, C., Okamoto, A. Y., and Shan, B. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 6660–6665
15. Inagaki, T., Choi, M., Moschetta, A., Peng, L., Cummins, C. L., McDonald, J. G., Luo, G., Jones, S. A., Goodwin, B., Richardson, J. A., Gerard, R. D., Repa, J. J., Mangelsdorf, D. J., and Kliewer, S. A. (2005) *Cell Metab.* **2,** 217–225
16. Goodwin, B., Jones, S. A., Price, R. R., Watson, M. A., McKee, D. D., Moore, L. B., Galardi, C., Wilson, J. G., Lewis, M. C., Roth, M. E., Maloney, P. R., Willson, T. M., and Kliewer, S. A. (2000) *Mol. Cell* **6,** 517–526
17. Lee, Y. K., Dell, H., Dowhan, D. H., Hadzopoulou-Cladaras, M., and Moore, D. D. (2000) *Mol. Cell. Biol.* **20,** 187–195
18. Lu, T. T., Makishima, M., Repa, J. J., Schoonjans, K., Kerr, T. A., Auwerx, J., and Mangelsdorf, D. J. (2000) *Mol. Cell* **6,** 507–515
19. Kim, I., Ahn, S. H., Inagaki, T., Choi, M., Ito, S., Guo, G. L., Kliewer, S. A., and Gonzalez, F. J. (2007) *J. Lipid Res.* **48,** 2664–2672
20. Lee, Y. K., Schmidt, D. R., Cummins, C. L., Choi, M., Peng, L., Zhang, Y., Goodwin, B., Hammer, R. E., Mangelsdorf, D. J., and Kliewer, S. A. (2008) *Mol. Endocrinol.* **22,** 1345–1356
21. Bookout, A. L., and Mangelsdorf, D. J. (2003) *Nucl. Recept. Signal.* **1,** e012
22. Makishima, M., Lu, T. T., Xie, W., Whitfield, G. K., Domoto, H., Evans, R. M., Haussler, M. R., and Mangelsdorf, D. J. (2002) *Science* **296,** 1313–1316

23. Makishima, M., Okamoto, A. Y., Repa, J. J., Tu, H., Learned, R. M., Luk, A., Hull, M. V., Lustig, K. D., Mangelsdorf, D. J., and Shan, B. (1999) *Science* **284,** 1362–1365
24. Willy, P. J., Umesono, K., Ong, E. S., Evans, R. M., Heyman, R. A., and Mangelsdorf, D. J. (1995) *Genes Dev.* **9,** 1033–1045
25. Jurutka, P. W., Thompson, P. D., Whitfield, G. K., Eichhorst, K. R., Hall, N., Dominguez, C. E., Hsieh, J. C., Haussler, C. A., and Haussler, M. R. (2005) *J. Cell. Biochem.* **94,** 917–943
26. Nehring, J. A., Zierold, C., and DeLuca, H. F. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 10006–10009
27. Russell, D. W. (2003) *Annu. Rev. Biochem.* **72,** 137–174
28. Dawson, P. A., Lan, T., and Rao, A. (2009) *J. Lipid Res.* **50,** 2340–2357
29. Shulman, A. I., Larson, C., Mangelsdorf, D. J., and Ranganathan, R. (2004) *Cell* **116,** 417–429
30. Hofmann, A. F., Mangelsdorf, D. J., and Kliewer, S. A. (2009) *Clin. Gastroenterol. Hepatol.* **7,** 1151–1154
31. Chiang, J. Y. (2009) *J. Lipid Res.* **50,** 1955–1966
32. Gascon-Barré, M., Demers, C., Mirshahi, A., Néron, S., Zalzal, S., and Nanci, A. (2003) *Hepatology* **37,** 1034–1042
33. Walters, J. R., Tasleem, A. M., Omer, O. S., Brydon, W. G., Dew, T., and le Roux, C. W. (2009) *Clin. Gastroenterol. Hepatol.* **7,** 1189–1194
34. Garland, C. F., Gorham, E. D., Mohr, S. B., and Garland, F. C. (2009) *Ann. Epidemiol.* **19,** 468–483
35. Lamprecht, S. A., and Lipkin, M. (2003) *Nat. Rev. Cancer* **3,** 601-614
36. Nagengast, F. M., Grubben, M. J., and van Munster, I. P. (1995) *Eur. J. Cancer* **31A,** 1067–1070
37. Yin, L., Grandi, N., Raum, E., Haug, U., Arndt, V., and Brenner, H. (2009) *Aliment. Pharmacol. Ther.* **30,** 113–125
38. Liu, S., and Quarles, L. D. (2007) *J. Am. Soc. Nephrol.* **18,** 1637–1647