Sumoylated PPARα mediates sex-specific gene repression and protects the liver from estrogen-induced toxicity in mice

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As most metabolic studies are conducted in male animals, understanding the sex specificity of the underlying molecular pathways has been broadly neglected; for example, whether PPARs elicit sex-dependent responses has not been determined. Here we show that in mice, PPARα has broad female-dependent repressive actions on hepatic genes involved in steroid metabolism and immunity. In male mice, this effect was reproduced by the administration of a synthetic PPARα ligand. Using the steroid oxysterol 7α-hydroxylase cytochrome P450 7b1 (*Cyp7b1*) gene as a model, we elucidated the molecular mechanism of this sex-specific PPARα-dependent repression. Initial sumoylation of the ligand-binding domain of PPARα triggered the interaction of PPARα with GA-binding protein α (GABPα) bound to the target *Cyp7b1* promoter. Histone deacetylase and DNA and histone methylases were then recruited, and the adjacent Sp1-binding site and histones were methylated. These events resulted in loss of Sp1-stimulated expression and thus downregulation of *Cyp7b1*. Physiologically, this repression conferred on female mice protection against estrogen-induced intrahepatic cholestasis, the most common hepatic disease during pregnancy, suggesting a therapeutic target for prevention of this disease.

Introduction

Many metabolic pathways impacting whole-body physiology are expressed in a sexually dimorphic manner in the liver. In fact, the mammalian liver is the center for sex-specific controls that contribute to differences in energy homeostasis, lipid and steroid hormone metabolism, and the degradation of xenobiotics. Furthermore, in both humans and mice, sex affects inflammatory responses and hepatocellular carcinoma development (1–5). The regulation of the events that shape sex-specific physiological and pathological phenotypes is not well understood. At the molecular level, many sex-specific control mechanisms are unknown. Many of the 1,600 hepatic genes differentially expressed between sexes are regulated by differences in pituitary growth hormone (GH) secretion, which is pulsatile in males and continuous in females. The hormone affects gene expression by activating STATs (6, 7). Additional transcription factors known to activate genes controlling androgen metabolism, inflammation, and energy homeostasis, such as GA-binding proteins (GABPs), also contribute to hepatic sexual dimorphism (8–12). Furthermore, epigenetic modifications, including DNA methylation and methylation-sensitive transcription factors, participate in male-specific gene expression. Regulatory DNA methylation sites have been found in the male-specific *Slp* and *Cyp2d9* promoters and other cytochrome P450 enzyme (*Cyp*) genes (8, 13). The CYPs metabolize diverse steroids, fatty acids, and many lipophilic drugs (14, 15). Though most pronounced in rat and mouse liver, sex differences in CYP expression also occur in humans (16). A prominent member of the CYP superfamily, oxysterol 7α-hydroxylase (CYP7B1), is enriched in male compared with female liver (17–21). The CYP7B1 protein represses androgen biosynthesis by modulating the availability of dehydroepiandrosterone (DHEA), the main precursor of the male hormone testosterone (18). In contrast to its negative effect on androgen production, CYP7B1 promotes estrogen receptor (ER) activity by catalyzing the clearance of 27-hydroxycholesterol, which functions as a competitive ER antagonist (22). The activated ER positively regulates the expression of *Cyp7b1*, which participates in estrogen-induced inflammation and hepatotoxicity, particularly in females (20, 21, 23). These pathologies affect susceptible women using estrogen-containing oral contraceptives or postmenopausal hormone replacement therapy (23). Moreover, estrogens cause intrahepatic cholestasis, the most common hepatic disease during pregnancy, which can result in intrauterine fetal death or spontaneous premature delivery (24). Therefore, understanding hepatic sexual dimorphism in steroid metabolism may help us to improve the efficacy of prevention measures and treatments based on steroid hormones. Similarly, knowledge of the molecular basis of sex-specific protective effects could be used for the development of treatments for inflammation- and hormone detoxification–related diseases.

It is likely that transcription factors other than those mentioned above play critical roles as determinants of sexual dimorphism, and their action is likely influenced by estrogen. In fact, signaling crosstalk between PPAR and ER have been documented (25), making PPAR an obvious candidate for involvement in the regulation of hepatic sexual dimorphism. Like the ER, the 3 different PPAR isotypes (PPARα, PPARβ [also called PPARδ], and PPARγ), belong to the nuclear receptor superfamily (26). The PPARα isotype regulates hepatic pathways associated with energy homeostasis, hepatic detoxification, the inflammatory response, and hepatocellular carcinogenesis (27–29). In addition to ligand binding, posttranslational modifications contribute to PPAR activity. Hence, sumoylation is implicated in the ligand-dependent transrepression of proinflammatory genes by PPARγ (30, 31). Sex-specific differences involving PPARα functions are poorly understood. Investigating sex-specific metabolic regulations may unveil novel functions of PPARα and

Conflict of interest: The authors have declared that no conflict of interest exists.
Citation for this article: *J. Clin. Invest.* **119**:3138–3148 (2009). doi:10.1172/JCI39019.

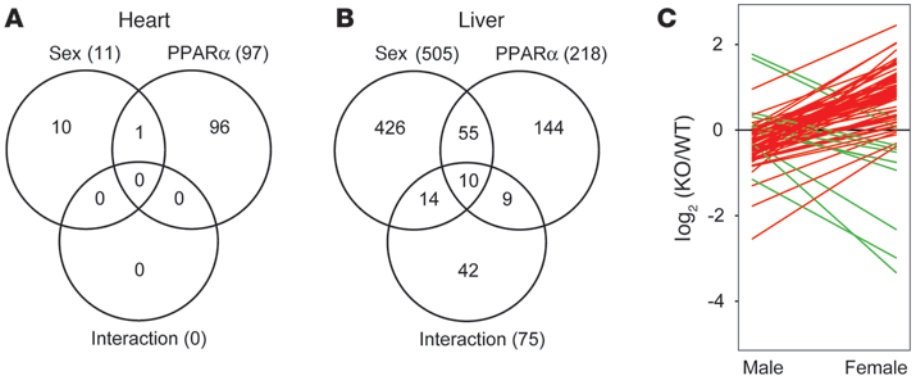

Figure 1
The hepatic sexual dimorphism of PPARα activity, with repressive functions in females. (**A** and **B**) Venn diagrams representing probe sets regulated in the heart (**A**) and liver (**B**). Microarray data were analyzed using a linear model with PPARα, sex, and their interaction as factors. For each factor, the total number of significant probe sets (FDR, <5%) is indicated in parentheses; the number of overlapping probe sets is indicated in the diagram. (**C**) Probe sets with a significant interaction factor. Log_2-fold differences between PPARα-null and WT for males and females are connected with a red line if the value is greater in females and a green line if the value is greater in males.

help in the design of new prevention measures and treatments for dysfunctions related to sex-associated steroid detoxification.

With this objective in mind, we studied how PPARα impacts female hepatic metabolism. We compared the mRNA expression profiles in the liver of male and female mice in the presence or absence of PPARα. We discovered a marked sex-dependent inhibitory action of the receptor in the female liver. This transcriptional repression depended on the sex-specific sumoylation of PPARα; which triggers a cascade of events, described for the first time to our knowledge in this study, that result in the downregulation of many promoters.

Results

PPARα-mediated inhibition of hepatic gene expression is more prevalent in females. We hypothesized that, among the genes differentially expressed in the male and female liver, a subset of genes is likely controlled by the hepatic regulator PPARα. To test this possibility, we compared the gene expression profiles of liver and heart, organs in which PPARα exerts regulatory functions, in female and male PPARα-null and WT mice. All animals were sacrificed at zeitgeber time 14 (ZT14; ZT00: lights on, ZT12: lights off), when the PPARα-stimulated genes were expected to reach maximum expression (32). Microarray data were analyzed using a linear model, with PPARα, sex, and their interaction as the factors examined (Supplemental Tables 1–5; supplemental material available online with this article; doi:10.1172/JCI39019DS1). Genes with a statistically significant interaction factor were differentially affected by sex in PPARα-null mice compared with WT animals. Although the ablation of PPARα affected both the liver and heart as expected, there was a marked differential effect between the sexes only in the liver (Figure 1, A and B). The majority of genes with a significant interaction (false discovery rate [FDR], <5%) exhibited greater differences between PPARα-null and WT mice for females compared with males (Figure 1C). An analysis of these differences suggested a broader impact of PPARα-dependent repression in females. In contrast, fewer genes were specifically induced by PPARα in females, for example *Rad51l1*, which is involved in DNA repair. Together, these data demonstrated a sex-dependent function of PPARα in the liver, but not the heart, mainly directed toward the repression of gene expression in the female.

Repression of genes involved in steroid biosynthesis and the immune response. We concentrated on the liver and searched for specific pathways exhibiting PPARα-dependent sexual dimorphism using a gene set enrichment analysis strategy. We looked for gene sets that were significantly up- or downregulated based on the moderated *t* statistic calculated for the interaction factor. Sets of genes that differed significantly between females and males were involved in hepatic steroid metabolism (including cholesterol biosynthesis), the NADPH cytochrome P450 reductase–regulated (CPR-regulated) pathway, and immunity via the complement pathway (Figure 2A and Supplemental Table 6). All of these genes were upregulated in PPARα-null females compared with WT females but remained unchanged in PPARα-null males compared with WT males. Notably, in both humans and mice, dysfunctional CPR expression is linked to impaired steroidogenesis (33).

Since hepatic steroidogenesis is a crucial component of sexual dimorphism, we tested whether the ablation of PPARα impacts the levels of plasma steroids, which are physiological markers of steroid biosynthesis dimorphism. Female PPARα-deficient mice had lower plasma androstenedione and testosterone levels than WT mice (Figure 2B), suggesting a positive effect of PPARα on androgen biosynthesis. This effect was confirmed by treatment with the PPARα agonist WY-14643, which resulted in a significant increase in the levels of androstenedione and testosterone in WT but not PPARα-null female mice (Figure 2B). In contrast, the same treatment had no effect in males, which had much higher levels of testosterone, as it is mainly produced in the testis (data not shown). These results indicated that PPARα enhances testosterone levels in females by either directly stimulating its biosynthesis or inhibiting the catabolism of an androstenedione and testosterone precursor.

The activation function–2 domain is essential in the repression of Cyp7b1 by PPARα. In microarrays, *CYP7B1* had one of the strongest positive interaction values, with a 4.1-fold increase in PPARα-null female livers compared with WT and no difference in males (FDR, 0.012; Supplemental Table 5). This result confirmed the sexual dimorphic expression pattern of a gene pivotal in steroid metabolism (17). In fact, CYP7B1 hydroxylates the testosterone precursor DHEA, diverting it from the sex hormone biosynthetic pathway in extragenital organs (20). Hepatic *Cyp7b1* downregulation by PPARα would result in more DHEA being available for sex hormone biosynthesis and maintaining physiological levels of circulating androstenedione and testosterone (Figure 3A). Because this gene is strongly downregulated in females, and because of its role in estrogen-induced liver disease in women, we used it as a model to study the molecular mechanism by which PPARα exerts female-specific repression. As expected from the gene profiling data, quantitative PCR (QPCR) confirmed the much higher expression (11-fold) of *Cyp7b1* in WT males than females (Figure 3B).

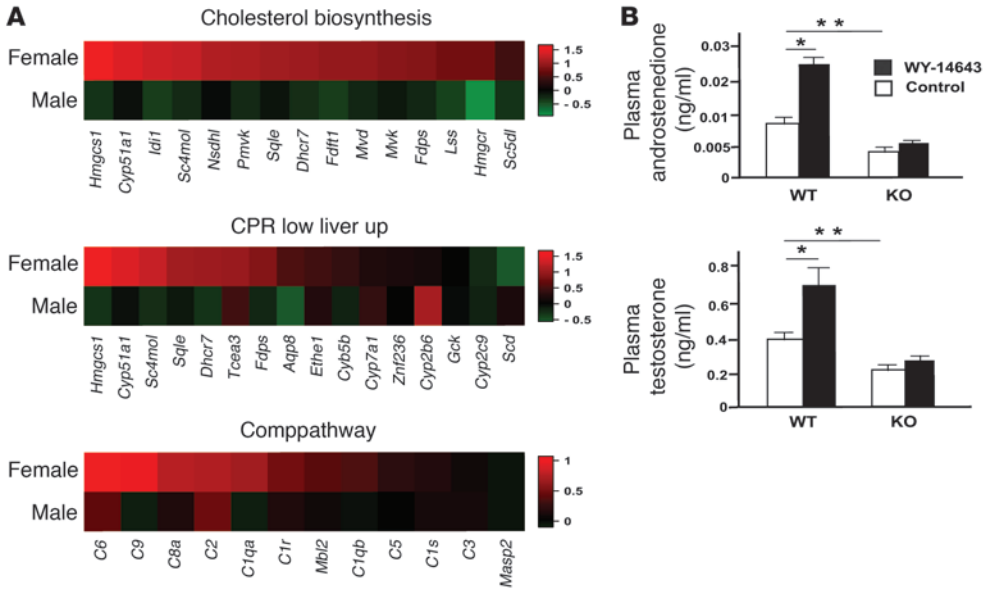

Figure 2

PPARα modulates hepatic steroid biosynthesis. (**A**) Heat maps of selected gene sets significantly enriched in genes with sexual dimorphism in PPARα-null mice. The $\log_2$-fold differences between PPARα-null and WT female and male mice are presented; for each gene set, the color correspondence is indicated on the right. Genes are ordered in each set by significance of the change in expression in females, with the most significant on the left. Cholesterol biosynthesis, cholesterol biosynthesis canonical pathway; CR low liver up, upregulated in liver from mice with reduced liver expression of NADPH–cytochrome P450 reductase (CPR) versus normal controls; Comppathway, both the classic and alternative immune complement pathways that promote inflammation, foreign cell lysis, and phagocytosis. For data, see Supplemental Table 4. (**B**) Female PPARα-null (KO) and WT mice ($n = 8$ per group) were treated (black bars) or not treated (white bars) for 5 days with WY-14643. Plasma androstenedione and testosterone levels were determined. Values are mean ± SEM. $^*P \leq 0.05$, $^{**}P \leq 0.01$.

Interestingly, in the absence of PPARα, PPARβ partially compensated for the repressive effect of the former on *Cyp7b1* expression in females. In fact, only in double PPARα/β-null female mice did *Cyp7b1* mRNA levels reach those of WT males (Supplemental Figure 1). After ligand treatment, expression was repressed more efficiently in males than females that presented lower basal expression (Figure 3, B and C). This inhibition was PPARα dependent, since it was not observed in PPARα-null mice.

A 1,086-bp fragment of the human *CYP7B1* promoter is known to mediate high basal activity in human kidney and liver cell lines (34). Using this promoter construct in NIH 3T3 cells, we reproduced the inhibitory effect of PPARα, which was enhanced by the PPARα ligand WY-14643, whereas a PPAR-positive reporter gene was stimulated (Figure 3D). Using chimeras containing either the N-terminal or ligand binding domain (LBD) of PPARα fused to the exogenous Gal4 DNA binding domain (DBD) in the presence or absence of PPARα ligand, we found that the LBD was responsible for the ligand-dependent repression of the *Cyp7b1* promoter (Figure 3E). The last 13 amino acids (helix 12) of the activation function–2 (AF-2) domain of the LBD were necessary for mediating repression (Figure 3F). Collectively, these data show that the addition of the PPARα ligand in males mimics the repression observed in females and that helix 12 of the PPARα AF-2 domain is necessary for mediating this inhibition.

The repressor effect of PPARα is sumoylation dependent. Previous studies have shown that some ligand-activated nuclear receptors use sumoylation (small ubiquitin-related modification) to repress gene expression (30, 31). We explored the possibility that this posttranslational modification is implicated in sex-dependent repression by PPARα. First, knockdown of ubiquitin-like protein SUMO-1 conjugating enzyme (Ubc9) and protein inhibitor of activated STAT1 (PIAS1), two sumoylation rate-limiting ligases, abolished PPARα transrepression (Figure 4A). Second, an analysis of the PPARα amino acid sequence highlighted 3 lysine residues embedded in putative motifs for SUMO conjugation (Figure 4B). These observations suggested that sumoylation is involved in the repressive effects of PPARα. Next, we mutated the lysine residue to arginine in each of the putative sumoylation sites and tested the ability of these PPARα mutants to repress the *Cyp7b1* promoter. The Lys358 mutation in helix 7 (Supplemental Figure 2) was sufficient to abolish the repressive activity of PPARα (Figure 4C), but the receptor conserved its transactivation potential (Figure 4D). Moreover, there was also a significant enhancement of PPARα sumoylation when small ubiquitin-related modifier 1 (SUMO1), SUMO2, and SUMO3 and PPARα were overexpressed in NIH 3T3 cells, demonstrating that PPARα has the potential to be modified by SUMO (Figure 4E).

To further explore the putative connection between PPARα sumoylation and hepatic sexual dimorphism, we measured the level of PPARα sumoylation in the liver of female and male mice. In untreated WT animals, sumoylated PPARα was detected in females but not males (Figure 4F). After treatment with the PPARα ligand, sumoylated PPARα increased to similar levels in both females and males (Figure 4F). These findings suggest that ligand-dependent PPARα sumoylation is determined by the agonist-induced conformation of the LBD, in which Lys358 is presented at the surface and therefore available for sumoylation, in contrast to the antagonist-induced conformation, in which case it is hidden (Supplemental

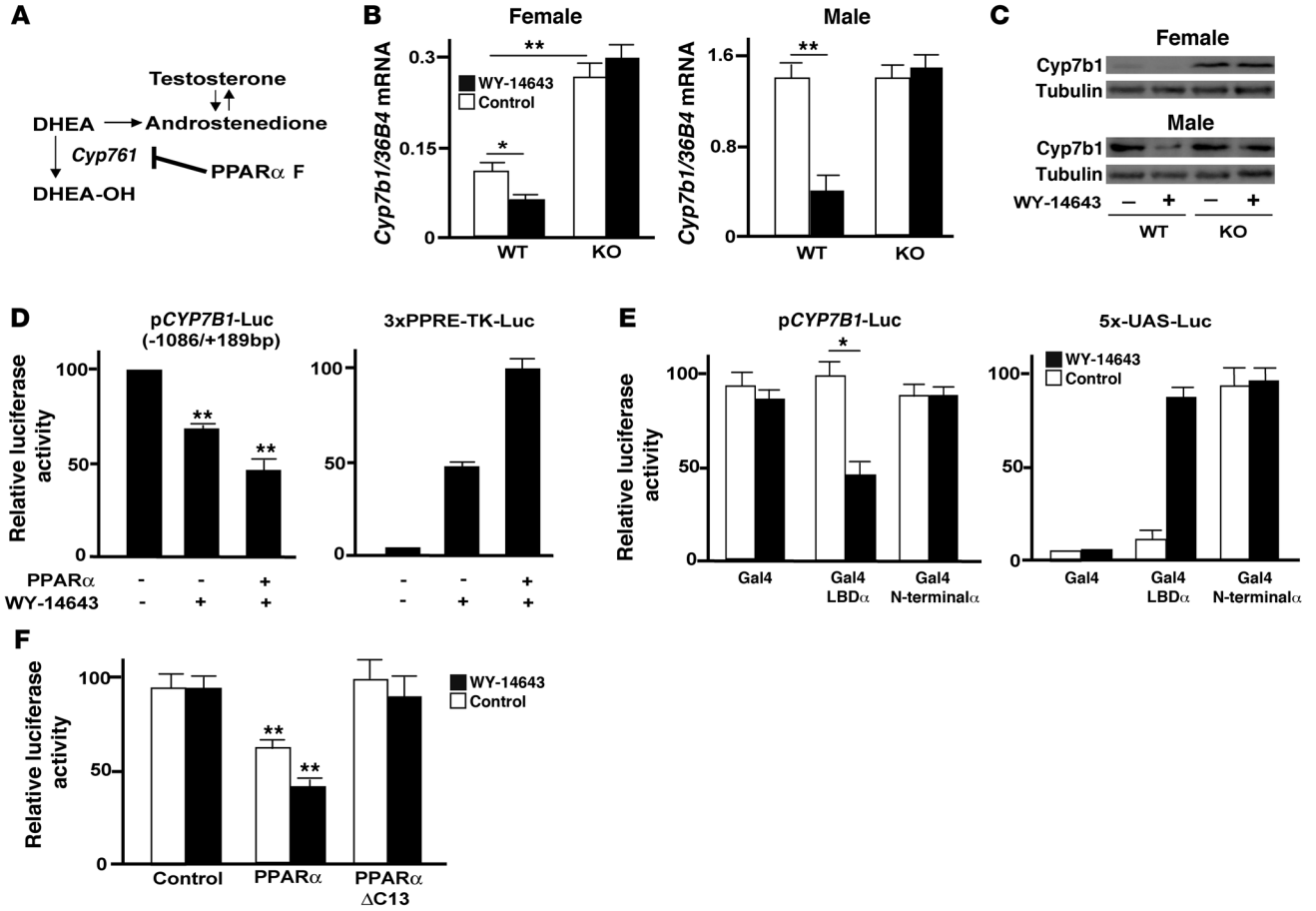

Figure 3

The AF-2 domain is essential for PPARα repression of *Cyp7b1*. (**A**) Schematic representation of PPARα-modulated androgen production through regulation of Cyp7b1 in females (F). (**B**) Age-matched female and male WT and PPARα-null mice were treated with WY-14643 for 5 days, and hepatic *Cyp7b1* expression was measured by quantitative real-time PCR ($n = 6$ per group) and (**C**) Western blotting. (**D**) Cotransfection of luc-reporter gene driven by a 1,086-bp *CYP7B1* promoter fragment [*pCYP7B1-luc*(–1086/+189bp)] and mouse PPARα expression vector. 3X-PPRE-TK-*Luc* was used as a positive control. (**E**) The LBDα or N-terminal domain of PPARα was fused to the Gal4 DBD. The chimeras were cotransfected with the *pCYP7B1-luc* reporter gene. 5X-UAS-TK-*Luc* was used as a control for the activity of the PPARα N-terminal domain and LBD chimera ($n = 4$ per group). (**F**) PPARα lacking the 13 C-terminal amino acids (PPARαΔC13) failed to repress *pCYP7B1-luc* activity. (**C**–**E**) After transfection, cells were treated with WY-14643 (1 μM) or DMSO (vehicle; Control) for 20 hours. Values are presented as mean ± SEM ($n = 3$). $^*P \leq 0.05$, $^{**}P \leq 0.01$ versus control.

Figure 2). This ligand-dependent conformational change is sufficient to explain why activated receptor is required for the repressive actions described in this study. Furthermore, the results indicate that sumoylation enzymes are similarly efficient in both sexes (Supplemental Table 3).

PPARα repression is mediated by an interaction with GABP. Transfection experiments identified the –144/–18 *CYP7B1* promoter region as a mediator of the PPARα repressive effect (Figure 5A). Because no peroxisome proliferator responsive element (PPRE) was found in this region, it is likely that PPARα exerts its effect via interaction with other *trans*-acting factor(s) and/or *cis*-regulatory element(s). In this region, we found 7 potential binding sites for GABPs, heterodimeric transcription factors with widespread tissue distribution, among which the 3 most distal sites are well conserved between mammals (Figures 5B and Supplemental Figure 3A). Simultaneous cotransfection of human GABPα, which is the DNA binding subunit of the heterodimer, and GABPβ1, the effector subunit, increased the activity of the *CYP7B1* promoter 8-fold, whereas separately transfecting GABPα and GABPβ1 had no effect (Figure 5C). Induction of the promoter by GABPα and GABPβ1 heterodimers was lost with the –18/+176 *CYP7B1* promoter construct, which lacks the GABP-binding sites (Figure 5C). Mutation analyses showed that the centrally positioned GABP-binding site was necessary, in combination with either the proximal or distal site, to mediate basal GABP activation (Supplemental Figure 3B). Because GABP proteins are known to interact with other transcription factors (35), we investigated whether PPARα physically interacts with GABPα and thus interferes with GABP activity. An interaction between PPARα and GABPα was demonstrated by coimmunoprecipitation experiments. The interaction required the AF-2 domain of PPARα and an intact K358 sumoylation site (Figure 5D).

Many nuclear receptor–interacting coactivators, including PGC-1, CBP, and RIP-140, contain one or more copies of the LXXLL motif necessary for their binding to nuclear receptors (29). We found

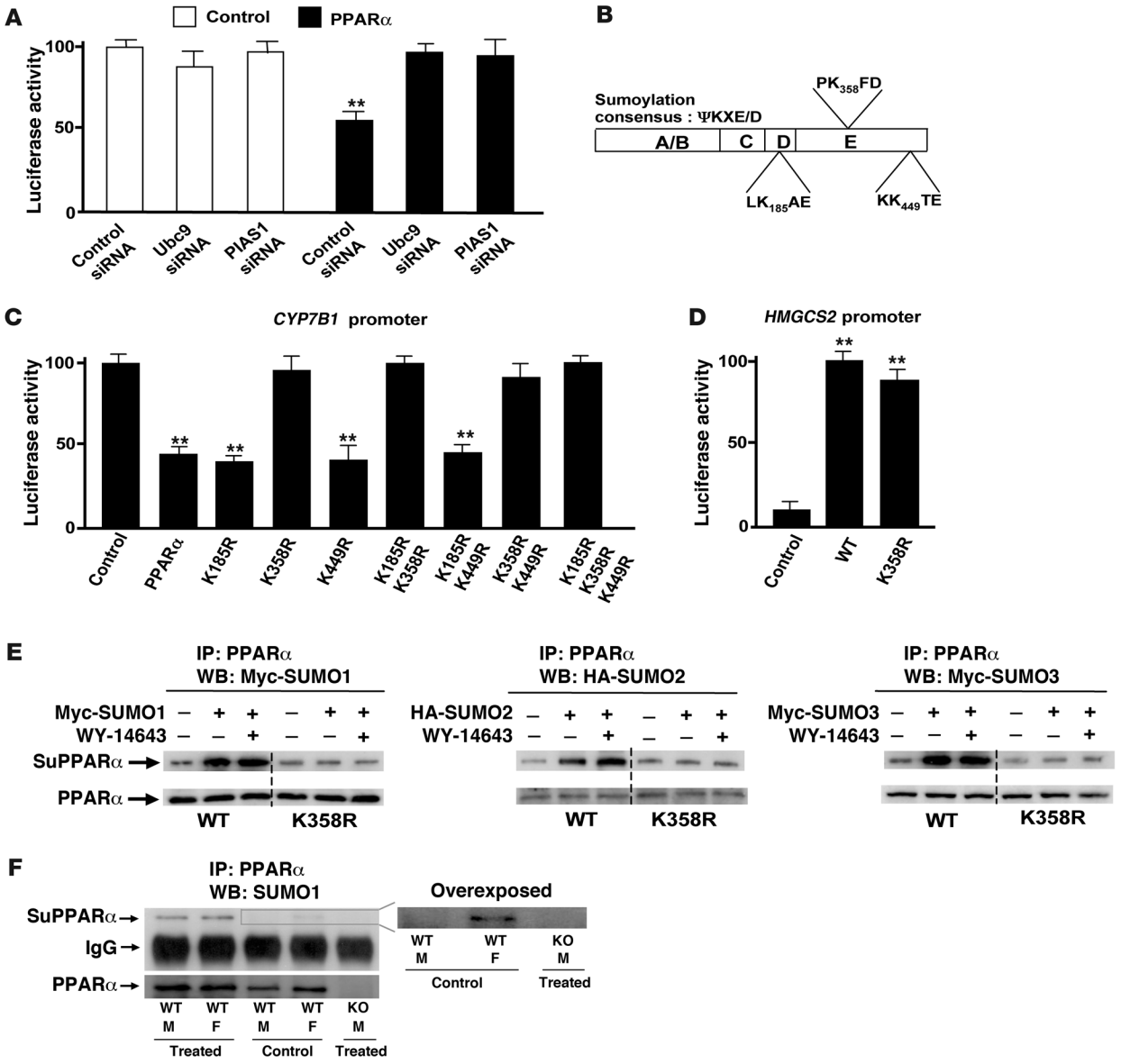

Figure 4

A sumoylation-dependent mechanism mediates PPARα repression of *Cyp7b1*. (**A**) NIH 3T3 cells transfected with control and Ubc9- and PIAS1-specific siRNAs and treated with WY-14643 for 48 hours. White bars, control plasmid; black bars, PPARα plasmid. (**B**) Schematic representation and localization of 3 putative sumoylation sites in the PPARα LBD.Ψ indicates a hydrophobic amino acid. (**C**) Sumoylation sites were modified by site-directed mutagenesis (K358R, K185R, and K449R). The activity of these mutants was compared with WT mouse PPARα by cotransfections with *pCYP7B1-Luc* or (**D**) p*HMGCS2-Luc* (reporter gene driven by the *HMGCS2* promoter), which is positively regulated by PPARα. For **A**, **C**, and **D**, values are presented as mean ± SEM (n = 3 per group). $^{**}P \leq 0.01$ versus control. (**E**) NIH 3T3 cells were cotransfected with expression vectors for WT or mutant (K358R) PPARα and c-myc-SUMO1, HA-SUMO2, or c-myc-SUMO3. Total proteins were immunoprecipitated using PPARα antibody and immunoblotted for c-Myc or HA-tag. After transfection, cells were treated with WY-14643 or vehicle (DMSO) for 20 hours. (**F**) Age-matched female (F) and male (M) PPARα-null and WT mice were treated with WY-14643 for 5 days. Hepatic nuclear proteins were immunoprecipitated with PPARα antibody and immunoblotted for SUMO1. The right panel presents an overexposure of the indicated area. Treated, WY-14643; Control, untreated.

that GABPα contains one such motif, LKKLL (amino acids 51–55), in its N-terminal domain. Strikingly, this motif is similar to the sequence found in the N-terminal region of PGC-1, a key energy metabolism coactivator known to interact with PPARα (36). Replacing leucines 54 and 55 with alanines impaired the ability of GABPα to interact with PPARα (Figure 5, E and F). Therefore, it is possible that PPARα uses GABPα to anchor itself to the *Cyp7b1* promoter in vivo. This possibility was tested by ChiP. Knockdown of GABPα impaired the recruitment of PPARα on the human *CYP7B1* promoter, indicating that GABPα is essential for PPARα-mediated repression (Figure 5G). Furthermore, we observed that PPARα and GABPα colocalized in vivo on this region of the mouse

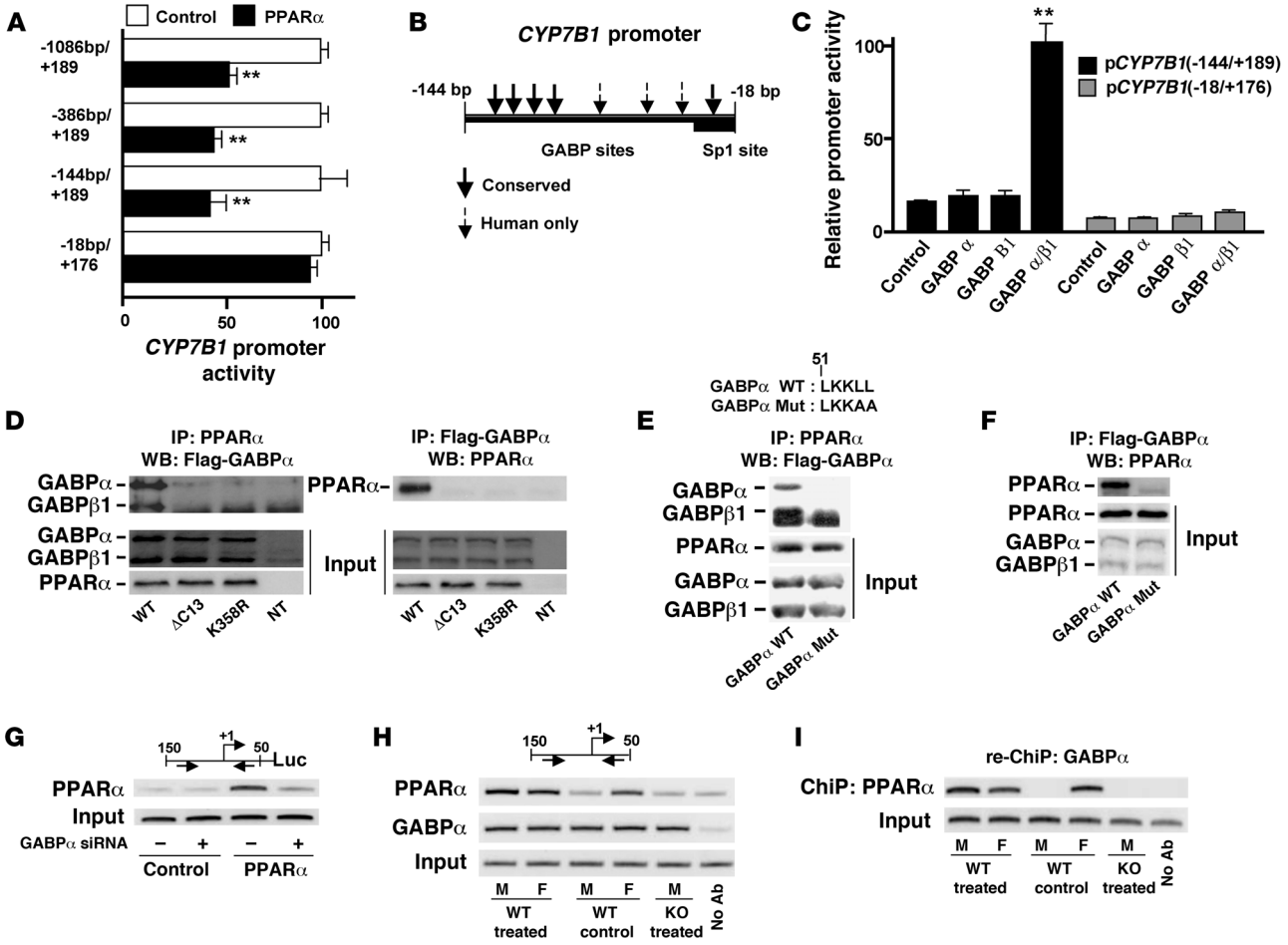

Figure 5

AF-2–dependent interaction between PPARα and GABP proteins on the *CYP7B1* promoter. (**A**) Cotransfection experiments in NIH 3T3 cells using *CYP7B1* promoter deletion constructs and a mouse PPARα expression vector or PSG5 as a control. Cells were then treated with WY-14643. (**B**) Schematic representation of the proximal *CYP7B1* promoter region, which confers PPARα-dependent repression, with the positions of the GABP sites and the Sp1 site indicated. Sites conserved between mouse and human and sites only found in human are indicated. (**C**) NIH 3T3 cells were cotransfected with pCDNA3-flag-GABP expression vectors and a reporter gene driven by the –144/+189 *CYP7B1* promoter region containing the GABP-binding sites or the –18/+176 promoter region, which lacks these sites. (**A** and **C**) Values are presented as mean ± SEM ($n = 3$ per group). $^{**}P \leq 0.01$. (**D**) NIH 3T3 cells were cotransfected with WT or mutant (Mut) PPARα (ΔC13 and K358R) expression vectors and the flag-GABP expression vector. Right: Reverse coimmunoprecipitation experiment. NT, nontransfected cells. (**E**) NIH 3T3 cells were cotransfected with a PPARα expression vector and Flag-GABPα expression vectors for WT or mutated LXXLL motif as indicated. (**F**) Same as in **E**, but IP was performed against Flag-tag, followed by Western blotting against PPARα. (**G**) Requirement of GABPα for mouse PPARα binding on the transfected human *CYP7B1* promoter was determined by a ChiP assay and GABPα siRNA. (**H**) In vivo binding of PPARα and GABPα on the *Cyp7b1* promoter was detected by a ChiP assay of hepatic nuclear protein using PPARα and GABPα antibodies. (**I**) Interaction of PPARα and GABPα on the *Cyp7b1* promoter was detected by a re-ChIP assay (see Methods). Treated, WY-14643; Control, untreated.

Cyp7b1 promoter (Figure 5, H and I). This experiment provided strong evidence for a GABPα LKKLL motif–mediated interaction between GABPα and the LBD of PPARα at the *Cyp7b1* promoter.

PPARα-dependent repression and DNA methylation. Epigenetic modifications, particularly DNA methylation, have been implicated in hepatic sexual dimorphism (8, 13). The structure of the *Cyp7b1* promoter, particularly the presence of an Sp1 site, which is known to stimulate the promoter, adjacent to the GABP sites suggests that its activity might be regulated by methylation (34). To test this possibility, we measured the expression of human *CYP7B1* in hepatic cells treated with increasing concentrations of the DNA methyltransferase (Dnmt) inhibitor 5-aza-2-deoxycytidine (5-aza-dC). Enhanced *CYP7B1* expression was observed (Figure 6A). To determine the role of Dnmt in PPARα-mediated repression, we performed transient transfection of a PPARα expression vector and a *CYP7B1* promoter reporter gene in the presence of 5-aza-dC. The inhibitory effect of PPARα on *CYP7B1* activity was abrogated by 5-aza-dC, suggesting that transcriptional repression is mediated by DNA methylation (Figure 6B). Moreover, knockdown of Dnmt3L, but not Dnmt1, increased *CYP7B1* expression (Figure 6C) and abolished PPARα-dependent repression (Figure 6D). The methylation pattern of the native promoter in liver was analyzed by bisulfite sequencing, which relies on the conversion of unmethylated cytosine residues to uracil. A change in the methylation level of the above-mentioned Sp1 site, which contains a CpG dinucle-

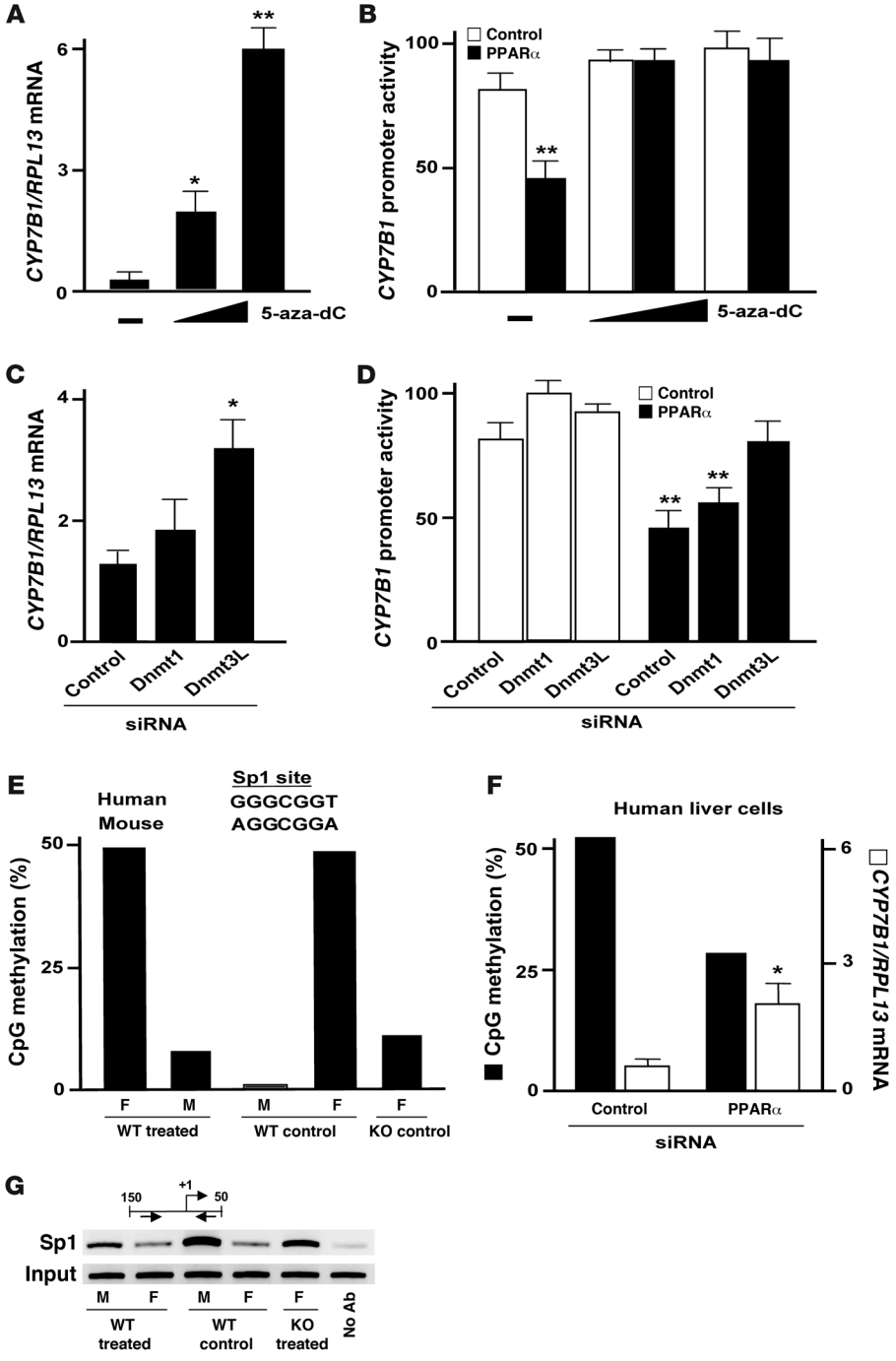

Figure 6

PPARα regulates epigenetic modification at the *CYP7B1* promoter. (**A**) QPCR shows that *CYP7B1* is activated in human hepatic cells (HepG2) treated with the Dnmt inhibitor 5-aza-dC (10 and 50 μM) for 4 days. (**B**) NIH 3T3 cells treated with WY-14643 and increasing concentrations of 5-aza-dC (10 and 50 μM) were transfected with a PSG5-mouse PPARα expression vector (PPARα) or the empty vector (control). (**C**) QPCR shows that knockdown of Dnmt3L, but not Dnmt1, stimulated *CYP7B1* in HepG2 cells. (**D**) Knockdown of Dnmt3L, but not Dnmt1, abolishes PPARα repression of *CYP7B1*. (**E**) Hepatic genomic DNA was treated with bisulfite and the Sp1 site methylation level recorded. (**F**) After knockdown of human PPARα in HepG2 cells, Sp1 site methylation level was recorded and compared with *CYP7B1* expression. Cells in **D** and **F** were treated with WY-14643 for 48 hours. In **A**–**D** and **F**, values are presented as mean ± SEM (n = 3). $^*P \le 0.05$, $^{**}P \le 0.01$ versus control. (**G**) In vivo binding of Sp1 to the *Cyp7b1* promoter was detected by a ChiP assay of hepatic nuclear proteins using Sp1 antibody. Primers encompassing the GABPα and Sp1 binding sites were used for PCR. Treated, WY-14643; Control, untreated.

otide, was observed. In untreated animals, methylation of the Sp1 site correlated with decreased expression levels of *Cyp7b1* in mouse liver (compare Figure 6E and Figure 3A) and human liver cells (Figure 6F). In agreement with the repressive effect of ligand-bound PPARα, CpG methylation increased in males after WY-14643 treatment. A ChiP assay indicated that there is an inverse correlation between CpG methylation at the Sp1 binding site and the binding of Sp1 itself (Figure 6G). It is known that DNA methylation and histone trimethylation systems can synergize in complexes including methyl-CpG binding protein, nuclear receptor corepressor (NCoR), associated histone deacetylases, and histone methyl transferases (37). Similarly, we found that histone deacetylases, NCoR, and the trimethylation of histone 3 lysine 9 were implicated in the PPARα-mediated repression of *Cyp7b1* (Supplemental Figure 4). Taken together, our observations suggest a functional interaction among sumoylated PPARα, epigenetic modifications, and chromatin remodeling in *Cyp7b1* repression.

PPARα protects against estrogen-mediated hepatotoxicity. The repression of *Cyp7b1* by PPARα suggests a possible protective effect of this receptor in estrogen-related liver diseases, including inflammation and cholestasis. Providing further support for this hypothesis, female PPARα-null mice displayed increased hepatic *Cyp7b1* and complement protein 6 (*C6*) mRNA levels after exposure to the estrogen ana-

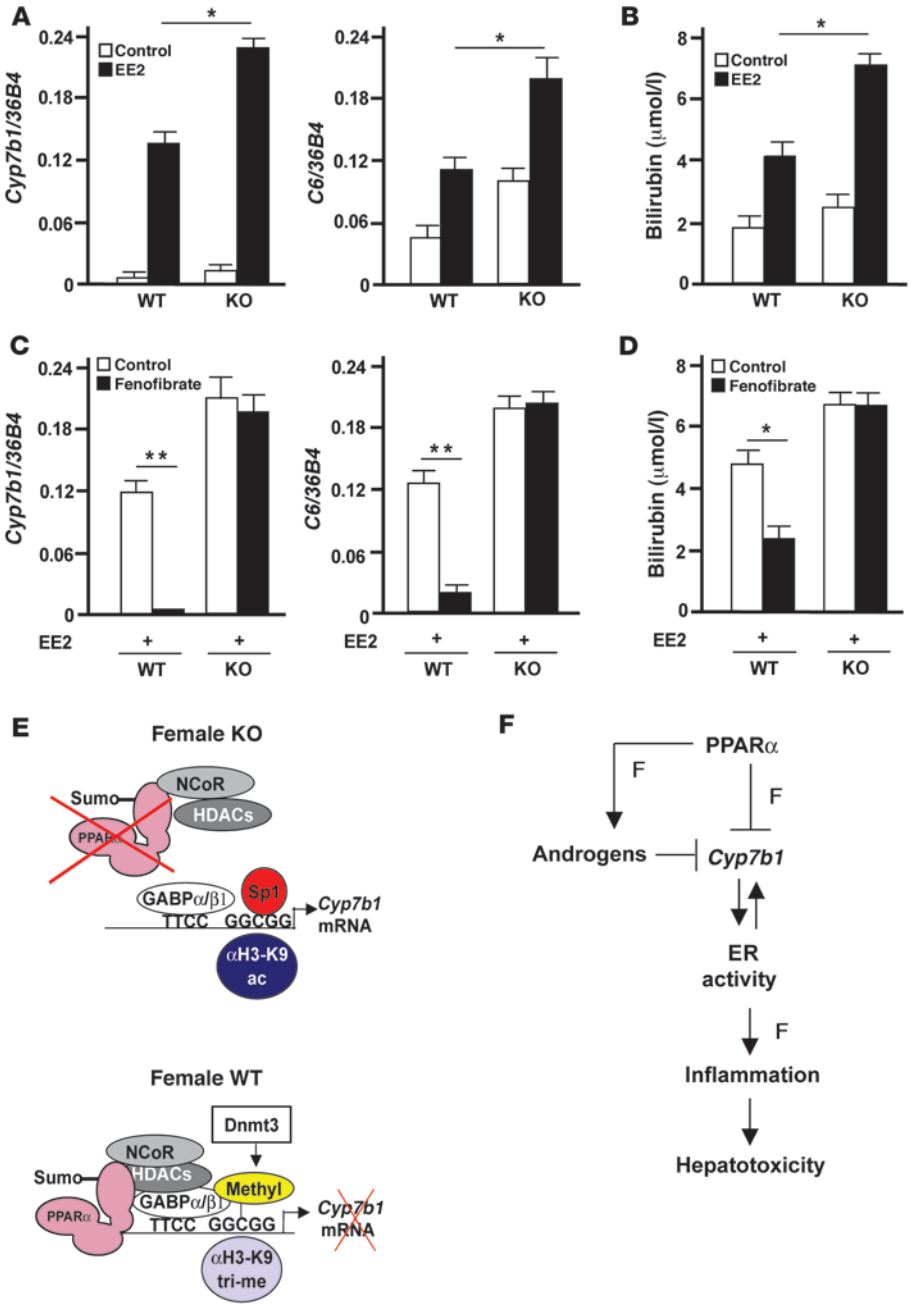

Figure 7

PPARα protects against estrogen-mediated hepatotoxicity in females. (**A** and **B**) Female PPARα-null and WT mice treated (black bars) or not treated (white bars) for 5 days with EE2. (**C** and **D**) Female PPARα-null and WT mice were treated for 5 days with EE2, with (black bars) or without (white bars) fenofibrate. (**A** and **C**) Hepatic *Cyp7b1* and *C6* mRNA levels were measured by QPCR and normalized to the 36B4 expression level. (**B** and **D**) Plasma bilirubin concentration. In **A**–**D**, values are presented as mean ± SEM ($n = 4$). $^*P \leq 0.05$; $^{**}P \leq 0.01$. (**E**) Schematic representation of the PPARα-induced repression of *Cyp7b1* in females. Sumoylation of PPARα promotes interaction with GABPs and HDACs, leading to DNA methylation by Dnmt3 and the displacement of Sp1 from its methylated core binding site. Removal of PPARα in female mice promotes the removal of the repression complex, which leads to *Cyp7b1* stimulation. (**F**) Schematic representation of the protective effect of PPARα against estrogen-mediated inflammation and hepatotoxicity in females (F). αH3-K9 ac, αhistone 3 acetylated at Lys9; αH3-K9 tri-me, αhistone 3 trimethylated at Lys9.

log 17-α-ethynylestradiol (EE2) (Figure 7A). These changes correlated with increased plasma bilirubin levels and liver weight, two common markers of xenobiotic stress (Figure 7B and data not shown). To assess the potential protective effect of PPARα against hepatic estrogen toxicity, we treated female PPARα-null and WT mice with fenofibrate, a well-known PPARα activator used in clinics to treat hyperlipidemia. Fenofibrate treatment abolished the induction of *Cyp7b1* and *C6* expression by estrogen (Figure 7C). Moreover, fenofibrate lowered the high plasma bilirubin levels in estrogen-treated WT mice, but it had no effect on PPARα-null mice (Figure 7D). The same protective effect was observed with WY-14463 treatment (data not shown). These results underscore the potential of PPARα and its ligands for protecting against estrogen-induced intrahepatic cholestasis.

Discussion

Here we provide evidence for a marked sex-dependent difference in the downregulation of key hepatic functions by PPARα. This nuclear receptor has a broader repressive effect on gene expression in females, especially for genes involved in hepatic steroid synthesis and the complement system. Because the repression seen in females was mimicked in males with the addition of PPARα ligand, we postulated a mechanism in which the nuclear receptor interferes with coregulatory proteins and promoter-bound transcription factors without direct, sequence-specific interactions with DNA, referred to as transrepression (38).

Among the many downregulated genes, *Cyp7b1* was chosen as a model because of its association with sex-related liver diseases.

Our results demonstrate that the PPARα repression of this gene in females requires sumoylation of the receptor, which was mimicked in males after treatment with the hypolipidemic drug fenofibrate, suggesting a crucial role for the LBD of PPARα in female-specific repression. Although sumoylation-dependent transrepression was already observed in cell cultures for another PPAR isotype, PPARγ (30, 31), our study is the first to our knowledge to demonstrate a repressive role for sumoylated PPARα in an in vivo context: the female liver under normal physiological conditions.

Furthermore, the PPARα transrepression mechanism has not, to our knowledge, been previously observed for any other member of the receptor superfamily. Sumoylated PPARα interacts with GABP on the *Cyp7b1* promoter via a functional nuclear receptor–interacting motif, LKKLL, in the N terminus of GABPα. The highly conserved LXXLL amphipathic α-helix motif, called the nuclear receptor box, is usually found in nuclear hormone receptor coactivators, suggesting the exclusive binding of GABPα or PPARα coactivators. In the unveiled transrepression mechanism, GABPα might neutralize coactivators when it recruits sumoylated PPARα together with NCoR, HDACs, and DNA and histone methylases to the *Cyp7b1* promoter, in which no PPRE was identified. The GABP regulates the transcription of many viral and cellular genes; in particular, it plays a key role in the immune system by regulating T cell function and lymphocyte development (9, 10). The PPARα protein is a key player in inflammation (39) and modulates immune functions. Our study suggests that interactions between PPARα and GABP may be involved in these processes. In support of this idea, the proximal promoter region of the complement system and hepatic steroidogenesis genes, which were downregulated by PPARα, have GABP-binding sites (Supplemental Figure 3, B and C, and Supplemental Figure 5, B and C). Future work with specific hepatic GABPα–null mice should establish this putative, more general GABP-mediated PPARα repression mechanism.

The GABP and Sp1 proteins are known to cooperate in the activation of several GABP target genes, including lineage-specific genes (40). In the *Cyp7b1* promoter, the GABP-binding sites are juxtaposed to an Sp1 site, which was methylated to a greater extent in female compared with male liver. Importantly, changes in DNA methylation have been associated with hepatic sexual dimorphism. Our finding establishes a link between PPARα sumoylation and the epigenetic modification of the *Cyp7b1* promoter that correlates with reduced gene expression. Although other studies have shown regulation of gene activity through the methylation of the CpG core sequence in Sp1 sites (41, 42), our study has unveiled a mechanism by which PPARα uses DNA methylation to exert its transrepression activity (Figure 7E). This mechanism hinders Sp1 from binding to its methylated site and thus from stimulating *Cyp7b1*.

The ligand-dependent sumoylation of the PPARα LBD suggests that in females, this process is triggered by endogenous PPARα activators and, possibly, coactivators. In line with this hypothesis, we observed that the expression of fatty acid translocase/CD36 occurred predominantly in females, which suggests an increased availability of fatty acids for PPARα activation in the female liver (Supplemental Table 3). Interestingly, this difference in CD36 expression has also been observed in humans (43). The identity of PPARα activators enriched in females, such as specific ligands and coactivators, is currently being investigated.

The model in Figure 7F illustrates two complementary and not mutually exclusive mechanisms by which PPARα might repress estrogen-mediated hepatotoxicity: directly through the repression of the *Cyp7b1* promoter or indirectly through its ability to increase testosterone levels, both leading to a reduction in ER activity. This model may help in the development of better treatments for estrogen-dependent liver diseases caused by high levels of estrogens, such as intrahepatic cholestasis of pregnancy (ICP), or for susceptible women undergoing postmenopausal hormone replacement therapy (44). In most areas of the world, ICP is observed in up to 1% of pregnancies, whereas it occurs in up to 15% of pregnancies in Chile and Bolivia. Today, the hydrophilic bile acid ursodeoxycholic acid is the most effective treatment for ICP (24), but it is not completely effective in preventing fetal death or premature delivery associated with fetal distress (45). Thus, due to its protective effect against estrogen-mediated hepatotoxicity, fenofibrate therapy might be an excellent alternative for treating ICP.

In addition to the fact that its corrective effects on estrogen decrease over time, postmenopausal hormone therapy is known to cause inflammation and liver damage in women (44, 46, 47). To prevent the deleterious effects of estrogen, androgen therapy is now a promising treatment for preventing menopausal problems in women, such as dyslipidemia, inflammation, and decreased libido (48–50). We demonstrated that the repressive effect of PPARα on *Cyp7b1* leads to an increase in plasma androgen in females, suggesting a potential positive role in preventing aging-related problems in women.

In conclusion, we unveiled a molecular and physiological mechanism that explains, at least in part, sex-specific PPARα functions. Due to the crosstalk between this receptor and hepatic steroid signaling, new perspectives are emerging on the treatment of hepatic complications related to estrogen dysfunction in women.

Methods

Animal care and treatment. Mice were maintained under germ-free conditions with a 12-hour light/12-hour dark cycle and free access to food and water. For in vivo treatment, 30 mg WY-14693, 30 mg ethynylestradiol, or 30 mg fenofibrate (all from Sigma-Aldrich) were added directly to 100 ml of water (0.3%) for 2 mice for 5 days. All animal procedures were performed at ZT14 (2100 hours). All animal care and handling procedures were approved by the Commission de Surveillance de l'Expérimention Animale of the Canton de Vaud, Switzerland.

RNA and protein isolation and analysis. Livers from 10- to 12-month-old mice were removed, frozen in liquid nitrogen, and stored at –80°C until use. For liver and human hepatic cell lines (HepG2.C3a), total RNA was extracted with TRIzol (Invitrogen). Mouse and human *CYP7B1* mRNA levels were quantified by SYBR green real-time RT-PCR. One microgram of liver whole-cell RNA was reverse transcribed using random hexamers and Multiscribe reverse transcriptase (Applied Biosystems). The cDNA equivalent of 10 ng of total RNA was amplified by PCR in a 7900HT fast real-time PCR system (Applied Biosystems). The primers used were as follows: mouse Cyp7b1 forward, 5′-GACGATCCTGAAATAGGAGCACA-3′ and reverse, 5′-AATGGTGTTTGCTAGAGAGGCC-3′; mouse C6 forward, 5′-CGAGCCAGTAATCCATACCG-3′ and reverse, 5′-TTTTGTCCTTTCATCCACTGA-3′; mouse 36B4 forward, 5′-ACCTCCTTCTTCCAGGCTTT-3′ and reverse, 5′-CCCACCTTGTCTCCAGTCTTT-3′; human Cyp7b1 forward, 5′-GCTGCAGTCAACAGGTCAAA-3′ and reverse, 5′-CAGTAGTCCCCGGTCTCTGA-3′; human RPL13 forward, 5′-AGGTATGCTGCCCCACAAAA-3′ and reverse, 5′-TGCCGTCAAACACCTTGAGA-3′. Isolation of total hepatic protein was carried out using TNE lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP-40) with protease inhibitor cocktail (Sigma-Aldrich). Antibody against Cyp7b1 protein was from Abcam.

Affymetrix oligonucleotide microarray analysis. Twenty-four WT (12 males and 12 females) and 24 knockout PPARα 129/Sv mice (12 males and 12 females)

approximately 10 to 12 weeks of age, were sacrificed at ZT14 and their livers and hearts quickly removed, frozen on liquid nitrogen, and stored at –80°C. The RNA pools for a given sex of each genotype were assembled by mixing an equal amount of RNA from 4 animals. Affymetrix Mouse Genome 430 2.0 array hybridizations were performed according to the manufacturer's protocol. One sample from a female PPARα-null mouse did not pass hybridization quality control because of high background noise. Normalized expression signals were calculated using the RMA normalization method implemented in the Bioconductor package "affy" (51, 52). Differential hybridized features were identified using the Bioconductor package "limma" (53), in which *P* values were obtained from moderated *t* statistics using empirical Bayesian methods. The *P* values were then adjusted globally for multiple testing with Benjamini and Hochberg's method (54) to control the FDR. Data are available at the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo; accession number GSE14395).

Gene set enrichment analysis. To test for sets of related genes systematically and differentially affected by sex in PPARα-null mice, we performed gene set analysis as previously described (55) but using moderated *t* statistics to calculate the gene set score. The motivation was to use a common statistic (moderated *t*) for both the individual gene and gene set analysis. Efron and Tibshirani (56) showed that the performance of this approach is comparable to the original method. We used a random permutation of the samples as well as randomization of the genes in the gene sets to estimate the null distribution and significance of the gene set scores. The predefined gene sets were obtained from the Molecular Signature Database (MSigDB, http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html; ref. 57). Gene sets from the curated portion of MSigDB (c2) with between 15 and 500 genes were considered for this analysis.

Plasma androgen hormones and bilirubin measurement. Blood was taken using intraorbital procedures. Androstenedione and testosterone were measured using ELISA (IBL International) according to the manufacturer's instructions, with slight modifications. Briefly, 50 μl of plasma were used instead of 25 μl. Plasma bilirubin was measured with a Hitachi robot (Roche) according to the manufacturer's instructions.

Plasmids and cell culture. Human *CYP7B1* promoter constructs were gifts from J.Y.L Chiang (Northeastern Ohio Universities College of Medicine, Rootstown, Ohio, USA). The 3x-PPRE-TK-*Luc* was a gift from R. Evans; Gal4-LBDα was a gift from B. Staels (Université Lille Nord de France, Inserm U545, UDSL, Lille, France); and Gal4-N-terminal domain α construct was a gift from T. Osumi (Graduate School of Life Science, Himeji Institute of Technology, University of Hyogo, Hyogo, Japan). The 5x-UAS-TK-*Luc* was purchased from Promega. Human *Hmgcs2* promoter reporter was a gift from E.F. Johnson (The Scripps Research Institute, La Jolla, California, USA); PPARαΔC13 was generated as described in ref. 58, and myc-SUMO1, HA-SUMO2 and myc-SUMO3 were gifts from C.K. Glass (UCSD, La Jolla, California, USA). The PPARα K185R, K358R, and K449R mutants and mutated GABP-binding sites were made using the Quickchange side-directed mutagenesis kit (Stratagene). The 3x-flag-GABPα and 3x-flag-GABPβ1 expression vectors were gifts from C.R. Mueller (Queen's Cancer Research Institute, Queen's University, Kingston, Ontario, Canada). For cell culture, NIH 3T3 cells were cultured in DMEM medium (GIBCO, Invitrogen) supplemented with 10% FBS without antibiotics.

Transient transfection. The NIH 3T3 mouse fibroblast cell line was transiently transfected with 1 μg of DNA: various regions of the human *CYP7B1* promoter reporter gene, 3x-PPRE-TK-*Luc* and 5x-UAS-TK-*Luc*. Firefly luciferase activity was normalized to CMV *Renilla* luciferase. For the transrepression experiments, plasmids were transfected using the SuperFect reagent (QIAGEN). For siRNA experiments, 50 nM of SMART-pool siRNAs (Dharmacon) were transfected using INTERFERin reagent (Polyplus-transfection) for 48 hours. Efficiency of knockdown was tested by quantitative PCR (Supplemental Figure 6).

Sumoylation assay. The in vitro sumoylation assay was performed as previously described (30). For in vivo sumoylation, liver nuclear proteins were prepared using the NaCl-Urea-NP40 (NUN) procedure (59) with *N*-ethylmaleimide (NEM) in all solutions (Cayman Chemical). PPARα was immunoprecipitated using anti-PPARα antibody (Cayman Chemical); sumoylated PPARα was detected using anti-Sumo1 antibody (Cell Signaling Technology).

Coimmunoprecipitation. The NIH 3T3 cells were transfected with WT or mutant PPARα and flag-GABPα or flag-GABPβ1 using SuperFect reagent. Cells were starved for 1 hour prior to addition of WY-14643 for 1 hour. Whole cell extracts were prepared using TNE lysis buffer. Immunoprecipitates were washed 3 times with TNE lysis buffer then boiled in 1× sample loading buffer for 10% SDS-PAGE.

Bisulfite DNA sequencing. Total DNA was extracted from liver using the Wizard genomic DNA extraction kit (Promega), then 4 μg of DNA was subjected to sodium bisulfite modification (MethylEasy kit; Human Genetic Signatures). Primers used for PCR amplification were as follows: mouse Cyp7b1 forward, 5′-CTCCATCTGGGGAAATCCTA-3′ and reverse, 5′-GGCTCTG-TAGGGAGCGATG-3′ and forward (nested), 5′-ATCCTTTCCCTCTTCCTT-GG-3′ and reverse (nested), 5′-TGCACAGTGCCCTCTCTTC-3′; human Cyp7b1 forward, 5′-CGCTGGGTCGTACCTAAGAG-3′ and reverse, 5′-AGGAATGTCACCGTGGTCTC-3′ and forward (nested), 5′-CCCTTCTA-ACCCCTTCCTTG-3′ and reverse (nested), 5′-AGGAATGTCACCGTG-GTCTC-3′. Products from the second round of nested PCR were subcloned into the pGEM-T-Easy vector (Promega) and the clones sequenced. The frequency of CpG methylation at the Sp1 site was calculated.

ChiP. ChIP and double ChiP (re-ChiP) were performed as described previously (60, 61). The mouse Cyp7b1 forward primer used was 5′-CTCCATCTGGGGAAATCCTA-3′, and the reverse primer was 5′-GGC-TCTGTAGGGAGCGATG-3′. Human Cyp7b1 forward primer was 5′-CGCTGGGTCGTACCTAAGAG-3′, and the reverse primer was 5′-AGGAAT-GTCACCGTGGTCTC-3′. Antibodies were purchased from Abcam (GABPα), Cayman Chemical (PPARα), and Santa-Cruz Biotechnology Inc. (Sp1).

Statistics. Statistical analysis was performed with 2-tailed Student's *t* test. Quantitative data are expressed as mean ± SEM. A *P* value less than 0.05 was considered significant.

Acknowledgments

We thank L. Michalik and N. Rotman for their comments on the manuscript. We are most grateful to D. Chollet and P. Descombes (Genomics Platform of the NCCR program Frontiers in Genetics) for their invaluable help with the Affymetrix transcription profiling studies; U. Schibler (University of Geneva) for help designing the transcription profiling experiment and stimulating discussions; M. Carrard (CIG, University of Lausanne) for measuring plasma bilirubin levels; C. Peter (CIG, University of Lausanne) for DNA sequencing; and V. Zoete (Molecular Modeling Platform, University of Lausanne) for providing Supplemental Figure 2. We are grateful to J.Y. Chiang, R.M. Evans, C.K. Glass, E.F. Johnson, C.R. Mueller, T. Osumi, and B. Staels for providing plasmids. This work was supported by the Swiss National Science Foundation, the Etat de Vaud, and the National Research Center "Frontiers in Genetics."

Received for publication February 24, 2009, and accepted in revised form July 1, 2009.

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