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Identification of oxysterol 7 α -hydroxylase (*Cyp7b1*) as a novel ROR α (NR1F1) target gene and a functional crosstalk between ROR α and LXR (NR1H3)

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Running Title: ROR α regulates *Cyp7b1*

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Abbreviations: ChIP, chromatin immunoprecipitation; *Cyp7b1*, oxysterol 7 α -hydroxylase;

EMSA, electrophoretic mobility shift assay; *Est*, estrogen sulfotransferase; LXR, liver X

receptor; LXRE, LXR response element; ROR, retinoid-related orphan receptor; RORE, ROR

response element; *Sult*, sulfotransferase; VP, viral protein 16

Abstract

The retinoid-related orphan receptors (RORs) and liver X receptors (LXRs) were postulated to have distinct functions. RORs play a role in tissue development and circadian rhythm, whereas LXRs are sterol sensors that impact lipid homeostasis. In this study, we revealed a novel function of ROR α (NR1F1) in regulating the oxysterol 7 α -hydroxylase (Cyp7b1), an enzyme critical for the homeostasis of cholesterol, bile acids and oxysterols. The expression of *Cyp7b1* gene was suppressed in the ROR α null (*ROR α ^{sg/sg}*) mice, suggesting ROR α as a positive regulator of *Cyp7b1*. Promoter analysis established *Cyp7b1* as a transcriptional target of ROR α and transfection of ROR α induced the expression of endogenous *Cyp7b1* in the liver. Interestingly, *Cyp7b1* regulation appeared to be ROR α -specific, as ROR γ had little effect. Reporter gene analysis showed that the activation of *Cyp7b1* gene promoter by ROR α was suppressed by LXR α (NR1H3), whereas ROR α inhibited both the constitutive and ligand-dependent activities of LXR α . The mutual suppression between ROR α and LXR was supported by the *in vivo* observation that loss of ROR α increased the expression of selected LXR target genes, leading to hepatic triglyceride accumulation. Reciprocally, mice deficient of LXR α and β isoforms showed activation of selected ROR α target genes. Our results have revealed a novel role for ROR α and a functional interplay between ROR α and LXR in regulating endo- and xenobiotic genes, which may have broad implications in metabolic homeostasis.

Introduction

Retinoid-related orphan receptors (RORs, or NR1F1-3), including the α , β and γ isoforms, were isolated based on their homology to the retinoid receptors (Jetten et al., 2001; Jetten and Joo, 2006). Each of the ROR isoforms has distinct tissue distribution patterns (Carlberg et al., 1994). ROR α is widely distributed, with its expression detectable in the cerebellar Purkinje cells, liver, thymus, skeletal muscle, skin, lung and kidney (Hamilton et al., 1996; Steinmayr et al., 1998). In contrast, ROR β has a more tissue-specific distribution, expressing in the brain, retina and pineal gland (Jetten et al., 2001; Andre et al., 1998). ROR γ is highly enriched in the thymus but its expression is also detectable in the kidney, liver and muscle (Jetten et al., 2001; Medvedev et al., 1996). The functional ligands of RORs remain elusive. It has been suggested that cholesterol and its sulfonated derivatives might function as ROR α ligands (Kallen et al., 2002). However, to our knowledge, none of those have been convincingly demonstrated to be physiological ROR agonists. RORs regulate gene expression by binding as monomers to the ROR response elements (ROREs) found in target gene promoters. A typical RORE is composed of a consensus AGGTCA half site preceded by an A/T-rich region (Giguere et al., 1994). ROR α has also been shown to bind DNA as homodimers (Harding et al., 1997).

Subsequent functional analyses, mainly through the creation and characterization of ROR deficient mice, have revealed diverse physiological function of RORs. ROR $\alpha^{-/-}$ null

mice had cerebellar ataxia, a behavioral phenotype also observed in the *Staggerer* (*sg/sg*) mutant mice, which contained a natural deletion in the ligand-binding domain of the ROR α gene due to a frame shift (Hamilton et al., 1996; Steinmayr et al., 1998). The *sg/sg* mice exhibited vascular dysfunction, muscular irregularities, osteoporosis, and immuno abnormalities (Jarvis et al., 2002). The *sg/sg* mice developed severe atherosclerosis and hypo- α -lipoproteinemia when maintained on an atherogenic diet (Mamontova et al., 1998). ROR β is believed to be involved in the processing of sensory information, as ROR $\beta^{-/-}$ mice showed significant phenotypes in circadian behaviors and retinal degeneration (Andre et al., 1998). ROR $\gamma^{-/-}$ mice lacked all lymph nodes and Peyer's patches, and contained reduced number of thymocytes (Kurebayashi et al., 2000), suggesting that ROR γ plays an essential role in lymphoid organogenesis and thymopoiesis. Although both ROR α and γ are expressed in the liver, their hepatic function is largely unknown.

Both liver X receptor (LXR) α and β are nuclear receptors that can be activated by the endogenous oxysterols, such as 22(*R*)-hydroxycholesterol; as well as synthetic agonists, such as T0901317 (TO1317) (Schultz et al., 2000) and GW3965 (Collins et al., 2002). LXRs exhibit diverse functions, ranging from cholesterol efflux to lipogenesis and anti-inflammation (Repa et al., 2002; Zelcer et al., 2006). LXRs have also been explored as therapeutic targets for atherosclerosis (Tontonoz et al., 2003), diabetes and Alzheimer's disease (Zelcer et al., 2007) in animal models. We have recently identified several novel LXR target genes. These

include the bile acid detoxifying sulfotransferase *Sult2a9/2a1* (Uppal et al., 2007), estrogen sulfotransferase (*Est/Sult1e1*) (Gong et al., 2007) and fatty acid transporter *Cd36* (J. Zhou and W. Xie, unpublished results). We showed that activation of *Sult2a9/2a1* by LXR was associated with increased bile acid detoxification and alleviation of cholestasis (Uppal et al., 2007). In the same study, the expression of *Cyp7b1* was found to be suppressed in LXR-activated mice but the mechanism for this suppression is unknown (Uppal et al., 2007). Activation of *Est/Sult1e1* by LXR led to functional estrogen deprivation and inhibition of estrogen-dependent breast cancer growth (Gong et al., 2007). More recently, we showed that *Cd36* is a LXR target gene and an intact expression of *Cd36* plays an important role in the steatotic effect of LXR agonists (J. Zhou and W. Xie, unpublished results).

In this report, we show that ROR α positively and directly regulates the expression of *Cyp7b1*. In addition, we have provided evidence for a functional crosstalk between ROR α and LXR in regulating *Cyp7b1* and other target genes controlled by these two receptors.

Materials and Methods

Animals. Heterozygous C57BL/6 *staggerer* (*RORa*^{+/*sg*}) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The *staggerer* (*RORa*^{*sg*/*sg*}) mice, a natural mutant mouse strain, contain a 6.5 kb deletion in the *RORa* gene resulting in a functional knockout of *RORa*. *RORa*^{*sg*/*sg*} mice of 8-10 weeks of age were used. All animal protocols followed the guidelines outlined by the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the NIEHS.

Plasmid Constructs and Cell Transfection. The *tk-Pcp2*/RORE-Luc and *tk-Cyp7b1*/RORE and its mutant variant were generated by insertion of corresponding annealed oligonucleotides into the tk-Luc vector. Three copies of the following response elements were used:

Pcp2/RORE 5'-GTTATAGTAACTGGGTCAGGGGACT-3', *Cyp7b1*/RORE WT

5'-TATTTTATGCAGGTCAGTGG-3', *Cyp7b1*/RORE Mutant

5'-TATTTTATGCACCTCAGTGG-3'. The 5' regulatory region (-3500 bp to +125 bp) of

mouse *Cyp7b1* was amplified by PCR using mouse liver genomic DNA as the PCR template

and the following oligonucleotides: *Cyp7b1* -3500 5'-TTTGTGAACTTGGCATGACAT-3'

and *Cyp7b1* +125 5'-TCCCGACGAGCTGGCGGCTC-3'. The PCR-amplified sequence

was cloned into the pGL3-basic vector (Promega). Site-directed mutagenesis was performed

by PCR overextension method and confirmed by DNA sequencing (Xie et al., 2000). HepG2

cells were transfected in 48-well plates using the polyethyleneimine (PEI) polymer transfection agent (Mu et al., 2005; Mu et al., 2006). For each three-well transfection, the pEI polymer complexes were formed by incubating 0.4 μ g of nuclear receptor expression vector or the CMX empty vector, 0.8 μ g of reporter gene, 0.3 μ g of CMX β -gal plasmid and 10 μ l of PEI at room temperature for 10 min in a total volume of 300 μ l of serum-free minimum Eagle's medium (MEM). The complexes were then diluted with additional 300 μ l of serum-free MEM, mixed, and applied at 200 μ l per well. After 12 h of incubation, the transfection medium was replaced with MEM supplemented with 10% fetal bovine serum and laced with DMSO solvent or drugs. The concentration for all drugs used in transfections is 10 μ M. Cells were lysed 24 h later and assayed for luciferase and β -galactosidase activities. The transfection efficiency was normalized against the β -gal activities. All transfections were performed in triplicate.

Human and Mouse Primary Hepatocyte Preparation and Transfection. Human livers were obtained through the Liver Tissue Procurement and Distribution System (LTPDS), and hepatocytes were isolated by three-step collagenase perfusion (Strom et al., 1999). Mouse primary hepatocytes and stellate cells were isolated from 8-week old female C57BL/6J mice by collagenase perfusion and differential centrifugation (Mu et al., 2005; Monga et al., 2005). Cells were plated on 6-well plates and maintained in Hepatocyte Maintenance Medium (HMM) from Cambrex Bio Science (Walkersville, MA) supplemented with dexamethasone

(10^{-7} M), insulin (10^{-7} M) and gentamycin (50 μ g/ml). Mouse primary hepatocytes on each well were transfected with 4 μ g of plasmid DNA using Lipofectamine 2000 (Invitrogen).

After 24 h of incubation, cells were replaced with fresh HMM for 16 h before RNA harvesting and real-time PCR analysis.

Real-Time RT-PCR Analysis. Total RNA was extracted with TRIZOL reagent (Invitrogen). The cDNA was synthesized from 1.6 μ g of total RNA by Superscript3 (Invitrogen), according to the manufacturer's protocol. Aliquots of cDNA were amplified on ABI 7300 Real-Time PCR System using the SYBR-Green PCR master mix (Applied Biosystem). The mRNA expression was normalized against the cyclophilin B expression (Zhou et al., 2006).

Electrophoretic Mobility Shift Assay (EMSA). Receptor proteins were prepared using the TNT *in vitro* transcription and translation system (Promega). The binding reactions were performed as previously described (Saini et al., 2004; Saini et al., 2005). Protein-DNA complexes were resolved by electrophoresis through 5% polyacrylamide gel in 0.5 x TBE at 4 °C for 1-3 h. For oligonucleotide competition experiments, unlabeled oligonucleotides were added to the reaction at 100-fold molar excess to the radio-labeled probes. EMSA probe sequences are labeled in the figures.

Hydrodynamic Liver Transfection. Six-week old CD-1 female mice purchased from Charles River (Wilmington, MA) were each injected with 5 μ g of plasmid DNA in 1.6 ml saline via tail vein (Zhou et al., 2006). Mice were sacrificed 6 h after the injection and their livers were harvested. Total RNA was extracted and subjected to real-time PCR analysis.

Chromatin Immunoprecipitation (ChIP) Assay. Three-week old C57BL/6J male mice were sacrificed and 30 mg of liver tissues from each mouse were subjected to ChIP assay as described previously (Zhou et al., 2006). Tissue lysates were incubated overnight with 1 μ g of anti-ROR α antibody (Cat # sc-6062) from Santa Cruz Biotechnology (Santa Cruz, CA) at 4 $^{\circ}$ C. Parallel samples were incubated with normal IgG as a negative control. The following PCR primers were used: *Cyp7b1* -1026, 5'-AACCTTAGGAAGGAGCCCATGAA-3'; *Cyp7b1* -902, TGATGAATACTCCATGTGTCAATGAGA-3'; *Cyp7b1* -2900, 5'-GTTTCAAATAATACATTCAGATCTT-3'; *Cyp7b1* -2776, 5'-AACAGGTAAAAGACTGATGGACAGGC-3'. ChIP assay using the SRC1 antibody was performed on primary mouse hepatocytes. In this experiment, cells were treated with DMSO or TO1317 (10 μ M) for 24 h before formaldehyde crosslinking. Cross-linked DNA was extracted from cells and ChIP assay was performed using an anti-SRC1 antibody (Cat # sc-8995) from Santa Cruz. The final DNA extracts were amplified by PCR using primer pairs encompassing the *Cyp7b1*/RORE, a distal control *Cyp7b1* promoter region, or the *Est*/LXRE

that we have previously described (Gong et al., 2007).

Measurement of Circulating and Tissue Lipid Levels. To measure circulating lipid levels, mice were fasted for 16 h prior to sacrificing and blood collection. The plasma levels of triglycerides and cholesterol were measured by using assay kits from Stanbio Laboratory (Boerne, TX). To measure liver lipids, tissues were homogenized and lipids were extracted as previously described (Zhou et al., 2006). The lipid pellets were then dissolved in a mixture of 60 μ l of tert-butanol and 40 μ l of Triton X-100 : methanol (2:1) mix. Triglyceride and cholesterol levels were then measured using the Stanbio assay kits.

Results

Mice deficient of $ROR\alpha$ had decreased expression of *Cyp7b1* in the liver

$ROR\alpha$ is expressed in the liver but its hepatic function is largely unknown. To understand the function of $ROR\alpha$ in the liver, we compared the hepatic gene expression between the wild type (WT) and $ROR\alpha^{sg/sg}$ males by microarray analysis using the Agilent mouse 20,000-oligo chips (Agilent Technologies). The $ROR\alpha^{sg/sg}$ mice contain a 6.5 kb deletion in the *RORa* gene, resulting in a functional knockout of the *RORa* gene (Hamilton et al., 1996; Steinmayr et al., 1998). We initially observed a 4.2 fold decrease in the expression of *Cyp7b1* in the male $ROR\alpha^{sg/sg}$ mice (Kang et al., 2007). The microarray results were confirmed and extended by real-time RT-PCR analysis. As shown in Fig. 1A, the hepatic expression of *Cyp7b1* was significantly decreased in both male and female $ROR\alpha^{sg/sg}$ mice. WT female mice had lower basal expression of *Cyp7b1*, consistent with previous reports (Uppal et al., 2007; Li-Hawkins et al., 2000). The down-regulation of *Cyp7b1* in $ROR\alpha^{sg/sg}$ males was also confirmed by Northern blot analysis (Fig. 1B).

To gain an insight into the hepatic function of $ROR\alpha$ and its potential regulation of *Cyp7b1*, we evaluated the expression of $ROR\alpha$ in isolated liver cell types, including the parenchymal hepatocytes and the mesenchymal/nonparenchymal stellate cells. As shown in Fig. 1C, $ROR\alpha$ is expressed in the stellate cells at a reduced (approximately 50% of the hepatocytes) but significant level. The expression of *Cyp7b1* and *LXR α* was substantially

lower (less than 10% of the hepatocytes) in the stellate cells. The identity of the stellate cells was confirmed by the near absence of *HNF4 α* and *Cyp3a11*, two hepatic differentiation markers; as well as an enriched expression of *Desmin* and *Gfap* (glial fibrillary acidic protein), two known stellate cell markers (Geerts et al., 2001; Morini et al., 2005).

***Cyp7b1* is a transcriptional target of ROR α**

The down-regulation of *Cyp7b1* in *ROR α ^{sg/sg}* mice suggested that this Cyp isoform might be under the positive control of ROR α . To determine whether *Cyp7b1* is a transcriptional target of ROR α , we cloned and analyzed the 3.5-kb 5' flanking region of the mouse *Cyp7b1* gene. As shown in Fig. 2A, this 3.5-kb *Cyp7b1* promoter was responsive to ROR α in transient transfection and reporter gene assay. The regulation appeared to be ROR isoform-specific, since co-transfection of ROR γ had little effect on the same promoter (Fig. 2A). Deletion analysis localized the ROR α responsive region to -1017 bp to -520 bp (Fig. 2A). Inspection of this region revealed a putative RORE characterized by an AGGTCA half site flanked by adjacent A/T-rich 6 nucleotides (Fig. 2B). Electrophoretic mobility shift assay (EMSA) was performed to determine the binding of RORs to *Cyp7b1*/RORE using synthesized receptor proteins and [³²P]-labeled oligonucleotide probe. ROR α bound to *Cyp7b1*/RORE efficiently (Fig. 2B), but not to the radiolabeled mutant *Cyp7b1*/RORE (data not shown). This binding was specific, as strong competition of binding was achieved by excess unlabeled wild

type *Cyp7b1*/RORE and *ApoA-V*/RORE, but not by the mutant *Cyp7b1*/RORE (Fig. 2B). *ApoA-V*/RORE is a prototypic RORE derived from the *ApoA-V* gene promoter (Lind et al., 2005). Interestingly, ROR γ could also bind to *Cyp7b1*/RORE, but the binding was substantially weaker compared to that of ROR α (Fig. 2B), consistent with the lack of *Cyp7b1* promoter activation by ROR γ (Fig. 2A). The binding of radio-labeled *ApoA-V*/RORE by ROR α and ROR γ was included as positive controls (Fig. 2B). EMSA analysis showed *Cyp7b1*/RORE was not bound by LXR α (data not shown).

Chromatin immunoprecipitation (ChIP) assay was used to determine whether the endogenous ROR α can be recruited onto the *Cyp7b1* promoter *in vivo*. As shown in Fig. 2C, immunoprecipitation on liver DNA lysate with an anti-ROR α antibody revealed the specific recruitment of ROR α to a 100-bp sequence encompassing the *Cyp7b1*/RORE. In contrast, no amplification was observed when the same precipitate was amplified using a pair of control primers designed for a distal region approximately 2-kb upstream of *Cyp7b1*/RORE (Fig. 2C).

Transfection-based assays were utilized to determine whether ROR α can transactivate through *Cyp7b1*/RORE. Synthetic luciferase reporter genes, containing three copies of the wild type or mutant *Cyp7b1*/RORE upstream of a minimal thymidine kinase (tk) promoter (*tk-Cyp7b1*/RORE), were constructed and transfected into HepG2 cells together with the expression vector for ROR α . As shown in Fig. 2D, co-transfection with ROR α activated the *tk-Cyp7b1*/RORE reporter gene and this activation was abolished when the *Cyp7b1*/RORE was

mutated. This RORE is also required for the activation of the natural 1-kb *Cyp7b1* promoter, as mutation of the RORE in this context also abolished the transactivation by ROR α (Fig. 2A).

Finally, we showed that overexpression of ROR α in primary hepatocyte cultures by transient transfection (Fig. 2E) or in wild type mouse livers by a hydrodynamic liver transfection method (Fig. 2F) induced the mRNA expression of the endogenous *Cyp7b1*, but not the control *Cyp3a11* gene. The overexpression of ROR α in transfected hepatocytes or livers was confirmed by real-time RT-PCR analysis (Fig. 2G). Taken together, our results strongly suggest that the mouse *Cyp7b1* gene is a transcriptional target of ROR α and this regulation is mediated by *Cyp7b1*/RORE.

The activation of *Cyp7b1* promoter by ROR α was negatively regulated by LXR α , and ROR α and LXR α were mutually suppressive in reporter gene assays

We have previously shown that the expression of *Cyp7b1* was suppressed in LXR-activated mice (Uppal et al., 2007). *CYP7B1* suppression was also seen in primary human hepatocytes treated with LXR agonists (Fig. 3A). However, the mechanism for LXR-mediated *Cyp7b1* suppression is unknown. Having established ROR α as a positive *Cyp7b1* regulator, we went on to examine whether LXR suppresses *Cyp7b1* by inhibiting ROR α activity. As shown in Fig. 3B, activation of the 1-kb *Cyp7b1* promoter (*pGL-Cyp7b1*) by ROR α was suppressed by co-transfection of LXR α , even in the absence of LXR agonists.

The inhibitory effect of LXR α was enhanced by the LXR agonist TO1317. Transfection of LXR α alone, even in the absence of LXR agonists, inhibited *Cyp7b1* promoter activity. The inhibitory effect of LXR α , in the presence or absence of TO1317, was largely abolished when the RORE was mutated (Fig. 3B), suggesting that the inhibition was mediated by ROR α . The suppression of ROR α by LXR α was also seen when a synthetic *tk-Pcp2*/RORE-Luc reporter was used. This reporter contains three copies of RORE derived from the Purkinje cell protein 2 (*Pcp2*) gene (Matsui, 1997). As shown in Fig. 3C, the activation of *tk-Pcp2*/RORE by ROR α was inhibited by ligand-free LXR α and this inhibition was enhanced by TO1317 or 22(*R*)-hydroxycholesterol, another LXR agonist. LXR α alone had little effect on the basal activity of *tk-Pcp2*/RORE reporter.

Interestingly, the LXR α activity was reciprocally suppressed by ROR α . As shown in Fig. 3D, co-transfection with LXR α activated the LXR-responsive *tk-MTV* reporter gene as expected (Willy et al., 1995). However, this activation was inhibited by co-transfection of a ROR α in a dose-dependent manner, whereas transfection of ROR α alone had little effect on *tk-MTV* reporter activity. The ligand-dependent activation of *tk-MTV* by LXR α was also inhibited by the co-transfection of ROR α (Fig. 3E). These results suggest that ROR α and LXR α are mutually suppressive.

The mutual suppression was also observed when the chimeric Gal4-LXR α and Gal4-ROR α receptors were transfected, together with the Gal4-responsive tk-UAS reporter

gene. The constitutive activity of Gal4-LXR α was inhibited by the wild type ROR α (CMX-ROR α) in a dose-dependent manner (Fig. 3F, left panel), whereas the constitutive activity of Gal4-ROR α was inhibited by the wild type LXR α (CMX-LXR α) in the absence of a ligand (Fig. 3F, right panel). ROR α is known to interact with nuclear receptor co-activators without an exogenously added ligand (Delerive et al., 2002). We showed that LXR α also exhibited ligand-independent interaction with the nuclear receptor co-activator SRC-1 as revealed by a mammalian two-hybrid assay, in which the VP fusion receptor of LXR α (VP-LXR α) was co-transfected with Gal4-SRC1 and tk-UAS-Luc (Fig. 3G). Moreover, the VP-LXR α -SRC1 interaction was inhibited by co-transfection of the wild type ROR α (Fig. 3G). ChIP assay on primary mouse hepatocytes showed that SRC1 was constitutively recruited onto *Cyp7b1*/RORE and this recruitment was decreased in the presence of TO1317 (Fig. 3H). In contrast, the recruitment of SRC1 onto *Est*/LXRE was increased by the TO1317 treatment (Fig. 3H). *Est*/LXRE is a DR-4 type LXR response element found in the mouse estrogen sulfotransferase (*Est*) gene promoter (Gong et al., 2007) and *Est* is a gene reciprocally activated in the ROR α null mice (see below in Fig. 4). The constitutive recruitment of SRC1 onto *Est*/LXRE was detectable when more PCR template was added or when the PCR cycle number was increased (data not shown). The ligand-independent recruitment of co-activator may account for the constitutive activities of both receptors, and co-activator competition may

represent a plausible mechanism for the mutual suppression of transcriptional activity between these two receptors.

Reciprocal and selective activation of target gene expression in mice deficient of ROR α and LXRs

The potential functional crosstalk between ROR α and LXR was further investigated *in vivo*. For this purpose, we measured the expression of LXR target genes and ROR target genes in the *ROR α ^{sg/sg}* and LXR DKO mice (Peet et al., 1998), respectively. As shown in Fig. 4A, among LXR target genes, the expression of *Est* (Gong et al., 2007), *Sult2a9* (Uppal et al., 2007), *Cd36* (J. Zhou and W. Xie, unpublished results), lipoprotein lipase (*Lpl*) (Zhang et al., 2001), aldo-keto reductase 1d1 (*Akr1d1*) (Volle et al., 2004), scavenger receptor BI (*SR-BI*) (Malerod et al., 2002) and acetyl CoA carboxylase 1 (*Acc-1*) was significantly induced, whereas the expression of *Srebp-1c* was significantly suppressed in female *ROR α ^{sg/sg}* mice. When the male *ROR α ^{sg/sg}* mice were analyzed, the activation of *Est*, *Cd36*, *Lpl* and *SR-BI*, but not *Sult2a9/2a1*, *Akr1d1* and *Acc-1*, remained significant (Fig. 4B). The lack of *Sult2a9* activation in males may be due to its nearly undetectable basal expression in this sex. Other gender differences include the male-specific activation of fatty acid synthase (*Fas*) and cholesterol 7 α -hydroxylase (*Cyp7a1*) and suppression of stearoyl CoA desaturase-1 (*Scd-1*). The suppression of *Srebp-1c* in *ROR α ^{sg/sg}* males was not statistically

significant (Fig. 4B). Loss of ROR α had little effect on the expression of *ApoE*, *Abcg5* and *LXR*s in either gender.

When the expression of ROR α target genes was measured in the LXR DKO mice, we found that the expression of *BMAL1* (Sato et al., 2004), *ApoA1* (Vu-Dac et al., 1997) and *p21* (Schrader et al., 1996) was induced in LXR DKO mice of both sexes (Fig. 4C and 4D).

IKK β (Delerive et al., 2001) was induced in female, but not male, LXR DKO mice. The expression of *ApoCIII* (Raspe et al., 2001), *Rev-erba* (Delerive et al., 2002) and *ROR α* was not significantly altered. The mutual activation of target gene expression in ROR α null and LXR DKO mice suggests that these two receptors are mutually suppressive *in vivo*, providing a plausible mechanism for the functional crosstalk between these two receptors.

ROR α null mice had increased liver triglyceride accumulation

LXR is known to activate lipogenic gene expression through Srebp-dependent (Repa et al., 2000) or independent (Chu et al., 2006; Cha et al., 2007) mechanisms. The activation of several LXR target genes in *ROR α ^{sg/sg}* mice prompted us to determine whether loss of ROR α affects hepatic lipid accumulation. We found that the average liver concentration of triglyceride was more than doubled in *ROR α ^{sg/sg}* mice of both genders, as compared to their age- and gender-matched wild type counterparts (Fig. 5A). In contrast, loss of ROR α had no significant effect on hepatic cholesterol levels in either sex (Fig. 5A). Interestingly, the

circulating levels of both triglycerides and cholesterol were significantly decreased in male $ROR\alpha^{sg/sg}$ mice (Fig. 5B), consistent with previous reports (Raspe et al., 2001; Kang et al., 2007). The decreases in circulating lipids in female $ROR\alpha^{sg/sg}$ mice were not statistically significant. These results suggest that the gene regulation in $ROR\alpha^{sg/sg}$ mice is functionally relevant by impacting hepatic triglyceride accumulation.

Discussion

In this study, we have established *Cyp7b1* as a novel ROR α target gene. Loss of ROR α decreased the basal expression of *Cyp7b1*, whereas transfection of ROR α activated *Cyp7b1* gene promoter and induced the expression of endogenous *Cyp7b1*. The activation of *Cyp7b1* gene promoter by ROR α was inhibited by LXR α . *Cyp7b1*, a key enzyme in the alternative pathway of cholesterol metabolism to form bile acids, plays an important role in the homeostasis of cholesterol, oxysterols and bile acids (Schwarz et al., 1998; Chiang et al., 2004). The oxysterol levels were increased in mice deficient of *Cyp7b1*, presumably due to a defect in the conversion of oxysterols to bile acids (Li-Hawkins et al., 2000). It would be interesting to know whether the decreased basal expression of *Cyp7b1* in the *ROR α ^{sg/sg}* mice is associated with accumulation of oxysterols, the endogenous LXR agonists.

The crosstalk between ROR α and LXR α is intriguing. This crosstalk was initially hinted by a remarkable overlap in gene regulation between the *ROR α ^{sg/sg}* mice and mice whose LXRs were genetically or pharmacologically activated. We then proposed that ROR α may normally function as a LXR suppressor, a notion that is supported by the activation of LXR target genes in the *ROR α ^{sg/sg}* mice. The suppression of LXR by ROR α may have broad physiological implications. LXRs are sterol sensors known to promote hepatic lipogenesis. Although lipogenesis is an essential function of the liver, over-activation of the lipogenic pathway is potentially harmful, leading to both local and systemic metabolic disorders. In this

regard, the constitutive activity of ROR α and its suppression on LXR activity may have offered a mechanism of “checks and balances” to prevent the over-activation of lipogenesis. Indeed, we showed that loss of this suppressor led to the accumulation of hepatic triglycerides (Fig. 5). Interestingly, the triglyceride accumulation in the *ROR α ^{sg/sg}* mice was independent of the activation of *Srebp-1c*, a master lipogenic transcriptional factor and primary target gene of LXR (Repa et al., 2000). Instead, the *ROR α ^{sg/sg}* mice had increased expression of *Cd36*, a fatty acid transporter known to play a role in PXR-mediated and Srebp-independent lipogenesis (Zhou et al., 2006). *Cd36* is also essential for LXR agonist-induced hepatic steatosis (J. Zhou and W. Xie, unpublished results). However, we cannot exclude the possibility that the regulation of genes other than *Cd36* may have also contributed to the accumulation of triglycerides in the *ROR α ^{sg/sg}* mice.

Reciprocally, loss of LXRs resulted in the activation of several ROR target genes, including the circadian-related *BMAL1* (Sato et al., 2004) and CDK inhibitor *p21* (Schrader et al., 1996). The *in vivo* consequences of ROR target gene activation in the LXR DKO mice remain to be determined. It is also interesting to note that the mutual activation of target gene expression in the *ROR α ^{sg/sg}* and LXR DKO mice are gene-specific. The mechanism for this selective gene regulation remains to be determined. Crosstalk between ROR α and LXR can involve several mechanisms, including competition for co-activators and DNA binding sites. The inhibition of ROR α -mediated *Cyp7b1* activation by LXR seemed to involve co-activator

competition, since the *Cyp7b1*/RORE was not bound by LXR α (data not shown) but was required for the inhibitory effect of LXR α (Fig. 3B). Repression of LXR target genes by ROR α may also involve an adjacent or distant RORE.

Another potential implication of the functional crosstalk between ROR α and LXR is the role of these two receptors in the survival of Purkinje cells. *Staggerer* mutant mice have a natural deletion in the LBD of ROR α , which is highly expressed in Purkinje cells. The *Staggerer* phenotype is similar to patients with Niemann-Pick disease type C who suffer from a significant loss of Purkinje cells and cerebellar ataxia (for a review, see Vanier and Millat, 2003). It has been shown that treatment with LXR agonists relieves Alzheimer's disease (Zelcer et al., 2007; Koldamova et al., 2005), a neurological disorder associated with cerebellar degeneration and loss of Purkinje cells (Sjobeck and Englund, 2001). For this reason, it was thought that activation of LXR might be beneficial to Purkinje cell survival. If loss of ROR α activates LXR, which was strongly suggested by hepatic gene regulation and steatosis in the *ROR α ^{sg/sg}* mice, one might expect that Purkinje cells may be protected in the *Staggerer* mice. Future studies are warranted to examine the role of ROR α and LXR in Purkinje cell survival and its implication in Niemann-Pick disease type C.

In summary, we have revealed a novel function of ROR α in regulating metabolic genes, including the cholesterol-metabolizing *Cyp7b1*. The effect of ROR α on metabolic gene expression can be achieved by its direct transcriptional regulation (such as *Cyp7b1*), as

well as through its functional crosstalk with the sterol receptor LXR. The metabolic regulatory role of ROR α is distinct to the previously known function of this orphan receptor in the structure and function of neuronal and immunological tissues and bones.

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Figure Legends

Fig. 1. Mice deficient of ROR α had decreased expression of *Cyp7b1* in the liver. (A)

Hepatic expression of *Cyp7b1* in the wild type and *ROR α ^{sg/sg}* mice was measured by real-time RT-PCR analysis. Results represent the averages and standard deviation from three mice per group. **, $P < 0.01$, compare to the same sex wild type (WT) control mice. (B) Expression of

Cyp7b1 in the WT and *ROR α ^{sg/sg}* male mice was measured by Northern blot analysis.

Ethidium bromide staining of the 28S and 18S rRNA serves as a loading control. Lanes represent RNA samples pooled from three individual mice of each genotype. (C) Gene

expression in isolated primary mouse hepatocytes and stellate cells as measured by real-time RT-PCR. Results represent averages and standard deviation from triplicate assays. The expression level of individual genes in the hepatocytes is arbitrarily set at 1.

Fig. 2. Regulation of the mouse *Cyp7b1* gene promoter by ROR α . (A) Reporter genes

containing various lengths of the mouse *Cyp7b1* gene promoter were transfected into HepG2 cells in the presence of expression vectors for ROR α or ROR γ . The position of the putative RORE is labeled. The transfection efficiency was normalized against the β -gal activity from the co-transfected CMX- β gal vector. Normalized luciferase activity in cells transfected with empty expression vector (CMX) was arbitrarily set at 1. Results shown represent the averages and standard deviation from triplicate assays. (B) Partial DNA sequence of the mouse

Cyp7b1 gene promoter. The putative *Cyp7b1*/RORE sequence is in capitals and the mutated nucleotides are underlined. The sequence of *ApoA-V*/RORE is also shown. The binding of ROR α and ROR γ proteins to ³²P-labeled *Cyp7b1*/RORE was demonstrated by EMSA. In the competition lanes, unlabeled probes were present in 100-fold molar excess relative to the radio-labeled probe. The binding of radio-labeled *ApoA-V*/RORE by ROR α and ROR γ was included as positive controls. (C) ChIP assay to demonstrate the recruitment of ROR α onto the *Cyp7b1* gene promoter. Formaldehyde cross-linked DNA was extracted from mouse liver tissues and ChIP assay was performed using an antibody against ROR α or control IgG. The final DNA extracts were amplified by PCR using the primer pairs encompassing either the *Cyp7b1*/RORE region (-902 bp to -1026 bp) or a distal control region (-2776 bp to -2900 bp) of the *Cyp7b1* gene promoter. Lanes represent individual mice. (D) The synthetic *tk-Cyp7b1*/RORE reporter or its mutant variant were transfected into HepG2 cells in the presence of ROR α . (E) Wild type mouse primary hepatocytes were transiently transfected with empty vector (CMX) or expression vectors for ROR α . Total RNA was isolated 40 h after transfection and subjected to real-time RT-PCR analysis to detect the expression of endogenous *Cyp7b1* and *Cyp3a11* (a non-target gene control). (F) Wild type mouse livers were transfected with empty vector (CMX) or expression vectors for ROR α by a hydrodynamic gene deliver method. Mice were sacrificed 6 h after transfection. Liver total RNA was extracted and subjected to real-time RT-PCR analysis to detect the expression of

endogenous *Cyp7b1* and *Cyp3a11*. (G) The expression of transduced ROR α in hepatocytes (in E) and mouse livers (in F) was confirmed by real-time RT-PCR using primers designed to detect total ROR α . Results shown in E-G represent averages and standard deviation of three independent experiments.

Fig. 3. ROR α and LXR α were mutually suppressive in reporter gene assays. (A)

Treatment with LXR agonists (10 μ M each) inhibited the expression of *CYP7B1* in primary human hepatocytes as measured by real-time PCR analysis. Patients 1 and 2 are a 41-year old Caucasian female and a 44-year old Caucasian male, respectively. TO, TO1317; 22(R), 22(R)-hydroxycholesterol; GW, GW3965. (B-E) The *pGL-Cyp7b1-Luc* or its RORE mutant variant (B), *tk-Pcp2/RORE-Luc* (C), and *tk-MTV-Luc* (D and E) reporter genes were transiently transfected into HepG2 cells in the presence of expression vectors for indicated receptors or their combinations. Where applicable, transfected cells were treated with DMSO or indicated drugs for 24 h before luciferase assay. Results shown are fold induction over CMX vector control and represent the averages and standard deviation from triplicate assays. Drug concentration is 10 μ M. (F) HepG2 cells were co-transfected with *tk-UAS* and indicated receptors or their combinations. Results shown are fold induction over reporter alone control and represent the averages and standard deviation from triplicate assays. (G) HepG2 cells were transfected with *tk-UAS*, Gal4-SRC1 and VP-LXR α , in the absence or presence of wild

type ROR α (CMX-ROR α). **(H)** ChIP assay on primary mouse hepatocytes to demonstrate the recruitment of SRC1 onto the *Cyp7b1* and *Est* gene promoters and the effect of LXR agonist TO1317 (TO) on SRC1 recruitment.

Fig. 4. Reciprocal and selective activation of target gene expression in mice deficient of

ROR α and LXRs. **(A and B)** Real-time PCR analysis on the hepatic expression of LXR

target genes in female **(A)** and male **(B)** *ROR α ^{sg/sg}* mice. **(C and D)** Real-time PCR analysis

on the hepatic expression of ROR target genes in female **(C)** and male **(D)** LXR double

knockout (LXR DKO) mice. Results represent the averages and standard deviation from three

(A and B) or five **(C and D)** mice per group. *, $P < 0.05$; **, $P < 0.01$, compare to the same sex

wild type (WT) control mice.

Fig. 5. ROR α null mice had increased liver triglyceride accumulation. Triglyceride and

cholesterol levels in the liver **(A)** and serum **(B)** of the WT and *ROR α ^{sg/sg}* mice of indicated

sexes. Results represent the averages and standard deviation from three mice per group. *,

$P < 0.05$; #, $P > 0.05$, compare to the same sex WT control mice.

Fig. 1

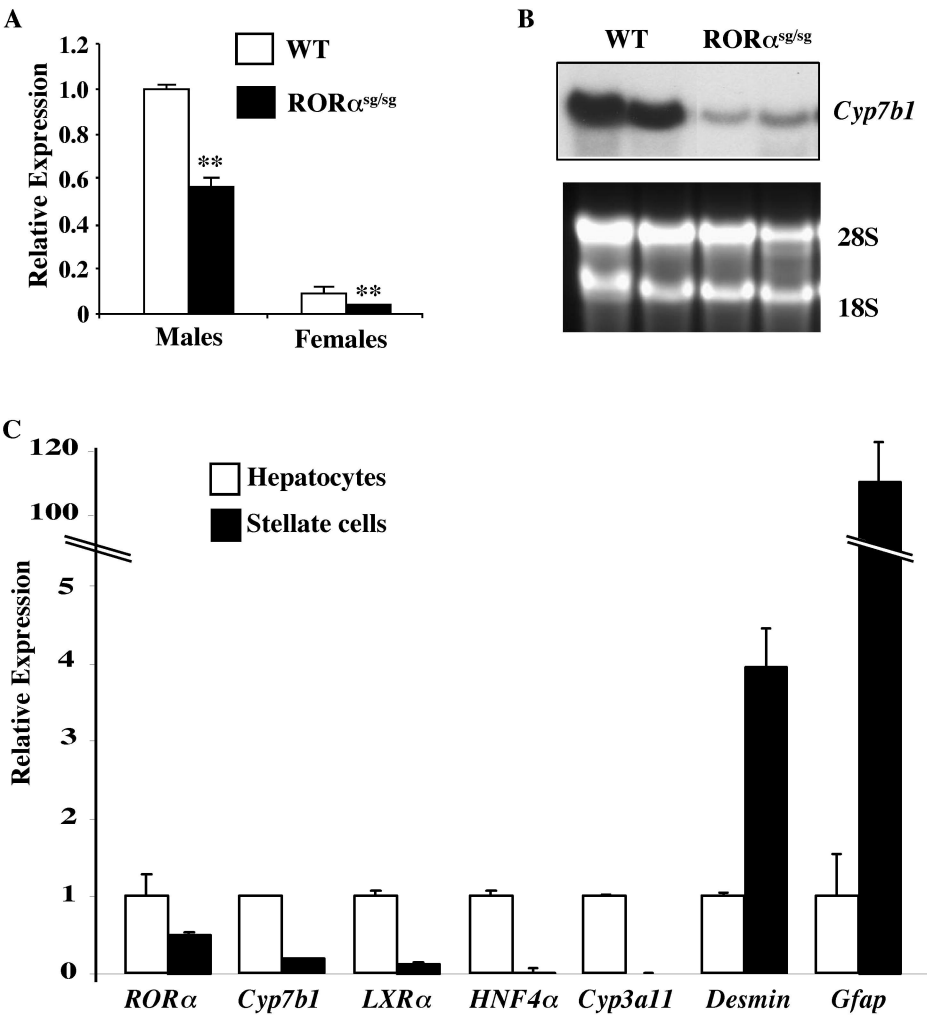


Fig. 2

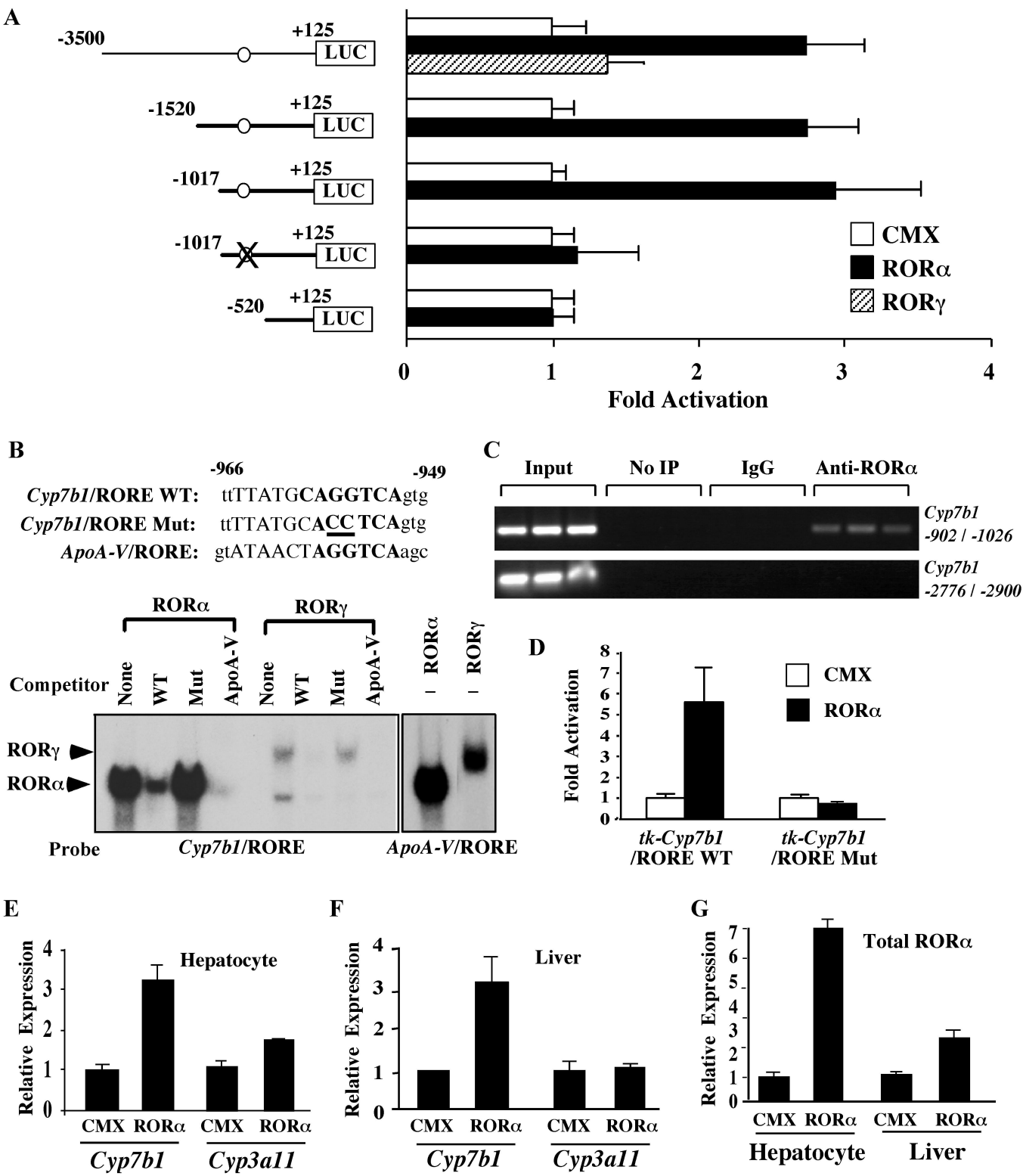


Fig. 3

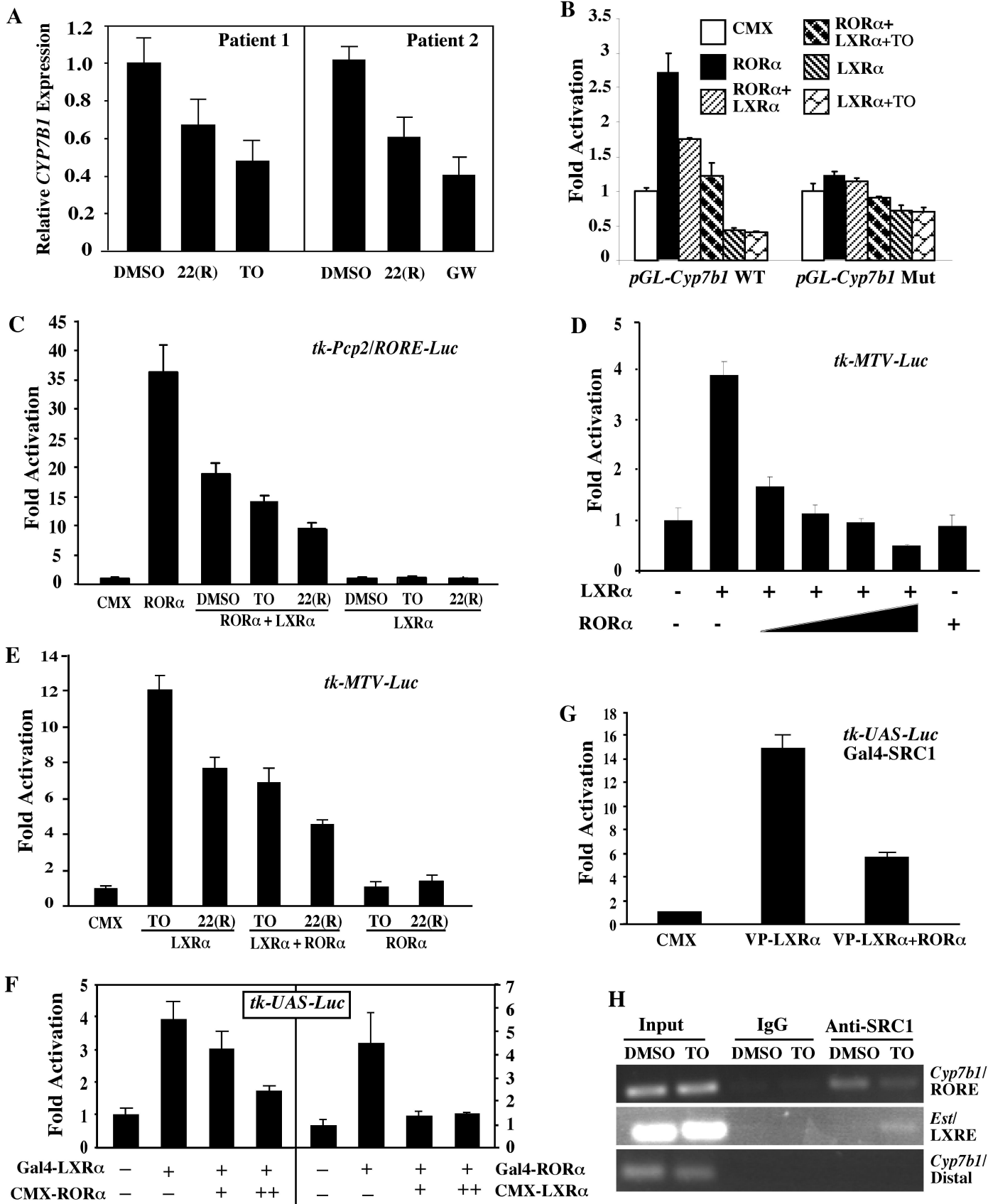


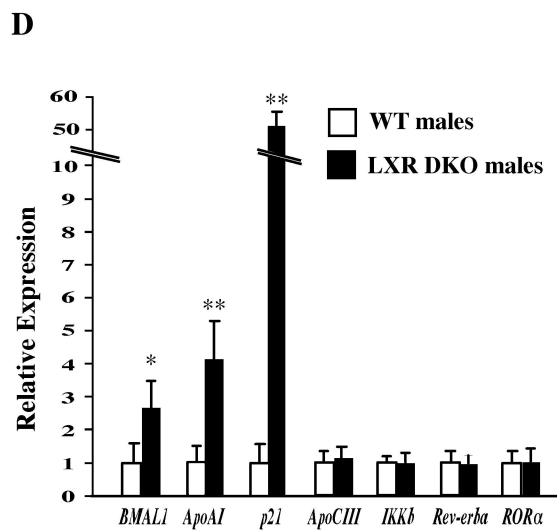
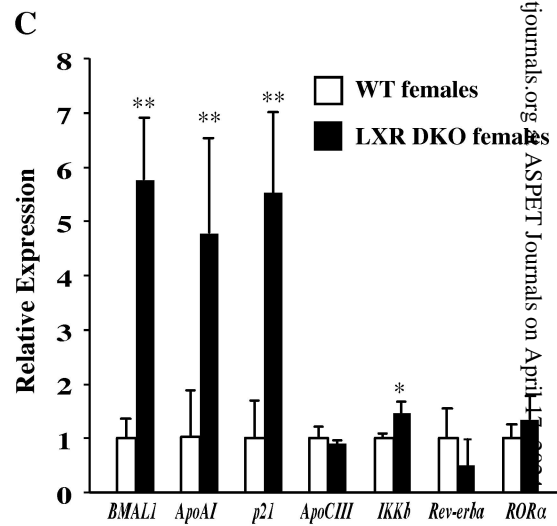
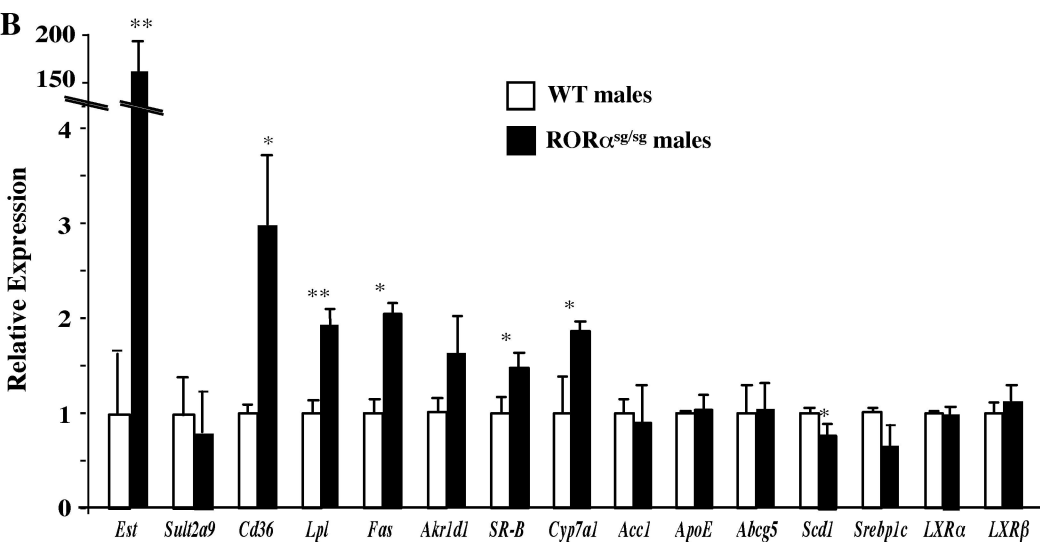
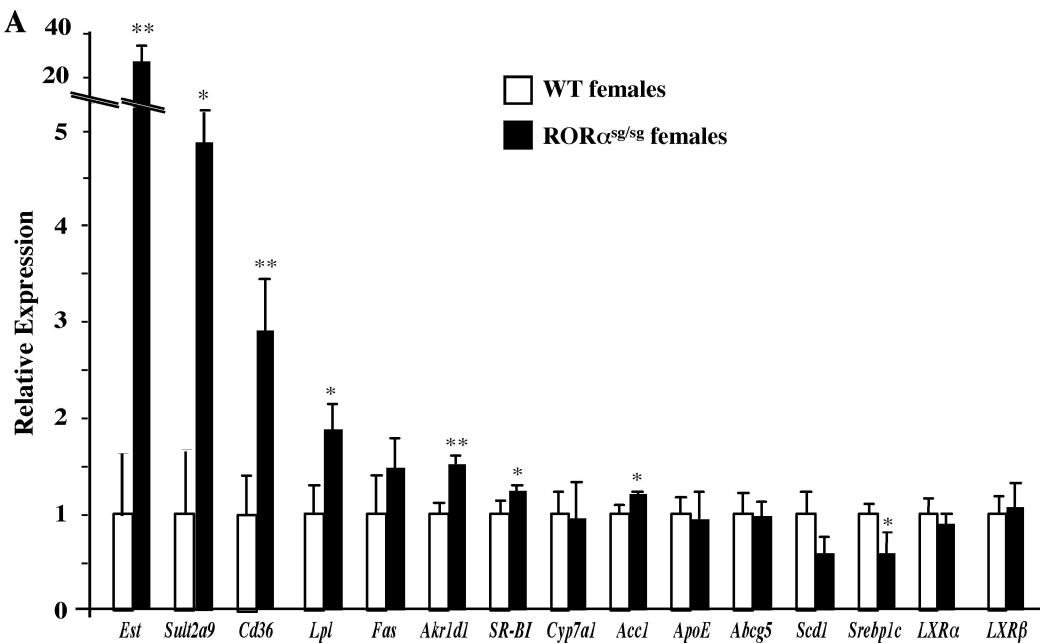
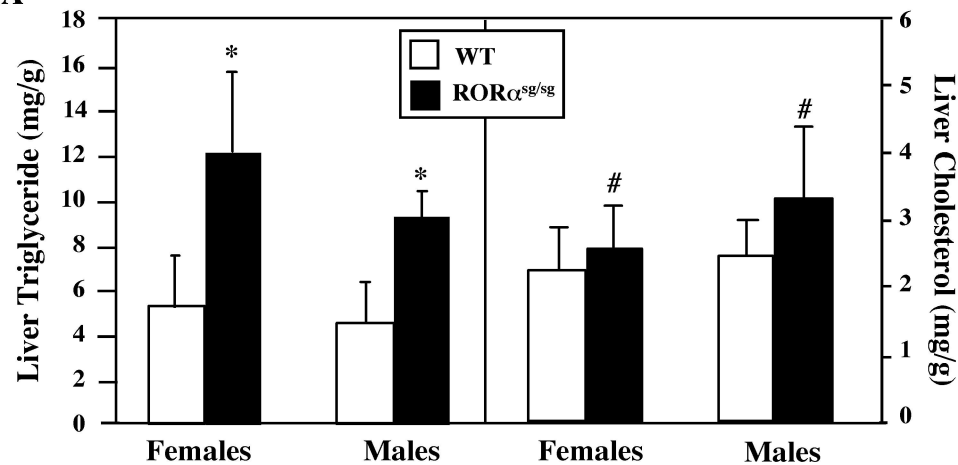
Fig. 4

Fig. 5**A****B**