

MOL 13375

# **Regulation and Binding of Pregnane X Receptor by Nuclear Receptor Corepressor SMRT**

David R. Johnson,\* Chia-Wei Li,\* Liuh-Yow Chen, Jagadish C. Ghosh, and J. Don Chen

Department of Pharmacology, University of Medicine & Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854

\*Co-first authors

J.D.C. (Corresponding Author) Department of Pharmacology, University of Medicine & Dentistry of New Jersey, 661 Hoes Lane, Piscataway, New Jersey, 08854; Email: chenjd@umdnj.edu; Tel: 732-235-3292.

D.R.J., Department of Medicine, Division of Rheumatology, University of Massachusetts Medical School, Worcester, MA 01605

L.Y.C., Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030

J.C.G., Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01605

MOL 13375

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**Corresponding Author:**

J. Don Chen, Ph.D. Department of Pharmacology, University of Medicine & Dentistry of New Jersey, 661 Hoes Lane, Piscataway, New Jersey, 08854; Email: chenjd@umdnj.edu; Tel: 732-235-3292, Fax: 732-235-3974.

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**Abbreviations:** PXR, Pregnane X Receptor; SMRT, Silencing Mediator of Retinoid and Thyroid Hormone Receptors; CYP, Cytochrome P450; Rif, Rifampicin; CTZ, Clotrimazole; CAR, Constitutive Androstane Receptor; PCB, Polychlorinated biphenols; PCN, pregnenolone-16 $\alpha$ -carbonitrile; DEX, dexamethasone; ONPG, o-nitrophenyl-beta-galactopyranoside; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride hydrate; RAC3, Receptor-Associated Coactivator 3.

MOL 13375

## ABSTRACT

The pregnane X receptor (PXR) is an orphan nuclear receptor predominantly expressed in liver and intestine. PXR coordinates hepatic responses to prevent liver injury induced by environmental toxins. PXR activates cytochrome P450 (CYP) 3A4 gene expression upon binding to rifampicin (Rif) and clotrimazole (CTZ) by recruiting transcriptional coactivators. Whether and how PXR regulates gene expression in the absence of ligand remains unclear. Here we analyzed interactions between PXR and the nuclear receptor corepressor SMRT (silencing mediator of retinoid and thyroid hormone receptors) and determined the role of SMRT in regulating PXR activity. We show that SMRT interacts with PXR in GST pull down, yeast two-hybrid and mammalian two-hybrid assays. The interaction is mediated through the ligand-binding domain of PXR and the SMRT ID2 domain. The PXR-SMRT interaction is sensitive to species-specific ligands, and Rif causes an exchange of the corepressor SMRT with the p160 coactivator RAC3. Deletion of the PXR's AF2 helix enhances SMRT binding, and abolishes ligand-dependent dissociation of SMRT. Coexpression of PXR with SMRT results in colocalization at discrete nuclear foci. Finally, transient transfection assays show that SMRT may inhibit PXR's transactivation of the CYP3A4 gene, and that silencing of SMRT by siRNA may enhance Cyp3A4 expression. Taken together, our results suggest that the corepressor SMRT may bind to and regulate the transcriptional activity of PXR.

MOL 13375

## INTRODUCTION

Cytochrome P450 3A4 (CYP3A4) is a member of the Cytochrome P450 superfamily of drug-metabolizing enzymes. CYP3A4 makes up a large portion of the total CYP concentration in liver and intestine (Guengerich, 1999). CYP3A4 metabolizes a vast array of endogenous, therapeutic, and xenobiotic chemicals, resulting in detoxification of 50-60% of all prescribed therapeutic drugs. CYP3A4 protein activity can be significantly increased by treatments with a wide range of chemical inducers. Pregnane X (or steroid and xenobiotics) receptor (PXR, SXR, NR1I2) binds to the promoter of Cyp3a4 gene and regulates its expression (Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998). PXR is a promiscuous nuclear receptor for xenobiotic chemicals and endogenous metabolites, and it coordinates hepatic responses with the constitutive androstane receptor (CAR, NR1I3) to prevent liver injury (Stedman et al., 2005). The human PXR (hPXR) is a 434-amino acid, 50-kDa protein primarily expressed in liver and intestine (Lehmann et al., 1998), the same organs that express CYP3A4. PXR heterodimerizes with the retinoic X receptor (RXR) on several DNA elements, including direct repeats separated by 3 nucleotides (DR3), everted motifs separated by 6 nucleotides (ER6), and inverted motifs separated by 6 or 8 nucleotides (IR6, IR8) (Bertilsson et al., 1998; Blumberg et al., 1998; Kast et al., 2002; Kliewer et al., 1998; Lehmann et al., 1998). Once activated, the PXR/RXR heterodimer recruits nuclear receptor coactivators like the p160 proteins to form a multiprotein complex that leads to the initiation of gene transcription (Kliewer et al., 1998; Lehmann et al., 1998).

MOL 13375

PXR can be activated by a diverse group of chemical compounds. The xenobiotic activators include the antibiotic rifampicin (Rif) (Goodwin et al., 1999; Lehmann et al., 1998), the antimycotic drug clotrimazole (CTZ) (Bertilsson et al., 1998; Lehmann et al., 1998), the chemotherapeutic agent paclitaxel (Synold et al., 2001), the antigluco-corticoid RU-486 (Bertilsson et al., 1998), and environmental contaminants such as polychlorinated biphenols (PCBs) (Schuetz et al., 1998). Some PXR activators are dehydroepiandrosterone (DHEA) and metabolites (Ripp et al., 2002), the bile acid lithocholic acid (LCA) (Staudinger et al., 2001), and the 5 $\beta$ -pregnane-3,20-dione and other pregnane derivatives (Bertilsson et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998). Some of these chemicals produce a species-specific effect on CYP3A up-regulation via PXR activation. This is most apparent with Rif and CTZ, which activate hPXR but not mouse PXR (mPXR), whereas the pregnenolone-16 $\alpha$ -carbonitrile (PCN) and dexamethasone (DEX) activate mPXR but not hPXR (Jones et al., 2000; Lehmann et al., 1998; Xie et al., 2000a). Most of these chemical inducers have been well characterized as having up-regulated CYP3A4.

Whether PXR plays a role in regulating gene expression in the absence of ligand remains controversial. It was reported that the basal expression of the mouse *Cyp3a11* (homologue of the human *Cyp3a4*) was significantly increased in PXR knockout mice (Staudinger et al., 2001). However, such an increase was not observed in another knockout mouse line (Xie et al., 2000a). If PXR is capable of repressing gene expression in the absence of ligand, it is possible that transcriptional repression by PXR may be due to protein-protein interaction with nuclear receptor corepressors such as SMRT and/or N-CoR (Chen and Evans, 1995; Horlein et al., 1995; Park et al., 1999), which facilitate the formation of a nuclear protein complex that represses transcription

MOL 13375

by recruiting histone deacetylases (Ordentlich et al., 2001). To understand whether and how PXR regulate basal expression of Cyp3a4, we have determined the intrinsic transcriptional activity of PXR and examined its interaction with SMRT. SMRT is a 270-kDa nuclear protein that interacts with nuclear receptors by two receptor interacting domains, ID-1 and ID-2, which contain extended L-XXX-L-XXX-I/L corepressor motifs (CoRNR motifs) (Hu and Lazar, 1999; Wu et al., 2001).

Here we show that PXR and SMRT form a tight complex *in vitro* and in yeast and cultured mammalian cells, and ligand binding causes dissociation of this complex. PXR selectively interacts with the SMRT ID2 motif, and the AF2 helix of PXR is involved in the mechanism of SMRT dissociation. We found that PXR colocalizes with SMRT at discrete nuclear foci. Overexpression of SMRT also repressed PXR activation on CYP3A4 promoter, and SMRT knockdown using siRNA up-regulated PXR transcriptional activity. These results suggest that the corepressor SMRT may bind to and regulate the transcriptional activity of PXR.

## **MATERIALS AND METHODS**

### **Chemicals and Reagents**

Rifampicin (Rif), clotrimazole (CTZ), pregnenolone-16 $\alpha$ -carbonitrile (PCN), dexamethasone (DEX), and *all-trans* retinoic acid were purchased from Sigma (St. Louis, MO). The anti-SMRT monoclonal antibody was a generous gift from GeneTex, Inc. (San Antonio, TX).

MOL 13375

The anti-HA polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals used were of the highest grade.

### **Plasmids Constructs**

Full-length pSG5-PXR $\Delta$ ATG was a generous gift from Steve Kliewer (Southwestern Medical Center, Dallas, TX). PXR was subcloned into pGBT9, pCMX-HA, and pCMX-Gal4 plasmids by restriction enzyme digestion (NEB, Beverly, MA). The  $\Delta$ AF2 mutant (aa 1-422), C-terminal fragment (C-term, aa 141-434), and N-terminal fragment (N-term, aa 1-204) of hPXR were generated by PCR and subcloned into various vectors. The human SMRTe (Park et al., 1999) corresponds to the SMRT $\tau$  isoform (GenBank # AY965853). The cSMRT, ID1, ID2, cSMRT-mID1, cSMRT-mID2, and cSMRT-mID1/2 were as previously described (Ghosh et al., 2002). The 13-kb CYP3A4-luc reporter was a generous gift of Christopher Liddle (University of Sydney at Westmead Hospital, Westmead, Australia).

### **GST-Pull Down Assay**

The GST-fusion proteins of cSMRT, ID1, ID2, mID1, mID2, and mID1/2 were as previously described (Ghosh et al., 2002). The various nuclear receptor proteins were synthesized in rabbit reticulocyte lysate and radiolabeled with [<sup>35</sup>S]-methionine using the TnT® Quick Coupled Transcription/Translation kit (Promega, Madison, WI). Glutathione Sepharose bead-bound GST-fusion protein (10  $\mu$ l) was blocked with 10 mg/ml bovine serum albumin (BSA) at room temperature for five minutes prior to mixing with BSA-blocked [<sup>35</sup>S]-receptor proteins.

MOL 13375

Proteins were mildly shaken at 4°C overnight in binding buffer (20 mM HEPES, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl<sub>2</sub>, 0.05% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM methionine, and 1 µg/ml BSA). The protein mixture was washed 3 times with fresh binding buffer, and the beads were collected by centrifugation at 3000 rpm for five minutes. After boiling for 10 minutes in SDS sample buffer, bound beads were subject to SDS-PAGE electrophoresis. Gels were then stained with Coomassie blue, dried, and detected by autoradiography.

### **Yeast Two-Hybrid Assay**

The yeast Y190 cells were co-transformed with indicated plasmids and 4 µg single-stranded salmon sperm DNA according to the manufacturer's protocol (Clontech, Palo Alto, CA). The transformed yeast cells were spread on selection plates and incubated at 30°C for 2 days. Colonies were picked from each plate and grown in tryptophan and leucine-deficient media for 48 hr at 30°C. Two aliquots (100 µl each) from each sample were taken and added to fresh selection media. For each sample, one aliquot received ligands and the other aliquot received vehicle control (DMSO). Samples were then shaken in a 30°C incubator for an additional 48 hr. Yeast cells were then harvested in Z-buffer containing 0.27% β-mercaptoethanol and analyzed for β-galactosidase activity by liquid assay using ONPG (o-nitrophenyl-beta-galactopyranoside) as substrate as previously described (Chen et al., 1996).

Statistical analyses were conducted by one-way ANOVA (Analysis of Variance) followed by post-hoc pair-wise comparisons of sample means via the conservative Tukey's HSD (Honestly Significant Difference) or the liberal LSD (Least Significant Difference) test. The

MOL 13375

ANOVA procedure was performed by the SAS statistical software (SAS Institute, Inc. Minneapolis, MN) or online at <http://faculty.vassar.edu/lowry/VassarStats.html> for samples less than five groups.

### **Immunofluorescence Colocalization Analysis**

COS-7 cells were seeded on coverglasses in 12-well plates one day prior to transfection by the standard calcium phosphate precipitation method. Twenty-four hours after transfection, cells were fixed in a methanol:acetone (1:1, v/v) mixture and processed for indirect immunofluorescence staining as previously described (Chen and Chen, 2003). Antibodies used against HA-tag and SMRT were from Santa Cruz (Santa Cruz, CA) and GeneTex (San Antonio, TX), respectively. After extensive washing, FITC-conjugated goat anti-rabbit and rhodamine-conjugated goat anti-mouse antibodies were added directly to the coverglasses. Cell nuclei were co-stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride hydrate; Sigma Chemical Co.). Coverglasses were mounted with a ProLong Antifade kit (Molecular Probes, Eugene, OR). Standard epifluorescence microscopy was performed on an inverted epi-fluorescence microscope (Axiovert 200, Zeiss, Thornwood, NY). The images were taken with a cool charge-coupled device camera (AxioCam) and analyzed by the Axiovision software (Zeiss).

### **Cell Culture and Transient Transfections**

Human Embryonic Kidney (HEK) 293 cells were maintained in DME medium containing 10% fetal bovine serum (FBS) and 5 µg/ml gentamycin (GibcoBRL, Carlsbad, CA).

## MOL 13375

Cells ( $2 \times 10^5$ ) were seeded in 12-well plates and transfected by the standard calcium phosphate method. The amounts of each DNA used in the transfection were as following unless otherwise stated: reporter plasmid 1  $\mu\text{g}$ , hPXR expression vector or other nuclear receptor 0.5  $\mu\text{g}$ , SMRT or other cotransfected expression vector 0.5  $\mu\text{g}$ , the internal control pCMX- $\beta$ -galactosidase 0.5  $\mu\text{g}$ , and the total amount of DNA was brought up to 2.5  $\mu\text{g}$  per well by pGEM7. Transfections proceeded overnight, and precipitates removed by washing cells with phosphate buffered saline (PBS) supplemented with calcium and magnesium. Cells were cultured for an additional 48 hrs, after which the cells were harvested and analyzed for luciferase activity and  $\beta$ -galactosidase activities as previously described (Chen et al., 1996).

HepG2 cells were maintained in phenol red-free DME medium containing 10% FBS and antibiotic/antimycotic (GibcoBRL, Carlsbad, CA). HepG2 cells ( $5 \times 10^5$ ) were seeded in 6-well plates and transfected with FuGENE-6 (Roche Diagnostic, Indianapolis, IN). Transfection with pSG5-PXR or empty vector, pCMX-hSMRTe, CYP3A4-luciferase gene promoter construct,  $\beta$ -galactosidase, and pGEM7 was allowed to proceed for 4 hours in serum-reduced Opti-MEM (GibcoBRL, Carlsbad, CA). The transfection media was then removed and replaced with phenol red-free DME media containing 10% charcoal-stripped FBS. Cells were then treated with vehicle control (DMSO) or 10  $\mu\text{M}$  Rif and incubated for an additional 48 hrs. Cells were then harvested and analyzed for luciferase and  $\beta$ -galactosidase activities.

The effect of SMRT siRNA on PXR transcriptional activity was analyzed in HEK293 and HepG2 cells. The SMRT and a negative control siRNAs were expressed from the pLL3.7 lentiviral vector (Rubinson et al., 2003). The targeted sequences for the SMRT siRNA is

MOL 13375

GGGTATCATCACCGCTGTG starting at nucleotide 5778 and amino acid 1875 of SMRT (Ishizuka and Lazar, 2003), and GGTCCGGCTCCCCCAAATG for the negative control siRNA that has no homologous sequences in the vertebrate transcriptome.

## RESULTS

### SMRT Interacts with PXR and Other Orphan Nuclear Receptors

The full-length hPXR contains 434 amino acids bearing 86% similarity with the mPXR (Kliwer et al., 1998). The SMRT sequence corresponds to that in hSMRTe (Park et al., 1999). The deletion and site-directed mutants of hPXR and SMRT used in this study are summarized in Fig. 1. We first tested the binding of hPXR and mPXR to GST-cSMRT by GST pull down assay (Fig. 2). As expected, both hPXR and mPXR formed a stable complex with cSMRT. Similarly, we observed strong bindings of cSMRT with orphan nuclear receptors hLXR $\beta$  (NR1H2) (Hu et al., 2003), mCOUP-TF1 (NR2F1) and mCOUP-TF2 (NR2F2) (Bailey et al., 1998; Shibata et al., 1997), v-erbA (Chen and Evans, 1995), and weakly with Rev-erbA $\alpha$  (NR1D1) (Downes et al., 1996) and mCAR. The cSMRT also bound strongly to the human farnesoid X-activated receptor (hFXR, NR1H4), but not the rat hepatocyte nuclear factor 4 (rHNF4), the *Drosophila* ultraspiracle (dUSP) or the hCAR. These data show that SMRT interacts with PXR and several orphan nuclear receptors.

### PXR Binds to the ID2 Domain of SMRT

MOL 13375

To investigate how SMRT interacts with PXR, we analyzed the interacting surface using SMRT deletion and site-directed mutants. Both hPXR and mPXR bound to the SMRT ID2 fragment strongly, but not to the ID1 fragment (Fig. 3A). Similarly, mPPAR $\gamma$  and hLXR $\beta$  bound to the ID2 fragment preferentially, while hRAR $\alpha$  preferred ID1. These results show that, while hRAR $\alpha$  prefers SMRT ID1, PXR, PPAR $\gamma$  and LXR $\beta$  interact with the ID2 of SMRT.

The contribution of each corepressor helix on SMRT-PXR interaction was further analyzed using site-directed mutants: mID1, mID2, and mID1/2 in the context of cSMRT (Fig. 3B). Again, both hPXR and mPXR strongly bound to cSMRT and ID2 instead of ID1. Consistently, the mID2 mutation weakened the interaction more profoundly than the mID1 mutation. Although the mID1 mutation also had an effect on PXR binding with cSMRT, this might be due to allosteric effect on the ID2-PXR interaction or a conformational change of cSMRT. The double mID1/2 mutation did not completely abolish PXR binding with cSMRT, suggesting that there could be additional PXR binding sites on SMRT. In contrast to PXR, the mID1 and mID1/2 mutations completely abolished hRAR $\alpha$  binding with cSMRT, whereas the mID2 and mID1/2 mutations completely abolished hLXR $\beta$  binding (Fig. 3B, right panel). In both cases, mutation of the non-binding ID (i.e., mID2 for hRAR $\alpha$ , or mID1 for hLXR $\beta$ ) had little or no effect. These data indicate that SMRT utilizes the ID2 helix in interaction with PXR.

We next determined the region within PXR that are responsible for interacting with cSMRT. Three hPXR deletion mutants were generated and tested for their bindings with cSMRT, SMRT-ID1, and SMRT-ID2 fragments (Fig. 3C). We found that the  $\Delta$ AF2 bound better to the cSMRT and ID2 fragments than to the wild type hPXR. The separated N-term and C-term

MOL 13375

fragments showed poor binding to cSMRT, ID1, or ID2 in this assay. In contrast, deletion of the N-terminus 39 amino acids from hPXR ( $\Delta$ AF1) had little effect on cSMRT or ID2 binding. These data suggest that both the LBD and DBD of hPXR are essential for strong binding with SMRT *in vitro*, while the AF2 helix and AF1 region are not essential.

### Effects of Ligands on PXR-SMRT Interaction *in vitro* and in Yeast Cells

Most nuclear receptors undergo conformational changes to cause release of corepressors and recruitment of coactivators upon ligand binding. Therefore, the effects of PXR ligands on PXR-SMRT interaction were examined. Increasing concentrations of Rif on hPXR binding to GST-cSMRT were examined by GST pull down assay. We observed a Rif dose-dependent dissociation of cSMRT at 10  $\mu$ M and higher concentrations (Fig. 4A). This dissociation of hPXR-cSMRT complex was specific because Rif at all concentrations tested had no effect on hLXR $\beta$ -cSMRT interaction. Similar results were observed using the ID2 fragment of SMRT (Fig. 4B), suggesting that Rif binding is indeed able to disrupt PXR-SMRT interaction *in vitro*.

We further analyzed the PXR-SMRT interaction and the effects of ligands by yeast two-hybrid assay. Consistent with the *in vitro* data, hPXR interacted strongly with SMRT in the absence of ligand (Fig. 5A). In comparison with SMRT, hPXR did not interact with hNCoR, consistent with a previous report (Takeshita et al., 2002). Rif at 10  $\mu$ M concentration caused a significant dissociation (approximately 2-fold) of the PXR-SMRT complex in this assay ( $p < .01$ ). In contrast, Rif enhanced the PXR-RAC3 association by approximately 3.3-fold ( $p < .01$ ).

MOL 13375

These data suggest that the unliganded PXR preferentially interacts with SMRT, whereas liganded PXR would prefer RAC3.

The ability of Rif to dissociate PXR-SMRT complex in the yeast two-hybrid assay was further analyzed in greater details by measuring the effects of Rif over a 5 orders of magnitude concentration range. As expected, Rif diminished the PXR-SMRT interaction and increased the PXR-RAC3 interaction in a concentration-dependent manner (Fig. 5B). When the Rif concentration is equal to or below 1  $\mu\text{M}$ , hPXR preferentially interacts with SMRT; whereas PXR binds stronger to RAC3 at 10  $\mu\text{M}$  or higher concentration of Rif. We estimated that Rif would cause an exchange of corepressor to coactivator at approximately 7  $\mu\text{M}$  in this assay. This concentration of Rif corresponds well to the  $\text{EC}_{50}$  that produced 50% of the maximal SMRT dissociation and RAC3 association with hPXR.

It is known that different ligands exert species-specific effects on PXR activation. Therefore, we tested the effects of various known PXR ligands on hPXR-SMRT interaction in yeast two-hybrid assay (Fig. 5C). Consistent with previous data, Rif at 10  $\mu\text{M}$  concentration caused an approximately 2.3-fold dissociation of the hPXR-SMRT complex ( $P < .01$ ). The human PXR-specific ligand CTZ caused an even greater dissociation (13.7-fold) of the hPXR-SMRT complex. In contrast, the mouse PXR-specific ligands PCN and DEX had no significant effect on the hPXR-SMRT interaction ( $P > .1$ ). These data confirms the species-specific effects of PXR ligands on disrupting PXR-SMRT interaction in yeast cells.

MOL 13375

The PXR fragments responsible for SMRT interaction and the effects of Rif on the interaction were then determined by the same assay (Fig. 5D). Again, Rif caused a consistent and significant 3-fold dissociation of the wild-type PXR-SMRT complex ( $p < .01$ ). The  $\Delta$ AF2 and C-term fragments interacted with cSMRT approximately 40-50% stronger than the full-length hPXR ( $p < .01$ ). Furthermore, Rif caused a significant dissociation of the C-term-SMRT interaction ( $p < .01$ ), while it had no effect on the  $\Delta$ AF2-SMRT interaction. The N-term fragment showed no interaction with cSMRT in this assay. These data suggest that the PXR-SMRT interaction is sensitive to Rif in yeast cells, that PXR interacts with SMRT through the C-terminal domain, and that the AF2 helix of PXR inhibits SMRT binding and is essential for Rif-dependent dissociation of SMRT.

### **PXR Colocalizes with SMRT in Mammalian Cells**

SMRT is localized in the nuclei of mammalian cells in a speckle pattern (Downes et al., 2000; Park et al., 1999; Wu et al., 2001). To further demonstrate the PXR-SMRT interaction, we tested colocalization of cotransfected hPXR and SMRT by double immunofluorescence staining (Fig. 6). When expressed alone, hPXR displays diffuse nuclear staining like many nuclear receptors (Kawana et al., 2003). Interestingly, when coexpressed with SMRT, the wild type,  $\Delta$ AF2 and C-term hPXR colocalized with SMRT at many nuclear foci (Fig. 6), indicating an association in the cultured mammalian cells. The N-term hPXR and hCAR showed no evidence of colocalization with SMRT. These data suggest that hPXR could interact with SMRT and be recruited to SMRT nuclear foci in mammalian cells.

MOL 13375

## SMRT Inhibits PXR Transcriptional Activity

To determine whether PXR regulates transcription in the absence of ligand, we analyzed its intrinsic transcriptional activity using Gal4 DBD-PXR fusion proteins. The full-length and various PXR domains were fused with Gal4 DBD, and the effects on a Gal4-dependent luciferase reporter were analyzed in HEK293 cells by transient transfection. As shown in Fig. 7A, the full-length PXR and all PXR fragments displayed significant repression activity in comparison with Gal4 DBD alone ( $p < .01$ ), although differences among various PXR fragments were less significant. We observed an approximately 7.9-fold repression by the full-length hPXR, a 10.2-fold repression by  $\Delta$ AF2, and 4.8-fold and 3.7-fold repression by C-term and N-term fragments, respectively. The stronger repression observed with  $\Delta$ AF2 correlates with its stronger interaction with SMRT (Fig. 5D), suggesting that repression by PXR is in part mediated by SMRT. In contrast, repression by the PXR N-term fragment could be due to interactions with other corepressors as the N-term fragment does not interact with SMRT. Rif partially reversed the repression mediated by full-length hPXR and C-term fragment by approximately 2.1-fold ( $p < .05$ ) and 2.8-fold ( $p < .01$ ), respectively; whereas Rif had no effect on  $\Delta$ AF2 and N-term fragment as expected as they do not contain ligand binding domain. These data strongly suggest that hPXR contains multiple repression domains, and Rif could partially release the repression activity mediated by PXR LBD domain.

Next, we examined the PXR-SMRT interaction in COS-7 cells by mammalian two-hybrid assay (Fig. 7B). Without ligand, VP16-hPXR interacted with Gal4-cSMRT by 2.3-fold ( $p < .05$ ). Deletion of AF2 helix from PXR enhanced the PXR-SMRT interaction by another 2.4-

MOL 13375

fold ( $p < .01$ ), suggesting that  $\Delta$ AF2 interacts with SMRT stronger than wild type PXR. Addition of Rif (10  $\mu$ M) disrupted the PXR-SMRT association by 2.9-fold ( $p < .05$ ), while Rif had no significant effect on the SMRT- $\Delta$ AF2 interaction ( $P > .05$  in ANOVA and Tukey HSD test). As control, cSMRT interacted with hRAR $\alpha$  strongly (4.6-fold), and *all-trans* retinoic acid disrupted the interaction completely. These results support our hypothesis that PXR could interact with SMRT in mammalian cells, that such an interaction is sensitive to Rif treatment, and that deletion of the AF2 helix enhanced PXR-SMRT interaction.

The functional consequence of PXR-SMRT interaction on transcriptional activity of hPXR was determined by transient transfection using a human primary hepatoma cell line HepG2. HepG2 cells were transfected with full-length hPXR, full-length SMRT, and a 13kb CYP3A4 promoter-driven luciferase reporter (Goodwin et al., 1999). In the absence of ligand, overexpression of hPXR alone activated the CYP3A4 promoter activity by approximately 6.6-fold ( $p < .01$ , Fig. 7C). Similar levels of PXR-dependent activation was observed previously (Takeshita et al., 2002). This effect may be due to endogenous PXR activators. Transfection of SMRT alone had a slight but non-significant effect on the CYP3A4 promoter activity. However, cotransfection of SMRT significantly attenuated the hPXR-mediated activation of the CYP3A4 promoter by 51% ( $p < .01$ ). These results suggest that SMRT could potentially inhibit hPXR-mediated transactivation of the CYP3A4 promoter in cultured hepatic cells.

To further support a role of SMRT in regulating CYP3A4 expression, we silenced SMRT expression in HEK293 cells by shRNA and analyzed the CYP3A4 promoter activity (Fig. 7D). As expected, Rif significantly activated the reporter gene (3.8-fold,  $p < .01$ ), and the control

MOL 13375

shRNA had no effect on CYP3A4 activation ( $p = .15$ ). In contrast, the SMRT shRNA enhanced Rif-activated reporter activity by 4.7-fold ( $p < .01$ ). Western blot analysis showed that the control shRNA had no effect on SMRT silencing, while the SMRT shRNA blocked the expression of a cotransfected SMRT fragment (aa 475-981) (Fig. 7D, inset). These data suggest that the effects of SMRT shRNA are due to silencing of endogenous SMRT expression and that SMRT silencing may lead to enhancement of PXR-stimulation of CYP3A4 promoter in hepatic cells.

## DISCUSSION

In this study, we investigated the physical and functional interactions between the orphan nuclear receptor PXR and the nuclear corepressor SMRT. We found that SMRT utilizes the ID2 domain to interact with the LBD of PXR. The interaction between PXR and SMRT could be disrupted by PXR ligands such as Rif. The AF2 helix of PXR appears to be inhibitory to SMRT binding. We also found that hPXR could be recruited by SMRT to discrete nuclear foci and showed that SMRT is inhibitory to the transcriptional activity of PXR. Taken together, these data provide strong evidence that SMRT plays a role in regulating the transcriptional activity of the orphan nuclear receptor PXR.

Using a GST pull-down assay, a direct interaction between PXR and SMRT was first observed in this study. This finding is consistent with previous reports using mammalian two-hybrid assays (Synold et al., 2001; Takeshita et al., 2002). In addition to PXR, SMRT also interacts with LXR $\beta$  and FXR, but not with CAR. The binding with LXR $\beta$  is consistent with a previous report (Hu et al., 2003). SMRT binds promiscuously to several nuclear receptors,

MOL 13375

utilizing either the ID1 or ID2 domain (Cohen et al., 2000; Ghosh et al., 2002; Li et al., 1997). We found that PXR and LXR $\beta$  both prefer ID2 (Fig. 3A). The mechanism for such a preference likely involves molecular recognitions between the cofactor-binding pocket on the LBD of the receptor, and specific configuration of the ID2 helix. Since disruption of the ID2 helix did not completely block PXR binding (Fig. 3A), we speculate that SMRT may contain additional binding surfaces for PXR and/or the mechanism of SMRT-PXR interaction may be different from other nuclear receptors.

The corepressor helix is known to bind to a site in the LBD of the receptor that overlaps with the coactivator-binding pocket formed by helices 3, 4 and 5 (Gampe et al., 2000; Shiau et al., 1998). It is known that the AF2 helix of the receptor could interfere with the corepressor binding, due to its ability to adopt an “active” conformation, which blocks corepressor binding. Similar to other nuclear receptors, deletion of the AF2 helix stabilizes PXR-SMRT interaction. Truncation of hPXR into the C-term LBD and N-term DBD fragments appears to diminish SMRT binding in the GST pull down assay (Fig. 3C), whereas strong interaction between the C-term LBD and SMRT could be observed in yeast (Fig. 5D) and by colocalization study (Fig. 6). It is possible that a proper intramolecular folding of the LBD in reticulocyte lysate requires the DBD, whereas chaperon proteins present in yeast and mammalian cells might compensate for such a requirement. Alternatively, the heterologous Gal4 DBD used in the two-hybrid assays might compensate for such a requirement.

The effect of PXR ligands on the PXR-SMRT interaction was also investigated. Activation of a nuclear receptor by its agonist ligand usually causes a release of corepressors,

MOL 13375

and allows recruitments of coactivators. Ironically, a prior report using mammalian two-hybrid assays showed that Rif enhanced the PXR-SMRT interaction by approximately 2-fold (Takeshita et al., 2002). In contrast, we found that Rif causes dissociation of the PXR-SMRT complex in multiple assays. This finding is in agreement with an earlier result showing paclitaxel disrupts PXR-SMRT interaction (Synold et al., 2001). We also found that the Rif-dependent dissociation of PXR-SMRT complex requires the AF2 helix of PXR, implicating a prerequisite conformational change of the AF2 helix before the release of corepressor. The EC<sub>50</sub> of Rif required for 50% dissociation of SMRT and 50% association of RAC3 was approximately 7 μM, similar to the concentration required for activation of PXR in mammalian cells (Lehmann et al., 1998; Xie et al., 2000b). We noticed that, in the mammalian two-hybrid assay, Takeshita et al. (2002) used the Gal4 DBD-PXR fusion, which could be activated by Rif. Such activation might have obscured the interpretation of their two-hybrid data. Both our study and the study by Synold et al. (2001) used the VP16-hPXR fusion, which could not activate the reporter. In addition to the Rif-induced dissociation of PXR-SMRT complex, the anticipated species-specific effects were also observed with hPXR and mPXR ligands in our yeast assay (Fig. 5C). These results strongly suggest that Rif disrupts PXR-SMRT interaction, resulting in release of corepressors and recruitment of coactivators.

The association of PXR and SMRT in mammalian cells was also demonstrated by immunofluorescence colocalization study (Fig. 6). Such colocalization reflects an interaction of the two proteins in mammalian cells. Both SMRT and PXR are known to shuttle between nucleus and cytoplasm under various conditions (Hong and Privalsky, 2000; Kawana et al., 2003; Zelko et al., 2001). Although recent studies have shown that unliganded endogenous mPXR may

MOL 13375

be located in the cytoplasm of mouse liver (Kawana et al., 2003; Squires et al., 2004), it is clear that when expressed in mammalian cells, PXR is localized in a diffuse pattern in the nucleus (Kawana et al., 2003; Zelko et al., 2001). SMRT is concentrated at discrete nuclear foci when expressed in culture cells (Hong and Privalsky, 2000; Park et al., 1999; Wu et al., 2001). Coexpression of SMRT and PXR resulted in recruitment of PXR into the SMRT nuclear foci (Fig. 6). This effect was most apparent with the  $\Delta$ AF2 mutant and the C-term fragment of hPXR. The recruitment of hPXR to SMRT nuclear foci appears to rely on interaction between PXR and SMRT. The function of the SMRT nuclear foci is currently unknown; although a prior report suggested that they could be sites of deacetylation (Downes et al., 2000). The accumulation of PXR to the SMRT nuclear foci might then serve a function to repress gene expression by tethering target promoters to the foci. Alternatively, PXR might be a substrate of the deacetylase, or that SMRT nuclear foci might be storage sites for PXR and other nuclear receptors.

The above data suggest that PXR might repress basal transcription in the absence of ligand. Indeed, Gal4 DBD-hPXR repressed basal promoter activity of a Gal4-dependent reporter (Fig. 7A). We found that removal of the AF2 helix slightly enhanced such a repressor activity of hPXR, correlating with an enhanced interaction with SMRT. Similarly, the C-term fragment also repressed basal transcription and interacted with SMRT. Both the full-length and C-term fragment of hPXR were able to stimulate transcription in response to Rif, correlating with the ability of Rif to dissociate their interactions with the corepressor. In contrast, the  $\Delta$ AF2 mutant could not respond to Rif because Rif could not cause dissociation with SMRT. Finally, we speculate that the repressor activity of the N-term fragment of hPXR might be due to interaction with other unknown corepressors.

MOL 13375

A previous PXR knockout mice study demonstrated that the *Cyp3a11* mRNA in the liver of the knockout mice was increased by ~ 4-fold compared to wild-type mice (Staudinger et al., 2001), although another independent PXR knockout mice study concluded that loss of PXR did not alter the basal expression of *Cyp3a* (Xie et al., 2000a). While the basis for this difference is unknown, the data presented by Xie et al. (2000a) seems to show a slight increase of *Cyp3a11* basal expression in the liver of the PXR-null mice (Fig. 1C in that study). It is possible that such a difference might arise from variations among different mouse lines, and PXR might differentially regulate basal expression of its target genes in different tissues. We further analyzed the effect of SMRT on PXR-regulated human *Cyp3a4* promoter activity in hepatic HepG2 cells. Transfection of hPXR enhanced the reporter activity by approximately 6.6-fold (Fig. 7C). The reporter we used contains a 13-kb promoter/enhancer element, thus its regulation must be under stringent control by other transcription factors such as the CCAAT enhancer-binding protein and hepatocyte nuclear factor-3, as described in a prior study (Rodriguez-Antona et al., 2003). Alternatively, it is possible that our stripped serum or culture media, or the HepG2 cells, might contain endogenous PXR ligands, because PXR is known to be promiscuous on the selection of ligands. Nonetheless, under this low level of activation, SMRT was able to suppress the activation of the *Cyp3a4* promoter by approximately 50% (Fig. 7C). In addition, silencing of SMRT expression by siRNA greatly enhanced *Cyp3a4* promoter activity (Fig. 7D). Taken together, these results suggest that PXR preferentially binds to the corepressor SMRT in the absence or low levels of ligand, and that SMRT may inhibit the transcriptional activity of PXR and the expression of PXR target promoter. However, we note that these results are limited to cultured mammalian cells and may not reflect what occurs with the CYP3A4 promoter in animal.

MOL 13375

Further investigations are thus need to establish a definitely role of SMRT in regulating CYP3A4 gene expression *in vivo*.

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MOL 13375

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## FOOTNOTE

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MOL 13375

## FIGURE LEGENDS

**Figure 1. Schematic of hPXR (A) and cSMRT (B) Constructs.** DBD, DNA binding domain; LBD, ligand binding domain, AF2, activation function –2;  $\Delta$ AF2, AF2 deletion; N-term, N-terminal fragment; C-term, C-terminal fragment;  $\Delta$ AF1, AF1 deletion. cSMRT, C-terminal domain of SMRT. ID1/ID2, nuclear receptor interacting domains 1/2. The wild type and mutated corepressor motif sequences are shown in ID1, ID2, mID1, and mID2. The mID1/2 contains both the mID1 and mID2 mutations.

**Figure 2. SMRT Interacts with Orphan Nuclear Receptors.** GST-pull down assays using GST-cSMRT and [<sup>35</sup>S]-orphan receptors are shown. GST alone was used as control for background binding. h, human; m, mouse.

**Figure 3. PXR Binds Preferentially to SMRT-ID2.** A) GST-pull down assay showing bindings of [<sup>35</sup>S]-labeled hPXR, mPXR, mPPAR $\gamma$ , hLXR $\beta$ , and hRAR $\alpha$  to GST, GST-cSMRT, GST-ID1, and GST-ID2. PXR, mPPAR $\gamma$ , and hLXR $\beta$  bound to cSMRT and ID2, but not ID1. In contrast, hRAR $\alpha$  preferentially bound to ID1. B) Bindings of hPXR, mPXR, hRAR $\alpha$ , and hLXR $\beta$  to cSMRT, ID1, ID2, mID1, mID2, and mID1/2 mutants of SMRT in GST-pull down assay. C) Relative bindings of various PXR fragments with cSMRT, SMRT-ID1, and SMRT-ID2.

**Figure 4. Rifampicin blocks PXR-SMRT Interaction.** A) Rifampicin inhibited hPXR binding to GST-cSMRT in a concentration-dependent manner. As control, rifampicin had no effect on

MOL 13375

hLXR $\beta$  binding to GST-cSMRT. Ls, rabbit reticulocyte lysate. **B)** Rifampicin inhibited hPXR binding to GST-SMRT-ID2 in a concentration-dependent manner.

**Figure 5. Interaction between PXR and SMRT in Yeast Two-Hybrid Assay.** **A)** hPXR

interacts with SMRT and RAC3 in yeast two-hybrid assay. Full-length hPXR was expressed as Gal4 DBD fusion in the pGBT9 vector (Clontech, Inc.). cSMRT, hNCoR (aa 2228-2440), and RAC3 (aa 1-1204) were expressed as Gal4 AD fusions in pACT2 or pGAD424 (RAC3) vectors.  $\beta$ -galactosidase activities were determined from three independent cultures treated with DMSO (solvent) or Rif (10  $\mu$ M) for 36 hours. **B)** Rif causes an exchange of SMRT to RAC3 in hPXR binding in yeast two-hybrid assay. Yeast colonies co-transformed with pGBT-hPXR and pACT-cSMRT or pGAD-RAC3 (1-1204) were analyzed for  $\beta$ -galactosidase expression after treatments with increasing concentrations of Rif. The exchange of cofactor binding occurs at approximately 7  $\mu$ M of Rif. **C)** Rif and CTZ disrupt hPXR-cSMRT interaction. Yeast cells co-transformed with pGBT-hPXR and pACT-cSMRT were treated with hPXR-specific ligands Rif or CTZ, and mPXR-specific ligands PCN or DEX (10  $\mu$ M each) for 48 hours.  $\beta$ -galactosidase activities were determined from three independent transformants. Both Rif and CTZ disrupted hPXR-cSMRT interaction, but PCN and DEX had no effect. **D)** PXR fragments that interact with SMRT in yeast. Yeast colonies containing pACT-cSMRT and pGBT-hPXR,  $\Delta$ AF2, C-Term fragment, or N-Term fragment were analyzed for  $\beta$ -galactosidase expression after solvent (DMSO) or Rif (10  $\mu$ M) treatment. Statistical analyses were conducted via one-way ANOVA and Tukey HSD test. Samples were grouped into statistically significantly different categories as labeled on top of each column.

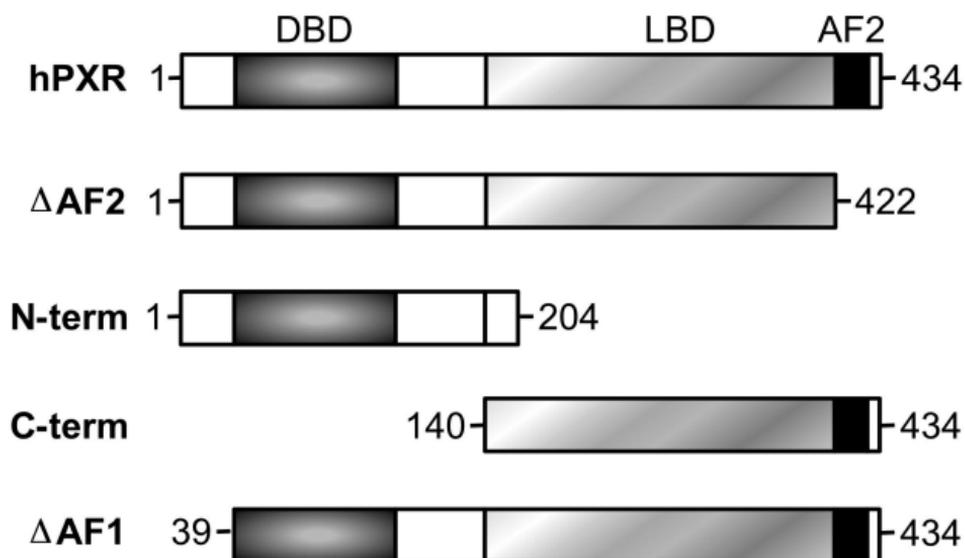
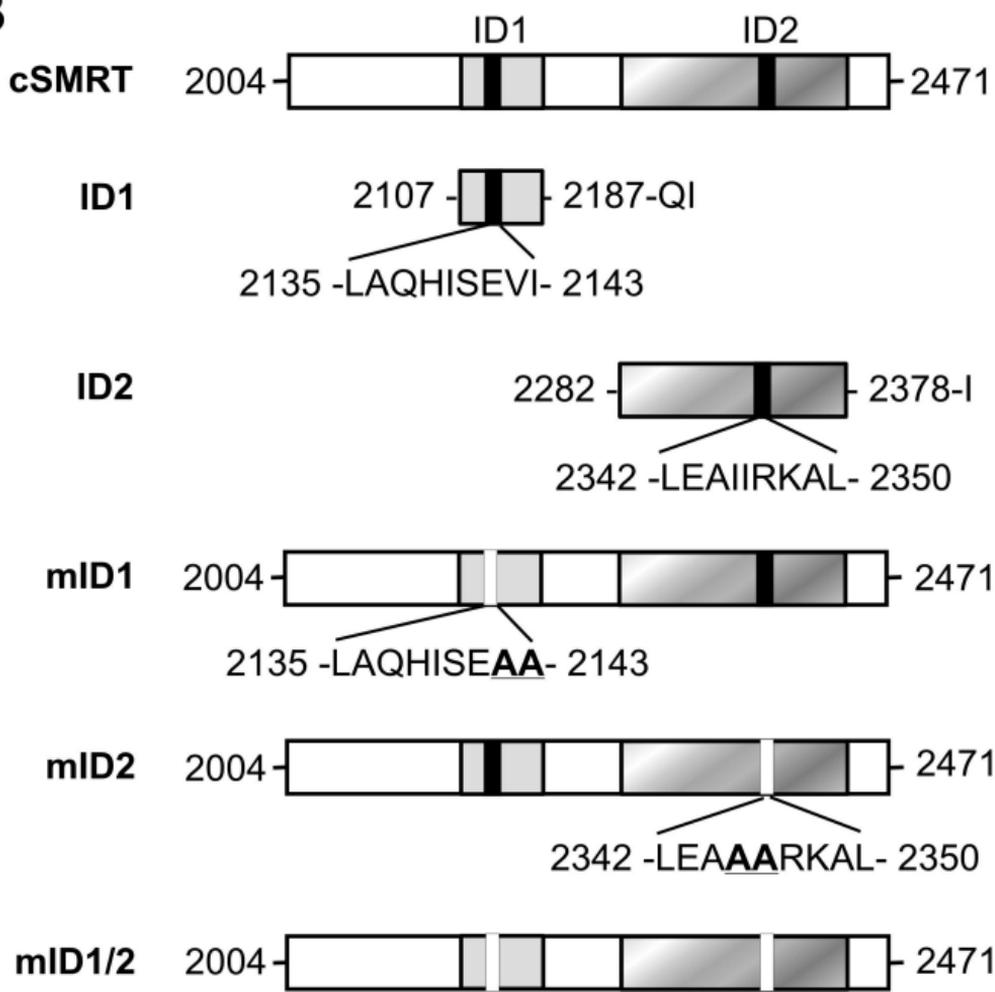
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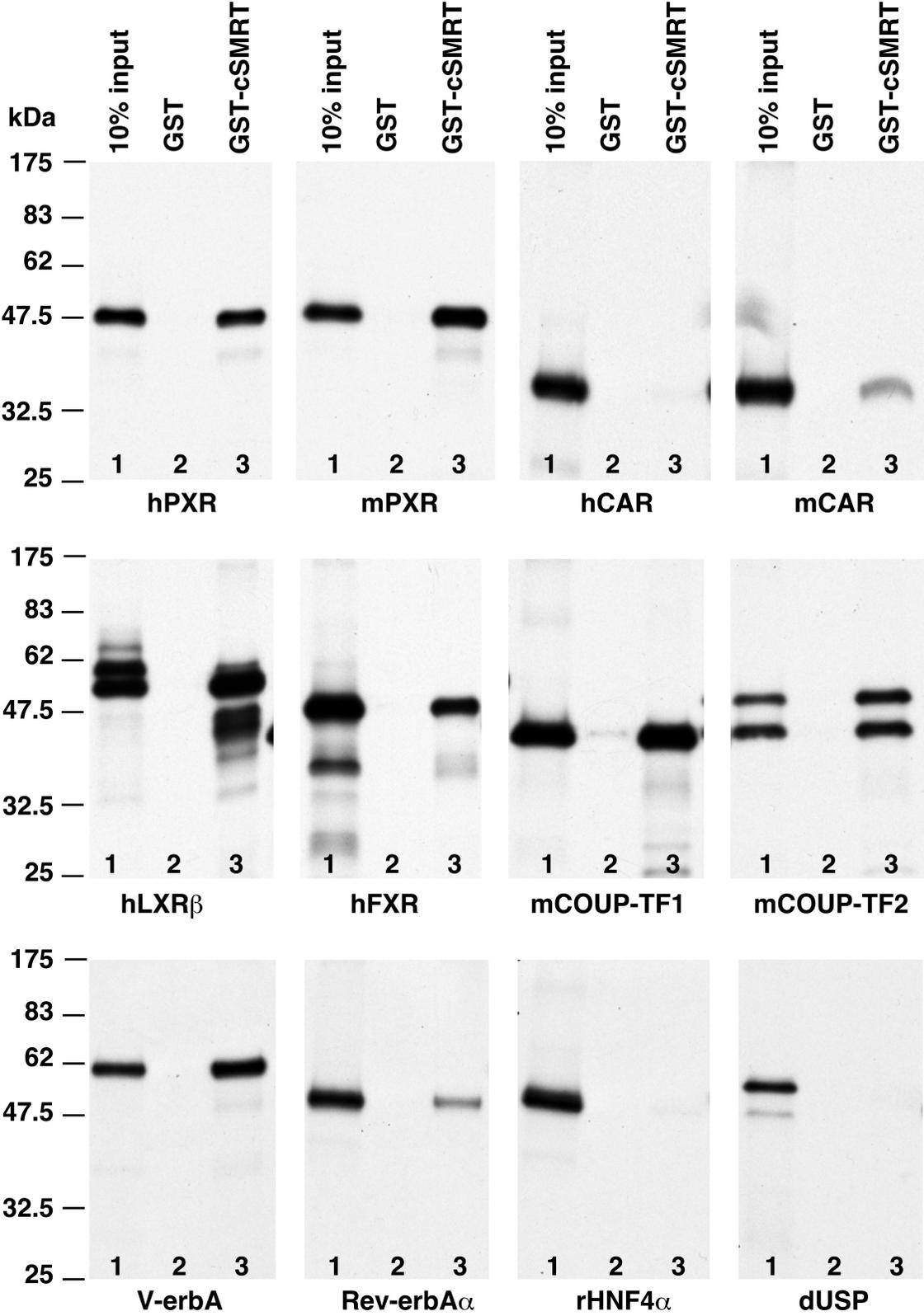
**Figure 6. PXR Colocalizes with SMRT in Mammalian Cells.** COS-7 cells were cotransfected with human SMRT and HA-hPXR, or indicated hPXR constructs including  $\Delta$ AF2, C-term, and N-term, and HA-hCAR. SMRT was detected with a mouse anti-SMRT monoclonal antibody (GeneTex, Inc.), followed by a rhodamine-conjugated goat anti-mouse secondary antibody. PXR and hCAR were detected by a rabbit anti-HA polyclonal antibody (Santa Cruz, Inc.), followed by a FITC-conjugated goat anti-rabbit secondary antibody. Cell nuclei were co-stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride hydrate). The wild type (W.T.) hPXR and its  $\Delta$ AF2 and C-term fragments show colocalization with SMRT at nuclear foci, while the hPXR N-term and hCAR were not recruited to the SMRT nuclear foci. The boxed cells were from different fields in the same slide.

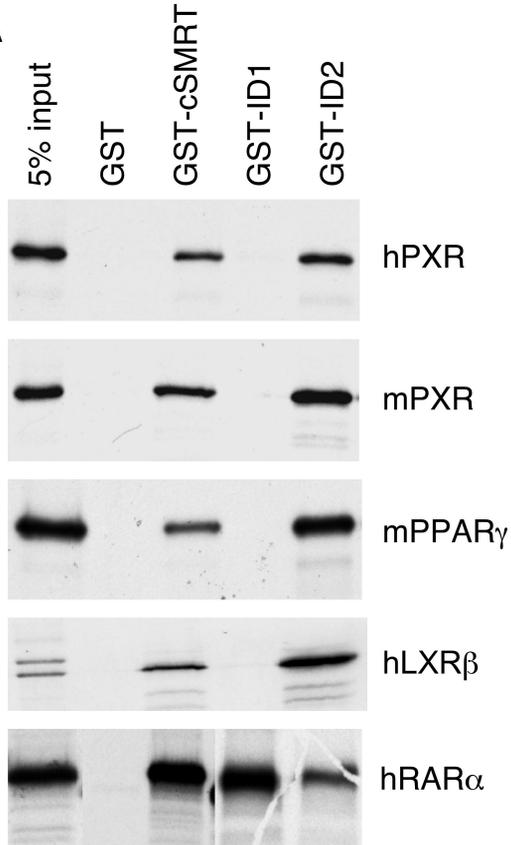
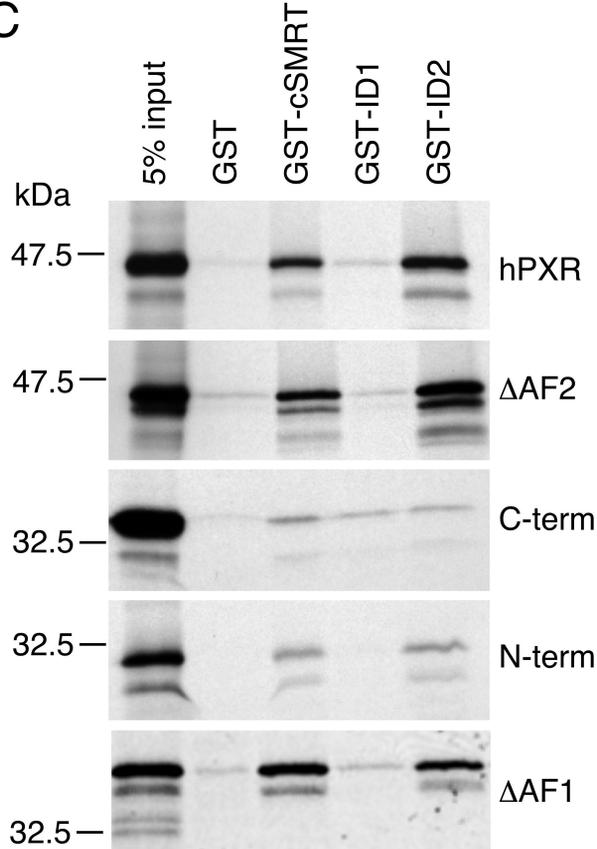
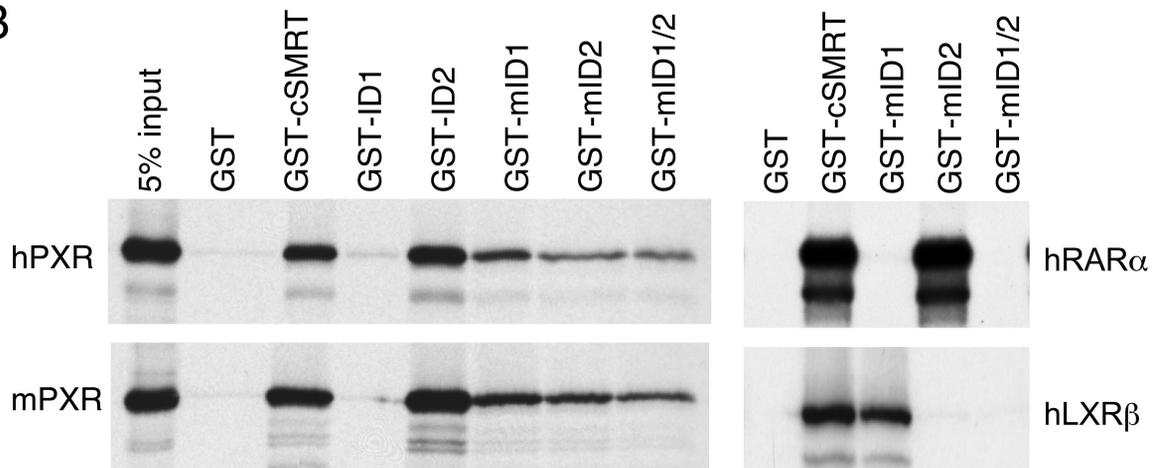
**Figure 7. Regulation of PXR Activity by SMRT.** **A)** Gal4 DBD-hPXR fusions repressed basal transcription. HEK293 cells were transfected with Gal4 DBD vector, or Gal4 DBD fusions of hPXR,  $\Delta$ AF2, C-term, or N-term fragment, together with the Gal4-dependent MH100-tk-luc reporter and a  $\beta$ -galactosidase expression vector as internal control. Transfected cells were treated with Rif (10  $\mu$ M) or solvent (DMSO) for 48 hrs. The fold repression was shown on top of each column as a negative value. The fold activation is also indicated in parentheses on top of each column. **B)** Mammalian two-hybrid assay showing interactions of Gal4 DBD-cSMRT with VP16-hPXR and VP16- $\Delta$ AF2 mutant. HEK293 cells were transfected with Gal4 DBD-cSMRT and indicated VP16 AD fusions, along with the Gal4-dependent MH100-tk-luc reporter and a  $\beta$ -galactosidase expression vector. After transfection, cells were refed with fresh media containing Rif (10  $\mu$ M for the vector, hPXR, and  $\Delta$ AF2) or *all-trans* retinoic acid (1  $\mu$ M for hRAR $\alpha$ ), or solvent (DMSO) alone as indicated. **C)** SMRT inhibits hPXR basal activity on CYP3A4

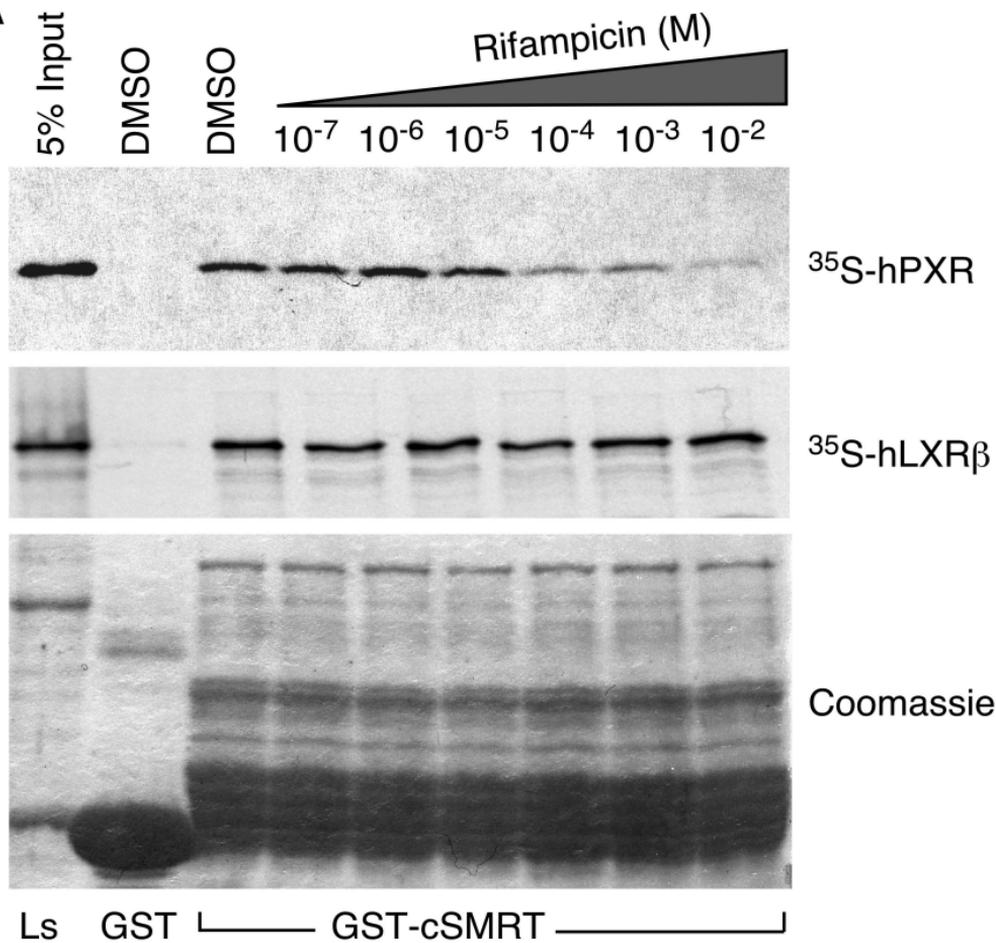
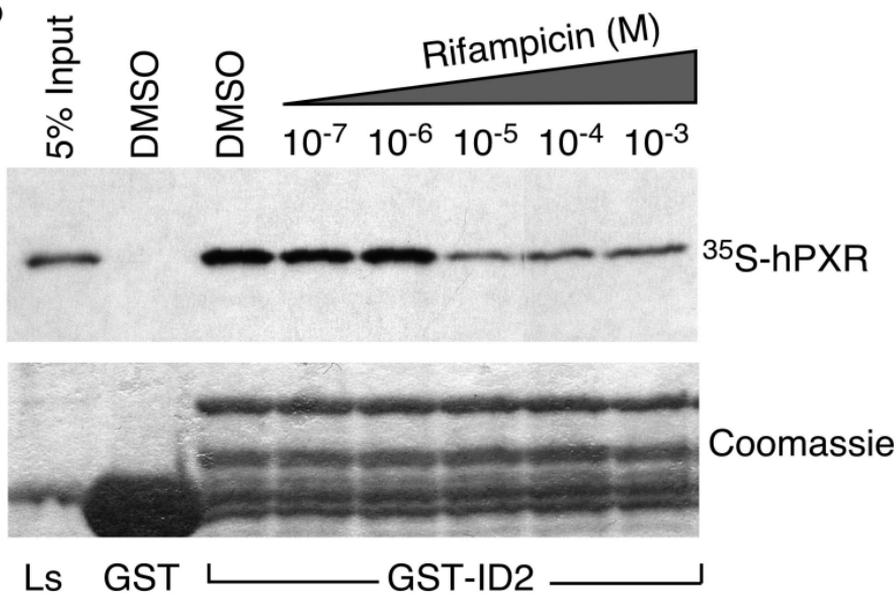
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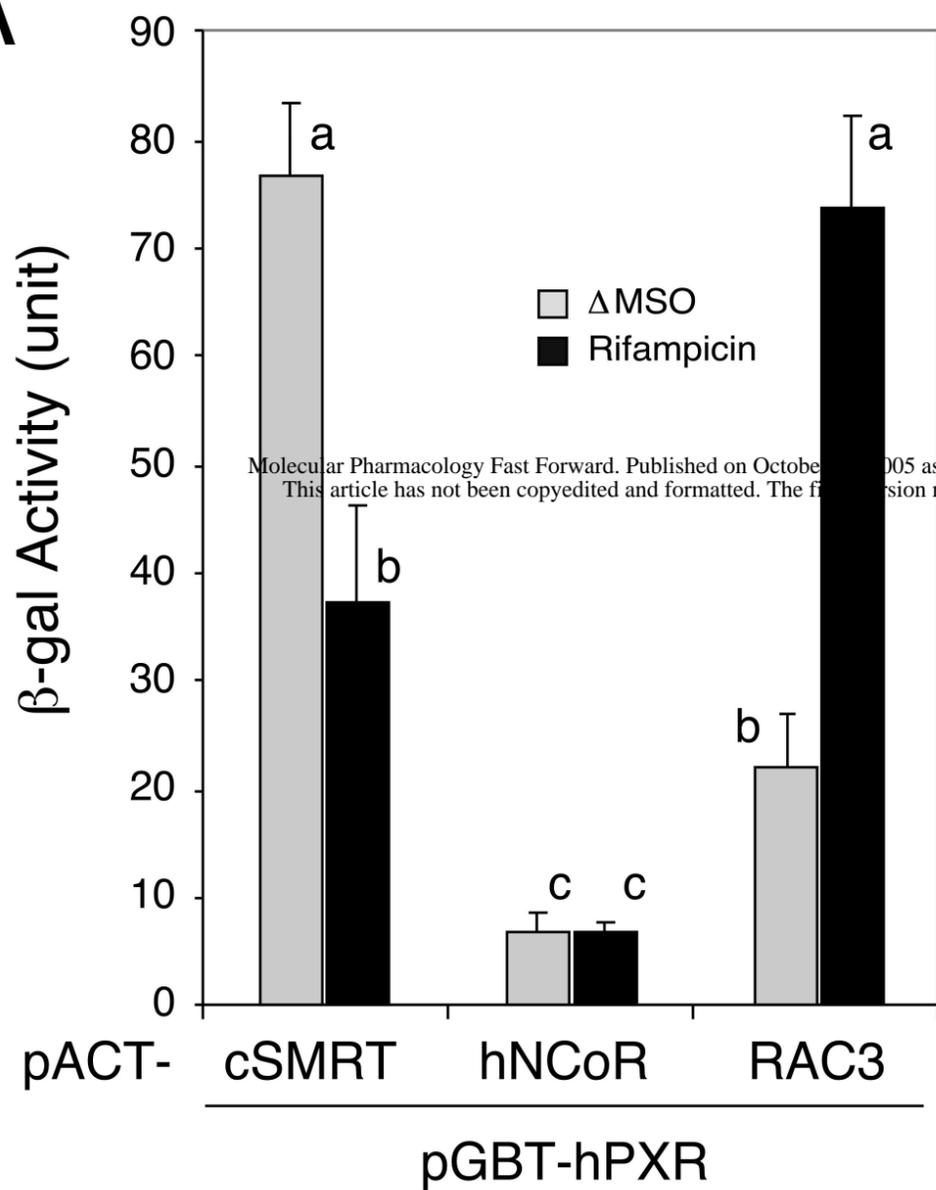
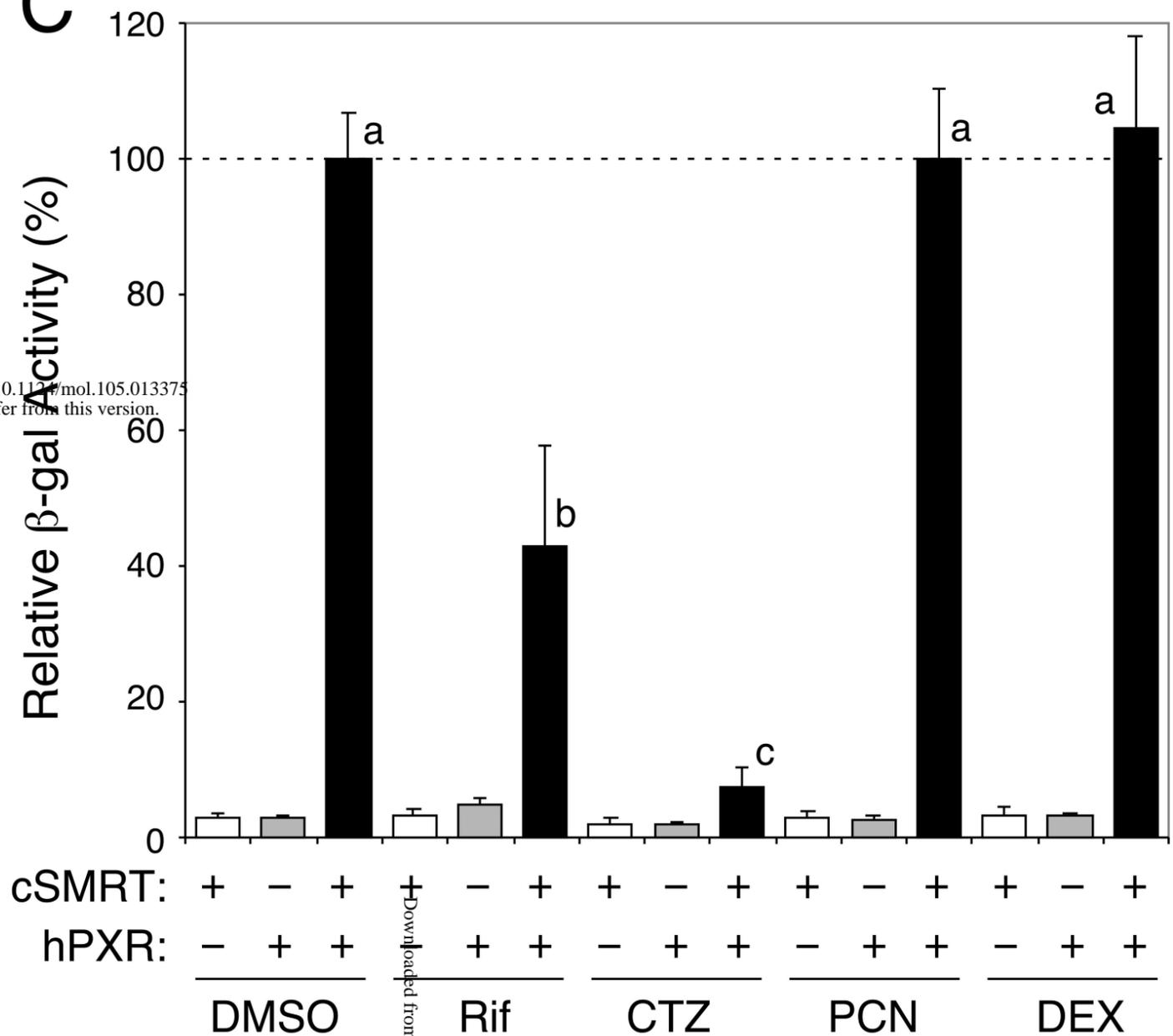
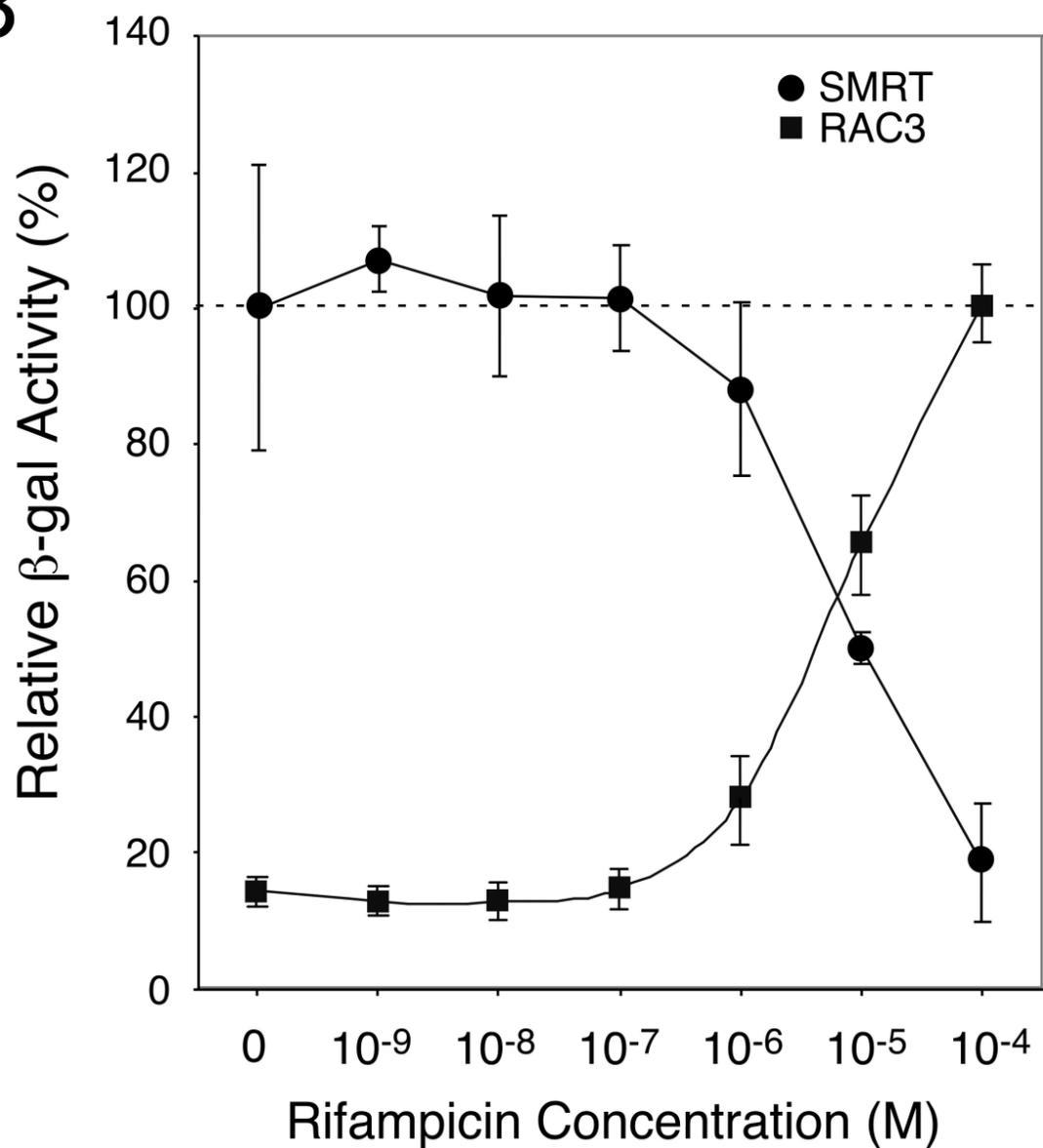
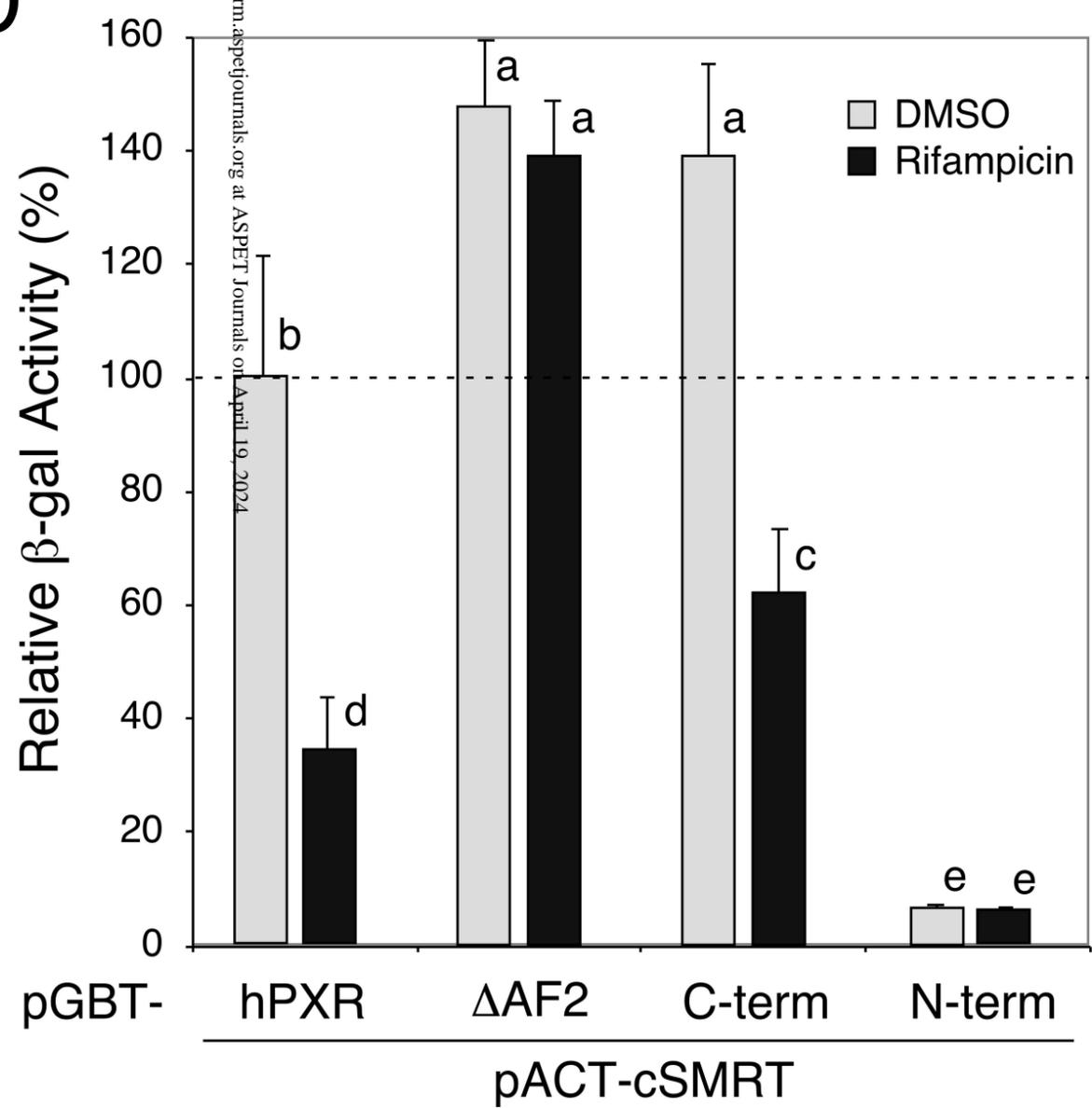
promoter in HepG2 cells. Cells were transfected with hPXR or empty vector with or without SMRT together with a CYP3A4-luciferase reporter and  $\beta$ -galactosidase control. Relative luciferase activities were determined from three independent experiments. Cotransfection of PXR stimulated CYP3A4 promoter activity by 6.6-fold, while coexpression of SMRT inhibits the PXR-dependent activation by 51% ( $p < .01$ ). **D**) Silencing of SMRT enhances PXR transcriptional activity. HEK293 cells were mock transfected (-) or with pLL3.7 vector (vector), a scramble control shRNA (ctrl), or the SMRT shRNA (SMRT) expression vector, along with the hPXR and CYP3A4-Luc reporter. Relative luciferase activities were determined from three independent transfections in the absence (-) or presence (+) of Rif (10  $\mu$ M). The inset shows that the SMRT shRNA blocked the expression of a cotransfected HA-tagged SMRT fragment (aa 475-981) that contains the RNAi target site. The “\*” denotes a non-specific band detected by Western blot with anti-HA antibody. The samples were grouped into statistically significantly different categories as labeled on top of each column. The difference between c and d groups in panel **A** are significant in LSD test, but nonsignificant in Tukey HSD test at .05 level. The difference between the two group a samples in panel **B** are not significant in Tukey HSD test, but significant in LSD test at .05 level.

**A****B**



**A****C****B**

**A****B**

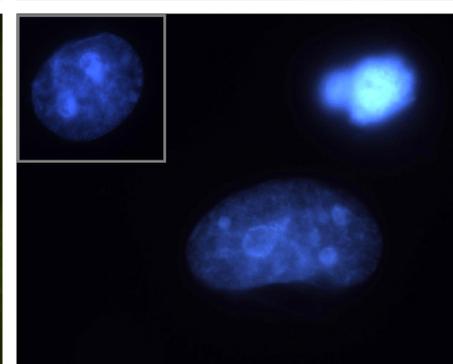
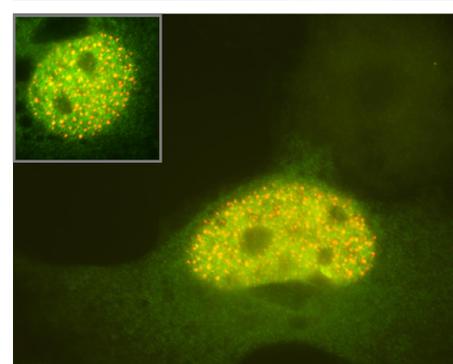
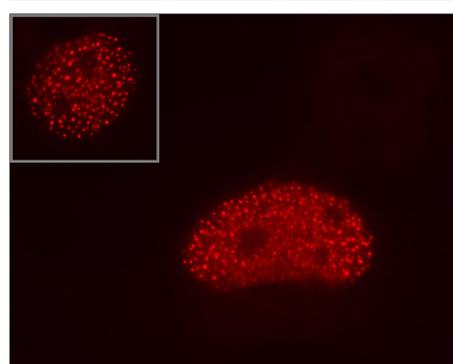
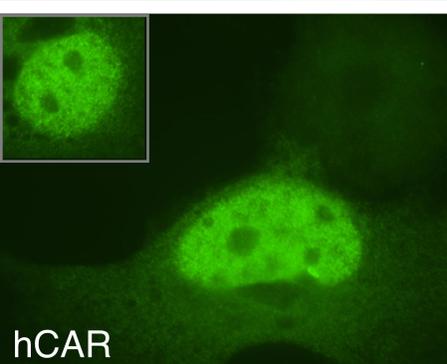
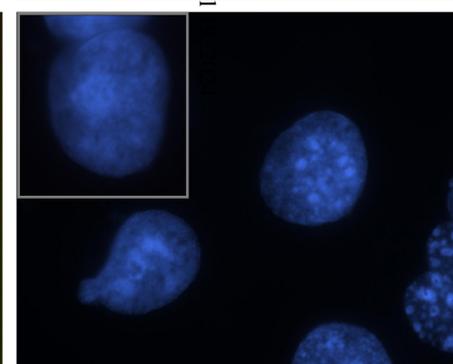
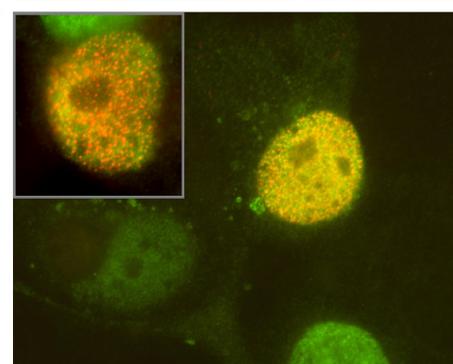
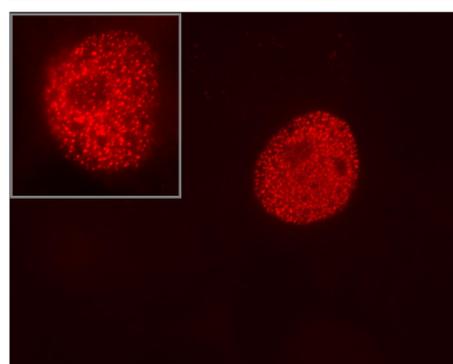
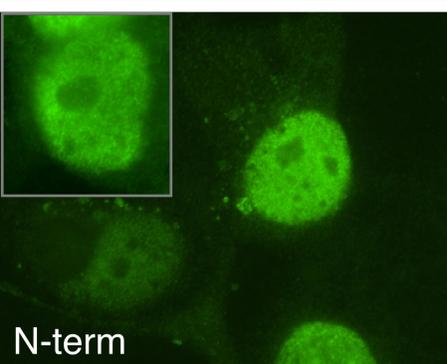
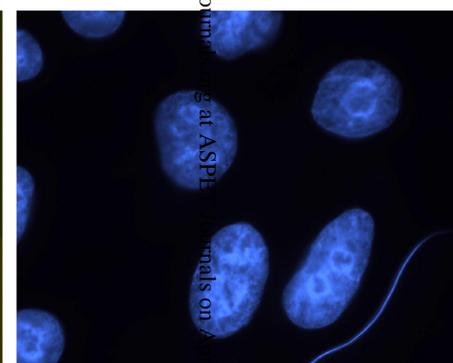
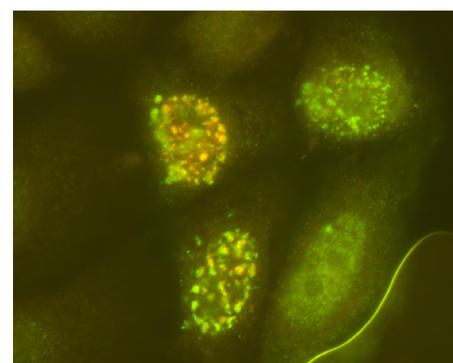
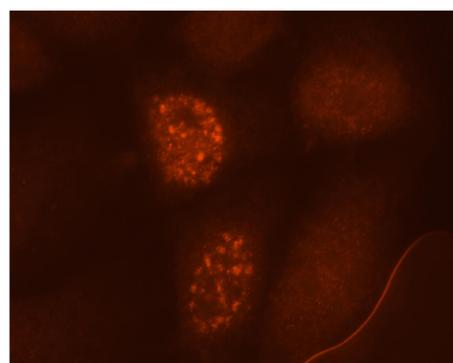
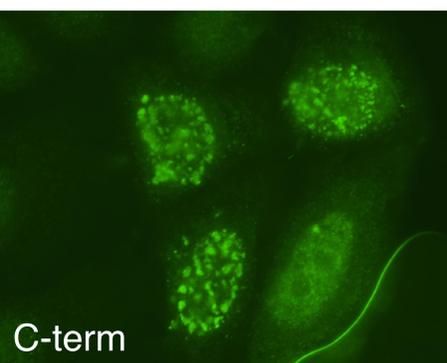
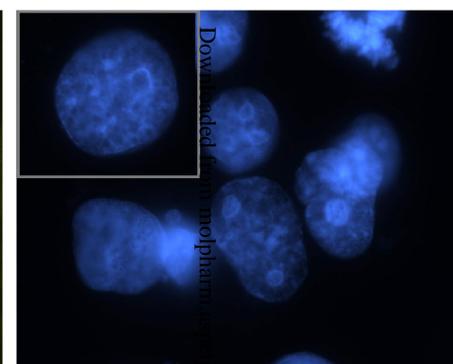
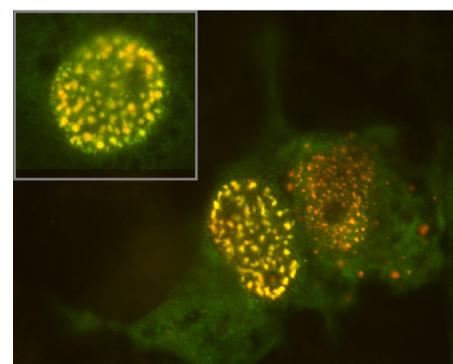
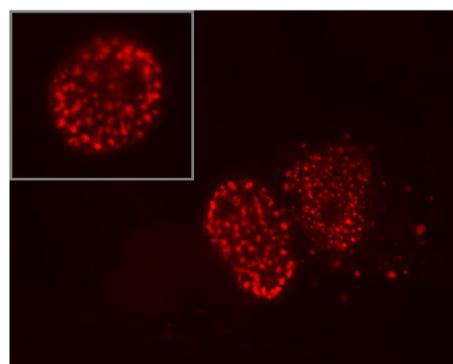
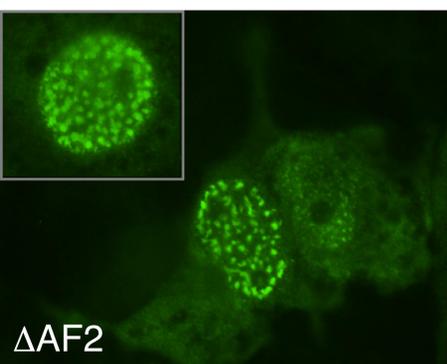
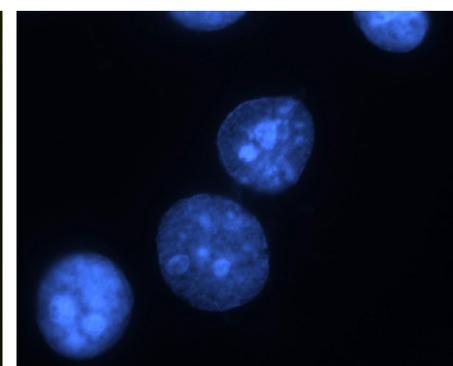
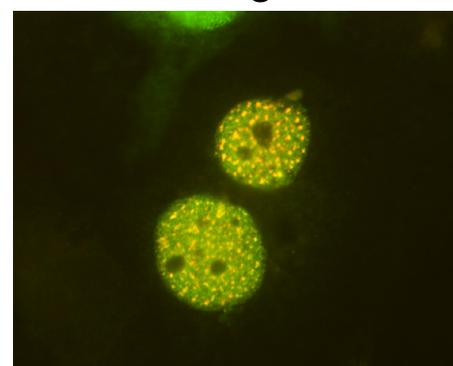
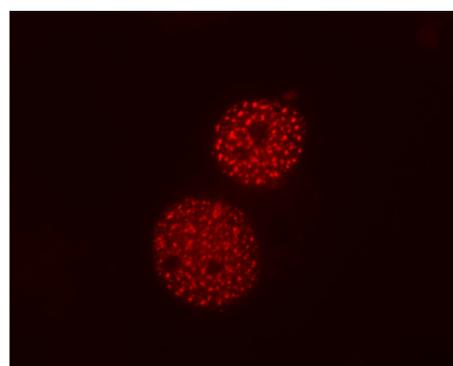
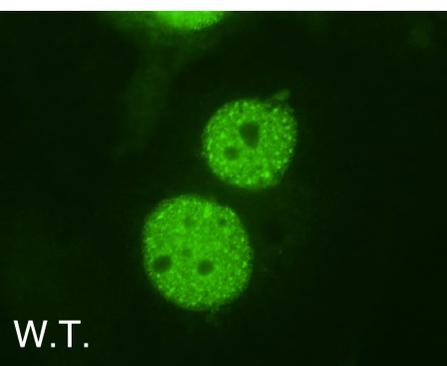
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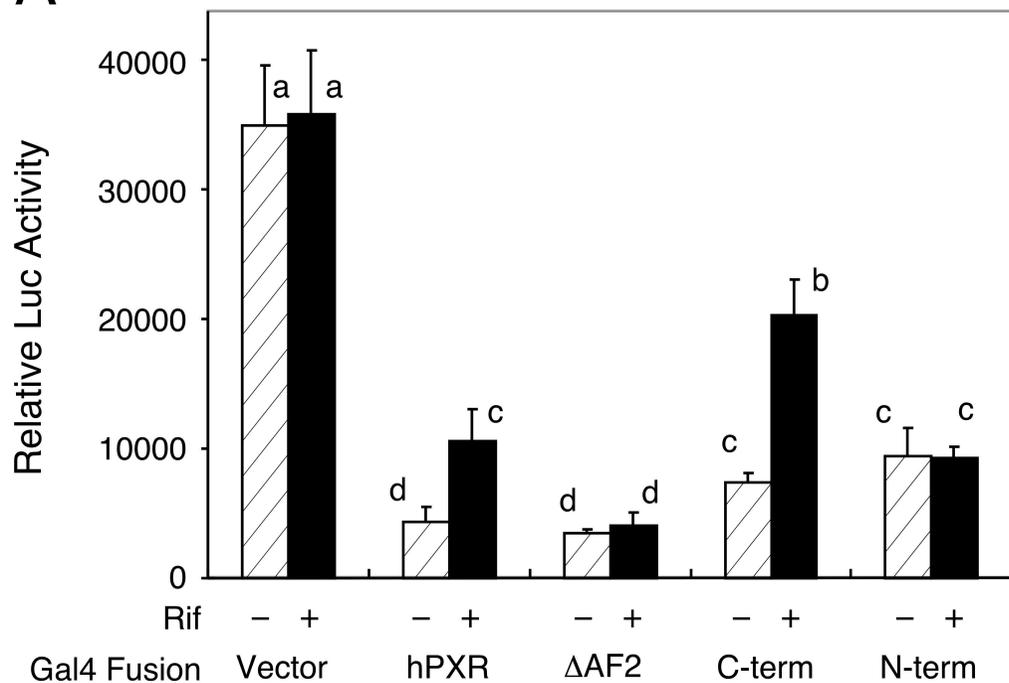
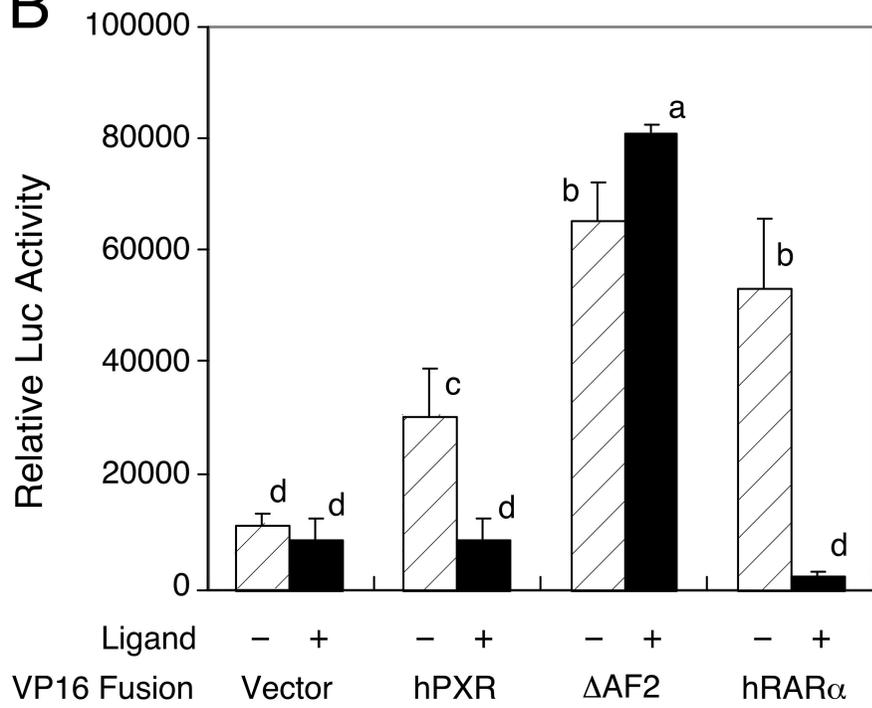
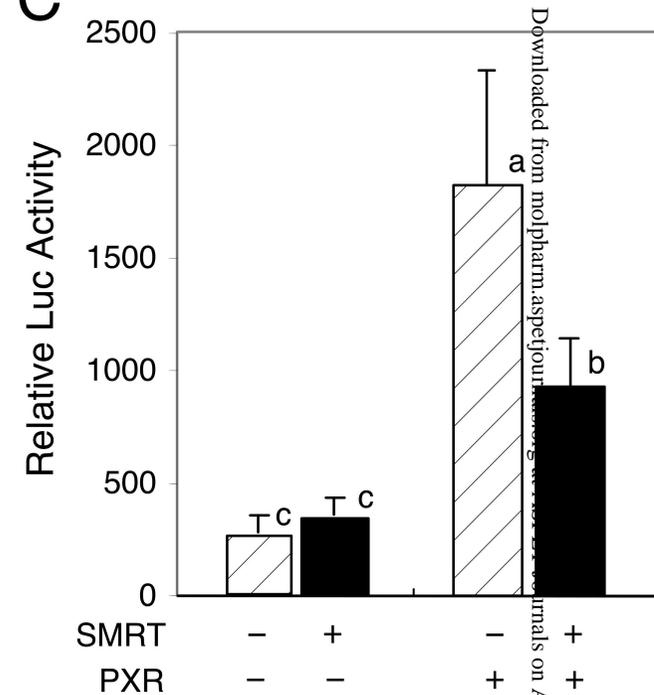
hPXR

SMRT

Merged

DAPI



**A****B****C****D**