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RBCK1, an E3 Ubiquitin Ligase, Interacts with and Ubiquinates the Human Pregnane X Receptor (hPXR)

Ritu Rana, Sherry Coulter, Harriet Kinyamu and Joyce A. Goldstein

Laboratory of Toxicology and Pharmacology (R.R., S.C., J.A.G.), Laboratory of Molecular Carcinogenesis (H.K.), National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina, 27709

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Corresponding author:

Dr. Joyce Goldstein, Laboratory of Toxicology & Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina, 27709. Phone: (919) 541-4495. Fax: (919) 541-4107. E-mail: goldste1@niehs.nih.gov

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ABBREVIATIONS:

RBCK1: RBCC protein interacting with protein kinase C1; RBCC: Ring-B-Box-Coiled-coil; hPXR: human pregnane X receptor; E3: E3 ubiquitin ligase; PKC-1: Protein Kinase C-1; CREB: cAMP response element-binding; PML : Promyelocytic leukemia; CYP: Cytochrome P-450; RXR: Retinoid X receptor; SRC-1: Steroid receptor coactivator-1; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; HNF4α: Hepatocyte Nuclear Factor 4 alpha; SHP: Small heterodimer partner; HA: Hemagglutinin; GR: glucocorticoid receptor; TBP: TATA binding-protein; GRIP-1:glucocorticoid receptor interacting protein/ steroid receptor coactivator-2.

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ABSTRACT:

The pregnane X receptor (PXR, NR1I2) plays a pivotal role in the disposition and detoxification of numerous foreign and endogenous chemicals by increasing transcription of numerous target genes including phase I and II drug-metabolizing enzymes and transporters. In the present study, yeast two-hybrid screening identified an E3 ubiquitin ligase, RBCK1 (RBCC (RING-B-box-Coiled-coil) protein interacting with PKC-1), as a hPXR-interacting protein. Coimmunoprecipitation studies confirmed the interaction between RBCK1 and hPXR when both were ectopically expressed in AD-293 cells. Domain mapping studies showed that the interaction between RBCK1 and hPXR requires different RBCK1 domains. We further demonstrate that RBCK1 ubiquitinates human PXR (hPXR), and this may target hPXR for degradation by the ubiquitin-proteasome pathway. Simultaneous ectopic overexpression of RBCK1 and PXR decreased PXR levels in AD-293 cells and this decrease was inhibited by the proteasomal inhibitor MG-132. Furthermore, overexpression of RBCK1 decreased endogenous levels of PXR in HepG2 cells. Importantly, ectopic overexpression and silencing of endogenous RBCK1 in primary human hepatocytes resulted in a decrease and increase, respectively, in endogenous PXR protein levels and in the induction of PXR target genes by rifampicin. These results suggest that RBCK1 is important for the ubiquitination of PXR and may play a role in its proteasomal degradation.

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Introduction

Protein degradation is an essential and versatile housekeeping function in eukaryotic cells that maintains cellular homeostasis. The discovery of the ATP/ubiquitin (Ub)-dependent 26S proteasomal system (Ub/26S) has revolutionized the concept of intracellular protein degradation from a non-specific scavenger process to a highly controlled and specific cellular process. This process is carried out by a complex cascade of enzymes via a three-step mechanism involving the ubiquitin-activating enzyme E1 activating ubiquitin, followed by the E2 (ubiquitin-conjugating enzyme) mediated transfer of ubiquitin from E1 to a member of the ubiquitin-protein ligase family, E3. E3 enzymes catalyze covalent attachment of ubiquitin to the specific substrate. The ubiquitination of protein serves as a marker for the protease for its eventual degradation (Glickman and Ciechanover, 2002).

RBCK1, RBCC (RING-B-box-Coiled-coil) protein interacting with PKC-1 (C20orf18 or HOIL-1, XAP3 OR UIP28), is a transcription factor which consists of a ubiquitin-like sequence (Tokunaga et al., 1998), two coiled-coil regions, a novel zinc finger motif (Meyer et al., 2002), and a RING-IBR domain (Marin and Ferrus, 2002). RBCK1 is localized in both the nucleus and cytoplasm, possessing a classical Leu-rich nuclear export signal as well as a nuclear localization signal (Tatematsu et al., 2005). Studies have shown that RBCK1 facilitates transcriptional coactivation upon HBV infection (Cong et al., 1997) and interacts with various proteins including UbcM4 E2 ubiquitin ligase (Martinez-Noel et al., 1999), protein kinase C (Tokunaga et al., 1998), CREB-binding protein, and PML (Tatematsu et al., 2005). It acts as an E3 ligase causing ubiquitin-dependent degradation of heme-oxidized IRP2 (iron regulatory protein-2) in iron metabolism (Yamanaka et al., 2003). The pregnane X receptor (PXR), also known as NR1I2 (nuclear receptor subfamily 1, group I, member 2), is a nu clear receptor which acts as a

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xenobiotic/metabolite sensor and regulates the expression of a broad array of genes involved in biotransformation and transport of endogenous substances, natural products, drugs and other xenobiotic chemicals (Chang, 2009). PXR is predominantly expressed in liver, although it has been detected in small intestine, colon, kidney, brain and mammary tissues (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998; Dotzlaw et al., 1999; Masuyama et al., 2001; Miki et al., 2005). PXR target genes include those encoding for various cytochrome P450 enzymes (*CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP3A5, CYP3A7, CYP4F12, and CYP27A1*), uridine diphosphate (UDP)-glucuronosyltransferases (*UGT1A1, UGT1A3, UGT1A4, UGT1A6*, and *UGT1A9*), sulfotransferases (*Sult2a1*), and the drug transporter genes *ABCB1* (P-glycoprotein), *Abcc2*, and *SLC21A6* (oatp2) (Rosenfeld et al., 2003; Stanley et al., 2006; Ong et al., 2011). The ligand-activated PXR forms a heterodimer with RXRα and binds to DNA response elements of a PXR target gene resulting in increased gene transcription (Lehmann et al., 1998; Geick et al., 2001). PXR interacts with various coactivators such as SRC-1 and PGC-1α, (Li and Chiang, 2006) and corepressors (e.g. nuclear receptor co-repressor 1 (Roth et al., 2008) and silencing mediator for retinoid or thyroid-hormone receptor) (Johnson et al., 2006) to regulate PXR target genes. PXR transcriptional activity is also influenced by other nuclear receptors (e.g. HNF4α (Li and Chiang, 2006) and GR (Pascussi et al., 2001)) which increase PXR levels. In contrast, SHP suppresses PXR activity (Li and Chiang, 2006).

Current efforts to understand the regulation of nuclear receptors have revealed that several steroid hormone receptors, including the glucocorticoid, progesterone, androgen and estrogen receptors, are tightly regulated by the ubiquitin-proteasome system (Kinyamu et al., 2005). Previous studies have indicated that binding of certain chemicals, some of which could be PXR ligands, increases its half-life, in part due to the disruption of its interaction with the

suppressor for gal1 (SUG1), a component of the proteasome (Masuyama et al., 2002; Masuyama et al., 2005). A recent study demonstrated an increase in ubiquitinated PXR following inhibition of the 26S proteasome with MG132. Proteasomal inhibition also resulted in the inhibition of PXR transactivation, suggesting interplay between PXR and the ubiquitin pathway (Staudinger et al., 2011).

In the present study, we show that the E3 ligase, RBCK1 interacts and ubiquitinates hPXR and this may in part target hPXR to proteasomal degradation. Since PXR plays an important role in the transcriptional regulation of genes involved in various metabolic pathways, its regulation by RBCK1 has important physiological and pharmacological consequences.

Materials and Methods

Yeast Two-Hybrid Screening

ProNet technologies automated two-hybrid screening was performed by Myriad Genetics (Salt Lake City, UT) as described previously (Garrus et al., 2001). Human liver and brain cDNA libraries were used to screen for prey proteins that interact with full length PXR or PXR domains. Plasmids isolated were transformed into yeast, and interactions were confirmed by liquid β-galactosidase assays. The identities of the prey were determined by DNA sequencing.

Antibodies and Chemicals

Antibodies to PXR (H160, catalog # sc-25381) and RBCK1 (P19, catalog # sc-49718) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Mouse monoclonal antibody to PXR (catalog # PP-H4417-00) was purchased from R&D Systems (Minneapolis MN). Rat anti-HA antibody (clone 3F10; catalog # 11867423001) was purchased from Roche Diagnostics. MG132 was obtained from Calbiochem, La Jolla; CA. Rifampicin was purchased from Sigma-Aldrich, St. Louis, MO.

Cell culture and transfections

AD-293 cells (Agilient Technologies, Santa Clara, CA) were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS). HepG2 cells were cultured in Eagle's minimum essential medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin-streptomycin at 37^{o}C under 5% CO_2. Primary human hepatocytes were obtained from CellzDirect/Life Technologies, Durham, NC, and maintained in William's E medium supplemented with ITS+1 (insulin, human transferrin,

sodium selenite, bovine serum albumin, and linoleic acid in Earle's balanced salt solution; Sigma-Aldrich, St. Louis, MO), HEPES, L-glutamine, and 100 nM dexamethasone. All transient transfections were carried out as described in the Lipofectamine™ 2000 protocol (Invitrogen, Carlsbad, CA). Twenty-four hours later, medium was replaced and drugs were added at the appropriate concentrations (0.1% DMSO or 10 μM rifampicin). Cells were then treated with 10 μM MG-132 or vehicle for 6 h after 42 h of transfection.

Plasmids and siRNAs

Full length RBCK1 was cloned from MGC clone EX-U1469-M02 into pcDNA3.1 vector. Full length RBCK1 (1-470 aa) and the deletion constructs RBCK1-I (1-260 aa), RBCK1-II (172-350 aa), and RBCK1-III (254-470 aa) were then cloned in pGEX-4T1. All constructs were verified by sequencing. PXR was cloned in pCR3 by PCR amplification as described earlier (Chen et al., 2005). pMT123-HA-Ub was a kind gift from Dr. Andrew Wallace (NC State University, Raleigh, NC). For silencing RBCK1 expression, RBCK1 Stealth Select RNAi™ siRNA siRBCK1-I and Stealth RNAi™ siRNA Negative Control Hi GC (12935-400) as control siRNA (siC) were purchased from Invitrogen. siRNA transfections were carried out using a final concentration of 50-100 nmol/L oligo (at 40–60% confluence) using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's recommendation.

GST pulldown assays

GST fusion proteins of full length and RBCK1 deletion constructs were expressed in *E.coli* BL-21 and were purified on GSH-sepharose beads (Amersham, Louisville, Colorado). The PXR protein was translated *in vitro* in rabbit reticulocyte lysate (Promega, Madison, Wisconsin), using a T NT Quick coupled transcription and translation system with radiolabeled ^{35}S-

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methionine (MP Biomedicals, Solon Ohio). Expression and purification of recombinant GST fusion proteins and interaction assays were as described previously (Surapureddi et al., 2008).

Immunoblotting

Forty-eight hours after transfection, cells were lysed using M-PER mammalian protein extraction reagent (Thermo Scientific, Waltham, MA) supplemented with complete protease inhibitor mixture (Roche, Nutley, NJ). After a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) to determine protein concentration, equal amounts (30 μg) of whole cell extracts were resolved by Nu-PAGE gel electrophoresis, transferred to a nitrocellulose membrane (Invitrogen), and analyzed using the appropriate antibodies including actin as a loading control. Proteins were detected using horseradish peroxidase-conjugated secondary antibodies and a SuperSignal West Femto kit (Thermo Scientific). Membranes were stripped between antibody probes using Restore Western Blot Stripping Buffer (Thermo Scientific).

Immunoprecipitations and Ubiquitination assays

Cells were cotransfected with PXR and RBCK1. After 48 h, whole-cell lysates were prepared using M-PER mammalian protein extraction reagent (Thermo Scientific) supplemented with complete protease inhibitor mixture (Roche). The lysates were immunoprecipitated using agarose beads coupled with rabbit anti-PXR or goat anti-RBCK1 antibodies using the manufacturer's protocol. The eluted proteins were run on a 10% NuPAGE gel (Invitrogen) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk and probed with mouse anti-PXR or goat anti-RBCK1 IgG (1:1000) for 2 h at room temperature. After washing, the blot was incubated for 1 h with appropriate horseradish peroxidase-conjugated secondary antibody (Promega). Detection was achieved using a SuperSignal West

Femto kit. Similarly, endogenous PXR protein from HepG2 cells was immunoprecipitated using rabbit anti-PXR antibody and immunoprecipitates were probed with mouse anti-PXR or goat-RBCK1 antibody. For ubiquitination assays, cells were transfected with PXR, RBCK1 and HA-tagged ubiquitin. Cell lysates were prepared as described earlier, immunoprecipitated with rabbit-PXR antibody and eluted proteins were probed with anti-HA-Ub antibody.

Quantitative PCR (qPCR)

Total RNA was extracted using RNeasy mini prep system (QIAGEN, Valencia, CA) following the manufacturer's procedure. RT-PCR analysis was performed as described earlier (Rana et al., 2010). Briefly, cDNAs were prepared using Superscript II (Invitrogen) reverse transcriptase. qPCR analyses were then performed on an ABI 7900 using Taqman® Universal PCR Master Mix (Applied Biosystems, Bedford, MA) Taqman gene expression assays for CYP2C9, CYP3A4, UGT1A9, ABCB1, GSTA2, ABCC2, with TBP as the internal control. The relative quantities were calculated as $2^{-\Delta\Delta Ct}$.

Statistical analysis

For qPCR results and the effect of ectopic RBCK1 expression in AD-293 cells and HepG2 cells) pairwise comparisons were made using the Holm-Sidak method subsequent to two-way analysis of variance in Sigma Plot version 11. Values for protein expression of Ubiquitin, PXR, and RBCK1 and the effects of siRBCK1 were compared using a paired, two-tailed t-test in GraphPad Prism.

Results

RBCK1 is a novel interaction of PXR

For the first time, we identified RBCK1 as a PXR interacting protein in a yeast two hybrid screening. Fourteen different full length or partial domains of PXR were used as baits. A human liver cDNA library was used as the prey to identify PXR interacting proteins. Five out of 14 baits showed interaction with various proteins, including some known PXR interacting proteins such as RXRα, SRC1 and GRIP1. In addition, the PXR domain containing amino acids 107-437 from a brain library interacted with RBCK1 (also known as C20orf18 or HOIL-1, XAP3 and UIP2). RBCK1 is an E3 ubiquitin ligase and is known to target certain proteins for degradation in a proteasomal-dependent manner. To confirm that RBCK1 is a PXR binding protein, we first ectopically transfected AD-293 cells (which do not normally express PXR) with plasmid expression constructs for PXR and RBCK1. Cells were lysed and subjected to immunoprecipitation with non-specific IgG or antibody to PXR or RBCK1 and subjected to Western blot analysis. As shown in Fig. 1A, RBCK1 was immunoprecipitated with PXR antibody and conversely, PXR was immunoprecipitated with RBCK1 antibody. The interaction of RBCK1 with PXR was further examined by GST-pull down assays. We generated a series of RBCK1 deletion constructs (Fig. 1B) including RBCK1-I (1-260 aa, containing NZF and CC), RBCK1-II (172-350 aa, containing NZF, CC and RING1) and RBCK1-III (254-470 aa, containing –RING and IBR). GST-pull down assays indicated that all three constructs interacted with PXR (Fig. 1C). These results suggest that both the RING1 and the coiled coil domains contribute to the interaction of RBCK1 interaction with PXR.

RBCK1 downregulates PXR protein levels in a proteasome-dependent manner

It has been shown previously that RBCK1 acts as an E3 ubiquitin ligase and regulates the expression of various proteins involved in iron metabolism and inflammatory signaling pathways in a proteasome-dependent manner (Tian et al., 2007; Zhang et al., 2008). Here, we examined the possible role of RBCK1 in regulating PXR protein expression. We transfected AD-293 cells with expression constructs for RBCK1, PXR, and HA-tagged ubiquitin. Western blot analysis indicated that ectopic overexpression of RBCK1 specifically down regulates ectopic expression of PXR protein (Fig. 2A). To indicate whetherRBCK1 regulates PXR in a proteasome-dependent manner, we transfected AD-293 cells with ubiquitin, PXR plus ubiquitin, or PXR, ubiquitin and RBCK1 for 48 h. During the last 6 hours we treated the transfected cells with the proteasomal inhibitor, MG-132 (10 μM) or vehicle control. Treatment with MG-132 increased PXR protein levels in the PXR-transfected cells and partially reversed the effect of RBCK1 on PXR levels in cells transfected with RBCK1, PXR and ubiquitin (Fig. 2B). These results suggest that RBCK1 down-regulates PXR through the proteasomal-dependent pathway. Since RBCK1 is a known E3 ubiquinating ligase, we examined whether RBCK1 ubiquitinates PXR using AD-293 cells transfected with expression plasmids for RBCK1, PXR, and HA-ubiquitin. Cell lysates were immunoprecipitated with anti-PXR antibody and the immunoprecipitates were analyzed by Western blot with anti-HA antibody to detect HA-tagged ubiquitin (Fig. 2C). These results indicated that RBCK1 targets PXR protein for the proteasomal pathway by ubiquitination.

To investigate whether PXR ligands affect RBCK1 regulation of PXR, we coexpressed PXR, RBCK1 and HA-Ubiquitin in AD-293 cells in the presence or absence of a prototypical human PXR ligand (10 μM rifampicin). RBCK1 increased ubiquitination of PXR as demonstrated by Western blot analysis, but rifampicin had no effect on the ubiquitination of PXR by RBCK1 (Fig. 2D). In addition, RBCK1 transfection decreased PXR protein levels as

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demonstrated by Western blotting and subsequent densitometric analysis (Figs. 2E and F) but rifampicin had no significant effect on PXR degradation.

Overexpression of RBCK1 down regulates PXR genes in HepG2 cells and primary human hepatocytes.

We then investigated the effect of RBCK1 on endogenous PXR protein levels in HepG2 cells. A statistically significant decrease in endogenous levels of PXR protein was observed by Western blot analysis followed by densitometric analysis when RBCK1 was ectopically over expressed in HepG2 cells ($p<0.001$) but rifampicin had no further effect on this decrease (Figs. 3A and B). HepG2 cells were also lysed and subjected to immunoprecipitation with non-specific IgG or antibody to PXR and analyzed by Western blot analysis using antibody to RBCK1. Western blot analysis of the immunoprecipitates showed that endogenous RBCK1 was immunoprecipitated with antibody to PXR, showing interaction of the endogenous levels of PXR and RBCK1 in HepG2 cells (data not shown).

Importantly, in primary human hepatocytes endogenous RBCK1 and PXR were clearly detected by Western blot analysis of three pooled wells from a 24-well plate, and ectopic overexpression of RBCK1 decreased endogenous PXR protein expression (Fig. 3C). Using qPCR as described in methods, we examined the effect of RBCK1 on the expression of mRNAs for PXR-target genes that are involved in metabolizing xenobiotic compounds, including phase I and II enzymes and transporter genes. Overexpression of RBCK1 significantly reduced rifampicin-mediated up regulation of mRNA for various PXR-regulated genes such as CYP2C9 (47% decrease), CYP3A4 (44% decrease), UGT1A9 (38% decrease), GSTA2 (30% decrease), and two of the multidrug resistance reporters, ABCB1 (P-glycoprotein) (33% decrease) and ABCC2 (32% decrease).

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Silencing RBCK1 up regulates expression of PXR target genes in primary human hepatocytes

We then silenced endogenous RBCK1 expression in primary human hepatocytes. We used two siRBCK1 oligos along with si-control (si-C) at two different concentrations (50 and 100 ng). As indicated by Western blot analysis in Figs. 4A and B, 100 ng of siRBCK1-1 effectively down -regulated RBCK1 expression significantly ($p<0.05$). Endogenous PXR levels were increased 2-3-fold when RBCK1 expression was silenced with 50 or 100 ng of siRBCK1 as compared to untransfected cells or those transfected with si-C (Figs. 4A and B). In addition, silencing RBCK1 significantly enhanced rifampicin induction of the PXR target genes CYP2C9 and CYP3A4 (phase I enzymes), UGT1A9 and GSTA (phase II enzymes), and ABCB1 and ABCC2 (transporters) in primary human hepatocytes (Figs. 4C-H).

Discussion

In the present study, RBCK1, an E3 ubiquitin ligase was shown to interact with PXR in yeast two hybrid screens, GST-pull down assays and immunoprecipitation studies. Importantly, we describe the involvement of RBCK1 in the ubiquination of PXR presumably targeting it for degradation by the ubiquitin-proteasome system. PXR, a xenobiotic-sensing receptor, upregulates a large number of drug- metabolizing enzymes and transporters (Tolson and Wang ; Wang et al., 2011). PXR has also been reported to be involved in lipid homeostasis and atherosclerosis (Sui et al., 2011). A recent study demonstrated that PXR activation induces tumor aggressiveness in humans and mice through increased cellular proliferation and drug resistance (Wang et al., 2011). Though PXR regulates many target genes, its regulation is not fully understood. Here, we have identified RBCK1 as a novel interacting protein of PXR, which is involved in the regulation of hPXR protein expression. Domain mapping studies indicate that PXR exhibits strong interaction with full length RBCK1 and the domain containing the NZF, CC and RING-IBR region. Previously, studies have shown that the N-terminus of RBCK1, containing the NZF and CC domains, is essential for its interaction with various proteins such as TAB2/3 (Tian et al., 2007) and IRF3 (Zhang et al., 2008) but show some interaction with other domains. Our data show that the N-terminal region of RBCK1 containing a ubiquitin-like domain (UBL), NZF and CC interacts with PXR as well as the CC and RING-IBR regions. The RING-IBR region was previously shown to be required for ubiquitin ligase activity (Tian et al., 2007). Similar observations were also reported in a study involving another E3 ubiquitin ligase protein, Parkin, which interacts with its target protein, Septin 5 (SEPT5_v2) through either the N-terminal ubiquitin-like domain or the RING-IBR region (Choi et al., 2003). Future studies

would be required to determine whether the binding of PXR to this region is also important for the ability of RBCK1 to ubiquitinate PXR

Our results show that RBCK1 down regulates PXR protein expression. RBCK1 acts as an E3 ligase, facilitating ubiquitination and thus presumably targets PXR for eventual degradation. The proteasomal inhibitor MG-132 caused a marked increase in PXR protein levels when RBCK1, PXR and ubiquitin were ectopically expressed in AD-293 cells, suggesting a role for RBCK1 in proteasomal degradation of PXR. However, we failed to completely restore PXR protein expression with MG-132. Similar results were also observed for the effect of RBCK1 on the degradation of IRF3 under similar experimental conditions to those in our study (Zhang et al., 2008). Our results suggest that RBCK1 down regulates PXR expression by promoting proteasomal-dependent degradation, but we cannot exclude degradation by other mechanisms or effects on synthesis. Pulse-labeling experiments will be required to determine whether RBCK1 increases degradation of PXR. Our results are consistent with previous studies demonstrating that RBCK1 acts as an E3 ubiquitin ligase which regulates the degradation of heme-oxidized iron regulatory protein-2 (IRP2)(Yamanaka et al., 2003). Other studies have shown that RBCK1 is involved in negative regulation of TNF and IL-1-induced inflammatory signaling by targeting TGF-β-activated kinase 1 (TAK1) binding protein 2/3 (TAB2/3) for degradation (Tian et al., 2007).

One study (Masuyama et al., 2002) reported that some steroids (progesterone and dexamethasone) modify PXR protein levels through the proteasomal pathway in the mouse. Although the authors suggested these compounds might be PXR ligands, they are not prototypical PXR ligands. We used the prototype human PXR agonist rifampicin to analyze its potential to affect PXR degradation in AD-293 cells or HepG2 cells. We found ubiquitination of

PXR in AD-293 cells to be unaffected by the PXR ligand rifampicin after ectopic expression of PXR and RBCK1 in this cell line. In addition, over expression of RBCK1 in HepG2 cells caused down regulation of endogenous levels of PXR independent of rifampicin treatment. Our findings are consistent with those of Sh eflin and coworkers (Sh eflin et al., 2000) who found that the androgen receptor is degraded via the proteasome in the absence of ligand.

Importantly, RBCK1 decreases endogenous levels of PXR in pr imary human hepatocytes. Since human PXR is an essential regulator of a large number of drug disposition genes (Staudinger et al., 2001a; Staudinger et al., 2001b; Maglich et al., 2002; Rosenfeld et al., 2003; Hartley et al., 2006), we examined the impact of overexpression and silencing of RBCK1 on induction of various PXR-regulated genes in primary human hepatocytes by rifampicin, including phase I enzymes such as CYP enzymes, phase II enzymes such as UGTs, glutathione-S-transferases, sulfotransferases, and two members of the ATP-binding cassette (ABC) multidrug transistance transporters, ABCC2 and ABBC1 (P-glycoprotein). Western blot analysis indicated that overexpression of RBCK1 down regulated endogenous PXR levels in pr imary human hepatocytes, while silencing the expression of RBCK1 by RNAi resulted in increased PXR levels. RBCK1-mediated decreases in PXR protein levels down regulated the rifampicin-mediated PXR induction of CYP2C9, CYP3A4, UGT1A9, SULT2A1, GSTA2, ABCB1 and ABCC2 mRNAs in primary human hepatocytes. Conversely, silencing endogenous RBCK1 expression in primary human hepatocytes resulted in increased induction of PXR target genes by rifampicin.

In conclusion, we identified RBCK1 as a new PXR-interacting protein which regulates PXR protein expression via increased ubiquitination and presumably targets it for proteasomal degradation in human primary hepatocytes and other cell lines. Ectopic expression of RBCK1

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increased PXR degradation and thus decreased induction of various genes involved in drug metabolism. Silencing RBCK1 in primary hepatocytes increased rifampicin induction of these genes. Our study provides an important insight in the role of RBCK1 in the ubiquitination and presumably the targeting of PXR for degradation by the proteasomal system and thereby the regulation of PXR and various PXR target genes.

Authorship Contributions

Participated in research design: Rana, Kinyamu, and Goldstein

Conducted Experiments: Rana, and Coulter

Performed data analysis: Rana and Coulter

Wrote or contributed to the writing of the manuscript: Rana, Goldstein, Kinyamu, and Coulter

References

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P and Berkenstam A (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci U S A* 95:12208-12213.

Blumberg B, Sabbagh W, Jr., Juguilon H, Bolado J, Jr., van Meter CM, Ong ES and Evans RM (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev* 12:3195-3205.

Chang TK (2009) Activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR) by herbal medicines. *Aaps J* 11:590-601.

Chen Y, Kissling G, Negishi M, Goldstein JA (2005) The nuclear receptors constitutive androstane receptor and pregnane X receptor cross-talk with hepatic nuclear factor 4alpha to synergistically activate the human CYP2C9 promotor *J Pharmacol Exp Ther* 314:1125-33.

Choi P, Snyder H, Petrucelli L, Theisler C, Chong M, Zhang Y, Lim K, Chung KK, Kehoe K, D'Adamio L, Lee JM, Cochran E, Bowser R, Dawson TM and Wolozin B (2003) SEPT5_v2 is a parkin-binding protein. *Brain Res Mol Brain Res* 117:179-189.

Cong YS, Yao YL, Yang WM, Kuzhandaivelu N and Seto E (1997) The hepatitis B virus X-associated protein, XAP3, is a protein kinase C-binding protein. *J Biol Chem* 272:16482-16489.

Dotzlaw H, Leygue E, Watson P and Murphy LC (1999) The human orphan receptor PXR messenger RNA is expressed in both normal and neoplastic breast tissue. *Clin Cancer Res* 5:2103-2107.

Garrus JE, von Schwedler UK, Pornillos OW, Morham SG, Zavitz KH, Wang HE, Wettstein DA, Stray KM, Cote M, Rich RL, Myszka DG and Sundquist WI (2001) Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* 107:55-65.

Geick A, Eichelbaum M and Burk O (2001) Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem* 276:14581-14587.

Glickman MH and Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* 82:373-428.

Hartley DP, Dai X, Yabut J, Chu X, Cheng O, Zhang T, He YD, Roberts C, Ulrich R, Evers R and Evans DC (2006) Identification of potential pharmacological and toxicological targets differentiating structural analogs by a combination of transcriptional profiling and promoter analysis in LS-180 and Caco-2 adenocarcinoma cell lines. *Pharmacogenet Genomics* 16:579-599.

Johnson DR, Li CW, Chen LY, Ghosh JC and Chen JD (2006) Regulation and binding of pregnane X receptor by nuclear receptor corepressor silencing mediator of retinoid and thyroid hormone receptors (SMRT). *Mol Pharmacol* 69:99-108.

Kinyamu HK, Chen J and Archer TK (2005) Linking the ubiquitin-proteasome pathway to chromatin remodeling/modification by nuclear receptors. *J Mol Endocrinol* 34:281-297.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T and Lehmann JM (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* 92:73-82.

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT and Kliewer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* 102:1016-1023.

Li T and Chiang JY (2006) Rifampicin induction of CYP3A4 requires pregnane X receptor cross talk with hepatocyte nuclear factor 4alpha and coactivators, and suppression of small heterodimer partner gene expression. *Drug Metab Dispos* 34:756-764.

Maglich JM, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT and Kliewer SA (2002) Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharmacol* 62:638-646.

Marin I and Ferrus A (2002) Comparative genomics of the RBR family, including the Parkinson's disease-related gene parkin and the genes of the ariadne subfamily. *Mol Biol Evol* 19:2039-2050.

Martinez-Noel G, Niedenthal R, Tamura T and Harbers K (1999) A family of structurally related RING finger proteins interacts specifically with the ubiquitin-conjugating enzyme UbcM4. *FEBS Lett* 454:257-261.

Masuyama H, Hiramatsu Y, Mizutani Y, Inoshita H and Kudo T (2001) The expression of pregnane X receptor and its target gene, cytochrome P450 3A1, in perinatal mouse. *Mol Cell Endocrinol* 172:47-56.

Masuyama H, Inoshita H, Hiramatsu Y and Kudo T (2002) Ligands have various potential effects on the degradation of pregnane X receptor by proteasome. *Endocrinology* 143:55-61.

Masuyama H, Suwaki N, Tateishi Y, Nakatsukasa H, Segawa T and Hiramatsu Y (2005) The pregnane X receptor regulates gene expression in a ligand- and promoter-selective fashion. *Mol Endocrinol* 19:1170-1180.

Meyer HH, Wang Y and Warren G (2002) Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4. *Embo J* 21:5645-5652.

Miki Y, Suzuki T, Tazawa C, Blumberg B and Sasano H (2005) Steroid and xenobiotic receptor (SXR), cytochrome P450 3A4 and multidrug resistance gene 1 in human adult and fetal tissues. *Mol Cell Endocrinol* 231:75-85.

Ong S, Wang Y, Chai S and Chen T (2011) Pregnane X Receptor in Drug Development, in: *Drug Development-A Case Study Based Insight into Modern Strategies* (Runfield C ed), pp 337-364.

Pascussi JM, Drocourt L, Gerbal-Chaloin S, Fabre JM, Maurel P and Vilarem MJ (2001) Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor. *Eur J Biochem* 268:6346-6358.

Rana R, Chen Y, Ferguson SS, Kissling GE, Surapureddi S and Goldstein JA (2010) Hepatocyte nuclear factor 4{alpha} regulates rifampicin-mediated induction of CYP2C genes in primary cultures of human hepatocytes. *Drug Metab Dispos* 38:591-599.

Rosenfeld JM, Vargas R, Jr., Xie W and Evans RM (2003) Genetic profiling defines the xenobiotic gene network controlled by the nuclear receptor pregnane X receptor. *Mol Endocrinol* 17:1268-1282.

Roth A, Looser R, Kaufmann M and Meyer UA (2008) Sterol regulatory element binding protein 1 interacts with pregnane X receptor and constitutive androstane receptor and represses their target genes. *Pharmacogenet Genomics* 18:325-337.

Sheflin L, Keegan B, Zhang W and Spaulding SW (2000) Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels. *Biochem Biophys Res Commun* 276:144-150.

Stanley LA, Horsburgh BC, Ross J, Scheer N and Wolf CR (2006) PXR and CAR: nuclear receptors which play a pivotal role in drug disposition and chemical toxicity. *Drug Metab Rev* 38:515-597.

Staudinger J, Liu Y, Madan A, Habeebu S and Klaassen CD (2001a) Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor. *Drug Metab Dispos* 29:1467-1472.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH and Kliewer SA (2001b)

The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* 98:3369-3374.

Staudinger JL, Xu C, Biswas A and Mani S (2011) Post-translational modification of pregnane x receptor. *Pharmacol Res* 64:4-10.

Sui Y, Xu J, Rios-Pilier J and Zhou C (2011) Deficiency of PXR decreases atherosclerosis in apoE-deficient mice. *J Lipid Res* 52:1652-1659.

Surapureddi S, Rana R, Reddy JK and Goldstein JA (2008) Nuclear receptor coactivator 6 mediates the synergistic activation of human cytochrome P-450 2C9 by the constitutive androstane receptor and hepatic nuclear factor-4alpha. *Mol Pharmacol* 74:913-923.

Tatematsu K, Yoshimoto N, Koyanagi T, Tokunaga C, Tachibana T, Yoneda Y, Yoshida M, Okajima T, Tanizawa K and Kuroda S (2005) Nuclear-cytoplasmic shuttling of a RING-IBR protein RBCK1 and its functional interaction with nuclear body proteins. *J Biol Chem* 280:22937-22944.

Tian Y, Zhang Y, Zhong B, Wang YY, Diao FC, Wang RP, Zhang M, Chen DY, Zhai ZH and Shu HB (2007) RBCK1 negatively regulates tumor necrosis factor- and interleukin-1-triggered NF-kappaB activation by targeting TAB2/3 for degradation. *J Biol Chem* 282:16776-16782.

Tokunaga C, Kuroda S, Tatematsu K, Nakagawa N, Ono Y and Kikkawa U (1998) Molecular cloning and characterization of a novel protein kinase C-interacting protein with

structural motifs related to RBCC family proteins. *Biochem Biophys Res Commun* 244:353-359.

Tolson AH and Wang H (2010) Regulation of d rug-metabolizing enzymes by xenobiotic receptors: PXR and CAR. *Adv Drug Deliv Rev* 62:1238-1249.

Wang H, Venkatesh M, Li H, Goetz R, Mukherjee S, Biswas A, Zhu L, Kaubisch A, Wang L, Pullman J, Whitney K, Kuro-o M, Roig AI, Shay JW, Mohammadi M and Mani S (2011) Pregnane X receptor activation induces FGF19-dependent tumor aggressiveness in humans and mice. *J Clin Invest* 121:3220-3232.

Yamanaka K, Ishikawa H, Megumi Y, Tokunaga F, Kanie M, Rouault TA, Morishima I, Minato N, Ishimori K and Iwai K (2003) Identification of the ubiquitin-protein ligase that recognizes oxidized IRP2. *Nat Cell Biol* 5:336-340.

Zhang M, Tian Y, Wang RP, Gao D, Zhang Y, Diao FC, Chen DY, Zhai ZH and Shu HB (2008) Negative feedback regulation of cellular antiviral signaling by RBCK1-mediated degradation of IRF3. *Cell Res* 18:1096-1104.

Footnotes

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Address reprint requests to Joyce A. Goldstein, Maildrop A3-02, NIEHS, Research Triangle Park, NC, 27709, USA. E-mail: goldste1@niehs.nih.gov

Current affiliation-Ritu Rana,, Associate Research Scientist, Department of Pediatrics, Yale University School of Medicine, New Haven, CT 06520-8064

Figure Legends

Fig. 1. RBCK1 is identified as a novel PXR interacting protein. (A) RBCK1 interaction with PXR. AD-293 cells were transfected with ectopic PXR and RBCK1 constructs as indicated. The cell extracts were immunoprecipitated using PXR antibody and probed with RBCK1 (left panel) and reciprocally, the extracts were immunoprecipitated with RBCK1 antibody and probed with PXR antibody (right panel). (B) Schematic representation of RBCK1 along with generated fragments for domain mapping with PXR. The numbers indicate the amino acid (aa) residues, and the length of the recombinant protein fragments. RBCK1 consists of the N-terminal ubiquitin-like (UBL) domain, two coiled-coil (CC) domains, a novel zinc finger (NZF) domain, two RING fingers and an in-between-RING fingers (IBR) domain. (C) Full length RBCK1 (FL) and the three fragments were expressed as GST fusion proteins and incubated with full-length *in vitro*-translated ^{35}S-labeled PXR.

Fig 2. RBCK1 regulates PXR protein expression in a proteasome-dependent manner and degradation is not affected by treatment with the PXR ligand rifampicin. (A) RBCK1 down-regulates ectopically expressed PXR protein in AD-293 cells. Cells were transfected with PXR, HA-ubiquitin and RBCK1 as indicated. After 48h, cell lysates were analyzed by Western blotting with anti-PXR, anti-RBCK1or β-actin antibodies. (B) The decrease of PXR expression in cells cotransfected with RBCK1 was partially blocked by pretreatment with MG-132, a proteasomal inhibitor. AD-293 cells were transfected with the indicated plasmids, and after 40h, cells were treated with 10 μM MG-132 for 6h. Expression levels of PXR protein were compared using Western blot analysis. (C) RBCK1 ubiquitinates PXR. AD-293 cells were transfected with PXR, PXR and HA-ubiquitin, or PXR, HA-ubiquitin and RBCK1 as indicated. After 40h, cells were treated with MG-132 (10 μM) for 6h. Cell lysates were immunoprecipitated with PXR

antibody, subjected to gel electrophoresis and probed with anti-HA antibody to detect ubiquitination of PXR. The ubiquinated ladder was much more pronounced when the cells were transfected with PXR, ubiquitin, and RBCK1 compared to those transfected with PXR and ubiquitin alone. (D) The PXR ligand rifampicin had no effect on RB CK1-mediated ubiquitination of PXR protein. AD-293 cells were transfected with PXR, HA-ubiquitin and RBCK1 plasmids as indicated. After 24h, cells were treated with 10 μM rifamipicin or DMSO. Cell lysates were immunoprecipitated using PXR antibody and analyzed for polyubiquitinated PXR protein using anti-HA antibody. (E) Western blot analysis showed that RBCK1 decreased the expression of PXR and this decrease was independent of rifampicin treatment. AD-293 cells were transfected with the indicated plasmids. After 24h, cells were treated with 10 μM rifampicin (+) or DMSO controls (-). Cell lysates were subjected to Western blot analysis using antibodies to PXR or β-actin. (F) Densitometric analysis of the protein bands on Western blots relative to actin loading controls indicated that the relative amounts of ectopically expressed PXR were significantly decreased (**, $P<0.01$; ***, $P<0.001$) when PXR was coexpressed with RBCK1. Data represent the means ± SE (n=3).

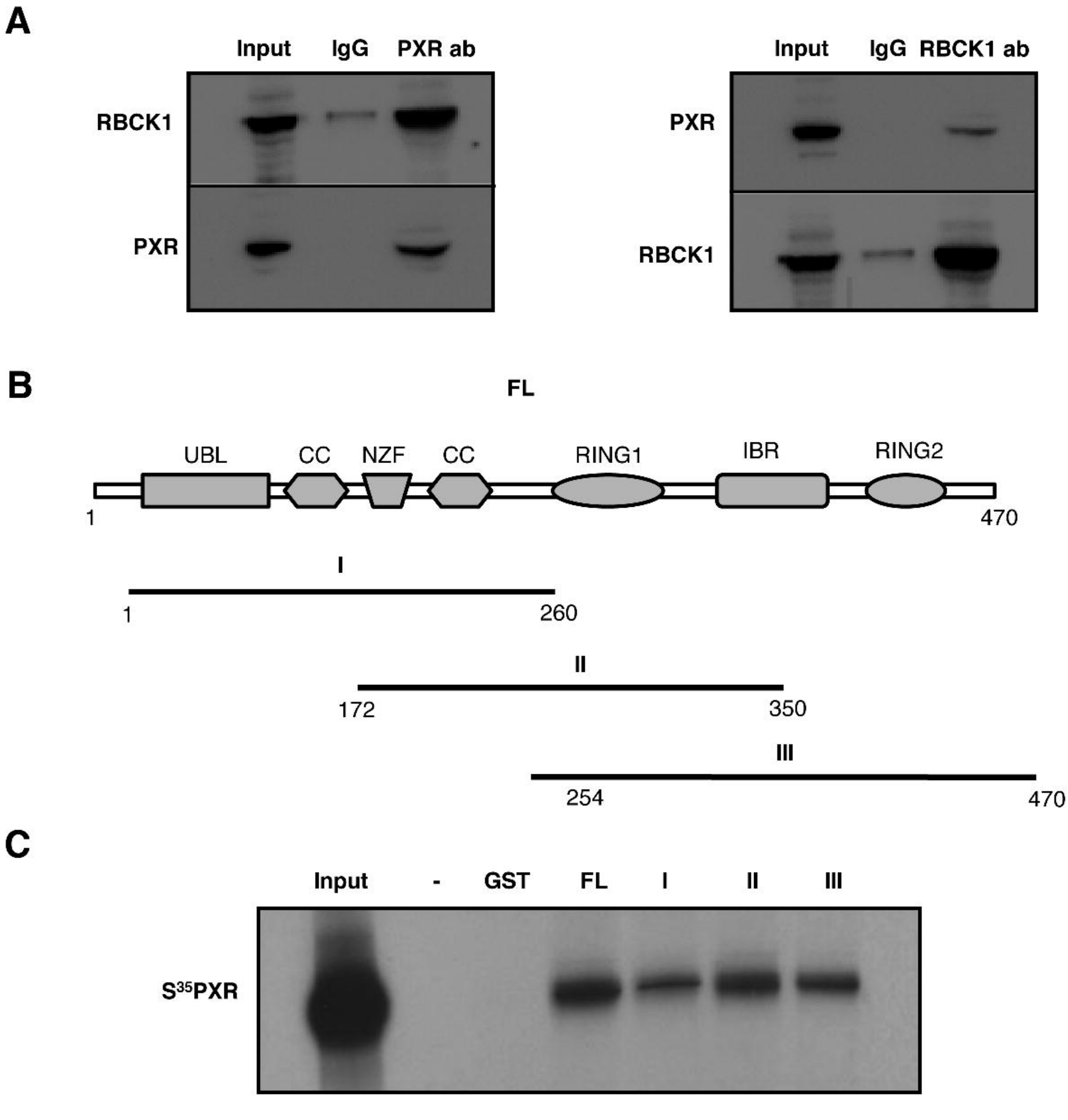
Fig. 3. RBCK1 down-regulates expression of PXR protein in HepG2 cells and human hepatocytes as well as PXR target mRNAs in primary human hepatocytes. (A) HepG2 cells were transfected with RBCK1 plasmid or the empty vector pcDNA3.1 and treated with DMSO (-) or rifampicin (10 μM) (+) for 24h. Cell lysates were analyzed by Western blot analysis using antibodies for PXR or β-actin as a loading control. Ectopic expression of RBCK1 resulted in decreased endogenous levels of PXR protein and there was no further effect of rifampicin treatment. (B) Densitometric scanning of the blots indicated a significant decrease in PXR levels after expression of RBCK1 relative to actin controls ($p<0.001$). (C) Ectopic over expression of

RBCK1 in primary human hepatocytes decreases endogenous PXR levels. Primary hepatocytes were transfected with RBCK1 and PXR and RBCK1 protein expression relative to actin were analyzed by Western blot 48h later of a pooled sample from three wells of a 24-well plate. Results indicate a single analysis (D-I) Overexpression of RBCK1 also decreases the induction of mRNA of PXR target genes in primary human hepatocytes as measured by qPCR. Values are expressed relative to TBP internal control. The relative quantities were calculated by the $2^{-\Delta\Delta Ct}$ method with rifampicin values expressed relative to vehicle controls set to 1. Black bars represent cells treated with rifampicin and white bars cells treated with the vehicle DMSO. Data represent means ± SE (n = 3). Rifampicin-treated cells had significantly higher amounts of the indicated mRNAs than DMSO controls (**P< 0.01, ***P<0.001). Rifampicin induction in RBCK1-transfected cells was significantly lower than in control cells ($^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.001).

Fig. 4. Silencing endogenous RBCK1 protein in primary human hepatocytes increases PXR expression and rifampicin induction of PXR target genes. (A) Western blot analysis of RBCK1, PXR and actin in primary human hepatocytes transfected with 50 or 100 ng of RBCK1 siRNA oligos (siRBCK1-I), or Control oligos (si-C) B. Quantitation was performed by densitometry of Western blots from two different experiments and normalized to actin (N=2). The effects of siRBCK1 on RBCK1 levels were compared using a paired, two-tailed t-test in GraphPad Prism. *, P<0.05. (C-H) Silencing RBCK1 also enhanced rifampicin-induction of PXR target genes. Primary human hepatocytes were transfected with 100 ng of siRBCK1-I or si-C and treated with 10 μM rifampicin or DMSO for 24h. mRNA for PXR target genes was analyzed by qPCR as above. Black bars indicate rifampicin-treated cells, white bars represent DMSO controls. Values are expressed relative to TBP and then normalized to untreated controls. Rifampicin increased the indicated mRNAs over the levels in DMSO controls (means ± SE (n = 3), ***P < 0.001,

while treatment with siRBCK1 significantly increased rifampicin induction over si-C-treated controls, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$.

Figure 1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PXR ab
RBCK1
PXR
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RBCK1
B
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UBL
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CC
RING1
IBR
RING2
1
470
I
1
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II
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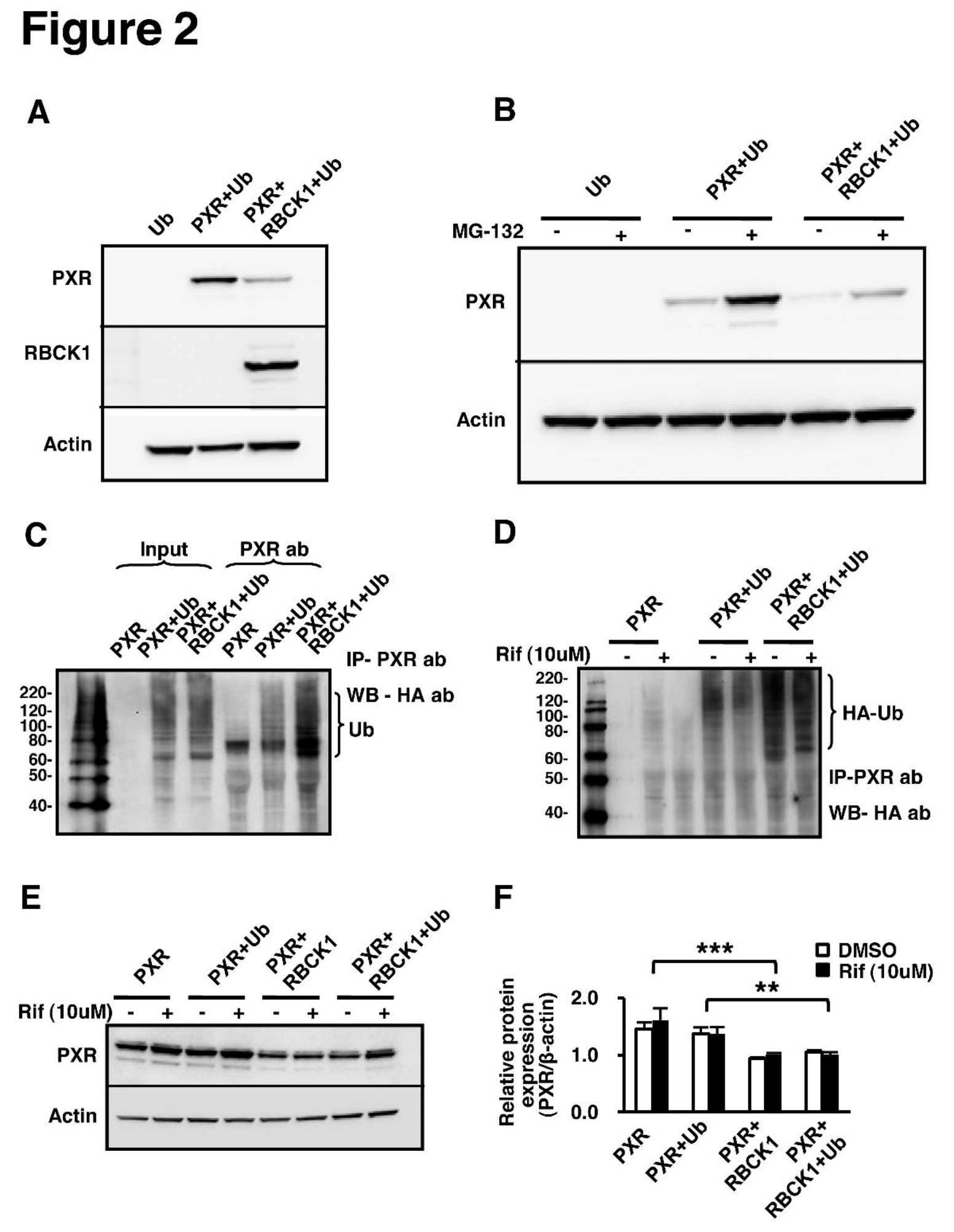
350
III
254
470
C
Input
-
GST
FL
I
II
III
S35PXR

Figure 2


A
Ub
PXR+Ub
PXR+ RBCK1+Ub
PXR
RBCK1
Actin
B
Ub
PXR+Ub
PXR+ RBCK1+Ub
MG-132
- + - + - +
PXR
Actin
C
Input
PXR ab
PXR
PXR+Ub
PXR+ RBCK1+Ub
IP- PXR ab
WB - HA ab
Ub
220-
120-
100-
80-
60-
50-
40-
D
PXR
PXR+Ub
PXR+ RBCK1+Ub
Rif (10uM)
- + - + - +
HA-Ub
IP-PXR ab
WB- HA ab
E
PXR
PXR+Ub
PXR+ RBCK1
PXR+ RBCK1+Ub
Rif (10uM)
PXR
Actin
F
DMSO
Rif (10uM)
***
**
Relative protein expression (PXR/β-actin)
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1.0
0.0
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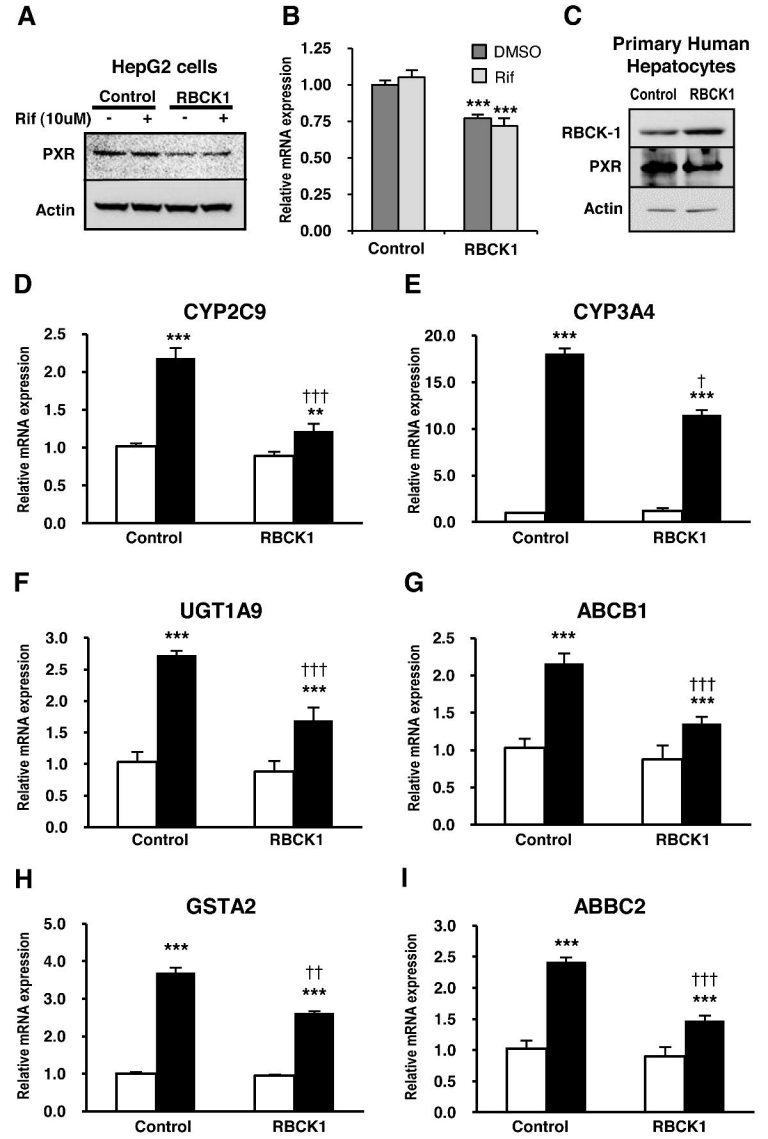
PXR+Ub
PXR+ RBCK1
PXR+ RBCK1+Ub

Figure 3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HepG2 cells
Control
RBCK1
Rif (10uM)
- + - +
PXR
Actin
B
Relative mRNA expression
1.25
1.00
0.75
0.50
0.25
0.00
DMSO
Rif
***
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Control
RBCK1
C
Primary Human Hepatocytes
Control
RBCK1
RBCK-1
PXR
Actin
D
CYP2C9
Relative mRNA expression
2.5
2.0
1.5
1.0
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†††
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Control
RBCK1
E
CYP3A4
Relative mRNA expression
20.0
15.0
10.0
5.0
0.0
***
†
***
Control
RBCK1
F
UGT1A9
Relative mRNA expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
†††
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Control
RBCK1
G
ABCB1
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
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†††
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Control
RBCK1
H
GSTA2
Relative mRNA expression
5.0
4.0
3.0
2.0
1.0
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††
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Control
RBCK1
I
ABBC2
Relative mRNA expression
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2.0
1.5
1.0
0.5
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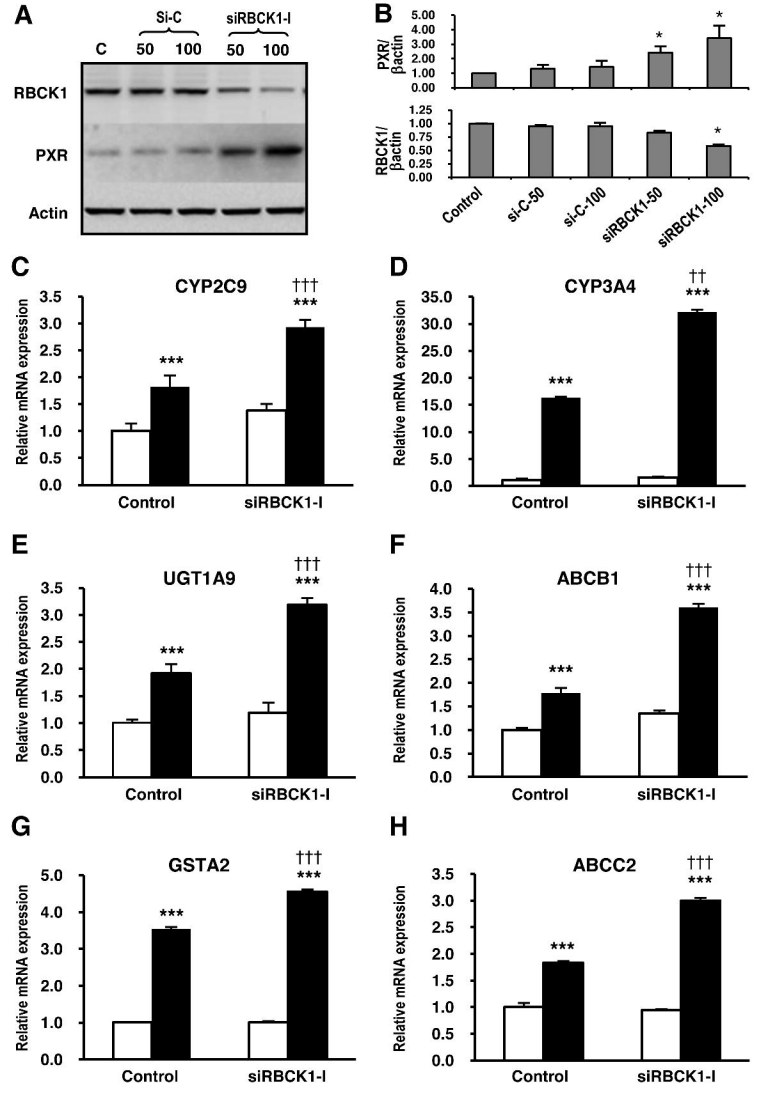
†††
***
Control
RBCK1

Figure 4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Si-C
siRBCK1-I
C
50
100
50
100
RBCK1
PXR
Actin
B
PXR/βactin
RBCK1/βactin
Control
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si-C-100
siRBCK1-50
siRBCK1-100
C
CYP2C9
D
CYP3A4
E
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F
ABCB1
G
GSTA2
H
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Relative mRNA expression
Control
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