Post-Translational Modifications of Pregnane X Receptor

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Abstract

Pregnane X Receptor (PXR, NR1I2) is a member of the nuclear receptor (NR) superfamily of ligand-activated transcription factors. Expression levels of PXR are highest in the liver and intestine. The activation of PXR can be achieved by exposure to a myriad of xenobiotic compounds and prescription drugs to regulate the expression of genes that encode key enzymes and membrane transporter proteins. Collectively, these PXR-target genes encode gene products that function in a coordinate manner and comprise a vital xenobiotic detoxification pathway in these tissues. In this way, PXR activation by these compounds functions as a 'xeno-sensor' of foreign substances in our body to positively regulate the transcription of genes such as cytochrome P450 (CYP) 3A4, and the drug efflux transporter multiple drug resistant protein 1 (MDR1/P-gp), as well as other drug metabolizing enzymes and drug transporter proteins. Xenobiotic-mediated activation of PXR in humans also represents the molecular mechanism of CYP3A4-triggered adverse drug-drug interactions in which the induction of the expression of this broadly selective drug-metabolizing enzyme increases the metabolism of many other co-administered substrates. Activation of PXR also appears to be involved at some level in the MDR1-mediated acquired resistance to chemotherapeutic agents in multiple cancer types.

Beyond the canonical physiology of ligand-mediated PXR activation in the regulation of drug metabolism, accumulating evidence clearly indicates that PXR exerts a trans-repressive activity towards the inflammatory response in both the liver and intestine in humans. A broad spectrum of evidence suggests the involvement of post-translational modifications (PTMs) in the regulation of the trans-repressive transcriptional effects of many liver-enriched NR proteins. Previous studies in our laboratory have revealed that PXR is the molecular target of several PTMs including phosphorylation, ubiquitination, and SUMO- (small ubiquitin-like modifier)

modification (SUMOylation). Moreover, our research shows that PTMs that target PXR likely regulate its biological activity through sophisticated system of networking or 'crosstalk'. Crosstalk in this sense is defined as how various PTMs interact with each other on a given protein target to produce a specific biological outcome. The current study is focused on the mechanism of crosstalk between the PTMs and their effect upon the regulation of PXR-mediated trans-repression phenomenon.

In the first chapter of this dissertation I provide an introduction to the topic of NR signaling in general, followed by an explanation of canonical PXR signaling in detail. In Chapter 2, the role of crosstalk between the SUMOylation and ubiquitination pathways is examined and its effect upon the regulation of PXR biology in primary hepatocytes is discussed. Tumor necrosis factor-alpha (TNFα)-triggered SUMO(1)ylation of PXR is well-known to inhibit the expression of inflammatory genes in liver and intestine. I show in this dissertation that treatment with the PXR activators, such as Rifampicin (Rif), promotes the SUMO3-modification of PXR. Further, I show that the SUMO(3)ylation of PXR subsequently increases the ubiquitination of PXR, likely to promote proteasomal degradation of this important transcription factor. In Chapter 3, the crosstalk between SUMOylation and acetylation was investigated. I found that pharmacological inhibition of histone deacetylase 3 (HDAC3) activity in cell line-based assays significantly promotes the SUMOylation of PXR, which subsequently impairs the ability of PXR to interact with its canonical corepressor multi-protein complex HDAC3/SMRT (silencing mediator for retinoid or thyroid–hormone receptor). Taken together, the results presented in this dissertation provide novel insight into the likely molecular mechanisms that regulate the clinically observed PXR-mediated trans-repression phenomenon. Specifically, my results suggest that this phenomenon is controlled by an SUMO-acetyl 'functional switch' in

which PXR acetylation marks PXR as competent for its subsequent SUMOylation, given the correct physiological extracellular condition, namely inflammation. In Chapter 4, the molecular details of the role of phosphorylation in the regulation of PXR-initiated transcription, and its effect upon the interaction with PXR accessory proteins were examined. Utilizing a liquid chromatography tandem mass spectrometry (LC-MS/MS)-based proteomic approach, two phosphorylation sites (T135 and S221) in PXR were identified in primary mouse hepatocytes. Phosphorylation at identified sites inhibits the trans-activation capacity of PXR through interrupting PXR-RXRα hetero-dimerization and PXR association with coactivator proteins. In conclusion, PTMs modulate different aspects of PXR biological activity in the liver and is especially essential for PXR-originated trans-repression of the inflammatory response in liver and intestine. Collectively, the data presented in this dissertation sheds new light upon the molecular mechanisms governing PXR-mediated suppression of inflammation, and could be expected to provide innovative strategies to target the PXR protein for the treatment of inflammatory diseases.

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List of Abbreviations

AF-1	Activation Function Domain 1
AF-2	Activation Function Domain 2
AP-1	Activator Protein 1
AR	Androgen Receptor
BAG3	Bcl2-Associated Athanogene 3
CAR	Constitutive Androstane Receptor
CBP	CREB-Binding Protein
CDK	Cyclin-Dependent Kinase
CK2	Casein Kinase II
COUP-TF1	Chicken Ovalbumin Upstream Promoter-Transcription Factor 1
CREB	cAMP Response Element-Binding Protein
CYP	Cytochrome P450
CYP450	Cytochrome Protein 450
DAX-1	Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, on chromosome X, gene 1
DBD	DNA Binding Domain
DR-3	Direct Repeat separated by 3 nucleotides
DRIP	Vitamin D Receptor-Interacting Protein
DSS	Dextran Sulfate Sodium
DUB	Deubiquitinating Enzyme
ER	Estrogen Receptor
ER-6	Everted Repeat separated by 6 nucleotides

FoxM1	Forkhead Box M1
FXR	Farnesoid X Receptor
GFP	Green Fluorescent Protein
GR	Glucocorticoid Receptor
GRIP1	Glucocorticoid Receptor-Interacting Protein 1
GSK3	Glycogen Synthase Kinase 3
GSK3	Glycogen Synthase Kinase 3
HAT	Histone Acetyltransferase
HCC	Hepatocellular carcinoma
HDAC	Histone Deacetylase
HIF1 alpha	Hypoxia Inducible Factor 1α
HNF-4	Hepatocyte Nuclear Factor 4
HSP	Heat Shock Protein
IBD	Inflammatory Bowel Disease
IL1β	Interleukin 1 beta
IMAC	Immobilized Metal Affinity Chromatography
IκBα	Inhibitor of Transcription Factor NF-κB alpha
LBD	Ligand Binding Domain
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LXR	Liver X Receptor
MDR1	Multiple Drug Resistant Gene 1
Mef2	Myocyte Enhancer Factor-2
MNK	Mitogen-Acivated Kinase-Interacting Serine/Threonine-protein Kinase

MR	Mineralocorticoid Receptor
NCoA	Nuclear Receptor Coactivator
NCoR	Nuclear Receptor Corepressor
NEMO	NF-κB Essential Modulator
NF-κB	Nuclear Factor kappa-light-chain-enhancer of Activated B cells
NR	Nuclear Receptor
nTRE	Negative Thyroid Response Element
P-gp	P-Gylcoprotein
P-gp1	P-Glycoprotein 1
p/CAF	p300/CBP Associated Factor
PBP	PPAR binding protein
PCN	Pregnenolone-16α-carbonitrile
PCNA	Proliferating Cell Nuclear Antigen protein
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PIAS	Protein Inhibitor of Activated STAT
PKA	Protein Kinase A
PKC	Protein Kinase C
PPAR	Peroxisome Proliferator-Activated Receptor
PR	Progesterone Receptor
prPXRE	Proximal PXR Response Element
PTM	Post-Translational Modification
PXR	Pregnane X Receptor
PXR-KO	PXR Knockout

RanBP2	Ran Binding Protein 2
RAR	Retinoic Acid Receptor
RFP	Red Fluorescent Protein
Rif	Rifampicin
RNF4	Ring Finger Protein 4
RXR	Retinoic X Receptor
SAE	SUMO Activating Enzyme
SENP	Sentrin/SUMO-specific Protease
SIM	SUMO-Interacting Motif
SIRT1	Sirtuin 1
SMRT	Silencing Mediator for Retinoid or Thyroid-hormone Receptor
SRC	Steroid Receptor Coactivator
SRF	Serum Response Factor
STUbL	SUMO-Targeted Ubiquitin Ligase
SUMO	Small Ubiquitin-like Modifier
TGFβ	Transforming Growth Factor β
TIF2	Transcriptional Intermediary Factor 2
TNFα	Tumor Necrosis Factor alpha
TR	Thyroid Receptors
TRAC1	T3 Receptor-Associating Cofactor 1
TRAP	TR associated proteins
TSA	Trichostatin A
UPS	Ubiquitin Proteasome System

VDR	Vitamin D Receptor
XREM	Xenobiotic-Responsive Enhancer Module

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Chapter 1: Introduction

1.1 NUCLEAR RECEPTORS

1.1.1 General Remarks

Three decades ago, the successful cloning of receptors for glucocorticoid, estrogen, and thyroid led to rapid recognition of the nuclear receptor (NR) superfamily that composed of 48 members in the human genome(1-3). Historically, the discovery of the action of NRs in the field of endocrinology was initially uncovered during studies of metabolism, development, and reproduction(4). All NR family members share an evolutionary conserved structural template and possess similar functional features. The principle function of NR is sensing hormones to exert direct regulation of tissue-specific gene expression. The hormone that binds to the NR and activates NR-specific transcriptional event is commonly called ligand. Typical ligands include steroidal molecules (progesterones, estrogens, androgens, glucocorticoids, and mineralocorticoids), vitamin D_3, thyroid hormone, retinoids, as well as other newly discovered hormones, including bile acids, dietary lipids, and xenobiotic compounds.

According to their cognate ligands, NRs can be classified in three groups. The first class (Class I) of NRs were identified and named after their tissue-specific ligands, the traditional hormones. Glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), androgen receptor (AR), estrogen receptors (ERs), retinoic acid receptors (RARs), thyroid receptors (TRs), and vitamin D receptor (VDR) all fall into the Class I NRs. After the identification of Class I NRs, a group of evolutionarily related proteins were discovered while their high-affinity endogenous ligands were unknown. These related proteins have thus acquired the name as orphan NRs. Several non-steroid hormones, such as bile acids, fatty acids, and

xenobiotic compounds, were then discovered to bind to specific orphan NRs with high affinity. These 'adopted' orphan NRs include retinoic x receptors (RXRs), peroxisome proliferator-activated receptors (PPARs), farnesoid x receptor (FXR), liver x receptor (LXR), pregnane x receptor (PXR), and constitutive androstane receptor (CAR). While 'adopted' orphan NRs are termed Class II NRs, the rest of the orphan NRs sort into the third class of NRs.

The NR initiated transcription activation and repression require two prerequisite actions, DNA binding and recruitment of coregulator proteins. As transcription factors, NRs depend on sequence-specific DNA binding to initiate the trans-activation of target genes. In response to ligand stimulation, Class I NRs bind to response elements as homodimers, whereas Class II NRs bind to DNA as half of a heterodimer with their preferred counterpart RXR(5-11). Besides DNA binding, NR-mediated transcription activities require coordinate interactions with a group of coregulator proteins, which are responsible for converting the NR from a silent state to an active state. Despite the fact that NRs share high-levels of homology among members, they exert varies modes of actions towards the regulation of transcription. For instance, NRs can either directly bind to DNA or through interaction with other types of transcription factors to regulate gene activation in a ligand-dependent manner. Additionally, several NRs suppress gene expression in response to ligand activation by binding to negative response elements or by antagonizing the transcription activity of other transcription factors(12-14).

1.1.2 Structure of Nuclear Receptors

The NR superfamily members share a conserved overall structure, which consists of five homologous domains **(Figure 1-1)**. From N-terminus to C-terminus, the functional domains include an N-terminal activation function domain 1 (AF-1), a zinc-finger-type DNA binding domain (DBD), a flexible hinge domain that separates the DBD from the ligand-binding domain

(LBD), and a C-terminal activation function domain 2 (AF-2). The AF-1 domain is a ligand-independent function domain that can be modified by phosphorylation and other types of post-translational modifications (PTMs). The DBD of NRs is the most conserved domain, and its core function is to recognize and bind specific sequence in the DNA. Specifically, the two zinc fingers buried in DBD intercalate into the major groove of DNA in a sequence-specific manner. The flexible hinge domain forms as a bridge to connect DBD and the multifunctional LBD. The hinge region is considered essential for the ligand-mediated conformational change of NRs. Also, growing evidence demonstrated that the hinge region contains motifs important for the subcellular localization of NRs(15-19). LBD of NRs contains the ligand-binding pocket, the homo- and heterodimerization interfaces, and a co-regulator binding region. The primary role of LBD is to act as a molecular switch by deciphering the ligand structure into conformational changes, which transforms the NR into a transcription activator or repressor(20). The AF-2 domain is buried in the LBD at the C-terminal region, serves as a scaffold for ligand-dependent recruitment of coactivator proteins(21). The structure and function of AF-2 domain remains mysterious due to its high variability. Mutagenesis and functional studies suggest the activity of AF-2 depends on ligand activation and is dispensable.

Figure 1-1.

Figure 1-1. Evolutionarily conserved structure of nuclear receptors.

1.1.3 Nuclear Receptors Dimerization

The first and most critical step in the NR-mediated transcription regulation is the dimerization. In response to ligand activation, the Class I steroid NRs (GR, PR, AR, and ER) form homodimers to bind to response elements constituted as palindromes composed of two hexad nucleotide sequences separated by three base pairs(5). However, non-steroid NRs (RAR, VDR, and TR) tend to bind to response elements configured as tandem repeats of two hexad half-sites sequences(22-24). Another type of dimerization that is strikingly distinct from the homodimerization of Class I NRs is the heterodimerization of class II non-steroid NR with a common partner RXR(6-11). The paradigm of RXR heterodimerization is a universal feature in most of the orphan NRs that include PPARs, LXRs, FXR, PXR, and CAR. Three highly conserved and functionally identical isoforms of RXR have been discovered, RXRα, RXRβ, and RXRγ, and at least one of them is expressed in every tissue type(17). Thus, the joint heterodimerization with RXR offers a simple but elegant mechanism to the evolution of target gene specificity.

1.1.4 The Nuclear Receptor Coregulator Proteins

It is currently well known that accessory coregulator proteins are recruited to NR family members to both suppress and enhance gene activation. In general, NR coregulator proteins include two small gene families of coactivator and corepressor proteins termed nuclear receptor coactivator proteins (NCoA) and NR corepressor proteins (NCoR), respectively. Based on their literal meaning, NCoA proteins are the accessory proteins in the nucleus that interact with ligand-activated NRs to help produce target gene activation, whereas NCoR proteins are accessory factors that interact with non-liganded or antagonist-suppressed NRs to lower the level of target gene activation(25). Silenced or non-liganded NR is recruited to the promoter region

together with NCoR in the absence of ligand or signal; and this multi-protein complex obscures interaction with basal transcription machinery, thereby lowering target gene expression(26). In order to suppress gene expression, the NR-NCoR complex requires the involvement of histone deacetylase (HDAC) enzymes(27). Therefore, the NR-NCoR complex maintains the repressive state of NR mediated transcription(28). Once the NRs are stimulated by ligand or kinase-mediated signaling pathways, NCoR proteins will detach following a conformational shift to allow NCoA proteins to interact with the liganded receptor to initiate gene activation. Histone acetyltransferase (HAT) enzyme activity appears to be required for liganded NR-mediated gene activation(29). In addition to interaction with NR superfamily members, it is now well recognized that NCoRs and NCoAs comprise two small families of structurally conserved proteins that are capable of interacting with other signal-dependent transcription factors including myocyte enhancer factor-2 (Mef2), c-Jun, c-Fos, cAMP response element-binding protein (CREB), and others(25,30,31). Additional studies revealed that HAT and HDAC enzyme activity is associated with coregulator proteins to mediate their functional activity(32-34). Histone modifications are therefore intimately involved in gene regulation by NRs and other signal-dependent transcription factors(35).

1.1.4.1 NR Coactivator Proteins

Most NR coactivator proteins interact with NRs in a ligand-dependent manner, and this interaction directly leads to NR-target gene activation. The NCoAs typically contain one or more “NR box” motifs, LXXLL or FXXLL (where X stands for any amino acid), which forms an amphipathic helix that supports interaction with the receptor(36-40). NCoAs are expressed at extremely low levels in most cell types, thus gene activation by one liganded NR effectively competes for this protein in the presence of another liganded NR to produce lower gene

activation of the second NR than would otherwise be achieved. This phenomenon is termed the "squelching effect". In addition, the NCoA proteins not only regulate gene expression, but also play key roles in driving alternative mRNA splicing, altered NR subcellular localization, altered NR protein stability, and various NR protein post-translational modifications.

To date, hundreds of NCoAs have been discovered. However, the main group is steroid receptor coactivator proteins (SRC family), which include SRC1, SRC2, and SRC3, as well as their various splice variants. The SRC family is a group of essential proteins for liganded NRs to fulfill their *in vivo* functions(39). All three of the SRC family members possess intrinsic HAT activity. Each of the SRC family members exhibit a strong preference for differing NR-binding partners. SRC1 interacts strongly with PR, ER, GR, TR, RXR(41), hepatocyte nuclear factor 4 (HNF-4)(42), and PPAR(43). In addition, they can also interact with some other types of transcription factors including activator protein 1 (AP-1)(44), serum response factor (SRF)(45), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)(46), and p53(47). The SRC-2 coactivator protein, alternatively referred to as glucocorticoid receptor-interacting protein 1 (GRIP1), transcriptional intermediary factor 2 (TIF2), and NCoA-2 in the literature, exhibits strong ligand-dependent interaction with RARα, ERs and PXR to stimulate target gene activation. The SRC-3 coactivator protein was the last to be discovered and has a large number of splice variants. The SRC-3 coactivator proteins can broadly activate the largest number of liganded NRs including RARα, RXR, TR, GR(48), PR(49) and ER(50). In addition, SRC-3 activates other types of signal-dependent transcription factors including the CREB(39) to enhance transcription of its target genes.

Besides SRC family coactivator proteins for liganded-NR, there are a myriad of other coactivator proteins that help induce gene activation in a ligand-dependent manner. In particular,

CREB-binding protein (CBP) can interact directly with SRC family members to enhance histone acetylation by liganded NRs(29). Moreover, the p300/CBP-associated factor (p/CAF) interacts with SRC family members to enhance their transcription efficiency in a similar manner(29,51). TR associated proteins/vitamin D receptor-interacting proteins (TRAP/DRIP complex) interact with TR/vitamin D receptor (VDR) and many NRs to induce trans-activation on target genes in a ligand-dependent fashion(52). The PPAR binding protein (PBP) that functions as a transcription mediator protein, is able to interact with thyroid, retinoid, vitamin D3 receptors and other select NRs including PXR in a ligand-dependent manner(53,54).

1.1.4.2 NR Corepressor Proteins

In general, NCoRs interact with non-liganded NRs to maintain the silent state of NR-target gene activation. The two family members are termed NCoR1 and silencing mediator for retinoic and thyroid hormone receptor (SMRT). Both NCoR1 and SMRT are unable to interact with steroid hormone receptor NR superfamily members in the presence of cognate ligands(55-58). The NCoR family members impart a scaffold function to recruit HDAC enzyme activity to select NRs in the absence of ligand(59,60). Specifically, they promote histone deacetylation through recruitment of the HDAC3 enzyme to enhance chromatin compaction and subsequent target gene repression(61,62). Similar to coactivator proteins, NCoR proteins also exhibit the "squelching effect" due to their relatively low overall cellular expression level.

NCoR1 is the first identified NR corepressor protein. It specifically interacts with TRα, RARα, COUP-TF1 (Chicken Ovalbumin Upstream Promoter-Transcription Factor 1), RevErb (NR1D1 and NR1D2), and DAX-1 (Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, on chromosome X, gene 1), but not RXR, VDR, ER or GR. Since NCoR1 regulates non-liganded NR-mediated gene repression, the vague role it plays in a metabolic homeostasis is

paramount. High serum glucose levels and the resulting increase in insulin produce elevation on NCoR1 expression level. In contrast, low glucose level and high fatty acid levels inhibit the expression of NCoR1(63). Signal-dependent activation of NRs increases the PTMs of NCoR1 and decreases their recruitment and exports corepressor proteins out of nucleus. Such PTMs of NCoR1 include phosphorylation, ubiquitination and SUMOylation(59,60).

SMRT was originally identified by its ability to interact with RAR and TR, the two well-known NRs that mediate strong non-liganded active suppression of target genes. SMRT interacts with RAR and TR in a ligand-reversible manner. However, SMRT interaction with RXR is not ligand-reversible(4,64,65). The ligand activation of most NRs accelerates the dissociation of SMRT from both RAR and TR. In contrast, reports show that the ability of SMRT to associate with RXR-PXR heterodimers is constitutive; in other words, it never leaves the heterodimer(65). TRAC1 (T_3 receptor-associating cofactor 1), which is a truncated SMRT splice variant, functions as a dominant negative suppressor of SMRT. It is noteworthy that SMRT does not always impart a repressive function, as it can interact with negative thyroid response elements (nTREs) to induce trans-activation of select target genes(66). Like NCoR1 and SRC family members, SMRT also interacts with various signal-dependent transcription factors(67).

While NCoR and SMRT share a similar structure, their respective biological functions differ in several key aspects, and they each exhibit specific and strong NR-binding preferences. For instance, one orphan NR called DAX-1 can only interact with NCoR1, but is unable to interact with SMRT(68). In a similar manner, PXR apparently exhibits a preference for SMRT on its prototypical target gene CYP3A(69). Like the SRC family members, NCoR1 and SMRT associate not only with NRs but also with diverse set of other signal-dependent transcriptional

factors such as NF-κB, AP-1, and SRF to mediate gene repression. Moreover, gene knockout studies indicate that both NCoR1 and SMRT are involved in cell development, metabolic homeostasis, inflammation, and cancer(70-72).

1.2 PREGNANE X RECEPTOR

1.2.1 Overview of PXR

PXR (NR1I2) belongs to the Class II NR superfamily and particularly activated by a broad spectrum of xenobiotic substances, including drug metabolizing enzymes and transporters. PXR is predominantly expressed in the liver and intestines and is also expressed in other tissues include kidney, stomach, brain, bone, lung, uterus, heart, adrenal glands, bone marrow, skeletal muscle, and testis to a lesser extent. As a direct regulator of drug metabolism and efflux, PXR is essential for the hepatic detoxification system that protects organisms against potential harmful xenobiotic and endobiotic chemicals(73-75). On a structural perspective, PXR comprises a large and flexible binding cavity in the LBD allowing PXR to bind to a variety of structurally diverse ligands, which is distinct from other NRs. Ligands for PXR range from endobiotics, such as steroid hormone metabolites, vitamins, and bile acids, to xenobiotic molecules, those include herbals, macrolide antibiotics, antifungals, and environmental pollutants(76,77). It is worth noting that PXR exhibits various ligand activation profiles across species which is primarily due to its relatively low homology (50 - 75% identity) in LBDs(78), which is very different from the high level of homology in the DBDs (approximately 95%). This sequence diversity accounts for the major pharmacological differences across species.

PXR-mediated transcription activation is initiated by binding to a regulatory DNA sequence within the promoter of its target gene, called xenobiotic-responsive enhancer module

(XREM). XREM is composed of two distal NR-interacting motifs (-7836 ~ -7617 on CYP3A4 gene), DR-3 (direct repeat separated by 3 nucleotides) and ER-6 (everted repeat separated by 6 nucleotides), respectively. Both of these binding sites are indispensable for PXR to exert full regulation of transcription activity. In addition, PXR can bind to a proximal response element (prPXRE)(79,80). The sequence of prPXRE varies in different PXR target genes, which can be either DR-3 or ER-6. Upon ligand activation, the PXR-RXRα heterodimer can bind to all three sites and thus capable of responding to divergent but overlapping groups of xenobiotic compounds.

It is well established that the coregulator protein exchange is critical for the activation and the termination of PXR activities. Unliganded PXR rests in a silent state via association with NCoR (NCoR1, SMRT). The NCoR functions as a scaffold protein for PXR to interact with histone deacetylase proteins (HDACs). In response to ligand stimulation, PXR dissociates from preoccupied corepressor protein complex and simultaneously recruits coactivator proteins. SRC family members (SRC1, SRC2/GRIP1, SRC3) and the transcription mediator PBP interacts with liganded PXR and promotes the recruitment of transcription machinery at the promoter of target genes by decondensing the chromatin structure(81). Through the regulation of coregulator protein exchange, physiological and pathophysiological signals define the outcome of PXR-mediated transcription events. Moreover, PTMs, such as phosphorylation, acetylation, and SUMOylation, are expected to contribute to the signal-dependent PXR-mediated transcription activities.

1.2.2 PXR in the Regulation of Drug Metabolism

Emerging evidence suggests the association of PXR in many human diseases such as metabolic diseases, inflammatory liver diseases, inflammatory bowel diseases (IBD), and many

cancer types. The canonical mechanism of action of PXR is to directly regulate the expression of genes encoding the drug metabolizing enzymes (phase I, phase II, and phase III). In humans, PXR directly activates the expression of cytochrome protein 450 (CYP) 3A4, CYP2B6 and UGT1A1 (UDP glucuronosyltransferase 1 family, polypeptide A1), which together are responsible for the metabolism of approximately 80% of FDA approved drugs. Induction of these drug-metabolizing enzymes results in increased drug turnover, which leads to clinically important drug-drug interactions. Of note, PXR-mediated adverse drug-drug interactions account for 10-17% of medical symptoms for hospital admissions for senior patients(82). In particular, PXR activation can lead to antagonistic effect on co-administrated drugs, such as anti-HIV protease inhibitors, oral contraceptive, thiazolidinediones, and benzodiazepines(83). PXR-activation induced drug-drug interactions can also lead to liver toxicity(84-87). Constitutive activation of PXR affects cholesterol metabolism and thus becomes a driving force of hepatic steatosis(77,88,89). Another aspect of PXR-caused clinical outcomes is drug resistance. PXR directly regulates the gene expression of multiple drug resistance gene 1 (MDR1), which functioned as a drug efflux pump for xenobiotic compounds. Overexpression of the MDR1-encoding p-glycoprotein (P-gp) leads to drug resistance and tumor progression in many tissue types. For instance, PXR activation promotes the growth of cancerous cells by inducing the expression of fibroblast growth factor 19, which results in subsequent colon cancer progression(90). The exact outcome of PXR activation in different cancer types can be tissue-specific. Activation of PXR accelerates drug clearance together with increased chemotherapeutic drugs resistance in colon cancer and prostate cancer, whereas PXR activation inhibits the progression of breast cancer(86,90,91). Taken together, PXR is a great therapeutic target in preventing adverse drug-drug interactions and chemoresistance in certain cancer types.

1.2.3 PXR in the Regulation of Inflammatory Response

Another significant aspect of PXR biology is its ability to regulate an inflammatory response in the liver and intestines. The clinical relevance of this effect is that patients suffering from chronic inflammation in the liver and intestines exhibit impaired drug metabolizing capability, which leads to increased cytotoxicity in these organs. Intensive studies on this topic indicate that a mutual trans-repression exhibited in drug metabolism and inflammation in the liver and intestines where PXR is the interface regulator. NF-κB is a fundamental transcription factor that mainly regulates innate and adaptive immune responses. The target genes of NF-κB consist of pro-inflammation cytokines and anti-inflammation cytokines. A precise and balanced regulation of the cytokines with opposing purposes is the major function of NF-κB. Dysregulation of NF-κB often contributes to a variety of human disease states including inflammatory diseases, autoimmune and metabolic disorders(94-97). Accumulating evidence indicates that activation of PXR exerted suppressive effects on NF-κB signaling and relieves the dextran sulfate sodium (DSS)-induced IBD symptoms in mice(98,99). These discoveries suggest the therapeutic potential of targeting PXR in the treatment of IBD, which is the fifth most prevalent gastrointestinal disease in the United States(100). Additionally, PXR activation has emerged as having a role in many inflammatory-related liver diseases that include cholestasis, hepatic steatosis, and non-alcoholic fatty liver disease. PXR agonists exhibit therapeutic potential in the treatment of cholestasis regarding a detected association between PXR activation and hepatoprotective effect(101). Moreover, PXR activation prevents the progression of liver fibrosis through suppression of the profibrogenic cytokine transforming growth factor β (TGFβ)(102). In summary, activation of PXR ameliorates NF-κB signaling-mediated chronic inflammation in the liver and intestines.

1.2.4 Post-Translational Modifications of PXR

PTMs are well-known cellular events involved in the regulation of NRs. PTM is a signal-dependent functional modification that affects NR-mediated transcription event through altering the biological activities of NR. PTMs establish a pivotal mechanism for crosstalk between signaling pathways. Recent efforts revealed a clear role of PTMs in modulating PXR-mediated transcriptional events. Known PTMs of PXR include phosphorylation, acetylation, SUMOylation, and ubiquitination.

Phosphorylation is one of the best-characterized PTM of PXR. It is now well established that site-specific phosphorylation of PXR offers a sophisticated mechanism for PXR-initiated transcription events. Phosphorylation of PXR impedes multiple aspects of PXR biological functions, which include subcellular localization, DNA-binding, and coregulator interactions(103-108). The characterized kinases for PXR phosphorylation are protein kinase C (PKC)(103,106), protein kinase A (PKA)(104,106,107), cyclin-dependent kinase 1 (CDK1)(107), CDK2(109-111), CDK5(112), casine kinase 2 (CK2)(107), p70 S6K(106,108,113), and glycogen synthase kinase 3 (GSK3)(107). In the context of PXR, direct phosphorylation often causes repression towards PXR-mediated transcription activity. In particular, both the PKA and PKC signaling pathway can be activated by inflammation. The initiation of PKA/PKC-mediated PXR phosphorylation results in inhibition of transcriptional activation of drug metabolizing enzymes. Therefore, site-specific phosphorylation may be the essential mechanism for PXR-mediated trans-repression towards inflammatory responses.

An increasing body of evidence suggests that dynamic acetylation/deacetylation constitutes another PTM that regulates the biological functions of NRs in a context-specific manner(114). P300-induced acetylation enhances PR-mediated transcription activity, while

suppressing FXR-mediated trans-activation(115,116). According to recent findings, PXR is acetylated *in vivo* and rifampicin-mediated PXR activation stimulates its acetylation(117). Sirtuin 1 (SIRT1) is involved in the deacetylation of PXR independent of ligand activation. P300 catalyzes the acetylation of PXR at lysine 109 (K109) and subsequently hinders PXR transcriptional activity(118). It is worth noting that a functional crosstalk between acetylation and SUMOylation at the level of FXR has been reported(119), which provided innovative insights for understanding the complicated and signal-specific PTM regulatory network with respect to NR biology.

The ubiquitin-proteasome system (UPS) is an essential protein degradation pathway that is vital for maintaining protein homeostasis in the organism. This pathway is considered the disposal system that recycles the misfolded proteins and promotes amino acids turnover. Ubiquitination is a three-step enzymatic process that is catalyzed by E1 activating enzyme, E2 conjugation enzyme, and E3 ligase. Ubiquitination is a dynamic process that can be reversed by deubiquitinating enzymes (DUBs). The physiological function of ubiquitination is to target its substrate proteins and to generate poly ubiquitin-chains for further degradation of target proteins. According to the specific form of poly ubiquitin chain, target protein will be directed to different degradation pathways. In particular, lysine 48 (K48)-linked poly ubiquitin chain formation directs its target protein into the proteasomal degradation pathway, whereas K63-linked poly ubiquitin chain formation directs its target protein into the lysosomal degradation pathway. Recent studies from our lab have demonstrated that PXR is ubiquitinated in primary mouse hepatocytes. Inhibition of the proteasome with a pharmacological agent MG132 increased ubiquitination of PXR(120). Furthermore, ubiquitination targets multiple sites on PXR protein and primarily forms a K48-linked poly ubiquitin chain, which is the well-known signal for

subsequent proteasomal degradation(121). Therefore, ubiquitination of PXR is required for maintaining appropriate physiological functions of PXR in the liver.

SUMO is a member of the ubiquitin like protein family. SUMOylation has very broad functional implications in terms of modulating the target protein's biochemical function, subcellular localization, and stability. To date, four isoforms of SUMO have been identified in mammals and are termed SUMO1, SUMO2, SUMO3, and SUMO4(122-125). Because SUMO2 and SUMO3 share 98% of amino acid sequence homology, they are frequently referred as SUMO2/3. The function of SUMO4 is still unclear, as it is likely a pseudogene and also due to the presence of a proline residue (Pro90) that renders it non-activatable(126). It is also noteworthy that SUMO2/3 itself can be SUMOylated forming long chains that resemble those found during ubiquitination. Much like the process of ubiquitination, the SUMOylation pathway is comprised of a cascade of enzymatic reactions that catalyze three separate reactions and are termed SUMO activating enzyme (E1), conjugating enzyme (E2), and SUMO ligase (E3). The E1 enzyme is composed of a heterodimer of proteins called SAE1 (SUMO activating enzyme 1) and SAE2. The second enzyme in the pathway is called Ubc9 (E2) (homologous to yeast UBC9) and is the only E2 that has been identified to this point. In mammals, multiple SUMO E3 ligases facilitate SUMOylation in a substrate and sub-cellular compartment specific manner. Three different groups of E3 ligase enzymes have been classified to date. The family of protein inhibitor of activated STAT (PIAS), which consists of PIAS1, PIAS2, PIASxα, PIASxβ, and PIASy, is the largest group of SUMO E3 ligases. Ran binding protein 2 (RanBP2) and HDAC4 are characterized as the other two types of E3 ligase enzymes(127-129). While the E1, E2 and E3 enzymes control the SUMOylation cascade; dual function sentrin-specific proteases (SENPs) are responsible for regulating both SUMO maturation as well as the deSUMOylation process.

There are six SENP members in mammals (SENP1, 2, 3, 5, 6, 7), whereas two have been characterized in yeast (ubiquitin-like protein-specific protease 1 and 2, Ubl1 and Ubl2) to date(130). Basically, SENP1 and SENP2 carry out both functions as endopeptidase and isopeptidase, whereas SENP3, 5, 6, and 7 can only function as isopeptidase by de-conjugating SUMO2/3 from their substrates, as well as functioning to selectively remove the SUMO2/3 chains formation from target proteins (so-called chain editing function). The SUMOylation pathway plays an important and required regulatory role in many cellular processes including mitosis, cell development, cell differentiation, senescence, and apoptosis(131-133). A growing body of evidence has demonstrated that SUMOylation stimulates a plethora of cellular events that determine the biological fate of targeted NRs that include GR(134-138), LXR(139,140), FXR(119,141), HNF4(142), RXR(143-145), and PXR(146). Of particular importance, SUMOylation has emerged as a fundamental mechanism that converts NRs from a transcription activator to a transcription repressor in a signal-specific fashion. Our lab has previously reported that activation of inflammatory response increases the SUMOylation of PXR, and elevated PXR SUMOylation feedback suppresses the inflammatory response in hepatocytes(146). This novel observation suggests a potential mechanism by which SUMOylation negatively regulates inflammatory response through altering the biological fate of PXR in the liver.

1.3 DISSERTATION HYPOTHESIS

Despite the wealth of knowledge that has been collected on the canonical mechanism of PXR in regulating drug metabolism, the molecular details of PXR-mediated trans-repression of the inflammatory response remains unclear. While it is now well accepted that post-translational modifications are pivotal and prerequisite actions for NR signaling in the regulation of both

transcription activation (trans-activation) and transcriptional repression (trans-repression), the precise and sophisticated regulation of PTMs in response to physiological or pathophysiological conditions is, in my view, key to understanding the precise molecular interactions that occur between the pathways mediating endocrine/drug homeostasis and the inflammatory signaling pathway. In the specific case of PXR, accumulating evidence suggests that it is a promising therapeutic target for chronic inflammatory diseases in liver and intestinal tissues. This study is focused on the molecular details that regulate the clinically observed mutual trans-repression that occurs between PXR-activated xenobiotic response and NF-κB-mediated inflammatory response in the liver. We hypothesize that post-translational modifications including phosphorylation, acetylation, SUMOylation, and ubiquitination fine-tune the PXR-mediated trans-repression of the inflammatory response **(Figure 1-2)**. The role of each listed PTMs in regulating PXR biology was tested and is discussed in detail in the following chapters. In particular, how the crosstalk between PTMs modulates PXR-mediated trans-activation capacity is the primary interest of my research.

Figure 1-2.

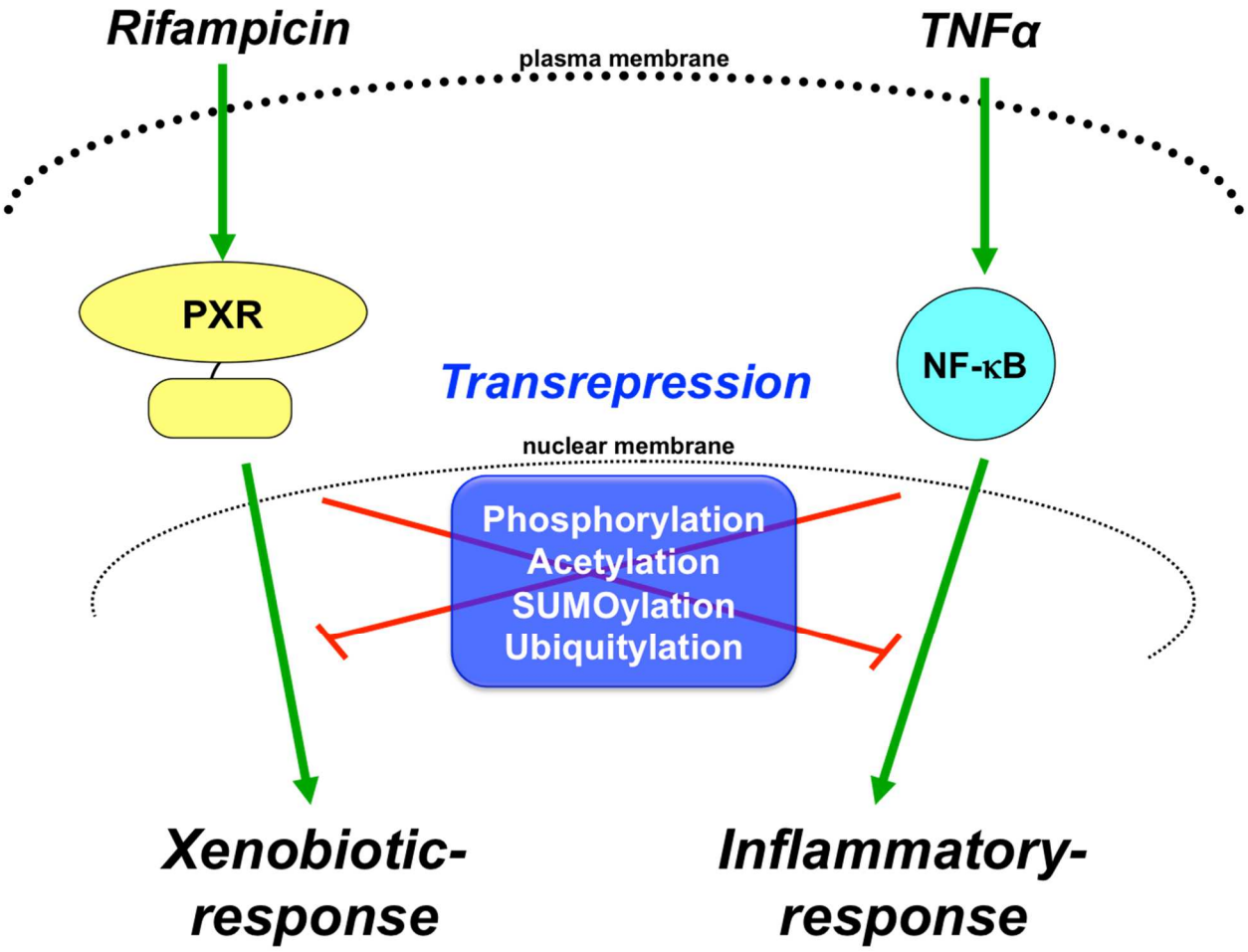


Figure 1-2. Working hypothesis. PTMs, including phosphorylation, acetylation, SUMOylation, and ubiquitination, fine-tune the PXR-mediated trans-repression of the inflammatory response

1.4 References

1. Green, S., Kumar, V., Krust, A., Walter, P., and Chambon, P. (1986) Structural and functional domains of the estrogen receptor. *Cold Spring Harb Symp Quant Biol* **51 Pt 2**, 751-758
2. Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G., and Evans, R. M. (1985) Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* **318**, 635-641
3. Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H., and Vennstrom, B. (1986) The c-erb-A protein is a high-affinity receptor for thyroid hormone. *Nature* **324**, 635-640
4. McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) Nuclear receptor coregulators: cellular and molecular biology. *Endocrine reviews* **20**, 321-344
5. Beato, M. (1991) Transcriptional control by nuclear receptors. *FASEB J* **5**, 2044-2051
6. Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K., and Rosenfeld, M. G. (1991) RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* **67**, 1251-1266
7. Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1992) Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature* **358**, 771-774
8. Bugge, T. H., Pohl, J., Lonnoy, O., and Stunnenberg, H. G. (1992) RXR alpha, a promiscuous partner of retinoic acid and thyroid hormone receptors. *EMBO J* **11**, 1409-1418
9. Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J. Y., Staub, A., Garnier, J. M., Mader, S., and et al. (1992) Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* **68**, 377-395
10. Marks, M. S., Hallenbeck, P. L., Nagata, T., Segars, J. H., Appella, E., Nikodem, V. M., and Ozato, K. (1992) H-2RIIBP (RXR beta) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. *EMBO J* **11**, 1419-1435
11. Zhang, X. K., Lehmann, J., Hoffmann, B., Dawson, M. I., Cameron, J., Graupner, G., Hermann, T., Tran, P., and Pfahl, M. (1992) Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. *Nature* **358**, 587-591
12. Langlais, D., Couture, C., Balsalobre, A., and Drouin, J. (2012) The Stat3/GR interaction code: predictive value of direct/indirect DNA recruitment for transcription outcome. *Mol Cell* **47**, 38-49
13. Ratman, D., Vanden Berghe, W., Dejager, L., Libert, C., Tavernier, J., Beck, I. M., and De Bosscher, K. (2013) How glucocorticoid receptors modulate the activity of other transcription factors: a scope beyond tethering. *Molecular and cellular endocrinology* **380**, 41-54
14. Surjit, M., Ganti, K. P., Mukherji, A., Ye, T., Hua, G., Metzger, D., Li, M., and Chambon, P. (2011) Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell* **145**, 224-241

15. Bernard, P., Ludbrook, L., Queipo, G., Dinulos, M. B., Kletter, G. B., Zhang, Y. H., Phelan, J. K., McCabe, E. R., Harley, V. R., and Vilain, E. (2006) A familial missense mutation in the hinge region of DAX1 associated with late-onset AHC in a prepubertal female. *Mol Genet Metab* **88**, 272-279
16. Burns, K. A., Li, Y., Arao, Y., Petrovich, R. M., and Korach, K. S. (2011) Selective mutations in estrogen receptor alpha D-domain alters nuclear translocation and non-estrogen response element gene regulatory mechanisms. *J Biol Chem* **286**, 12640-12649
17. Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakizuka, A., and Evans, R. M. (1992) Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev* **6**, 329-344
18. Shaffer, P. L., McDonnell, D. P., and Gewirth, D. T. (2005) Characterization of transcriptional activation and DNA-binding functions in the hinge region of the vitamin D receptor. *Biochemistry* **44**, 2678-2685
19. Tanner, T. M., Denayer, S., Geverts, B., Van Tilborgh, N., Kerkhofs, S., Helsen, C., Spans, L., Dubois, V., Houtsmuller, A. B., Claessens, F., and Haelens, A. (2010) A 629RKLKK633 motif in the hinge region controls the androgen receptor at multiple levels. *Cellular and molecular life sciences : CMLS* **67**, 1919-1927
20. Pawlak, M., Lefebvre, P., and Staels, B. (2012) General molecular biology and architecture of nuclear receptors. *Curr Top Med Chem* **12**, 486-504
21. Tsai, M. J., and O'Malley, B. W. (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annual review of biochemistry* **63**, 451-486
22. Koenig, R. J., Brent, G. A., Warne, R. L., Larsen, P. R., and Moore, D. D. (1987) Thyroid hormone receptor binds to a site in the rat growth hormone promoter required for induction by thyroid hormone. *Proc Natl Acad Sci U S A* **84**, 5670-5674
23. Naar, A. M., Boutin, J. M., Lipkin, S. M., Yu, V. C., Holloway, J. M., Glass, C. K., and Rosenfeld, M. G. (1991) The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell* **65**, 1267-1279
24. Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell* **65**, 1255-1266
25. Lonard, D. M., and O'Malley, B. W. (2012) Nuclear receptor coregulators: modulators of pathology and therapeutic targets. *Nat Rev Endocrinol* **8**, 598-604
26. Hudson, L. G., Santon, J. B., Glass, C. K., and Gill, G. N. (1990) Ligand-activated thyroid hormone and retinoic acid receptors inhibit growth factor receptor promoter expression. *Cell* **62**, 1165-1175
27. Ishizuka, T., and Lazar, M. A. (2003) The N-CoR/histone deacetylase 3 complex is required for repression by thyroid hormone receptor. *Molecular and cellular biology* **23**, 5122-5131
28. Kato, S., Yokoyama, A., and Fujiki, R. (2011) Nuclear receptor coregulators merge transcriptional coregulation with epigenetic regulation. *Trends Biochem Sci* **36**, 272-281
29. Korzus, E., Torchia, J., Rose, D. W., Xu, L., Kurokawa, R., McInerney, E. M., Mullen, T. M., Glass, C. K., and Rosenfeld, M. G. (1998) Transcription factor-specific requirements for coactivators and their acetyltransferase functions. *Science* **279**, 703-707
30. Lee, S. K., Na, S. Y., Jung, S. Y., Choi, J. E., Jhun, B. H., Cheong, J., Meltzer, P. S., Lee, Y. C., and Lee, J. W. (2000) Activating protein-1, nuclear factor-kappaB, and serum

response factor as novel target molecules of the cancer-amplified transcription coactivator ASC-2. *Molecular endocrinology* **14**, 915-925
31. Sartorelli, V., Huang, J., Hamamori, Y., and Kedes, L. (1997) Molecular mechanisms of myogenic coactivation by p300: direct interaction with the activation domain of MyoD and with the MADS box of MEF2C. *Molecular and cellular biology* **17**, 1010-1026
32. Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **387**, 43-48
33. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**, 403-414
34. Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L., and Evans, R. M. (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**, 373-380
35. Mottis, A., Mouchiroud, L., and Auwerx, J. (2013) Emerging roles of the corepressors NCoR1 and SMRT in homeostasis. *Genes Dev* **27**, 819-835
36. Ding, X. F., Anderson, C. M., Ma, H., Hong, H., Uht, R. M., Kushner, P. J., and Stallcup, M. R. (1998) Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. *Molecular endocrinology* **12**, 302-313
37. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* **387**, 733-736
38. Le Douarin, B., Zechel, C., Garnier, J. M., Lutz, Y., Tora, L., Pierrat, P., Heery, D., Gronemeyer, H., Chambon, P., and Losson, R. (1995) The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. *EMBO J* **14**, 2020-2033
39. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387**, 677-684
40. Voegel, J. J., Heine, M. J., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. (1998) The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates trans-activation through CBP binding-dependent and -independent pathways. *EMBO J* **17**, 507-519
41. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**, 1354-1357
42. Wang, J. C., Stafford, J. M., and Granner, D. K. (1998) SRC-1 and GRIP1 coactivate transcription with hepatocyte nuclear factor 4. *J Biol Chem* **273**, 30847-30850
43. Zhu, Y., Qi, C., Calandra, C., Rao, M. S., and Reddy, J. K. (1996) Cloning and identification of mouse steroid receptor coactivator-1 (mSRC-1), as a coactivator of peroxisome proliferator-activated receptor gamma. *Gene expression* **6**, 185-195
44. Jenster, G., Spencer, T. E., Burcin, M. M., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) Steroid receptor induction of gene transcription: a two-step model. *Proc Natl Acad Sci U S A* **94**, 7879-7884

45. Kim, H. J., Kim, J. H., and Lee, J. W. (1998) Steroid receptor coactivator-1 interacts with serum response factor and coactivates serum response element-mediated trans-activations. *J Biol Chem* **273**, 28564-28567
46. Na, S. Y., Lee, S. K., Han, S. J., Choi, H. S., Im, S. Y., and Lee, J. W. (1998) Steroid receptor coactivator-1 interacts with the p50 subunit and coactivates nuclear factor kappaB-mediated trans-activations. *J Biol Chem* **273**, 10831-10834
47. Lee, S. K., Kim, H. J., Kim, J. W., and Lee, J. W. (1999) Steroid receptor coactivator-1 and its family members differentially regulate trans-activation by the tumor suppressor protein p53. *Molecular endocrinology* **13**, 1924-1933
48. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* **90**, 569-580
49. Li, H., Gomes, P. J., and Chen, J. D. (1997) RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. *Proc Natl Acad Sci U S A* **94**, 8479-8484
50. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* **277**, 965-968
51. Blanco, J. C., Minucci, S., Lu, J., Yang, X. J., Walker, K. K., Chen, H., Evans, R. M., Nakatani, Y., and Ozato, K. (1998) The histone acetylase PCAF is a nuclear receptor coactivator. *Genes Dev* **12**, 1638-1651
52. Fondell, J. D., Ge, H., and Roeder, R. G. (1996) Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proc Natl Acad Sci U S A* **93**, 8329-8333
53. Yuan, C. X., Ito, M., Fondell, J. D., Fu, Z. Y., and Roeder, R. G. (1998) The TRAP220 component of a thyroid hormone receptor- associated protein (TRAP) coactivator complex interacts directly with nuclear receptors in a ligand-dependent fashion. *Proc Natl Acad Sci U S A* **95**, 7939-7944
54. Aouabdi, S., Gibson, G., and Plant, N. (2006) Transcriptional regulation of the PXR gene: identification and characterization of a functional peroxisome proliferator-activated receptor alpha binding site within the proximal promoter of PXR. *Drug Metab Dispos* **34**, 138-144
55. Jackson, T. A., Richer, J. K., Bain, D. L., Takimoto, G. S., Tung, L., and Horwitz, K. B. (1997) The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. *Molecular endocrinology* **11**, 693-705
56. Lavinsky, R. M., Jepsen, K., Heinzel, T., Torchia, J., Mullen, T. M., Schiff, R., Del-Rio, A. L., Ricote, M., Ngo, S., Gemsch, J., Hilsenbeck, S. G., Osborne, C. K., Glass, C. K., Rosenfeld, M. G., and Rose, D. W. (1998) Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci U S A* **95**, 2920-2925
57. Smith, C. L., Nawaz, Z., and O'Malley, B. W. (1997) Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. *Molecular endocrinology* **11**, 657-666

58. Xu, J., Nawaz, Z., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1996) The extreme C terminus of progesterone receptor contains a transcriptional repressor domain that functions through a putative corepressor. *Proc Natl Acad Sci U S A* **93**, 12195-12199
59. Perissi, V., Jepsen, K., Glass, C. K., and Rosenfeld, M. G. (2010) Deconstructing repression: evolving models of co-repressor action. *Nature reviews. Genetics* **11**, 109-123
60. Watson, P. J., Fairall, L., and Schwabe, J. W. (2012) Nuclear hormone receptor co-repressors: structure and function. *Molecular and cellular endocrinology* **348**, 440-449
61. Guenther, M. G., Barak, O., and Lazar, M. A. (2001) The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. *Molecular and cellular biology* **21**, 6091-6101
62. Yu, J., Li, Y., Ishizuka, T., Guenther, M. G., and Lazar, M. A. (2003) A SANT motif in the SMRT corepressor interprets the histone code and promotes histone deacetylation. *EMBO J* **22**, 3403-3410
63. Yamamoto, H., Williams, E. G., Mouchiroud, L., Canto, C., Fan, W., Downes, M., Heligon, C., Barish, G. D., Desvergne, B., Evans, R. M., Schoonjans, K., and Auwerx, J. (2011) NCoR1 is a conserved physiological modulator of muscle mass and oxidative function. *Cell* **147**, 827-839
64. Chen, J. D., and Evans, R. M. (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* **377**, 454-457
65. Hirooka-Masui, K., Lesmana, R., Iwasaki, T., Xu, M., Hayasaka, K., Haraguchi, M., Takeshita, A., Shimokawa, N., Yamamoto, K., and Koibuchi, N. (2013) Interaction of silencing mediator for retinoid and thyroid receptors with steroid and xenobiotic receptor on multidrug resistance 1 promoter. *Life sciences* **92**, 911-915
66. Konno, Y., Kodama, S., Moore, R., Kamiya, N., and Negishi, M. (2009) Nuclear xenobiotic receptor pregnane X receptor locks corepressor silencing mediator for retinoid and thyroid hormone receptors (SMRT) onto the CYP24A1 promoter to attenuate vitamin D3 activation. *Mol Pharmacol* **75**, 265-271
67. Muscat, G. E., Burke, L. J., and Downes, M. (1998) The corepressor N-CoR and its variants RIP13a and RIP13Delta1 directly interact with the basal transcription factors TFIIB, TAFII32 and TAFII70. *Nucleic acids research* **26**, 2899-2907
68. Crawford, P. A., Dorn, C., Sadovsky, Y., and Milbrandt, J. (1998) Nuclear receptor DAX-1 recruits nuclear receptor corepressor N-CoR to steroidogenic factor 1. *Molecular and cellular biology* **18**, 2949-2956
69. Johnson, D. R., Li, C. W., Chen, L. Y., Ghosh, J. C., and Chen, J. D. (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
70. Feng, X., Jiang, Y., Meltzer, P., and Yen, P. M. (2001) Transgenic targeting of a dominant negative corepressor to liver blocks basal repression by thyroid hormone receptor and increases cell proliferation. *J Biol Chem* **276**, 15066-15072
71. Jepsen, K., Hermanson, O., Onami, T. M., Gleiberman, A. S., Lunyak, V., McEvilly, R. J., Kurokawa, R., Kumar, V., Liu, F., Seto, E., Hedrick, S. M., Mandel, G., Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (2000) Combinatorial roles of the nuclear receptor corepressor in transcription and development. *Cell* **102**, 753-763
72. Li, P., Fan, W., Xu, J., Lu, M., Yamamoto, H., Auwerx, J., Sears, D. D., Talukdar, S., Oh, D., Chen, A., Bandyopadhyay, G., Scadeng, M., Ofrecio, J. M., Nalbandian, S., and Olefsky, J. M. (2011) Adipocyte NCoR knockout decreases PPARgamma

phosphorylation and enhances PPARgamma activity and insulin sensitivity. *Cell* **147**, 815-826
73. Kliewer, S. A., Goodwin, B., and Willson, T. M. (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocrine reviews* **23**, 687-702
74. Lehmann, J. M., McKee, D. D., Watson, M. A., Willson, T. M., Moore, J. T., and Kliewer, S. A. (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
75. Kliewer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Watson, M. A., Jones, S. A., McKee, D. D., Oliver, B. B., Willson, T. M., Zetterstrom, R. H., Perlmann, T., and Lehmann, J. M. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92**, 73-82
76. Chang, T. K., and Waxman, D. J. (2006) Synthetic drugs and natural products as modulators of constitutive androstane receptor (CAR) and pregnane X receptor (PXR). *Drug Metab Rev* **38**, 51-73
77. di Masi, A., De Marinis, E., Ascenzi, P., and Marino, M. (2009) Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. *Mol Aspects Med* **30**, 297-343
78. Reschly, E. J., and Krasowski, M. D. (2006) Evolution and function of the NR1I nuclear hormone receptor subfamily (VDR, PXR, and CAR) with respect to metabolism of xenobiotics and endogenous compounds. *Curr Drug Metab* **7**, 349-365
79. Barwick, J. L., Quattrochi, L. C., Mills, A. S., Potenza, C., Tukey, R. H., and Guzelian, P. S. (1996) Trans-species gene transfer for analysis of glucocorticoid-inducible transcriptional activation of transiently expressed human CYP3A4 and rabbit CYP3A6 in primary cultures of adult rat and rabbit hepatocytes. *Mol Pharmacol* **50**, 10-16
80. Ogg, M. S., Williams, J. M., Tarbit, M., Goldfarb, P. S., Gray, T. J., and Gibson, G. G. (1999) A reporter gene assay to assess the molecular mechanisms of xenobiotic-dependent induction of the human CYP3A4 gene in vitro. *Xenobiotica* **29**, 269-279
81. Rosenfeld, M. G., Lunyak, V. V., and Glass, C. K. (2006) Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev* **20**, 1405-1428
82. Beard, K. (1992) Adverse reactions as a cause of hospital admission in the aged. *Drugs Aging* **2**, 356-367
83. Biswas, A., Mani, S., Redinbo, M. R., Krasowski, M. D., Li, H., and Ekins, S. (2009) Elucidating the 'Jekyll and Hyde' nature of PXR: the case for discovering antagonists or allosteric antagonists. *Pharm Res* **26**, 1807-1815
84. Burk, R. F., Hill, K. E., Hunt, R. W., Jr., and Martin, A. E. (1990) Isoniazid potentiation of acetaminophen hepatotoxicity in the rat and 4-methylpyrazole inhibition of it. *Res Commun Chem Pathol Pharmacol* **69**, 115-118
85. Lee, W. M. (2004) Acetaminophen and the U.S. Acute Liver Failure Study Group: lowering the risks of hepatic failure. *Hepatology* **40**, 6-9
86. Pondugula, S. R., and Mani, S. (2013) Pregnane xenobiotic receptor in cancer pathogenesis and therapeutic response. *Cancer Lett* **328**, 1-9
87. Staudinger, J. L., Goodwin, B., Jones, S. A., Hawkins-Brown, D., MacKenzie, K. I., LaTour, A., Liu, Y., Klaassen, C. D., Brown, K. K., Reinhard, J., Willson, T. M., Koller, B. H., and Kliewer, S. A. (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* **98**, 3369-3374

88. Masson, D., Lagrost, L., Athias, A., Gambert, P., Brimer-Cline, C., Lan, L., Schuetz, J. D., Schuetz, E. G., and Assem, M. (2005) Expression of the pregnane X receptor in mice antagonizes the cholic acid-mediated changes in plasma lipoprotein profile. *Arterioscler Thromb Vasc Biol* **25**, 2164-2169
89. White, D. B., Batayneh, Z., and Bachmann, K. A. (2010) A synergism model for PPARalpha and PXR agonist effects on HDL-cholesterol and apoA1. *Front Biosci (Elite Ed)* **2**, 399-410
90. 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, A. I., Shay, J. W., 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
91. Chen, Y., Tang, Y., Wang, M. T., Zeng, S., and Nie, D. (2007) Human pregnane X receptor and resistance to chemotherapy in prostate cancer. *Cancer Res* **67**, 10361-10367
92. Sun, M., Cui, W., Woody, S. K., and Staudinger, J. L. (2015) Pregnane X receptor modulates the inflammatory response in primary cultures of hepatocytes. *Drug Metab Dispos* **43**, 335-343
93. Zhou, C., Tabb, M. M., Nelson, E. L., Grun, F., Verma, S., Sadatrafiei, A., Lin, M., Mallick, S., Forman, B. M., Thummel, K. E., and Blumberg, B. (2006) Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest* **116**, 2280-2289
94. Baker, R. G., Hayden, M. S., and Ghosh, S. (2011) NF-kappaB, inflammation, and metabolic disease. *Cell Metab* **13**, 11-22
95. Courtois, G., and Gilmore, T. D. (2006) Mutations in the NF-kappaB signaling pathway: implications for human disease. *Oncogene* **25**, 6831-6843
96. Karin, M. (2006) Nuclear factor-kappaB in cancer development and progression. *Nature* **441**, 431-436
97. Kumar, A., Takada, Y., Boriek, A. M., and Aggarwal, B. B. (2004) Nuclear factor-kappaB: its role in health and disease. *J Mol Med (Berl)* **82**, 434-448
98. Chalian, M., Ozturk, A., Oliva-Hemker, M., Pryde, S., and Huisman, T. A. (2011) MR enterography findings of inflammatory bowel disease in pediatric patients. *AJR Am J Roentgenol* **196**, W810-816
99. Shah, Y. M., Ma, X., Morimura, K., Kim, I., and Gonzalez, F. J. (2007) Pregnane X receptor activation ameliorates DSS-induced inflammatory bowel disease via inhibition of NF-kappaB target gene expression. *Am J Physiol Gastrointest Liver Physiol* **292**, G1114-1122
100. Long, M. D., Crandall, W. V., Leibowitz, I. H., Duffy, L., del Rosario, F., Kim, S. C., Integlia, M. J., Berman, J., Grunow, J., Colletti, R. B., Schoen, B. T., Patel, A. S., Baron, H., Israel, E., Russell, G., Ali, S., Herfarth, H. H., Martin, C., Kappelman, M. D., and ImproveCareNow Collaborative for Pediatric, I. B. D. (2011) Prevalence and epidemiology of overweight and obesity in children with inflammatory bowel disease. *Inflamm Bowel Dis* **17**, 2162-2168
101. Banerjee, M., Robbins, D., and Chen, T. (2015) Targeting xenobiotic receptors PXR and CAR in human diseases. *Drug Discov Today* **20**, 618-628
102. Haughton, E. L., Tucker, S. J., Marek, C. J., Durward, E., Leel, V., Bascal, Z., Monaghan, T., Koruth, M., Collie-Duguid, E., Mann, D. A., Trim, J. E., and Wright, M.

C. (2006) Pregnane X receptor activators inhibit human hepatic stellate cell transdifferentiation in vitro. *Gastroenterology* **131**, 194-209
103. Ding, X., and Staudinger, J. L. (2005) Repression of PXR-mediated induction of hepatic CYP3A gene expression by protein kinase C. *Biochem Pharmacol* **69**, 867-873
104. Ding, X., and Staudinger, J. L. (2005) Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway. *J Pharmacol Exp Ther* **312**, 849-856
105. Doricakova, A., Novotna, A., Vrzal, R., Pavek, P., and Dvorak, Z. (2013) The role of residues T248, Y249 and T422 in the function of human pregnane X receptor. *Arch Toxicol* **87**, 291-301
106. Lichti-Kaiser, K., Brobst, D., Xu, C., and Staudinger, J. L. (2009) A systematic analysis of predicted phosphorylation sites within the human pregnane X receptor protein. *J Pharmacol Exp Ther* **331**, 65-76
107. Lichti-Kaiser, K., Xu, C., and Staudinger, J. L. (2009) Cyclic AMP-dependent protein kinase signaling modulates pregnane x receptor activity in a species-specific manner. *J Biol Chem* **284**, 6639-6649
108. Pondugula, S. R., Dong, H., and Chen, T. (2009) Phosphorylation and protein-protein interactions in PXR-mediated CYP3A repression. *Expert Opin Drug Metab Toxicol* **5**, 861-873
109. Lin, W., Wu, J., Dong, H., Bouck, D., Zeng, F. Y., and Chen, T. (2008) Cyclin-dependent kinase 2 negatively regulates human pregnane X receptor-mediated CYP3A4 gene expression in HepG2 liver carcinoma cells. *J Biol Chem* **283**, 30650-30657
110. Sugatani, J., Osabe, M., Kurosawa, M., Kitamura, N., Ikari, A., and Miwa, M. (2010) Induction of UGT1A1 and CYP2B6 by an antimitogenic factor in HepG2 cells is mediated through suppression of cyclin-dependent kinase 2 activity: cell cycle-dependent expression. *Drug Metab Dispos* **38**, 177-186
111. Sugatani, J., Uchida, T., Kurosawa, M., Yamaguchi, M., Yamazaki, Y., Ikari, A., and Miwa, M. (2012) Regulation of pregnane X receptor (PXR) function and UGT1A1 gene expression by posttranslational modification of PXR protein. *Drug Metab Dispos* **40**, 2031-2040
112. Dong, H., Lin, W., Wu, J., and Chen, T. (2010) Flavonoids activate pregnane x receptor-mediated CYP3A4 gene expression by inhibiting cyclin-dependent kinases in HepG2 liver carcinoma cells. *BMC Biochem* **11**, 23
113. Pondugula, S. R., Brimer-Cline, C., Wu, J., Schuetz, E. G., Tyagi, R. K., and Chen, T. (2009) A phosphomimetic mutation at threonine-57 abolishes trans-activation activity and alters nuclear localization pattern of human pregnane x receptor. *Drug Metab Dispos* **37**, 719-730
114. Wang, C., Tian, L., Popov, V. M., and Pestell, R. G. (2011) Acetylation and nuclear receptor action. *J Steroid Biochem Mol Biol* **123**, 91-100
115. Chung, H. H., Sze, S. K., Tay, A. S., and Lin, V. C. (2014) Acetylation at lysine 183 of progesterone receptor by p300 accelerates DNA binding kinetics and trans-activation of direct target genes. *J Biol Chem* **289**, 2180-2194
116. Kemper, J. K., Xiao, Z., Ponugoti, B., Miao, J., Fang, S., Kanamaluru, D., Tsang, S., Wu, S. Y., Chiang, C. M., and Veenstra, T. D. (2009) FXR acetylation is normally dynamically regulated by p300 and SIRT1 but constitutively elevated in metabolic disease states. *Cell Metab* **10**, 392-404

117. Biswas, A., Pasquel, D., Tyagi, R. K., and Mani, S. (2011) Acetylation of pregnane X receptor protein determines selective function independent of ligand activation. *Biochem Biophys Res Commun* **406**, 371-376
118. Pasquel, D., Doricakova, A., Li, H., Kortagere, S., Krasowski, M. D., Biswas, A., Walton, W. G., Redinbo, M. R., Dvorak, Z., and Mani, S. (2016) Acetylation of lysine 109 modulates pregnane X receptor DNA binding and transcriptional activity. *Biochim Biophys Acta* **1859**, 1155-1169
119. Kim, D. H., Xiao, Z., Kwon, S., Sun, X., Ryerson, D., Tkac, D., Ma, P., Wu, S. Y., Chiang, C. M., Zhou, E., Xu, H. E., Palvimo, J. J., Chen, L. F., Kemper, B., and Kemper, J. K. (2015) A dysregulated acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity. *EMBO J* **34**, 184-199
120. Staudinger, J. L., Xu, C., Biswas, A., and Mani, S. (2011) Post-translational modification of pregnane x receptor. *Pharmacol Res* **64**, 4-10
121. Cui, W., Sun, M., Galeva, N., Williams, T. D., Azuma, Y., and Staudinger, J. L. (2015) SUMOylation and Ubiquitylation Circuitry Controls Pregnane X Receptor Biology in Hepatocytes. *Drug Metab Dispos* **43**, 1316-1325
122. Guo, D., Li, M., Zhang, Y., Yang, P., Eckenrode, S., Hopkins, D., Zheng, W., Purohit, S., Podolsky, R. H., Muir, A., Wang, J., Dong, Z., Brusko, T., Atkinson, M., Pozzilli, P., Zeidler, A., Raffel, L. J., Jacob, C. O., Park, Y., Serrano-Rios, M., Larrad, M. T., Zhang, Z., Garchon, H. J., Bach, J. F., Rotter, J. I., She, J. X., and Wang, C. Y. (2004) A functional variant of SUMO4, a new I kappa B alpha modifier, is associated with type 1 diabetes. *Nature genetics* **36**, 837-841
123. Kamitani, T., Kito, K., Nguyen, H. P., Fukuda-Kamitani, T., and Yeh, E. T. (1998) Characterization of a second member of the sentrin family of ubiquitin-like proteins. *J Biol Chem* **273**, 11349-11353
124. Lapenta, V., Chiurazzi, P., van der Spek, P., Pizzuti, A., Hanaoka, F., and Brahe, C. (1997) SMT3A, a human homologue of the S. cerevisiae SMT3 gene, maps to chromosome 21qter and defines a novel gene family. *Genomics* **40**, 362-366
125. Saitoh, H., and Hinchey, J. (2000) Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J Biol Chem* **275**, 6252-6258
126. Owerbach, D., McKay, E. M., Yeh, E. T., Gabbay, K. H., and Bohren, K. M. (2005) A proline-90 residue unique to SUMO-4 prevents maturation and sumoylation. *Biochem Biophys Res Commun* **337**, 517-520
127. Zhao, X., Sternsdorf, T., Bolger, T. A., Evans, R. M., and Yao, T. P. (2005) Regulation of MEF2 by histone deacetylase 4- and SIRT1 deacetylase-mediated lysine modifications. *Molecular and cellular biology* **25**, 8456-8464
128. Reverter, D., and Lima, C. D. (2005) Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. *Nature* **435**, 687-692
129. Pichler, A., Knipscheer, P., Saitoh, H., Sixma, T. K., and Melchior, F. (2004) The RanBP2 SUMO E3 ligase is neither HECT- nor RING-type. *Nature structural & molecular biology* **11**, 984-991
130. Geiss-Friedlander, R., and Melchior, F. (2007) Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol* **8**, 947-956
131. Deyrieux, A. F., Rosas-Acosta, G., Ozbun, M. A., and Wilson, V. G. (2007) Sumoylation dynamics during keratinocyte differentiation. *J Cell Sci* **120**, 125-136
132. Gill, G. (2005) SUMO changes Sox for developmental diversity. *Mol Cell* **20**, 495-496

133. Ihara, M., Koyama, H., Uchimura, Y., Saitoh, H., and Kikuchi, A. (2007) Noncovalent binding of small ubiquitin-related modifier (SUMO) protease to SUMO is necessary for enzymatic activities and cell growth. *J Biol Chem* **282**, 16465-16475
134. Holmstrom, S., Van Antwerp, M. E., and Iniguez-Lluhi, J. A. (2003) Direct and distinguishable inhibitory roles for SUMO isoforms in the control of transcriptional synergy. *Proc Natl Acad Sci U S A* **100**, 15758-15763
135. Holmstrom, S. R., Chupreta, S., So, A. Y., and Iniguez-Lluhi, J. A. (2008) SUMO-mediated inhibition of glucocorticoid receptor synergistic activity depends on stable assembly at the promoter but not on DAXX. *Molecular endocrinology* **22**, 2061-2075
136. Hua, G., Ganti, K. P., and Chambon, P. (2016) Glucocorticoid-induced tethered trans-repression requires SUMOylation of GR and formation of a SUMO-SMRT/NCoR1-HDAC3 repressing complex. *Proc Natl Acad Sci U S A* **113**, E635-643
137. Hua, G., Paulen, L., and Chambon, P. (2016) GR SUMOylation and formation of an SUMO-SMRT/NCoR1-HDAC3 repressing complex is mandatory for GC-induced IR nGRE-mediated trans-repression. *Proc Natl Acad Sci U S A* **113**, E626-634
138. Lin, D. Y., Huang, Y. S., Jeng, J. C., Kuo, H. Y., Chang, C. C., Chao, T. T., Ho, C. C., Chen, Y. C., Lin, T. P., Fang, H. I., Hung, C. C., Suen, C. S., Hwang, M. J., Chang, K. S., Maul, G. G., and Shih, H. M. (2006) Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Mol Cell* **24**, 341-354
139. Lee, J. H., Park, S. M., Kim, O. S., Lee, C. S., Woo, J. H., Park, S. J., Joe, E. H., and Jou, I. (2009) Differential SUMOylation of LXRalpha and LXRbeta mediates trans-repression of STAT1 inflammatory signaling in IFN-gamma-stimulated brain astrocytes. *Mol Cell* **35**, 806-817
140. Venteclef, N., Jakobsson, T., Ehrlund, A., Damdimopoulos, A., Mikkonen, L., Ellis, E., Nilsson, L. M., Parini, P., Janne, O. A., Gustafsson, J. A., Steffensen, K. R., and Treuter, E. (2010) GPS2-dependent corepressor/SUMO pathways govern anti-inflammatory actions of LRH-1 and LXRbeta in the hepatic acute phase response. *Genes Dev* **24**, 381-395
141. Vavassori, P., Mencarelli, A., Renga, B., Distrutti, E., and Fiorucci, S. (2009) The bile acid receptor FXR is a modulator of intestinal innate immunity. *J Immunol* **183**, 6251-6261
142. Zhou, W., Hannoun, Z., Jaffray, E., Medine, C. N., Black, J. R., Greenhough, S., Zhu, L., Ross, J. A., Forbes, S., Wilmut, I., Iredale, J. P., Hay, R. T., and Hay, D. C. (2012) SUMOylation of HNF4alpha regulates protein stability and hepatocyte function. *J Cell Sci* **125**, 3630-3635
143. Burrage, P. S., Schmucker, A. C., Ren, Y., Sporn, M. B., and Brinckerhoff, C. E. (2008) Retinoid X receptor and peroxisome proliferator-activated receptor-gamma agonists cooperate to inhibit matrix metalloproteinase gene expression. *Arthritis Res Ther* **10**, R139
144. Choi, S. J., Chung, S. S., Rho, E. J., Lee, H. W., Lee, M. H., Choi, H. S., Seol, J. H., Baek, S. H., Bang, O. S., and Chung, C. H. (2006) Negative modulation of RXRalpha transcriptional activity by small ubiquitin-related modifier (SUMO) modification and its reversal by SUMO-specific protease SUSP1. *J Biol Chem* **281**, 30669-30677

145. Lee, W. P., Jena, S., Rodriguez, E. P., O'Donovan, S. P., Wagner, C., Jurutka, P. W., and Thompson, P. D. (2015) Distinct functional modes of SUMOylation for retinoid X receptor alpha. *Biochem Biophys Res Commun* **464**, 195-200
146. Hu, G., Xu, C., and Staudinger, J. L. (2010) Pregnane X receptor is SUMOylated to repress the inflammatory response. *J Pharmacol Exp Ther* **335**, 342-350

Chapter 2: SUMOylation and Ubiquitination Circuitry Controls Pregnane X Receptor Biology in Hepatocytes

ABSTRACT

Several nuclear receptor (NR) superfamily members are known to be the molecular target of either the SUMO- or ubiquitin-signaling pathways. However, little is currently known regarding how these two post-translational modifications interact to control NR biology. We show that the SUMO and ubiquitin circuitry coordinately modifies Pregnane X Receptor (PXR, NR1I2) to play a key role in regulating PXR protein stability, trans-activation capacity, and transcriptional repression. The SUMOylation and ubiquitination of PXR is increased in a ligand- and tumor necrosis factor-alpha (TNFα)-dependent manner in hepatocytes. The SUMO-E3 ligase enzymes PIAS1 and PIASy drive high levels of PXR SUMOylation. Expression of PIAS1 selectively increases SUMO(3)ylation, as well as PXR-mediated induction of CYP3A and the xenobiotic response. The PIASy-mediated SUMO(1)ylation imparts a transcriptionally repressive function by ameliorating interaction of PXR with co-activator protein peroxisome proliferator-activated receptor gamma coactivator-1-alpha (PGC-1α). The SUMO-modification of PXR is effectively antagonized by the sentrin/SUMO-specific protease 2 (SENP2), whereas SENP3 and SENP6 proteases are highly active in removal of SUMO2/3-chains. The PIASy-mediated SUMO(1)ylation of PXR inhibits ubiquitin-mediated degradation of this important liver-enriched NR by the 26S proteasome. Our data reveal a working model that delineates the interactive role that these two post-translational modifications play in reconciling PXR-mediated gene activation of the xenobiotic response -versus- transcriptional repression of the pro-inflammatory response in hepatocytes. Taken together, our data reveal that the SUMOylation

and ubiquitination of PXR interface in a fundamental manner to direct its biological function in liver in response to xenobiotic or inflammatory stress.

2.1 INTRODUCTION

Ligand-dependent activation of Pregnane X Receptor (PXR, NR1I2) is associated with increased metabolism and clearance of a myriad of potentially toxic compounds from the body, and is thus thought of as a master-regulator of the protective xenobiotic response. However, clinical treatment with PXR activators can also lead to the repression or attenuation of other biochemical pathways including the inflammatory response in liver and intestine (1). It is now well-accepted that activation of PXR is associated with general suppression of the inflammatory response in these tissues (2-6).

Post-translational modification with the small-ubiquitin related modifier (SUMO) plays a key role in determining the biological fate and function of a myriad of transcription factors, including several liver-enriched NR superfamily members to alter inflammatory signaling pathways (7). There are a number of different SUMO-E3 ligase enzymes, and the best characterized family is the protein inhibitors of activated STAT (PIAS) family (8). SUMOylation is a reversible process through the action of a family sentrin proteases (SENPs) that function as isopeptidases to deconjugate SUMO from substrates (9).

The SUMO- and ubiquitin-signaling pathways share a high degree of commonality (10). A recent thrust of research indicates that these two signaling pathways not only share structural similarity, but they also share a multitude of functional interrelations. These interactions include two discreet and distinct modes. The first mode of interaction is characterized by a stress-dependent competition for shared target lysine residues on a given protein substrate, whereas the

second mode of interaction is characterized by a stress-induced formation of SUMOylation-dependent ubiquitin chains on unique lysine residues in close proximity in a given target protein (11-13). The first mode of competitive interaction between SUMO and ubiquitin occurs on lysine residues within the inhibitor of transcription factor NF-κB-alpha, also known as IκBα, as well as within the proliferating cell nuclear antigen protein, also called PCNA (14,15). An example of the second mode of stress-induced SUMOylation-dependent ubiquitination is exemplified by arsenic inducing PML-RARα SUMOylation and its subsequent ubiquitination/K48-linked chain-mediated degradation by the proteasome (16). Another example of a protein that undergoes SUMO-dependent ubiquitination is NF-κB essential modulator (NEMO), which is activated by consecutive modifications with SUMO and ubiquitin that initiates K48-linked degradation by the proteasome following genotoxic stress (17). In each case, the interaction between these two post-translational modifications determines the biological function and molecular fate of the resulting modified protein.

Our laboratory has previously demonstrated that PXR is SUMOylated to suppress the expression of tumor necrosis factor alpha (TNFα)-inducible interleukin 1 beta (IL1β) gene expression in hepatocytes (18). We and others have also previously shown PXR to be a target for the ubiquitin signaling pathway (19-21) and it is well-known that ubiquitination is an integral part of canonical NR-mediated gene expression (22). Several studies have shown that phosphorylation controls PXR biological function as well (23-29). Moreover, PXR has been shown to be a target for acetylation (30). While these respective post-translational modifications of PXR have been observed and characterized in isolation, there has been no examination of the potential biological role of the interaction between these key signaling pathways at the level of the PXR protein. Here, we characterize the enzymatic reactions that promote SUMOylation and

de-SUMOylation of PXR. Using primary cultures of hepatocytes and cell line-based assays to demonstrate that the molecular consequence of SUMO-ubiquitin interaction at the level of PXR defines its role in mediating canonical activation of the xenobiotic response, contrasted with PXR-mediated repression of the pro-inflammatory response.

2.2 MATERIALS AND METHODS

2.2.1 Plasmids and Chemicals.

The full-length human PXR expression vectors were previously described (31,32). To construct the FLAG-tagged human PXR expression vector the cDNA encoding human PXR was excised from pSG5-PXR expression vector using EcoRI and SalI sites and was inserted into pCMV-Tag 2B (Agilent) using EcoRI and SalI restriction sites. Expression vectors encoding $(His)_6$-tagged SUMO1, SUMO2 and SUMO3 were a kind gift of Dr. Ronald T. Hay and were previously described (33). Expression vectors encoding protein inhibitor of activated stat (PIAS) proteins were a kind gift from Dr. Ke Shuai (34) and were obtained from Addgene (plasmid numbers- 15206, FLAG-PIAS1; 15209, FLAG-PIASxα; 15210, FLAG-PIASxβ; 15207, FLAG-PIAS3; 15208, FLAG-PIASy). The expression vectors encoding the respective SENPs and the corresponding catalytically deficient mutant SENPs were a kind gift from Dr. Ed Yeh (35) and were obtained from Addgene (plasmid numbers- 17357, FLAG-SENP1; 17358, FLAG-SENP1m; 18047, FLAG-SENP2; 18713, FLAG-SENP2m; 18048, RGS-SENP3; 18714, RGS-SENP3m; 18053, RGS-SENP5; 18715, RGS-SENP5m; 18065, FLAG-SENP6; 18716, FLAG-SENP6m; 42886, 3XFLAG-SENP7). Expression vectors encoding HA-tagged wild type and K48R mutant ubiquitin constructs were previously described (36). Inserts encoding wild type and K48R mutant ubiquitin were excised using EcoRI and NotI restriction enzyme sites and

inserted into the EcoRI and NotI sites in pCDNA4-HisMax-A to create $(His)_6$-tagged forms of wild type and K48R $(His)_6$-ubiquitin. The single mutant $(His)_6$-K63R and double mutant $(His)_6$-K48,63R expression vectors were created using the primers listed in **Table 2-1** in a QuickChange site-directed mutagenesis reaction per manufacturer instructions (Agilent). The reporter plasmid $(ER\text{-}6)_3$-tk-Luc was generated by insertion of three copies of the double-stranded annealed oligonucleotide primers listed in **Table 2-1** into the Bgl II site of pGL3-Basic. The FLAG-tagged PIAS1 adenoviral expression vector was constructed using PCR primers listed in **Table 2-1** to introduce an Xho I site and was inserted into the pShuttle IRES-hrGFP expression vector (Agilent). The SUMO3 adenoviral expression vector was constructed using PCR primers listed in **Table 2-1** to insert Spe I and Xho I restriction sites into the 5' and 3' end of the open reading frame of $(His)_6$-SUMO3, respectively, and was inserted into pShuttle IRES-hrGFP expression vector (Agilent). The human PXR adenoviral expression vector was constructed using PCR primers to remove the STOP codon in PXR and introduce EcoRV and Xho I restriction sites into the open reading frame of PXR using $(His)_6$-tagged PXR as a template. The resulting PCR amplimer was inserted into the multiple cloning site in the pShuttle IRES-hrGFP expression vector and adenovirus was generated as described (37).

2.2.2 Cell-based Cobalt-Bead Affinity Pull-down Assay.

The Hepa1-6 cell line was utilized due to its proven utility in studies of hepatic gene expression and liver biochemistry (38). The general strategy for enrichment of SUMO- and ubiquitin-modified PXR was previously described (21).

Table 2-1. PCR Primers used in Cloning and Site-Directed Mutagenesis.

Ubiquitin K63R	Left Primer	5' gCT gTC TgA TTA CAA CAT TCA gAg ggA gTC CAC CCT 3’
	Right Primer	5’ Agg gTg gAC TCC CTC TgA ATg TTg TAA TCA gAC AgC 3’
CYP3A4-ER6-PXRE	Sense Primer	5’ gAT CAA TAT gAA CTC AAA ggA ggT CAg Tg 3’
	Antisense Primer	5’ gAT CCA CTg ACC TCC TTT gAg TTC ATA TT 3’
FLAG-tagged PIAS1	Left Primer	5’ gAC ggC CTC gAg ACC ATG GAC TAC AAG GAC GAC 3’
	Right Primer	5’ gAC ggC CTC gAg TCA gTC CAA TgA gAT AAT gTC Tgg 3’
SUMO3	Left Primer	5’ gAC ggC ACT AgT Cgg ACg gCC TCC gAA ACC ATG g 3’
	Right Primer	5’ gAC ggC CTC gAg CTA ACC TCC CgT CTg CTg CCg g 3’
(His)-tagged-PXR	Left Primer	5’ gAC ggC gAT ATC TTA ATA CgA CTC ACT ATA ggg Ag 3’
	Right Primer	5’ gAC ggC CTC gAg GCT ACC TGT GAT GCC GAA CAA CTC C 3’
PXR-K108R	Left Primer	5' CTg gAg AgC ggC ATg Agg AAg gAg ATg ATC ATg 3'
	Right Primer	5’ CAT gAT CAT CTC CTT CCT CAT gCC gCT CTC CAg 3’
PXR-K128R	Left Primer	5' CTT gAT CAA gCg gAA gAg AAg TgA ACg gAC Agg gA 3'
	Right Primer	5' TCC CTg TCC gTT CAC TTC TCT TCC gCT TgA TCA Ag 3'
PXR-K160R	Left Primer	5' gAT ggA CgC TCA gAT gAg AAC CTT TgA CAC TAC CT 3'
	Right Primer	5' Agg TAg TgT CAA Agg TTC TCA TCT gAg CgT CCA TC 3'
PXR-K170R	Left Primer	5' TAC CTT CTC CCA TTT CAg gAA TTT CCg gCT gCC Ag 3'
	Right Primer	5' CTg gCA gCC ggA AAT TCC TgA AAT ggg AgA Agg TA 3'

2.2.3 Isolation and Culturing of Primary Hepatocytes.

PXR knockout (PXR-KO) mice were generated as previously described (39). Hepatocytes were isolated from congenic (C57BL6) wild type and PXR-KO mice aged 6-10 weeks using a standard collagenase perfusion method as described previously (40). Hepatocytes isolated from either male or female mice were used throughout this study in order to identify any potential sex difference. Identical results were obtained in both sexes. The results shown are from the male mice. The hepatocytes were allowed to attach to the plate for 4 hours and the medium was then replaced with serum-free Williams E medium as described previously (40).

2.2.4 Total RNA Isolation, Reverse Transcription, and Real-Time Quantitative-Polymerase Chain Reaction Analysis.

Real time quantitative Polymerase Chain Reaction was performed as described (41).

2.2.5 Western Blot Analysis.

Western blot analysis was performed as described previously (42). Antibodies used include anti-PXR antibody (Santa Cruz, H-11), anti-SUMO1 antibody (Cell Signaling, C9H1), anti-SUMO2/3 antibody (Cell Signaling, 18H8), anti-Ubiquitin (Cell Signaling, P4D1), and an anti-β-actin (Chemicon, MAb1501).

2.2.6 LC-MS/MS Analysis.

LC/MS experiments were performed essentially as described (43). Data were processed using Thermo Proteome Discoverer software (Thermo Fisher Scientific, version 1.4) which workflow combined two complementary search engines, Sequest (44) and Mascot (Matrix Science, London, UK; version 2.5). The search parameters covered fragment ion mass tolerance of 0.8 Da, parent ion tolerance of 20 PPM, and cysteine carbamidomethylation as a fixed modification. The modification of lysine by -GlyGly- (+114.04), a tryptic remainder of ubiquitin

attachment to lysine, was included into the search. Protein fasta database was composed from all murinae entries of Uniprot (http://www.uniprot.org/). Search results were imported into the Scaffold software (version 4.4, Proteome Software Inc., Portland, OR) for further validation of MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm (45).

2.2.7 Statistical Analysis.

Where appropriate the statistical differences among an experimental group were determined using a one-way analysis of variance followed by the Duncan's multiple range post hoc test. Statistical differences between experimental groups were determined using the student's *t*-test.

2.3 RESULTS

2.3.1 PXR is the Molecular Target of Both the SUMO- and Ubiquitin-Signaling Pathways in Primary Hepatocytes.

Several type II liver-enriched NR superfamily members are SUMOylated to modify their trans-activation capacity. In most cases, the protein inhibitor of activated STAT-1 (PIAS1) functions as an E3 SUMO-ligase to enhance their modification (46-48). Previous research from our laboratory indicated that endogenous hepatic PXR was the molecular target of SUMO-signaling pathway (18). However, the specific SUMO-E3 ligase(s) that performed this function was not investigated. We therefore constructed several adenoviral expression vectors to examine whether PIAS1 could function as a SUMO-E3 ligase to enhance PXR-SUMO(3)ylation in primary hepatocytes (**Figure 2-1A**). Indeed, co-expression of PXR together with PIAS1 and

SUMO3 in hepatocytes produced robust SUMO3-modification of this NR family member, as detected using anti-PXR antibody in western blot analysis following metal affinity (cobalt beads) methods as described (21) (**Figure 2-1B**).

A recent report indicated that hepatocyte nuclear factor 4-alpha is the simultaneous target of SUMO2/3 and ubiquitin to regulate its stability and biological function (49). We therefore sought to determine the extent to which the endogenous SUMO- and ubiquitin-signaling pathways converge at the level of the PXR protein in hepatocytes. Wild type mouse hepatocytes were transduced with the blank virus (Ad-GFP) or the virus encoding a $(His)_6$-tagged form of PXR (Ad-PXR). Twenty-four hours post-transduction, cells were treated with rifampicin (10μM), a potent and efficacious human PXR ligand, for 24 hours. Following rifampicin treatment, hepatocytes were treated for an additional three hours with TNFα (10 ng/mL) alone, or were co-treated with rifampicin and TNFα together as indicated. Total PXR protein was enriched from whole cell lysates. Protein aliquots were resolved using SDS-PAGE, and subsequent western blot analysis was performed to detect PXR, SUMO1-, SUMO2/3-, ubiquitin-modified forms of PXR, and β-actin as a loading control (**Figure 2-1C**). The SUMO- and ubiquitin-signaling pathways modified the exogenously added PXR protein, with ligand- and TNFα-treatment both increasing the levels of detectable non-modified PXR, as well as the SUMO- and ubiquitin-modified forms of PXR. We note here that SUMO(1)ylation of PXR was observed with comparatively low stoichiometry when compared with SUMO(2/3)ylation and ubiquitination, as judged by their respective sensitivity in western blot analysis. These data reveal that PXR is the simultaneous target of both the SUMO- and ubiquitin-signaling pathways, and that it is targeted in both a ligand- and TNFα-dependent manner. It is likely that the interaction of SUMO and ubiquitin on PXR occurs in a manner similar to that previously

observed for hepatocyte nuclear factor 4-alpha (49), and further suggest that these two post translational modifications somehow alter PXR protein-stability, likely through regulating its entry into the 26S proteasome-mediated degradation machinery.

Figure 2-1.

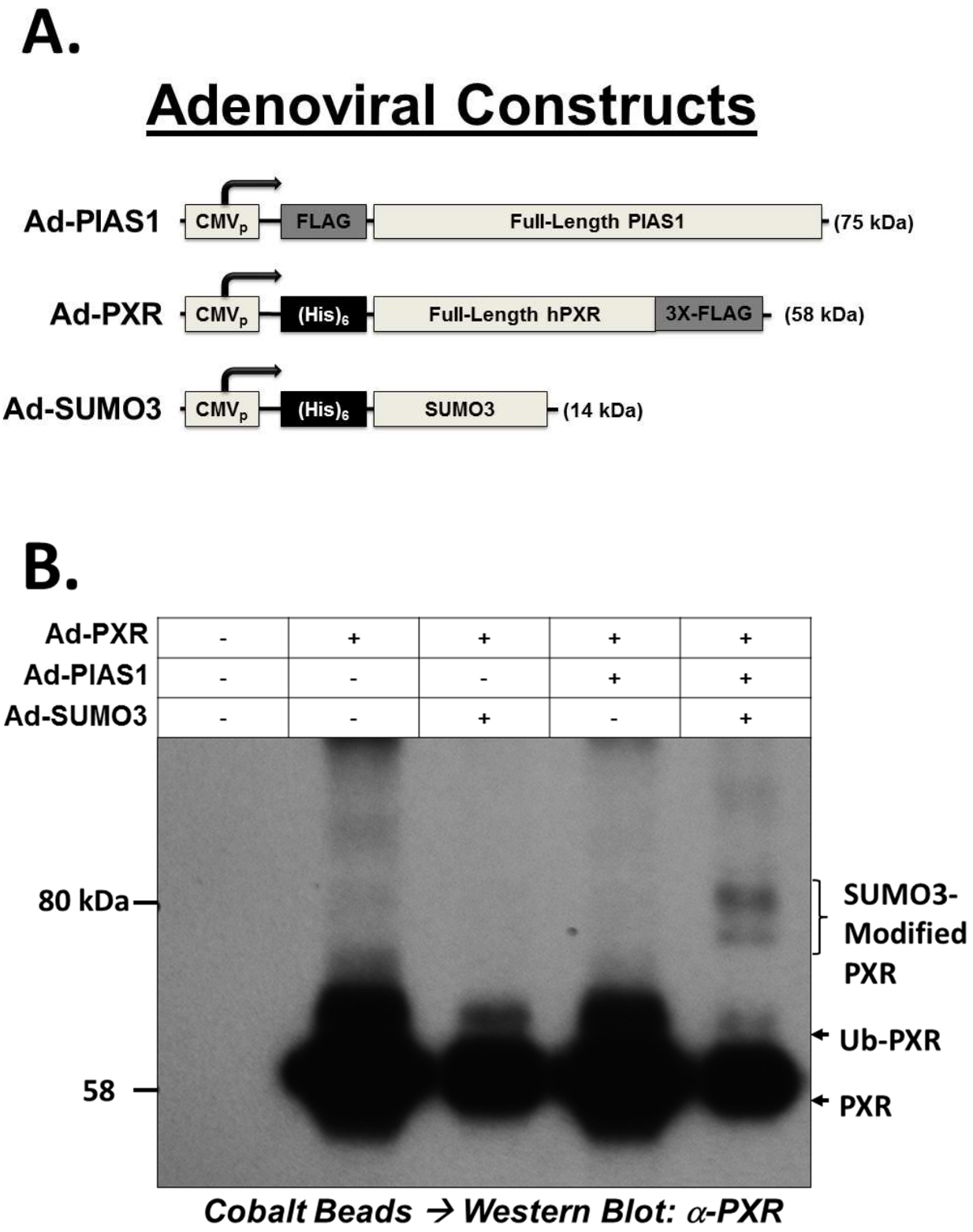

A.
Adenoviral Constructs
Ad-PIAS1
CMVp
FLAG
Full-Length PIAS1
(75 kDa)
Ad-PXR
CMVp
(His)6
Full-Length hPXR
3X-FLAG
(58 kDa)
Ad-SUMO3
CMVp
(His)6
SUMO3
(14 kDa)
B.
Ad-PXR - + + + +
Ad-PIAS1 - - - + +
Ad-SUMO3 - - + - +
80 kDa
58
SUMO3-Modified PXR
Ub-PXR
PXR
Cobalt Beads → Western Blot: α-PXR

Figure 2-1.

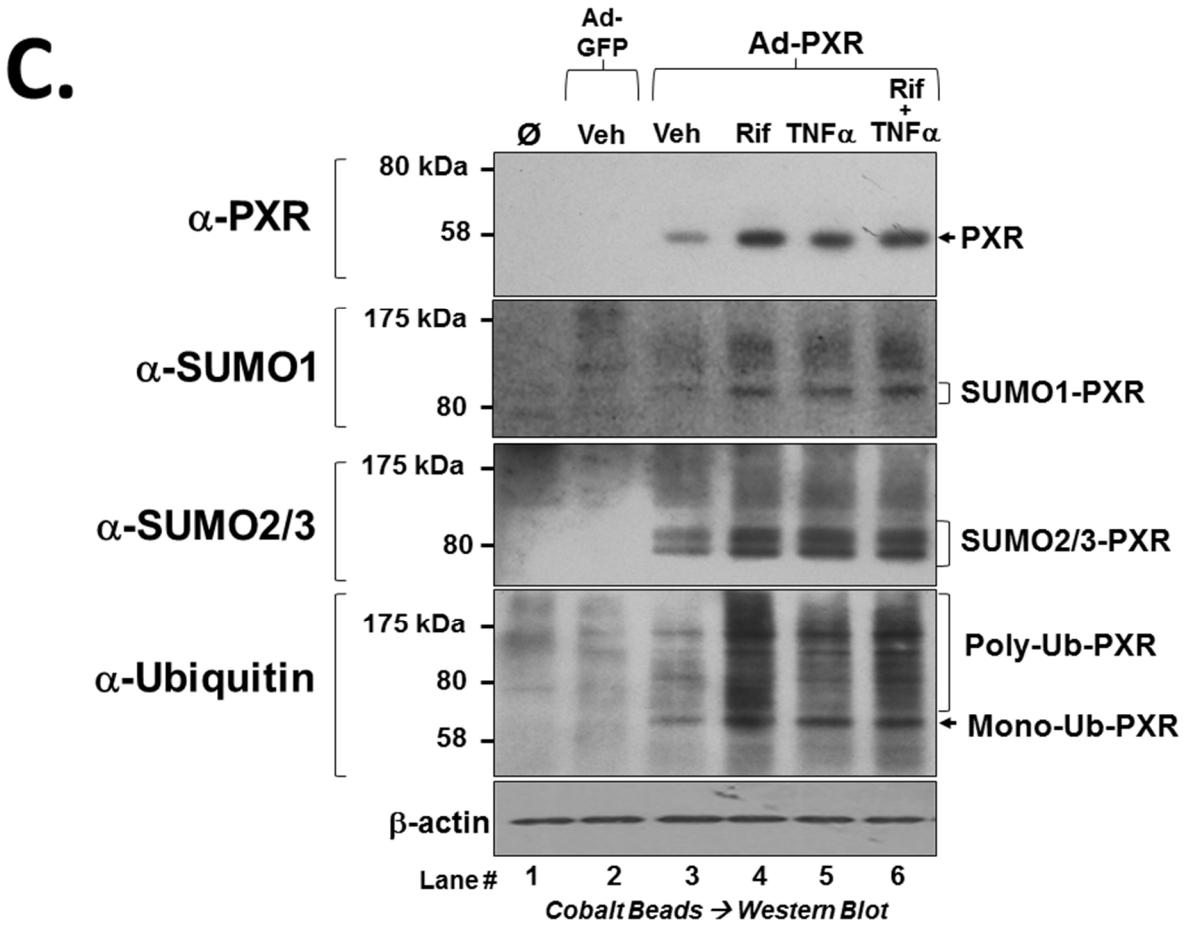


Figure 2-1. Adenoviral-Mediated Approach to Examine PXR Post-Translational Modifications. (A) Depiction of adenoviral constructs including Ad-PIAS1, Ad-PXR, and Ad-SUMO3. Note the use of FLAG epitope and $(His)_6$-affinity tags that increase the apparent molecular weight of the exogenously expressed proteins. **(B)** Primary hepatocytes isolated from wid type (C57Bl6) mice were left non-transduced or were transduced as indicated in the figure. Hepatocytes were lysed using strong denaturing conditions as described in Materials and Methods. Cell lysates were subjected to enrichment using cobalt beads and captured proteins were washed sequentially using guanidine-HCl and urea-based wash buffers. Proteins were eluted using 2X-Laemmli buffer and resolved using 10 % SDS-PAGE. Western blot analysis was performed with an anti-PXR antibody that detects all modified forms of the protein (Santa Cruz, H-11 monoclonal Ab). **(C)** Primary hepatocytes isolated from wid type (C57Bl6) mice were left non-transduced, or were transduced with either blank virus (Ad-GFP) or Ad-PXR. Hepatocytes were lysed using strong denaturing conditions, and western blot analysis was performed with an anti-PXR antibody (Santa Cruz, H-11 monoclonal Ab), anti-SUMO1 antibody (Cell Signaling, C9H1), anti-SUMO2/3 antibody (Cell Signaling, 18H8), anti-Ubiquitin (Cell Signaling, P4D1), or anti-□-actin (Chemicon, MAb1501) as a loading control.

2.3.2 Expression of PIAS1 Modulates PXR Activity in Primary Mouse Hepatocytes.

We next examined whether expression of PIAS1 altered PXR activity in liver cells. Primary hepatocytes were isolated from 8-week old male *pxr*-nullizygous (PXR-KO) mice and cultured overnight. The next day hepatocytes were transduced with purified adenoviral vectors encoding human PXR, PIAS1, or were co-transduced with both adenoviral expression vectors together. Forty-eight hours post-transduction, hepatocytes were treated with rifampicin (10 μM), a potent and efficacious human PXR ligand, for and additional twenty-four hours. Following rifampicin treatment, hepatocytes were treated as indicated with TNFα (10 ng/mL) alone, or were co-treated with rifampicin and TNFα together for an additional three hours as indicated. Total RNA was isolated and rt-QPCR analysis was performed to determine expression levels of PXR-target genes. As expected, induction of the well-known PXR-target gene, *Cyp3a11*, was absent following rifampicin treatment in both non-transduced as well as PIAS1 transduced PXR-KO hepatocytes (**Figure 2-2A**). In contrast, rifampicin treatment produced an approximately fourteen-fold induction of *Cyp3a11* gene expression levels in PXR-KO mouse hepatocytes expressing human PXR. Treatment of hepatocytes expressing of both PXR and PIAS1 with rifampicin produced an approximately thirty five-fold increase in *Cyp3a11* gene expression levels, indicating that PIAS1 has a co-activator effect on PXR with respect to the *Cyp3a11* promoter. Co-treatment of PXR-transduced hepatocytes, as well as PXR- and PIAS1-co-transduced hepatocytes, with rifampicin and TNFα produced significant repression of *Cyp3a11* expression when compared with rifampicin treatment alone. There was no modulation of *Cyp3a11* expression in PXR-KO hepatocytes by any treatment when the addition of exogenous PXR was omitted. Taken together, these data indicate that PIAS1 activity can enhance PXR trans-activation with respect to the prototypical xenobiotic response gene, *Cyp3a11*, and further

suggest that PXR is required for TNFα-mediated repression of *Cyp3a11* expression levels in hepatocytes.

Previous research from our laboratory and others indicates that PXR activation can suppress the cytokine-inducible expression of TNFα and IL-6 in liver and intestine (3,5,6,18,50,51). We therefore examined the role of PIAS1 in promoting this effect in a PXR-dependent manner in liver. Primary hepatocytes isolated from PXR-KO mice were transduced with PIAS1 alone, human PXR alone, or both PIAS1 and PXR together for twenty-four hours. Hepatocyte cultures were treated with either vehicle, rifampicin, TNFα, or with rifampicin and TNFα together as indicated. The expression levels of pro-inflammatory cytokines TNFα and IL-6 were subsequently examined (**Figure 2-2B and 2-2C**). As expected, in non-transduced PXR-KO hepatocytes, treatment with TNFα (10 ng/mL) for three hours increased the expression of TNFα messenger RNA approximately 16-fold. In contrast, co-treatment of non-transduced PXR-KO cells with rifampicin and TNFα together, or treatment with rifampicin alone had no significant effect on TNFα messenger RNA levels. Similarly, expression of PIAS1 alone did not modify the TNFα-inducible expression of TNFα messenger RNA. Expression of exogenous PXR significantly increased the basal levels of TNFα messenger RNA by approximately 8-fold when compared with vehicle treated non-transduced PXR-KO hepatocytes. This is consistent with our previous publication that indicates that hepatocytes lacking PXR exhibit a diminished capacity to mount a robust immune response following challenge with lipopolysaccharide (6). Hepatocytes expressing exogenous PXR that were co-treated with TNFα and rifampicin together exhibited significant repression of TNFα-inducible TNFα messenger RNA expression. The co-expression of PIAS1 and PXR further suppressed TNFα-inducible TNFα expression in a PXR-dependent manner, indicating that PXR and PIAS1 collaborate to suppress cytokine-inducible

TNFα expression in hepatocytes. A similar effect was noted when expression levels of IL-6 messenger RNA were examined as well (**Figure 2-2C**). Taken together, the data presented in **Figure 2-2** indicate that PXR is required for production of full and robust TNFα-inducible pro-inflammatory cytokines (TNFα and IL-6), and that PIAS1 participates in this PXR-dependent trans-repression phenomenon in hepatocytes.

Figure 2-2A.

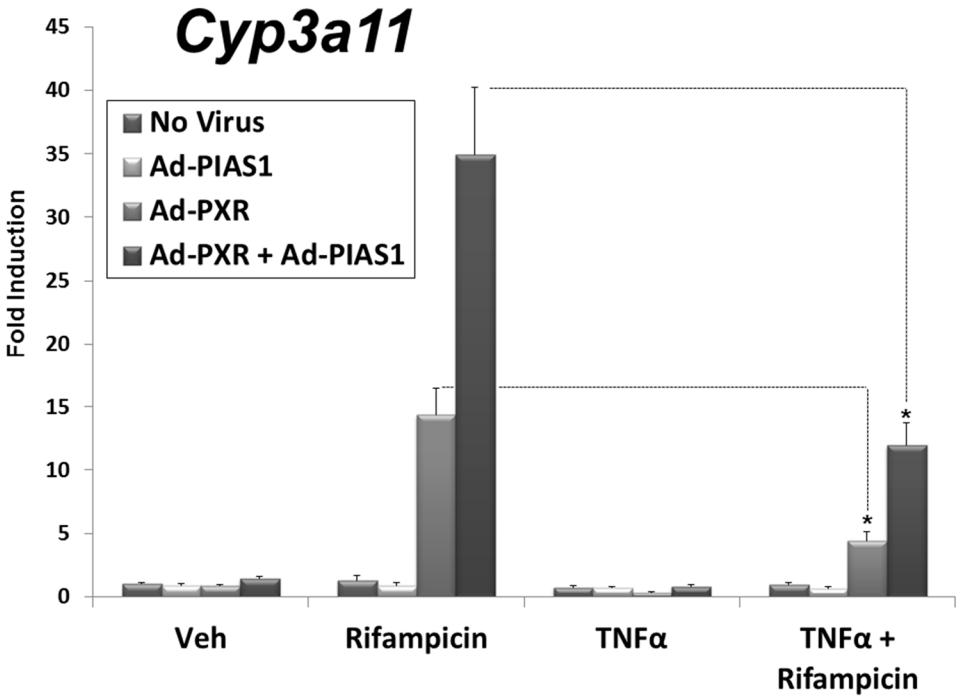


Figure 2-2B.

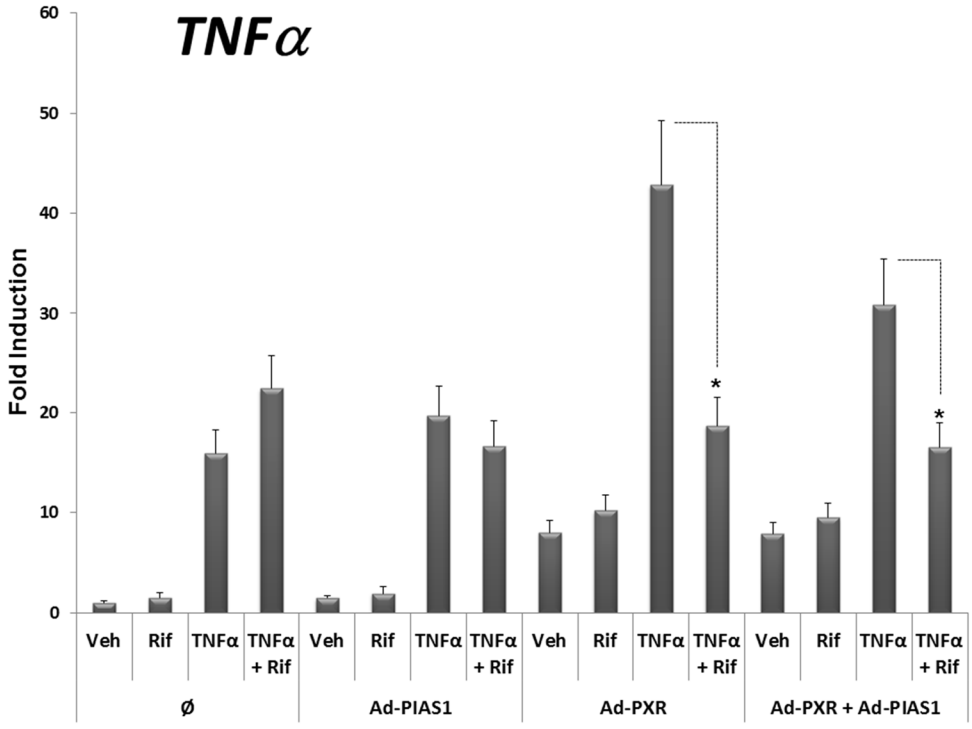


Figure 2-2C.

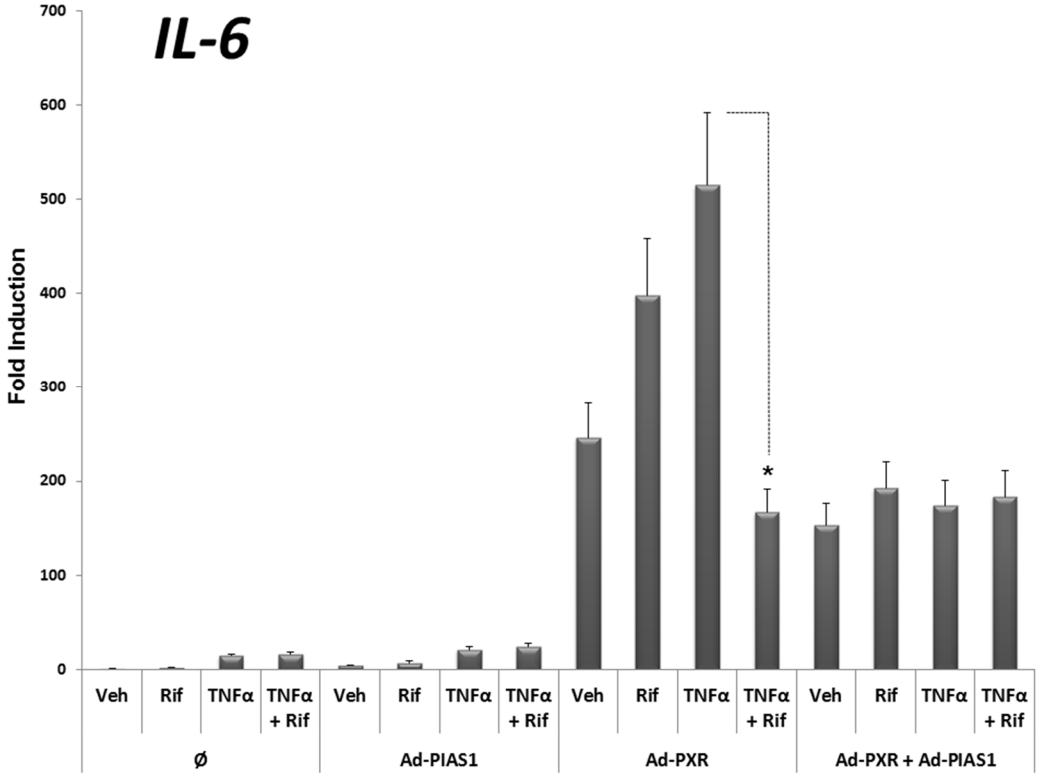


Figure 2-2. PIAS1-mediated Modulation of the Xenobiotic Response and Inflammatory Response in Hepatocytes. Primary hepatocytes isolated from *pxr nullizygous* (PXR-KO) mice were transduced as indicated. Following treatments, total RNA was isolated and the relative expression level of **(A)** Cyp3a11, **(B)** TNF□, and **(C)** IL-6 were determined. All data are normalized to β-actin levels and are presented as fold regulation. Asterisks indicate a statistical difference between treatment groups (n=3, and $p<0.05$).

2.3.3 Detection of the SUMOylation Machinery in Primary Hepatocytes.

To characterize the levels of SUMO and its associated enzymes in mouse liver, we first examined the expression levels of SUMO1, SUMO2/3, Sae1, Sae2, Ubc9, PIAS1, PIAS2, PIAS3, PIASy, SENP1, SENP2, SENP3, SENP5, SENP6, and SENP7 using cDNA generated using RNA isolated from primary cultures of wild type C57BL6 hepatocytes isolated from male animals aged 6-10 weeks. Initial studies using standard rt-PCR analysis revealed that all genes examined were expressed at detectable levels in mouse hepatocytes (**Figure 2-3**), except for Sentrin protease 3 (SENP3) (**Figure 2-3,** ***Arrow Lane 12***). Subsequent real-time quantitative PCR (rt-QPCR) analysis determined that twenty-four hour treatment with PXR ligand, pregnenolone 16α-carbonitrile (10μM), or three hour treatment with TNFα (10ng/mL) had no effect on expression levels of the genes encoding these enzymes (***data not shown***). These data indicate that primary hepatocytes express most of the key genes involved in regulating the SUMOylation of target proteins, and that neither PXR ligand nor pro-inflammatory cytokine (TNFα) has any effect on the expression level of genes encoding SUMO and its associated enzymes.

Figure 2-3.

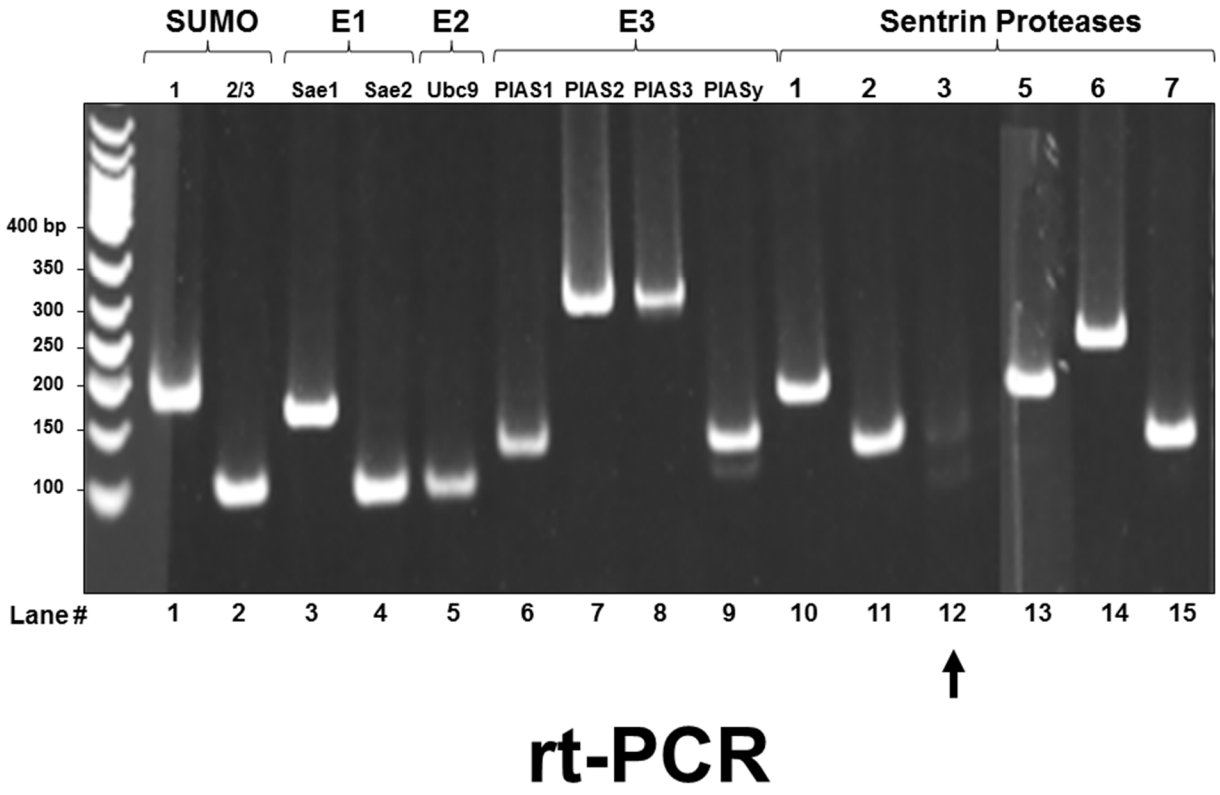


Figure 2-3. Detection of SUMO1, SUMO2/3, and SUMO-Associated Enzymes at the Level of Gene Expression in Hepatocytes. Primary hepatocytes isolated from wid type (C57Bl6) mice were isolated and cultured as described in Materials and Methods. Total RNA was isolated and the expression of indicated genes was determined using standard non-quantitative reverse transcriptase-PCR and agarose gel (2%) electrophoresis methods. The arrow (Lane 12) indicates equivocal detection of SENP3 in hepatocytes.

2.3.4 Identification of the SUMO-E3 Ligase Enzymes Important for SUMO-Modification of PXR.

To determine which PIAS family members could function as the most effective SUMO-E3 ligase towards PXR, the murine hepatoma-derived cell Hepa1-6 cells was used (38). Cultured cells were co-transfected with expression vectors encoding FLAG-tagged PXR together with either $(His)_6$-SUMO1 or $(His)_6$-SUMO3. An additional expression vector encoding a specific PIAS- family member was added as indicated (**Figure 2-4A**). The expression levels of all five PIAS proteins examined in this assay were roughly equivalent (data not shown). In the presence of PIASy, modification of PXR by SUMO1 was supported at least two sites as determined using western blot analysis with an anti-PXR antibody following enrichment with cobalt beads (**Figure 2-4A,** ***asterisks Lane 7***). The other four PIAS family members examined (PIAS1, PIASxα, PIASxβ, and PIAS3) promoted more modest SUMO1-modification of PXR. When $(His)_6$-SUMO3 was used in the assay, more robust SUMO-chain formation was observed with PIASy promoting robust SUMO(3)ylation of PXR (**Figure 2-4A,** ***Bracket Lanes 8, 11, and 12***). While both PIAS1 and PIAS3 promoted SUMO(3)ylation of PXR with high efficiency, PIASy was the most effective SUMO-E3 ligase examined with respect to SUMO(3)ylation of PXR. Of note, the intensity of the primary non-modified PXR band was increased in direct proportion to the level of SUMO-modified PXR (**Figure 4A,** ***arrow Lanes 7, 8, 11, and 12***). Taken together, the data presented in **Figures 2-3 and 2-4A** indicate that PIASy is the most effective SUMO-E3 ligase towards the PXR protein in this cell line-based assay. It is worth noting here that PIASy, PIAS1, and PIAS3 potentially play differential roles in SUMO-modification of PXR.

2.3.5 Identification of the Sentrin Protease Enzymes Important for De-SUMOylation of PXR.

There is increasing recognition that de-SUMOylation of SUMO-substrates by sentrin protease enzymes, or SENPs, represents a key regulatory step in the SUMO-signaling pathway (52,53). Similar to SUMO-E3 ligase enzymes, the specific SENP(s) that remove SUMO from PXR are currently unknown. We therefore sought to identify the specific SENPs capable of de-SUMOylating PXR using a variation of our transient transfection cell line-based assay. Expression vectors encoding FLAG-PXR, PIASy, and $(His)_6$-SUMO1 were introduced into Hepa1-6 cells together with selected SENPs as indicated (**Figure 2-4B**). Where available, the catalytically deficient mutant forms of each SENP (ΔSP1, ΔSP2, ΔSP3, ΔSP5, AND ΔSP6) were used as negative controls as indicated. Expression of SENP2 completely abolished SUMOylation of PXR, whereas, the catalytically deficient form of SENP2 (ΔSP2) was ineffective. While expression of SENP1 and SENP6 promoted de-SUMOylation of PXR to some extent, the removal was incomplete. It is noteworthy that the 52 kDa immunoreactive band that corresponds to non-modified PXR decreases in direct proportion to the level of PXR de-SUMOylation (**Figure 2-4B,** *arrow*). These data suggest that PIASy-mediated SUMOylation of PXR may stabilize the protein or inhibit its proteasome-mediated degradation. Identical experiments using SUMO3 indicate that the SENP1, SENP3, and SENP6 de-SUMOylating enzymes selectively remove SUMO-chains, while SENP2 is the most effective at removing all SUMO moieties from PXR (**Figure 2-4C**). These data suggest that SENP2 is the key de-SUMOylating enzyme for PXR, and also raise the possibility that other SENPs (SENP1, SENP3, and SENP6) may have differential effects on removal of SUMO chains or site-specific de-SUMOylase activity toward PXR.

Figure 2-4A.

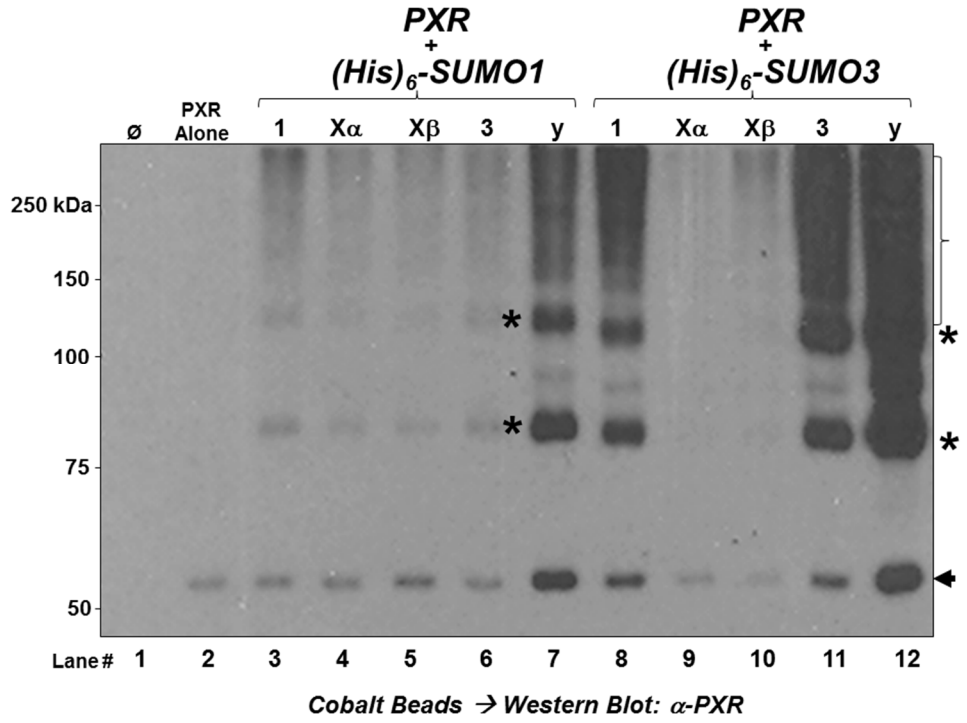


Figure 2-4B.

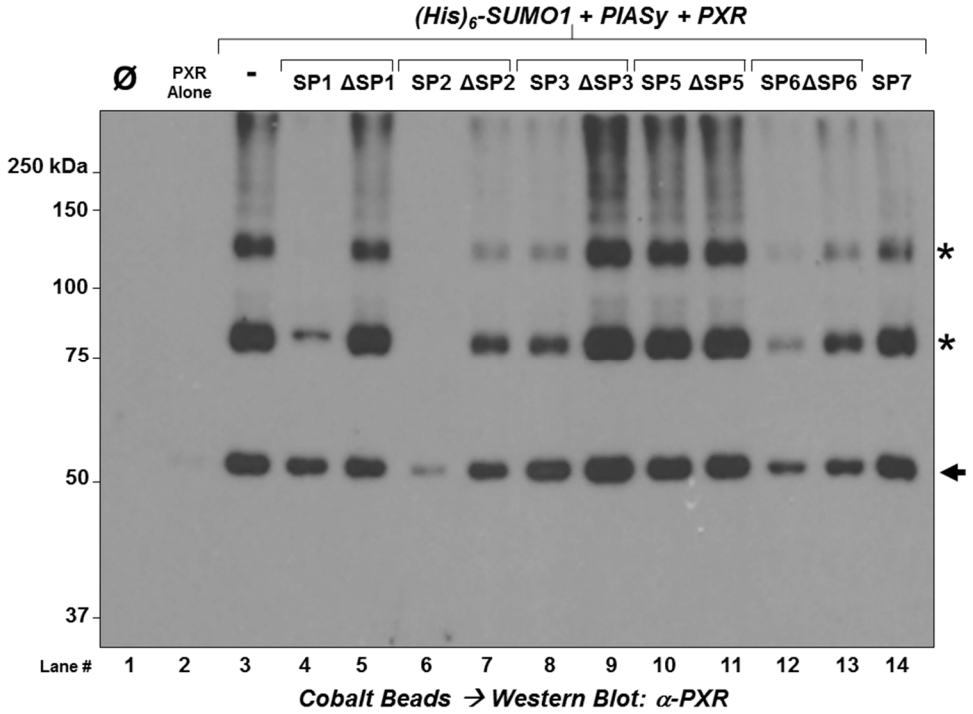


Figure 2-4C.

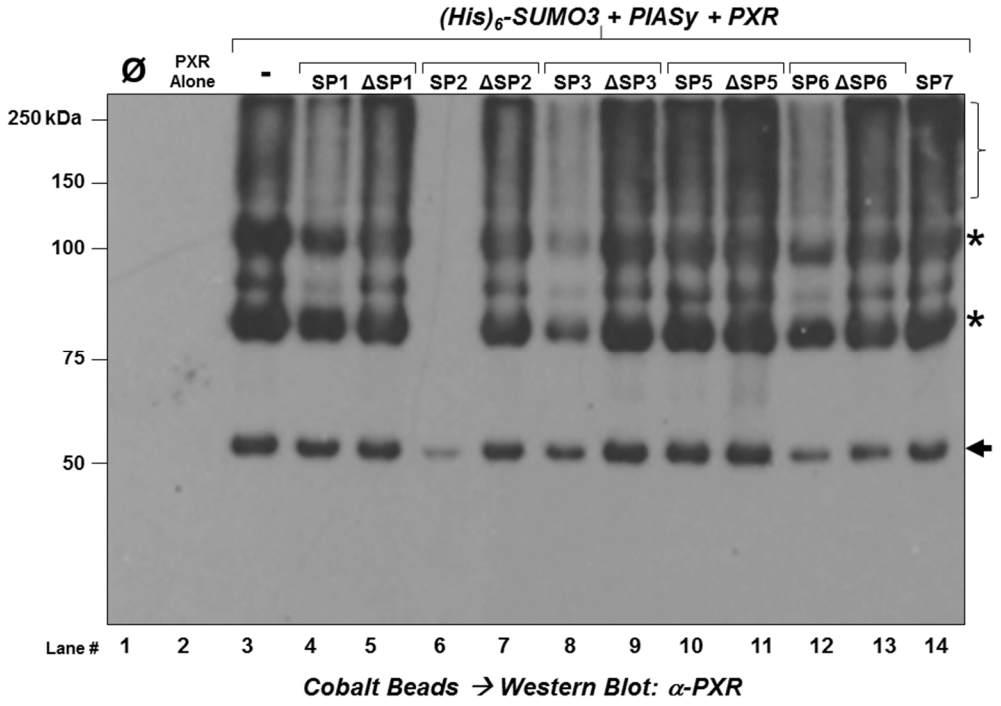


Figure 2-4. Characterization of SUMO E3-Ligases and SENPs Associated with PXR SUMOylation and De-SUMOylation. (A) Indicated expression vectors were transfected into Hepa1-6 cells. Forty-eight hr post-transfection cells were harvested and SUMOylated proteins were gathered. Proteins were subjected to SDS-PAGE and subsequent western blot analysis using an anti-PXR antibody. Asterisks (*) indicate modified forms of PXR and the arrow (←) indicates non-modified PXR protein. **(B and C)** Expression vectors encoding SENPs, control mutant catalytically deficient SENPs (ΔSP'X') were transfected into Hepa1-6 cells in combination with either SUMO1 or SUMO3, together with PXR and PIASy as indicated. Forty-eight hr post-transfection cells were harvested in denaturing buffer and SUMOylated proteins were captured. Proteins were subjected to SDS–PAGE and subsequent western blot analysis using anti-PXR antibody. Asterisks (*) indicate SUMO-modified forms of PXR and the arrow (←) indicates non-modified PXR protein. The brackets in **(A)** and **(C)** represent the formation of SUMO-chain on PXR.

2.3.6 PGC-1α-Mediated Trans-activation of PXR Is Regulated by PIASy-mediated SUMOylation.

Interaction of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) with liver X receptor-beta is attenuated by increased SUMO-signaling (54,55). The PXR NR family member also strongly interacts with, and is co-activated by PGC-1α (56). We therefore sought to determine the extent to which PGC-1α-mediated trans-activation of PXR is modulated by the SUMO1 or SUMO2/3-signaling pathways. To accomplish this we constructed a multimerized (3X) PXR-dependent (ER-6 PXR-enhancer) luciferase reporter gene [$(ER\text{-}6)_3$-tk-Luc] as described in Materials and Methods. Treatment of PXR-transfected CV-1 cells with rifampicin induced expression of this PXR-dependent reporter gene approximately 2.7-fold (**Figure 2-5**). The addition of PGC-1α significantly increased reporter gene activity in a PXR-dependent manner, while the addition of SUMO1 and PIASy together significantly inhibited PGC-1α-mediated trans-activation of PXR. Importantly, the de-SUMOylating enzyme SENP2 significantly restored the SUMO1/PIASy-mediated suppression of PXR/PGC-1α reporter gene activity when compared with cells expressing only PIASy and SUMO1, while SENP6 was less effective in this regard. When SUMO3 was used in place of SUMO1 in identical experiments the PIASy-mediated suppression of PXR activity was absent, suggesting a differential role for SUMO(1)ylation versus SUMO(3)ylation. However, addition of SENP2 had a significant positive effect upon both basal- and rifampicin-dependent PGC-1α-mediated trans-activation of PXR-dependent reporter gene activity in this case. It is interesting to note that SENP6, which has strong SUMO-chain editing activity and is ineffective at complete removal of SUMO3 from PXR (**Figure 2-4C)**, had little to no effect on PGC-1α-mediated trans-activation of PXR in the

face of SUMO3 and PIASy. These data suggest that strong de-SUMOylation signaling pathways mediated by SENP2 enhance PGC-1α-mediated co-activation of PXR.

Figure 2-5.

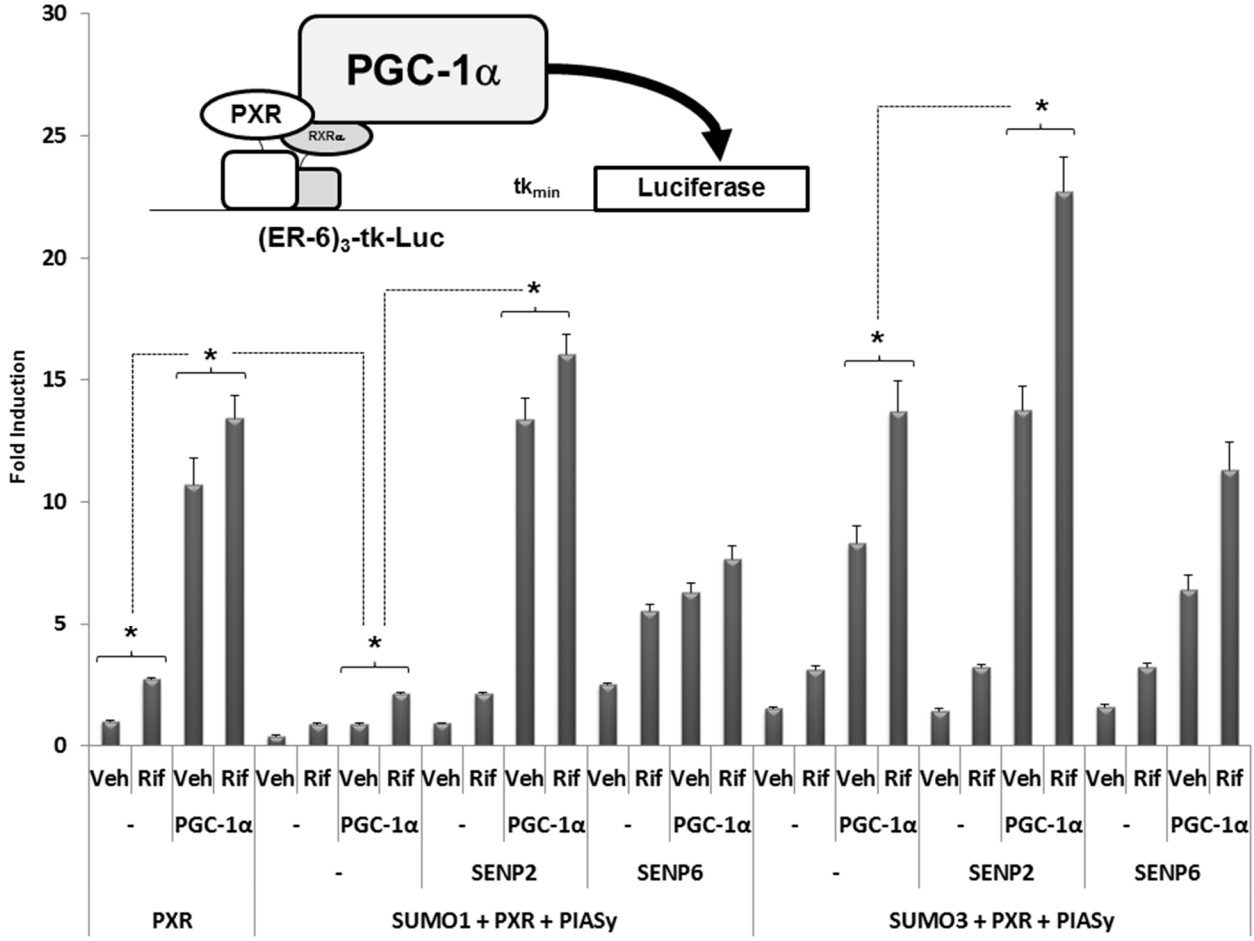


Figure 2-5. PIASy and SUMO1 Abrogate Association of PXR with PGC-1α to Attenuate Its Trans-activation Capacity. CV-1 cells were transfected with a PXR-dependent luciferase reporter gene $(ER6)_3$-tk-Luc and expression vectors encoding PXR, PGC-1α, PIASy, SUMO1, SUMO3, SENP2, or SENP6 as indicated. Twenty-four hours post-transfection cells were treated with vehicle (0.1% DMSO) or Rif (10μM) for an additional 24 hr. Luciferase activity was normalized to β-gal controls and data are presented as fold induction ± SEM. Asterisks indicate statistically significant differences between relevant treatment groups ($p \leq 0.05$).

2.3.7 The SUMO- and Ubiquitin-signaling Pathways Interface at the Level of PXR.

Ubiquitination of PXR has previously been demonstrated by our group and others (19-21), and pharmacological inhibition of the 26S proteasome in cells inhibits PXR function (21). However, the precise molecular nature of the ubiquitin chain formation, the specific lysine residue on PXR that is the target of ubiquitin and the biological significance of ubiquitin- SUMO interaction at the level of PXR are not currently well defined. The novel ubiquitin expression vector we constructed adds approximately 17 kDa to the size of the PXR protein due to the presence of an extended N-terminus (**Figure 2-6A**). We engineered several key features into the N-terminus of ubiquitin including a $(His)_6$-metal-affinity tag for bead-based enrichment, as well as both an Xpress-epitope tag and an HA-epitope antibody tag for enrichment and western blot strategies. We have termed this expression vector His-Ub. The His-Ub expression vector was used as a template to create expression constructs that contain mutations at key lysine residues including (1) lysine 48 mutated to arginine (His-K48R), (2) lysine 63 mutated to arginine (His-K63R), and (3) both lysine 48 and lysine 63 mutated to arginine (His-K48R,K63R). Using His-Ub and the mutant ubiquitin expression vectors in our cell line-based assay together with a plasmid encoding FLAG-PXR (52 kDa) in transfection-based experiments, we detect heavily mono-ubiquitinated PXR at the predicted 69 kDa molecular weight (**Figure 2-6B, *lanes 3, 4, 5, and 6***). When His-K48R was used co-expressed with PXR, chain formation was dramatically reduced. In contrast, when the His-K63R mutant was co-expressed with PXR, chain formation was completely intact. When the double mutant His-K48,K63R construct was used in the assay, ubiquitin chain formation was completely lacking. Taken together, these data suggest that lysine 48 in ubiquitin supports chain formation that is linked to a single lysine residue in the PXR protein.

To directly examine the nature of ubiquitin chains and site of PXR ubiquitination using LC-MS/MS-based methods, we took advantage of our adenoviral expression vector encoding $(His)_6$ -tagged human PXR to achieve high level of expression and relative ease of purification. Primary hepatocytes were transduced with an appropriate amount of PXR virus. Forty-eight hours post-transduction, cells were treated with either vehicle (0.1% DMSO) or rifampicin for an additional twenty-four hours. Hepatocytes were lysed using denaturing conditions, and total PXR was isolated using cobalt-bead affinity methods (21). Following SDS-PAGE of PXR-enriched protein lysates, LC-MS/MS methods were used to probe the site of PXR-ubiquitination (**Figure 2-6C)**, and the precise nature of observed ubiquitin chains (**Figure 2-6D**). This analysis detected lysine 170 (K170) as the site of PXR ubiquitination, and also confirmed our previous analysis indicating a high level of K48-linked ubiquitin chains. It is widely recognized that ubiquitin-chain formation linked through K48 that is anchored to a single lysine in the substrate is a canonical signal for targeting proteins for proteasome-mediated degradation (57). These data confirm that PXR is the target of the ubiquitin-signaling pathway, and further confirm the precise site (K170) and nature of PXR ubiquitination (K48-linked ubiquitin chains). Taken together, these data indicate that PXR post-translational modification by ubiquitin is highly implicated in regulation of PXR protein stability, likely through the activity of the 26S proteasome.

Figure 2-6A.

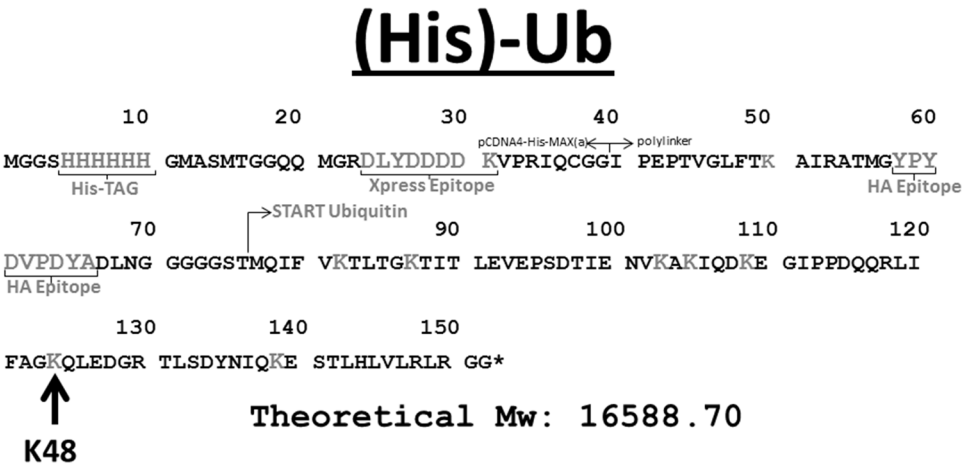


Figure 2-6B.

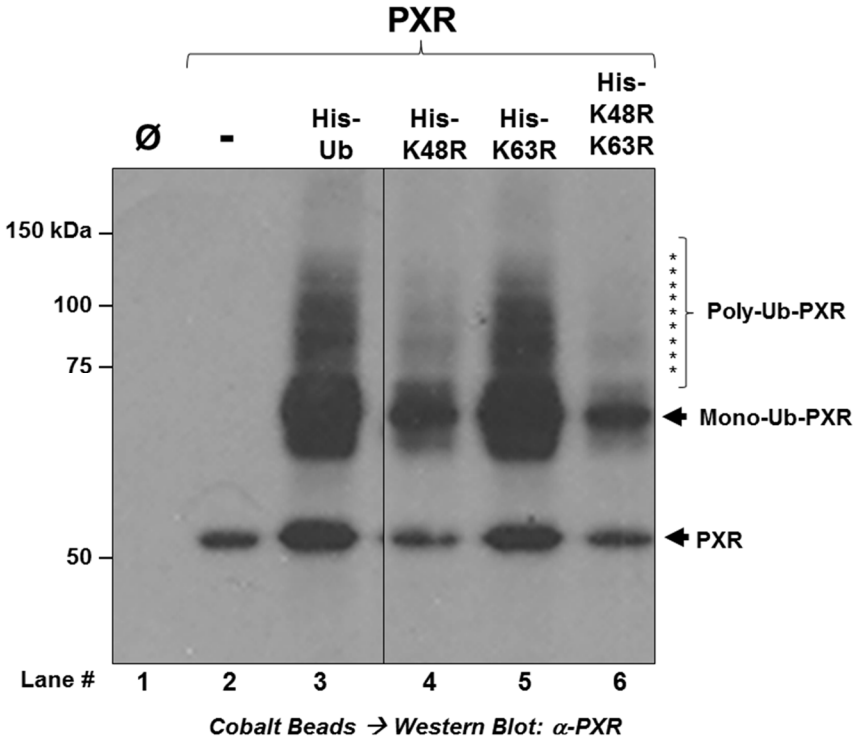


Figure 2-6C.

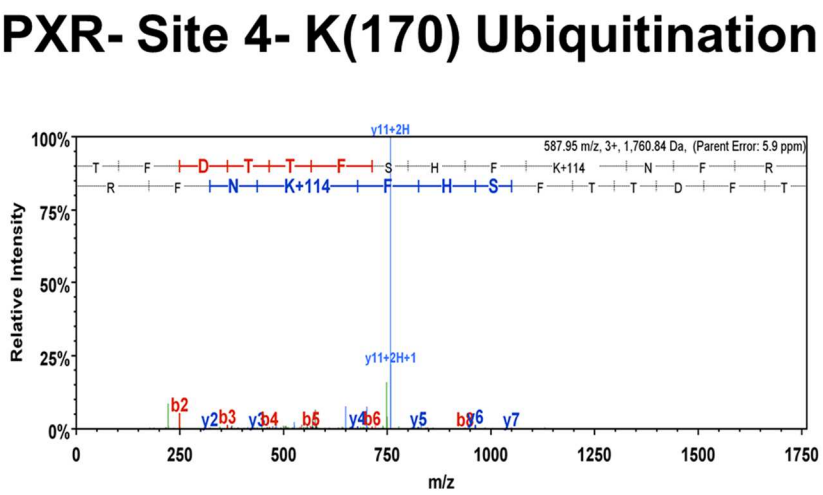


Figure 2-6D.

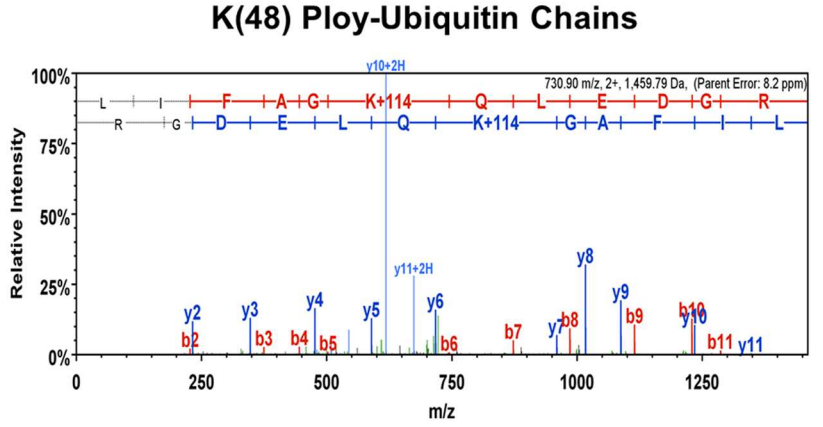


Figure 2-6. K48-Linked Ubiquitin chain formation on a Single Lysine Residue (K170) within PXR. (A) Single letter amino acid representation of the open reading frame of $(His)_6$-tagged wid type ubiquitin. **(B)** Hepa1-6 cells were transfected with expression vectors encoding PXR alone, or the wild type and indicated mutant forms of ubiquitin together with PXR as shown. Cells were lysed and total $(His)_6$-tagged ubiquitinated proteins were captured using cobalt beads. Captured proteins were subjected to SDS-PAGE and subsequent western blot analysis was performed using an anti-PXR antibody. Asterisks (*) indicate ubiquitin-modified forms of PXR and the arrow (←) indicates non-modified PXR protein. The bracket and asterisks indicate poly-ubiquitinated forms of PXR. **(C)** Lysine residue (K170) in PXR was identified based on assignment of multiple product ions (b and y ions) in the MS/MS scan of the precursor ion at M/z 587.95 to the PXR tryptic peptide sequence with a mass addition of 114 at the lysine residue (ubiquitin di-glycine post-tryptic digestion). **(D)** MS/MS spectrum assigned to ubiquitin tryptic peptide sequence showed lysine residue (K48) carried a modification with a mass of 114 that indicates poly-ubiquitination.

We next sought to determine whether promoting SUMO(1)ylation of PXR in cells can affect its modification by ubiquitin. Transfection of Hepa1-6 cells with expression vectors encoding His-Ub and PXR produces detectable forms of ubiquitinated PXR, both in the absence and presence of the PXR ligand (**Figure 2-7, *lanes 3 and 4***). Co-expression of PIASy and PXR in the absence of His-Ub produces increased levels of unmodified PXR (**Figure 2-7, *lanes 5 and 6***). When His-Ub was co-expressed with PIASy and SUMO1 together, the modification of PXR by ubiquitin was dramatically increased (**Figure 2-7, *lanes 3 and 4 versus lanes 7 and 8***). These data indicate that PIASy-mediated SUMO(1)ylation of PXR stabilizes the protein, likely through prevention of ubiquitin-mediated degradation by the 26S proteasome.

Figure 2-7.

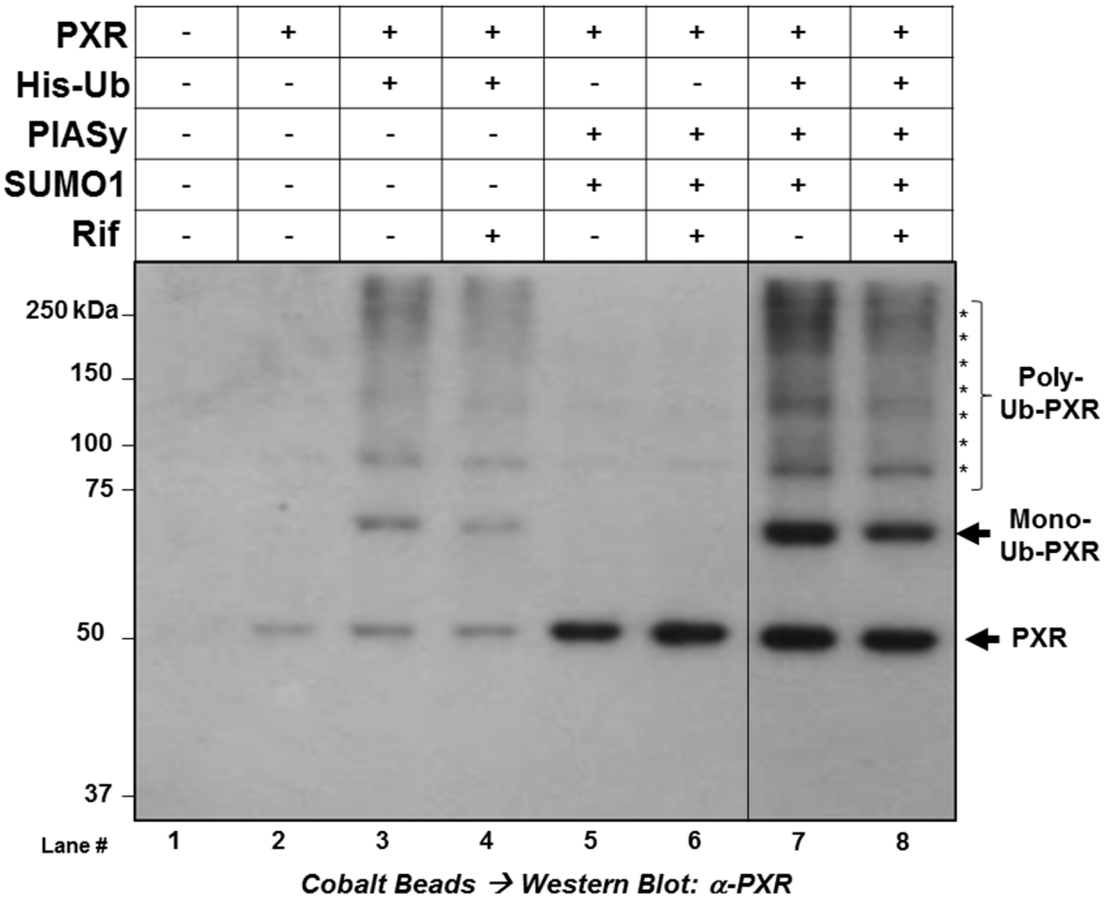


Figure 2-7. PIASy Increases Levels of Ubiquitinated PXR. Hepa1-6 cells were transfected with expression vectors as indicated. Ubiquitinated proteins were captured using cobalt-linked agarose beads. Captured proteins were subjected to SDS-PAGE and the blots were probed for PXR immunoreactivity. Asterisks (*) indicate ubiquitin-modified proteins.

2.3.8 Identification of Site 1 and Site 2 as the Primary Sites of SUMO-Modification.

The PXR protein contains several lysine residues that are predicted to serve as acceptor sites for SUMOylation (18). Among the four sites (labeled Site1, Site 2, Site 3, and Site 4) is one 'high probability' type I consensus site (Ψ-K-x-D/E; where Ψ=hydrophobic residue) at lysine 108 (Site 1, K108) (**Figure 2-8A**). The other three predicted sites have a lower probability to serve as SUMO-acceptor sites. We created a series of mutant PXR expression vectors as indicated at these four potential sites of SUMOylation shown in **Figure 2-8B**. Co-transfection of Hepa1-6 cells with $(His)_6$ -SUMO1, PIASy, and PXR together produced two clear sites of modification following enrichment and western-blot with an anti-PXR antibody (**Figure 2-8B, *asterisks***). Consistently, wherever mutation of Site 1 appears (**K108R**, ***lanes 4, 5, 6, and 8***), the upper band disappears. No other lysine to arginine mutation examined in Site 2, 3, or 4 appeared to support SUMO(1)ylation in this analysis. When $(His)_6$ -SUMO3 was used in place of $(His)_6$ -SUMO1, both Site 1 and Site 2 in PXR appeared to support SUMO(3)ylation and SUMO-chain formation in a cooperative manner, with adjacent lysine residues 128 and 129 serving as a likely sites of further SUMO(3)ylation. It is interesting to note that both Site 1 (-MKKE-) and Site 2 (-KKSE-) contain di-lysine residues (-KK-) embedded within the predicted SUMO-acceptor sites. These data indicate that both Site 1 and Site 2 contribute differentially to SUMO-modification of PXR, and further suggest that SUMO1 may modify PXR in a manner that is distinct from that observed with SUMO2/3.

Figure 2-8A.

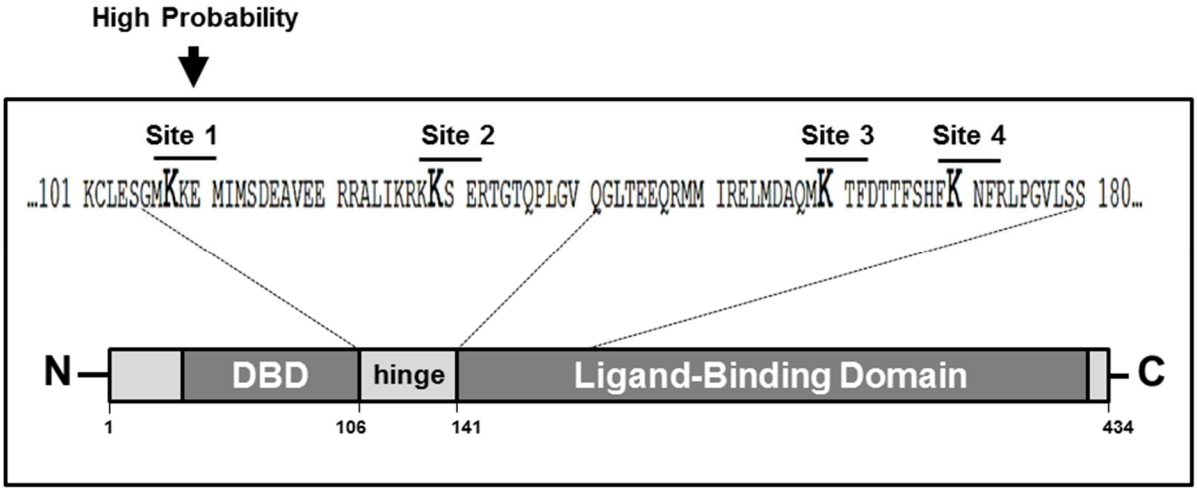


Figure 2-8B.

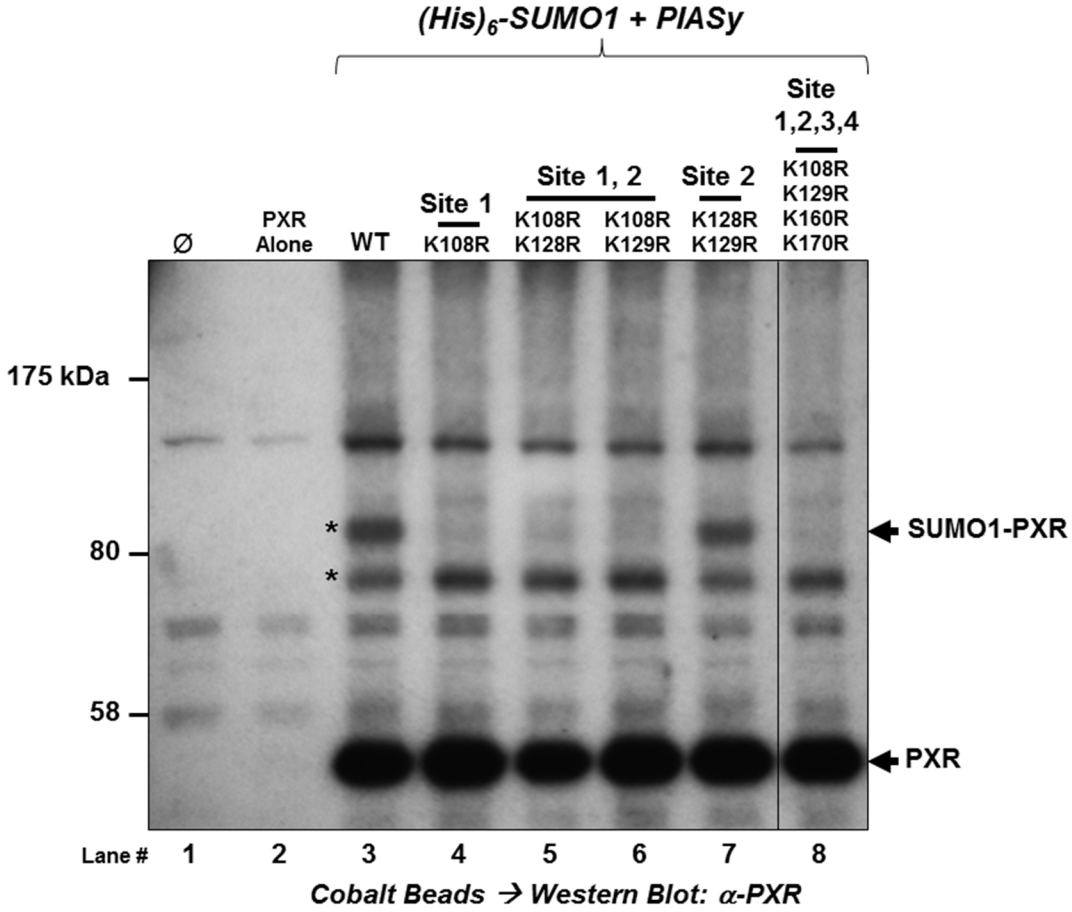


Figure 2-8C.

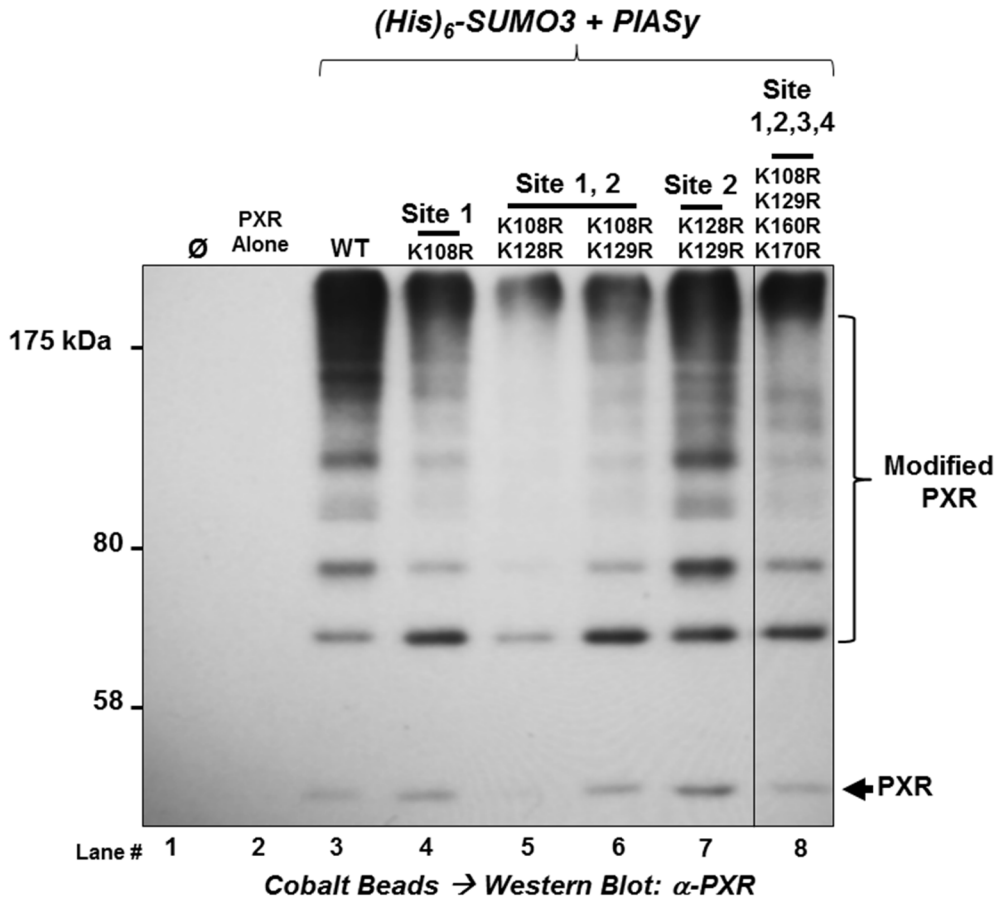


Figure 2-8. Site-Directed Mutagenesis Study of Potential Sites of SUMOylation of PXR. **(A)** Four probable sites of SUMO modification were identified using SUMOplot (http://www.abgent.com/sumoplot) and SUMPsp (http://sumosp.biocuckoo.org/) prediction analysis servers. **(B)** Site-directed mutagenesis was performed and the indicated mutant PXR proteins were transfected together with PIASy and **(C)** SUMO1 or **(C)** SUMO3. SUMOylated proteins were captured using cobalt-linked agarose beads. Captured proteins were subjected to SDS-PAGE and the blots were probed for PXR immunoreactivity. Asterisks (*) and brackets indicate modified PXR proteins.

2.4 DISCUSSION

SUMO proteins are transcribed as immature precursor molecules with an extended C-terminus that first need to be cleaved by a SENP to expose the C-terminal di-glycine motif (58). Following cleavage of SUMO at its C-terminus by SENPs, the SUMO-E1 activating enzyme heterodimeric protein (SAE1/SAE2) utilizes an ATP-dependent process to activate the SUMO for entry into the SUMO-signaling pathway. Next, the activated SUMO is transferred to the catalytic Cys residue (C93) of the E2 enzyme Ubc9 to form a thioester-linkage at the C-terminal di-glycine motif of SUMO. The E2-SUMO activated complex is conjugated to a specific lysine residue in the substrate by a SUMO-E3 ligase enzyme that catalyzes the transfer of SUMO from E2 to its specific substrate. Like protein ubiquitination, protein SUMOylation is regulated by two opposing reactions. The first reaction is conjugation, which is carried out by a three-step cascade of enzymes that activate SUMO and selectively couple it to its target substrate proteins. The second reaction is deconjugation, which is carried out by a specialized family of SUMO-protease enzymes called SENPs (53,58).

While the SUMOylation of liver-enriched NR family members is strongly associated with suppression of the acute phase response, there are important molecular differences governing their anti-inflammatory effect. One key distinction is evident with respect to ligand-dependence of the SUMOylation reaction. On the one hand, SUMOylation of farnesoid x receptor, liver receptor homologue-1, and liver x receptor-β are all enhanced by ligand (Balasubramaniyan et al., 2013; Venteclef et al., 2010), whereas the SUMOylation of peroxisome proliferator activated receptor-α is decreased by ligand (Pourcet et al., 2010). Additional differences exist in whether NRs are modified by SUMO1 or SUMO2/3. Most NRs are reported to be modified by either SUMO1 or SUMO2/3, whereas in this study, modification

of PXR was observed with both SUMO1 and SUMO2/3. The modification of PXR was stimulated by ligand and pro-inflammatory signaling. We also found that multiple PIAS family members are capable of promoting SUMO-modification of PXR, and may thus play distinct roles in modulating its function. Further, we found that different SENPs have differing activity with respect to the deSUMOylation of PXR. Hence, our data indicate that it is likely that SUMO1 and SUMO2/3 play differing roles in regulating PXR biological function. It is also worth noting that inflammatory mediators increase SUMOylation of RXR α, a critical heterodimeric partner of PXR (59,60). This is particularly interesting in light of the fact that not only can RXRα function as a partner for the xenobiotic sensor PXR, but it also functions as an obligate heterodimeric partner for many other NR family members including those for retinoic acid, thyroid hormone, vitamin D, prostanoids, oxysterols, and bile acids.

Protein modification by SUMO was historically thought of as a post-translational modification that largely regulates the biological function and subcellular localization of many cellular proteins (61). Recent evidence indicates that SUMOylation is often a prerequisite for, or a competitor of shared substrate protein ubiquitination (12). Hence, SUMOylation was proposed as a post-translational modification that can possibly influence the degradation of shared SUMO-ubiquitin substrate proteins. More recently, SUMO2/3-dependent ubiquitin-proteasome proteolysis has been clearly demonstrated to play a critical role in the regulation of the biological function and fate of key tumor suppressor proteins involved in the development of Fanconi's anemia and other yet to be identified proteins (11-13).

In this study we identified a key role for interaction between SUMOylation and ubiquitination post-translational modifications of PXR that likely play a crucial role in the regulation of hepatic function during the xenobiotic and inflammatory responses. The PXR

protein is SUMOylated by SUMO1 and SUMO2/3 and ubiquitinated in both primary hepatocytes, as well as in cell line-based assays. We found SUMO1, SUMO2/3 and all of the SUMO-E3-ligase and key SENP enzymes to be highly expressed in mouse liver. Using novel expression and affinity purification methods combined with western blot analysis we revealed that both SUMOylation and ubiquitination of PXR was increased in a ligand-dependent fashion in cultured primary hepatocytes. Expression of PIAS1, an effective PXR-dependent SUMO-E3 ligase enzyme, and PXR in PXR-KO hepatocytes revealed that PIAS1 increases ligand-dependent expression of the prototypical xenobiotic response gene- *Cyp3a11*. In contrast, expression of PIAS1 and PXR endowed hepatocytes with rifampicin-dependent suppression of TNFα-inducible pro-inflammatory cytokine gene expression. In cell line-based assays, both PIAS1 and PIASy functioned as SUMO-E3 ligase enzymes to modify PXR, with PIASy promoting effective modification of PXR by either SUMO1 or SUMO2/3. In contrast, the PIAS1 promoted strong SUMO(1)ylation of PXR. These data raise the intriguing possibility that PIASy and PIAS1 may play differing respective roles in regulating PXR biology through the selective promotion of SUMO2/3 versus SUMO1 modification of this NR family member. The SENP2 SUMO-protease effectively removed all SUMO-modification from PXR, whereas expression of SENP1, SENP3, and SENP6 reduced the SUMO2/3-chain formation on this SUMO-substrate. Interaction of PXR with the strong co-activator protein PGC-1α was completely abrogated by PIASy-mediated SUMO(1)ylation, and this effect was reversed by co-expression of the SENP2 and SENP6 PXR-de-SUMOylase enzymes. This repressive effect was largely absent when PIASy-driven SUMO(3)ylation of PXR was examined in cell line-based reporter gene assays. The expression of SENP2 in cultured cells (i.e., strong de-SUMOylation of

PXR) increased co-activation of PXR by PGC-1α as determined using a multimerized PXR-response element (ER-6).

Four potential sites SUMO- and ubiquitin-modification of PXR were identified (Site 1, Site 2, Site 3, and Site 4), and were examined using site-directed mutagenesis and cell line-based assays. Using LC-MS/MS and cell-based assays we identified Site 4 (K170) as a primary site of ubiquitination of PXR. Our LC-MS/MS analysis combined with ubiquitin mutant expression vectors revealed the formation of K48-linked ubiquitin chains on the PXR protein through a single lysine residue (K170). These data indicate a likely role for ubiquitination of PXR in regulating its degradation by the 26S proteasome, as K48-linked ubiquitin chains on a single substrate lysine comprises the canonical signal for marking proteins for proteasome-mediated degradation (57). In addition, PIASy-mediated SUMO(1)ylation of PXR strongly increased the presence of both ubiquitinated and non-modified forms of this NR, suggesting a key interaction between these two post-translational modifications at the level of PXR. Mutation of the well-conserved type I (-Ψ-K-x-D/E-) SUMOylation consensus site at lysine 108 (Site 1- M**K**KE-) abolished a discrete form of PXR SUMO(1)ylation. In contrast, modification of PXR by SUMO2/3 and subsequent chain formation on PXR required mutation at both Site 1 and Site 2. Mutation of Site 2 alone had no effect on PXR SUMOylation, whereas mutation of all four sites strongly reduced the capacity of PXR to support chain formation. These data suggest that SUMO2/3-modification at Site 1 and Site 2 affect ubiquitination at Site 4, likely through the formation of mixed SUMO-ubiquitin chains or SUMO-dependent ubiquitination at Site 4 to promote proteasome-mediated degradation of the PXR protein (**Figure 9**). Taken together, these results indicate that Site 1, Site 2, and Site 4 serve as the principal attachment sites for SUMO1,

SUMO2/3, and ubiquitin to regulate its degradation during the xenobiotic response, and to mediate PXR-dependent repression of the pro-inflammatory response.

An increasing number of proteins have been shown to bind SUMO or SUMOylated proteins non-covalently through SUMO-interacting motifs (SIM). The PXR protein also contains a SIM consensus amino acid sequence, but how this contributes to SUMO-dependent PXR biological function is yet to be determined. Regardless, there are multiple proteins for which SUMOylation is dependent on the presence of a SIM in the substrate (62,63). The current thought is that SUMO binding to the SIM domain comprises the initial association with its target protein, which precedes SUMO conjugation to the SUMO consensus motif. Another possibility is that the SIM allows modified proteins to interact with new and novel protein partners, or allows SUMO-substrates to interact with themselves following SUMO-modification. Alternatively, there are many transcription factors and co-activator proteins such as PGC-1α involved with PXR trans-activation that are also subject to SUMOylation, thereby allowing the formation of SUMO-dependent multi-protein complexes. Our data indicate that it is likely that PXR interacts with PGC-1α and that modification by SUMO1, but not SUMO2/3, prevents this protein-protein interaction. The functional significance of this and other complex regulatory networks will require additional studies.

In vivo, SUMOylation can influence single or multiple properties of a target protein including its stability, localization, or activity. In most cases SUMO1 modification inhibits transcriptional activity of a NR, as we have now shown here with PXR towards the inflammatory response. Synergistic or antagonistic cross-talk among different types of post-translational modifications can occur, and our data clearly show that PXR modification by SUMO2/3 likely promotes its ubiquitination to play a pivotal role in facilitating PXR protein degradation, and

thereby facilitates another round of messenger RNA production. These data are consistent with the ubiquitin-mediated 'promoter clearance' hypothesis put forth by Dennis and O'Malley (22). However, our data indicate that PXR is likely ubiquitinated in a SUMO2/3-dependent manner, and that SUMO(1)ylated PXR is refractory to this phenomenon. More importantly, our data suggest that a very low stoichiometric amount of PXR is in fact modified by SUMO1 in ligand- and TNFα-stimulated primary cultures of hepatocytes, furthermore, that this particular post-translational modification results in PXR-mediated repression of the pro-inflammatory response in a ligand-dependent manner. Unraveling the details of how phosphorylation and acetylation, or other post-translational modifications on PXR and associated protein cofactors, influence PXR biology in the context of SUMOylation and ubiquitination will undoubtedly require further effort, and represent interesting issues for the future.

Figure 2-9.

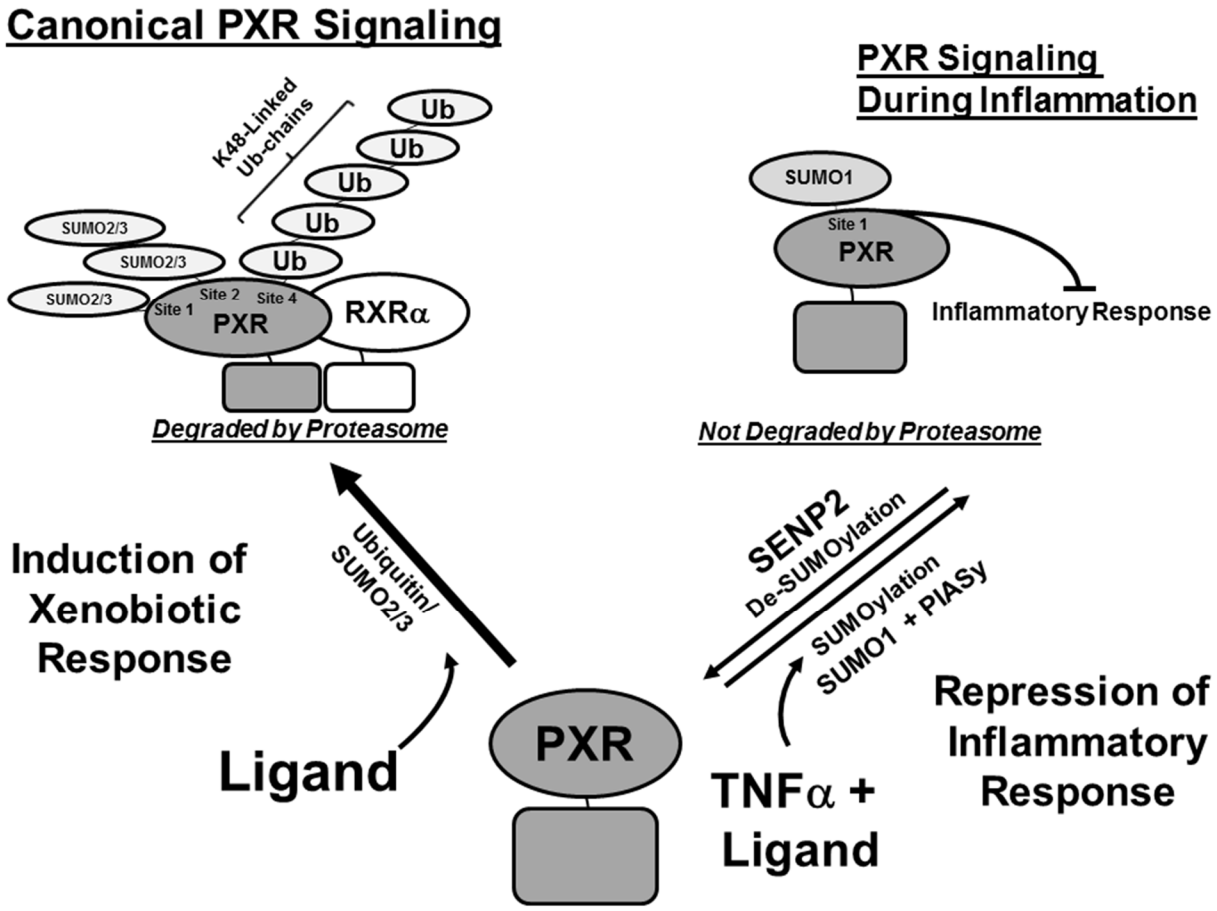


Figure 2-9. Working Model and Hypothesis of the Role of Ubiquitin- and SUMO-Signaling in Regulation of PXR Biology. A schematic representation of the molecular basis of the interface between canonical PXR activation and the xenobiotic response (Left Half of Diagram), and the molecular basis of the role of SUMO-PXR in suppression of the pro-inflammatory response (Right Half of Diagram).

2.5 REFERENCES

1. Moreau, A., Vilarem, M. J., Maurel, P., and Pascussi, J. M. (2008) Xenoreceptors CAR and PXR activation and consequences on lipid metabolism, glucose homeostasis, and inflammatory response. *Mol Pharm* **5**, 35-41
2. Cheng, J., Shah, Y. M., and Gonzalez, F. J. (2012) Pregnane X receptor as a target for treatment of inflammatory bowel disorders. *Trends in pharmacological sciences* **33**, 323-330
3. Dou, W., Mukherjee, S., Li, H., Venkatesh, M., Wang, H., Kortagere, S., Peleg, A., Chilimuri, S. S., Wang, Z. T., Feng, Y., Fearon, E. R., and Mani, S. (2012) Alleviation of gut inflammation by Cdx2/Pxr pathway in a mouse model of chemical colitis. *PLoS One* **7**, e36075
4. Dou, W., Zhang, J., Li, H., Kortagere, S., Sun, K., Ding, L., Ren, G., Wang, Z., and Mani, S. (2014) Plant flavonol isorhamnetin attenuates chemically induced inflammatory bowel disease via a PXR-dependent pathway. *The Journal of nutritional biochemistry*
5. Shah, Y. M., Ma, X., Morimura, K., Kim, I., and Gonzalez, F. J. (2007) Pregnane X receptor activation ameliorates DSS-induced inflammatory bowel disease via inhibition of NF-kappaB target gene expression. *Am J Physiol Gastrointest Liver Physiol* **292**, G1114-1122
6. Sun, M., Cui, W., Woody, S. K., and Staudinger, J. L. (2015) Pregnane X receptor modulates the inflammatory response in primary cultures of hepatocytes. *Drug Metab Dispos* **43**, 335-343
7. Treuter, E., and Venteclef, N. (2011) Transcriptional control of metabolic and inflammatory pathways by nuclear receptor SUMOylation. *Biochim Biophys Acta* **1812**, 909-918
8. Shuai, K., and Liu, B. (2005) Regulation of gene-activation pathways by PIAS proteins in the immune system. *Nat Rev Immunol* **5**, 593-605
9. Drag, M., and Salvesen, G. S. (2008) DeSUMOylating enzymes--SENPs. *IUBMB Life* **60**, 734-742
10. Glickman, M. H., and Ciechanover, A. (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **82**, 373-428
11. Gibbs-Seymour, I., Oka, Y., Rajendra, E., Weinert, B. T., Passmore, L. A., Patel, K. J., Olsen, J. V., Choudhary, C., Bekker-Jensen, S., and Mailand, N. (2015) Ubiquitin-SUMO circuitry controls activated fanconi anemia ID complex dosage in response to DNA damage. *Mol Cell* **57**, 150-164
12. Praefcke, G. J., Hofmann, K., and Dohmen, R. J. (2012) SUMO playing tag with ubiquitin. *Trends Biochem Sci* **37**, 23-31
13. Schimmel, J., Larsen, K. M., Matic, I., van Hagen, M., Cox, J., Mann, M., Andersen, J. S., and Vertegaal, A. C. (2008) The ubiquitin-proteasome system is a key component of the SUMO-2/3 cycle. *Mol Cell Proteomics* **7**, 2107-2122
14. Desterro, J. M., Rodriguez, M. S., and Hay, R. T. (1998) SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. *Mol Cell* **2**, 233-239
15. Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419**, 135-141

16. Lallemand-Breitenbach, V., Jeanne, M., Benhenda, S., Nasr, R., Lei, M., Peres, L., Zhou, J., Zhu, J., Raught, B., and de The, H. (2008) Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. *Nat Cell Biol* **10**, 547-555
17. Huang, T. T., Wuerzberger-Davis, S. M., Wu, Z. H., and Miyamoto, S. (2003) Sequential modification of NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress. *Cell* **115**, 565-576
18. Hu, G., Xu, C., and Staudinger, J. L. (2010) Pregnane X receptor is SUMOylated to repress the inflammatory response. *The Journal of pharmacology and experimental therapeutics* **335**, 342-350
19. 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
20. Rana, R., Coulter, S., Kinyamu, H., and Goldstein, J. A. (2013) RBCK1, an E3 ubiquitin ligase, interacts with and ubiquinates the human pregnane X receptor. *Drug Metab Dispos* **41**, 398-405
21. Staudinger, J. L., Xu, C., Biswas, A., and Mani, S. (2011) Post-translational modification of pregnane x receptor. *Pharmacol Res* **64**, 4-10
22. Dennis, A. P., and O'Malley, B. W. (2005) Rush hour at the promoter: how the ubiquitin-proteasome pathway polices the traffic flow of nuclear receptor-dependent transcription. *J Steroid Biochem Mol Biol* **93**, 139-151
23. Elias, A., High, A. A., Mishra, A., Ong, S. S., Wu, J., Peng, J., and Chen, T. (2014) Identification and characterization of phosphorylation sites within the pregnane X receptor protein. *Biochem Pharmacol* **87**, 360-370
24. Lichti-Kaiser, K., Brobst, D., Xu, C., and Staudinger, J. L. (2009) A systematic analysis of predicted phosphorylation sites within the human pregnane X receptor protein. *J Pharmacol Exp Ther* **331**, 65-76
25. Lichti-Kaiser, K., Xu, C., and Staudinger, J. L. (2009) Cyclic AMP-dependent protein kinase signaling modulates pregnane x receptor activity in a species-specific manner. *J Biol Chem* **284**, 6639-6649
26. Pondugula, S. R., Brimer-Cline, C., Wu, J., Schuetz, E. G., Tyagi, R. K., and Chen, T. (2009) A phosphomimetic mutation at threonine-57 abolishes trans-activation activity and alters nuclear localization pattern of human pregnane x receptor. *Drug Metab Dispos* **37**, 719-730
27. Smutny, T., Mani, S., and Pavek, P. (2013) Post-translational and post-transcriptional modifications of pregnane X receptor (PXR) in regulation of the cytochrome P450 superfamily. *Curr Drug Metab* **14**, 1059-1069
28. Sugatani, J., Hattori, Y., Noguchi, Y., Yamaguchi, M., Yamazaki, Y., and Ikari, A. (2014) Threonine-290 regulates nuclear translocation of the human pregnane X receptor through its phosphorylation/dephosphorylation by Ca2+/calmodulin-dependent protein kinase II and protein phosphatase 1. *Drug Metab Dispos* **42**, 1708-1718
29. Sugatani, J., Uchida, T., Kurosawa, M., Yamaguchi, M., Yamazaki, Y., Ikari, A., and Miwa, M. (2012) Regulation of pregnane X receptor (PXR) function and UGT1A1 gene expression by posttranslational modification of PXR protein. *Drug Metab Dispos* **40**, 2031-2040

30. Biswas, A., Pasquel, D., Tyagi, R. K., and Mani, S. (2011) Acetylation of pregnane X receptor protein determines selective function independent of ligand activation. *Biochem Biophys Res Commun* **406**, 371-376
31. Kliewer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Watson, M. A., Jones, S. A., McKee, D. D., Oliver, B. B., Willson, T. M., Zetterstrom, R. H., Perlmann, T., and Lehmann, J. M. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92**, 73-82
32. Lehmann, J. M., McKee, D. D., Watson, M. A., Willson, T. M., Moore, J. T., and Kliewer, S. A. (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *The Journal of clinical investigation* **102**, 1016-1023
33. Tatham, M. H., Rodriguez, M. S., Xirodimas, D. P., and Hay, R. T. (2009) Detection of protein SUMOylation in vivo. *Nat Protoc* **4**, 1363-1371
34. Liu, B., Liao, J., Rao, X., Kushner, S. A., Chung, C. D., Chang, D. D., and Shuai, K. (1998) Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci U S A* **95**, 10626-10631
35. Cheng, J., Kang, X., Zhang, S., and Yeh, E. T. (2007) SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. *Cell* **131**, 584-595
36. Lim, K. L., Chew, K. C., Tan, J. M., Wang, C., Chung, K. K., Zhang, Y., Tanaka, Y., Smith, W., Engelender, S., Ross, C. A., Dawson, V. L., and Dawson, T. M. (2005) Parkin mediates nonclassical, proteasomal-independent ubiquitination of synphilin-1: implications for Lewy body formation. *J Neurosci* **25**, 2002-2009
37. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* **95**, 2509-2514
38. Darlington, G. J., Bernhard, H. P., Miller, R. A., and Ruddle, F. H. (1980) Expression of liver phenotypes in cultured mouse hepatoma cells. *J Natl Cancer Inst* **64**, 809-819
39. Staudinger, J. L., Goodwin, B., Jones, S. A., Hawkins-Brown, D., MacKenzie, K. I., LaTour, A., Liu, Y., Klaassen, C. D., Brown, K. K., Reinhard, J., Willson, T. M., Koller, B. H., and Kliewer, S. A. (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* **98**, 3369-3374
40. Staudinger, J. L., Madan, A., Carol, K. M., and Parkinson, A. (2003) Regulation of drug transporter gene expression by nuclear receptors. *Drug Metab Dispos* **31**, 523-527
41. Ding, X., and Staudinger, J. L. (2005) Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway. *J Pharmacol Exp Ther* **312**, 849-856
42. Xu, C., Wang, X., and Staudinger, J. L. (2009) Regulation of tissue-specific carboxylesterase expression by pregnane x receptor and constitutive androstane receptor. *Drug Metab Dispos* **37**, 1539-1547
43. Rankin, C. A., Galeva, N. A., Bae, K., Ahmad, M. N., Witte, T. M., and Richter, M. L. (2014) Isolated RING2 domain of parkin is sufficient for E2-dependent E3 ligase activity. *Biochemistry* **53**, 225-234
44. Eng, J. K., McCormack, A. L., and Yates, J. R. (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* **5**, 976-989

45. Keller, A., Nesvizhskii, A. I., Kolker, E., and Aebersold, R. (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem* **74**, 5383-5392
46. Lee, J. H., Park, S. M., Kim, O. S., Lee, C. S., Woo, J. H., Park, S. J., Joe, E. H., and Jou, I. (2009) Differential SUMOylation of LXRalpha and LXRbeta mediates trans-repression of STAT1 inflammatory signaling in IFN-gamma-stimulated brain astrocytes. *Mol Cell* **35**, 806-817
47. Lu, Y., Zhou, Q., Shi, Y., Liu, J., Zhong, F., Hao, X., Li, C., Chen, N., and Wang, W. (2013) SUMOylation of PPARgamma by rosiglitazone prevents LPS-induced NCoR degradation mediating down regulation of chemokines expression in renal proximal tubular cells. *PLoS One* **8**, e79815
48. Pascual, G., Fong, A. L., Ogawa, S., Gamliel, A., Li, A. C., Perissi, V., Rose, D. W., Willson, T. M., Rosenfeld, M. G., and Glass, C. K. (2005) A SUMOylation-dependent pathway mediates trans-repression of inflammatory response genes by PPAR-gamma. *Nature* **437**, 759-763
49. Zhou, W., Hannoun, Z., Jaffray, E., Medine, C. N., Black, J. R., Greenhough, S., Zhu, L., Ross, J. A., Forbes, S., Wilmut, I., Iredale, J. P., Hay, R. T., and Hay, D. C. (2012) SUMOylation of HNF4alpha regulates protein stability and hepatocyte function. *J Cell Sci* **125**, 3630-3635
50. Koutsounas, I., Theocharis, S., Patsouris, E., and Giaginis, C. (2013) Pregnane X receptor (PXR) at the crossroads of human metabolism and disease. *Current drug metabolism* **14**, 341-350
51. Teng, S., and Piquette-Miller, M. (2005) The involvement of the pregnane X receptor in hepatic gene regulation during inflammation in mice. *J Pharmacol Exp Ther* **312**, 841-848
52. Bawa-Khalfe, T., and Yeh, E. T. (2010) The in vivo functions of desumoylating enzymes. *Sub-cellular biochemistry* **54**, 170-183
53. Mikolajczyk, J., Drag, M., Bekes, M., Cao, J. T., Ronai, Z., and Salvesen, G. S. (2007) Small ubiquitin-related modifier (SUMO)-specific proteases: profiling the specificities and activities of human SENPs. *J Biol Chem* **282**, 26217-26224
54. Zhang, Y., Gan, Z., Huang, P., Zhou, L., Mao, T., Shao, M., Jiang, X., Chen, Y., Ying, H., Cao, M., Li, J., Li, J., Zhang, W. J., Yang, L., and Liu, Y. (2012) A role for protein inhibitor of activated STAT1 (PIAS1) in lipogenic regulation through SUMOylation-independent suppression of liver X receptors. *J Biol Chem* **287**, 37973-37985
55. Ghisletti, S., Huang, W., Ogawa, S., Pascual, G., Lin, M. E., Willson, T. M., Rosenfeld, M. G., and Glass, C. K. (2007) Parallel SUMOylation-dependent pathways mediate gene- and signal-specific trans-repression by LXRs and PPARgamma. *Molecular cell* **25**, 57-70
56. Bhalla, S., Ozalp, C., Fang, S., Xiang, L., and Kemper, J. K. (2004) Ligand-activated pregnane X receptor interferes with HNF-4 signaling by targeting a common coactivator PGC-1alpha. Functional implications in hepatic cholesterol and glucose metabolism. *J Biol Chem* **279**, 45139-45147
57. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* **243**, 1576-1583
58. Mukhopadhyay, D., and Dasso, M. (2007) Modification in reverse: the SUMO proteases. *Trends in biochemical sciences* **32**, 286-295

59. Choi, S. J., Chung, S. S., Rho, E. J., Lee, H. W., Lee, M. H., Choi, H. S., Seol, J. H., Baek, S. H., Bang, O. S., and Chung, C. H. (2006) Negative modulation of RXRalpha transcriptional activity by small ubiquitin-related modifier (SUMO) modification and its reversal by SUMO-specific protease SUSP1. *J Biol Chem* **281**, 30669-30677
60. Schneider Aguirre, R., and Karpen, S. J. (2013) Inflammatory mediators increase SUMOylation of retinoid X receptor alpha in a c-Jun N-terminal kinase-dependent manner in human hepatocellular carcinoma cells. *Mol Pharmacol* **84**, 218-226
61. Yeh, E. T., Gong, L., and Kamitani, T. (2000) Ubiquitin-like proteins: new wines in new bottles. *Gene* **248**, 1-14
62. Kerscher, O. (2007) SUMO junction-what's your function? New insights through SUMO-interacting motifs. *EMBO Rep* **8**, 550-555
63. Song, J., Durrin, L. K., Wilkinson, T. A., Krontiris, T. G., and Chen, Y. (2004) Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *Proc Natl Acad Sci U S A* **101**, 14373-14378

Chapter 3: A SUMO-Acetyl Switch in PXR Biology

ABSTRACT

Post-translational modification (PTM) of nuclear receptor superfamily members regulates various aspects of their biology to include sub-cellular localization, the repertoire of protein-binding partners, as well as their stability and mode of degradation. The nuclear receptor Pregnane X Receptor (PXR, NR1I2) is a master-regulator of the drug-inducible gene expression in liver and intestine. The PXR-mediated gene activation program is primarily recognized to increase drug metabolism, drug transport, and drug efflux pathways in these tissues. The activation of PXR also has important implications in significant human diseases including inflammatory bowel disease and cancer. Our recent investigations reveal that PXR is modified by multiple PTMs to include phosphorylation, SUMOylation, and ubiquitination. Using both primary cultures of hepatocytes and cell line-based assays, we show here that PXR is modified through acetylation on lysine residues. Further, we show that increased acetylation of PXR stimulates its increased SUMO-modification to support active transcriptional suppression. Pharmacologic inhibition of lysine de-acetylation using trichostatin A (TSA) alters the sub-cellular localization of PXR in cultured hepatocytes, and also has a profound impact upon PXR trans-activation capacity. Both the acetylation and SUMOylation status of PXR is affected by its ability to associate with the lysine de-acetylating enzyme histone de-acetylase (HDAC) 3 in a complex with silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). Taken together, our data support a model in which a SUMO-acetyl 'switch' occurs such that acetylation of PXR likely stimulates SUMO-modification of PXR to promote the active repression of PXR-target gene expression.

3.1 INTRODUCTION

Pregnane X Receptor (PXR, NR1I2) was initially described as a master-regulator of drug-inducible xenobiotic detoxification pathways in the enterohepatic system [1, 2]. However, there is increasing recognition that PXR activation has a multiplicity of 'non-canonical' roles. For example, recent evidence indicates that PXR activation in liver impacts regulation of glucose and lipid metabolism [3, 4], and may affect the development of multi-drug resistance in certain solid tumor types [5]. Moreover, a fundamental role for PXR activation in ameliorating pro-inflammatory signals and loss of intestinal barrier permeability in the inflamed condition has been identified [6, 7].

Numerous studies reveal that acetylation of transcriptional regulatory proteins blends together with phosphorylation, ubiquitination, SUMOylation to form complex programs of gene activation [8]. More recently, attention has been given to the notion that a complex interplay between post-translational modifications (PTMs) occurs to allow alterations of PXR biology depending upon the physiological context [9, 10]. It is abundantly clear that different PTMs form a complex regulatory network with interactions and integrated features that resemble a refined language. We feel that such a complex and interwoven regulatory program of gene expression is likely to play a pivotal role in disease pathogenesis and progression. The discovery of the repertoire of PTMs that target the PXR protein and how they interact with each other is thus an important and newly emerging field of research.

Our recent efforts combined with that of others indicate that phosphorylation, ubiquitination, and SUMOylation of PXR play a pivotal and likely interactive role in regulating the biological function of this nuclear receptor protein [11-18]. However, where the PTMs occur on the protein and how they might interact with each other in live cells to regulate its

complex biology in liver and intestine is only beginning to be understood. There is a clear recognition that both SUMOylation and acetylation of numerous transcriptional regulatory proteins and histone proteins occurs in a coordinated and unified manner [8, 19, 20]. We therefore sought to investigate whether SUMOylation and acetylation have the ability to determine aspects of PXR biology, and whether they might interact with each other to affect PXR activity in hepatocytes.

Using an immunoprecipitation approach, we detect acetylation of PXR in primary hepatocytes and this is reduced by treatment with the PXR ligand rifampicin (Rif). The acetylation of PXR is increased in hepatocytes following treatment with the class I and class II de-acetylation inhibitor TSA. Further, we identify the lysine/histone deacetylase HDAC3-SMRT co-repressor multi-protein complex as a likely regulator of ligand-dependent PXR acetylation. The co-expression of fluorescently tagged HDAC3 and PXR proteins in hepatocytes indicate that PXR and HDAC3 co-localize in mouse hepatocytes, and that the increased acetylation of PXR produced by treatment with TSA alters their sub-cellular localization. Treatment of transfected cells with TSA produces synergistic trans-activation of a PXR-dependent reporter gene when combined with Rif. Enzymatic de-acetylation of PXR with the HDAC3-SMRT co-repressor complex inhibits SUMO-modification of PXR, while pharmacological promotion of acetylation with TSA promotes high levels of SUMO-modification of PXR. The acetylated and SUMOylated forms of PXR differentially associate with the HDAC3-SMRT co-repressor complex. The covalent attachment of SUMO proteins to PXR produces a strong repressive function that is functionally separate from its interaction with the HDAC3-SMRT co-repressor complex. Taken together, our data presented here support a model in which a SUMO-acetyl

'switch' occurs at the level of PXR, such that acetylation is prerequisite to promote SUMO-modification of PXR to support active repression of PXR-target gene expression.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and Plasmids

Rifampicin (Rif), Trichostatin A (TSA), and Pregnenolone-16α-carbonitrile (PCN) were purchased from Sigma-Aldrich. All other reagents including culture medium for primary hepatocytes and mammalian cell lines were purchased from standard sources. The expression vectors encoding FLAG-tagged full length human PXR, $(His)_6$-tagged SUMO3, and protein inhibitor of activated STAT-1 (PIAS1) were as previously described (Cui et al., 2015). The expression vector encoding FLAG-tagged HDAC3 is a kind gift from Dr. Eric Verdin and purchased from Addgene (plasmid #13819) [21]. The expression vector encoding HDAC3-GFP was a kind gift of Dr. Eric Olson [22]. The expression vector encoding full length SMRT was a kind gift of Dr. J.D. Chen [23]. The RFP-PXR expression vector was constructed by using the following primers to amplify human PXR to add a HindIII site: Left Primer- 5' GACGGCCAAGCTTCGATGGAGGTGAGACCCAAAG 3'; Right Primer: 5' GACGGCAAGCTTTCAGCTACCTGTGATGCCG 3'. The resulting amplimer was inserted into the pM-Cherry-C1 expression vector using the HindIII site (ClonTech). To generate $(His)_6$-PXR-SUMO1 and $(His)_6$-PXR-SUMO3 linear fusion construct, we used the following PCR primers that introduce XhoI restriction sites and both adds a STOP codon and removes one C-terminal glycine from the SUMO1 and SUMO3: SUMO1- left primer- 5' GACGGCCTCGAGGCCATGTCTGACCAGGAGGCAAAA 3'; SUMO1- right primer- 5' GACGGCCTCGAGCTACCCCGTTTGTTCCTGATAAAC 3'; SUMO3- left primer- 5'

GACGGCCTCGAGCCATGTCCGAGGAGAAGCCCAAG 3'; SUMO3- right primer 5' GACGGCCTCGAGCTATCCCGTCTGCTGCTGGAACAC 3'. The modified SUMO1 and SUMO3 sequences were amplified and then sub-cloned into pShuttle-$(His)_6$-PXR-$(FLAG)_3$ expression vector using the XhoI restriction site that exists in between the last amino acid of PXR and the triple FLAG tag in this expression vector [11]. The FLAG-SUMO3-PXR construct was generated using the following PCR primers to introduce EcoRI restriction sites and removes the STOP codon and one glycine residue from SUMO3: SUMO3- left primer- 5' GACGGCGAATTCATGTCCGAGGAGAAGCCCAAG 3'; SUMO3- right primer- 5' GACGGCGAATTCTCCCGTCTGCTGCTGGAACAC 3'. The resulting amplimer was digested with EcoRI and inserted into the EcoRI site that exists between the FLAG epitope and PXR in the previously described pCMV-Tag human PXR expression construct [11]. All expression vectors were sequenced on both strands to ensure the integrity of the resulting open-reading frames.

3.2.2 Isolation and Culturing of Primary Mouse Hepatocytes

Primary hepatocytes were isolated from C57BL6 mice at age 6-10 weeks with a classic collagenase perfusion procedure as previously described (Staudinger, 2003). Potential sex differences were determined throughout the study, and identical results were acquired from both male and female mice. The representative results were obtained from male mice.

3.2.3 Total RNA Isolation, Reverse Transcription, and Real-Time Quantitative-Polymerase Chain Reaction Analysis

Real-time (RT) quantitative polymerase chain reaction (qPCR) was executed as previously described (Ding, 2005).

3.2.4 Immunoprecipitation Assay

Primary hepatocytes or Hepa1-6 cells cultured in 10 cm dishes and were harvested in 1 mL of a Lysis Buffer containing 150 mM NaCl, 50 mM Tris-Cl (pH 8.0), 1% Triton X-100, 20 mM N-Ethylmaleimide (NEM), and 1% Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Samples were disrupted through sonication and subsequently centrifuged at 18,000 x *g* for 10 minutes to remove the insoluble substances. A fraction of the supernatant (5%) was saved as a loading control for western blot analysis, while the rest of sample was subjected to pre-clearing with 5% Protein A/G Sepharose beads at 4 °C. The pre-cleared cell lysates were separated from beads by centrifugation and then incubated with the appropriate antibody. The anti-acetylated lysine antibody cocktail was previously described and consisted of equal masses of four separate monoclonal antibodies from Novus Biologicals-15G10, Santa Cruz-AKL5C1, Cell Signaling-Ac-K-103, and Thermo Scientific-1C6 [24]. An equal amount of each antibody was added to the mixture, and the final antibody concentration was 4 μg per 1 mL of total cell lysate. The antibody cocktail was mixed together with a 5% volume of Protein A/G Sepharose beads and was applied to cell lysates for immunoprecipitation of acetylated proteins overnight at 4 °C with shaking. FLAG-tagged-HDAC3 and associated proteins were immunoprecipitated using antibody-agarose conjugated beads (Anti-FLAG M2 Affinity Gel, Sigma Aldrich, A2220). Subsequently, the beads were pelleted gently and were washed 3 times with Lysis Buffer containing 20 mM NEM, and 1% Halt™ Protease and Phosphatase Inhibitor Cocktail. Immunoprecipitated proteins were eluted in 30 μL 2X-Laemmli buffer and heated at 95 °C for 10 minutes for western blot analysis.

3.2.5 Cell-Based Immobilized Metal Affinity Pull-Down Assay

SUMO-modified proteins were enriched with cobalt beads using a modification of a previously described approach [25]. Briefly, 10 cm dishes containing primary cultures of mouse

hepatocytes or Hepa1-6 cells were harvested using 1 mL of a strong denaturing lysis buffer 6M Guanidine-Cl (pH 8) according to specified experimental treatment. The whole cell lysates were then applied to 30 μL of cobalt beads and incubated on a rotor at room temperature for two hours with shaking. The gathered proteins were collected via centrifugation and washed twice with lysis buffer, three times with 8M Urea buffer (pH 6.5), and once with 1 x PBS. Proteins were removed from the beads using 30 μL of 2 x Laemmli buffer and heated at 95 °C for 10 minutes for subsequent western blot analysis.

3.2.6 Fluorescence Microscopy

Primary cultured mouse hepatocytes were transfected using Lipofecamine 2000 (Invitrogen) and maintained in *William's* E Media prior to image analysis. Twenty-four hours post-transfection, hepatocytes were washed once with 1x PBS and subsequently stained with Hoechst 33432 for an additional 30 minutes. To visualize, mouse hepatocytes were washed three times with 1x PBS and then maintained in Opti-MEM® I Reduced Serum Media during fluorescence protein image analysis. Fluorescent proteins were imaged with a 30x air objective, and excited at either 400 nm (GFP) or 561 nm (RFP). The nuclei were visualized using Hoechst 33432 staining under ultraviolet light.

3.2.7 Luciferase Reporter Gene Assay

CV-1 cells were seeded at 1 x 10^4 cells per well (n=8) in 96-well plate and transfected using lipofectamine 2000. Twenty-four hours post-transfection, cells were treated with either 10 μM rifampicin (Rif), 0.5 μM trichostatin A (TSA), or both for an additional 24 hours. Cells were lysed using standard conditions at 32 μL per well of lysis buffer (100 mM KPO_4, pH 7.8; 0.2% Triton X-100; 1 mM DTT) and subjected to luciferase activity analysis (12 μL) using a standard manufacturer's protocol (Promega). The transfection efficiency was quantified according to the

activity of β-galactosidase (20 μL). Fold change among experimental groups were normalized to control group with relative luciferase units/ β-galactosidase readouts.

3.2.8 Western Blot Analysis

Western blot analysis was conducted as described previously (Xu et al., 2009). Purchased antibodies include the mouse monoclonal anti-PXR antibody (H-11, Santa Cruz), rabbit monoclonal anti-SUMO1 (C9H1, Cell Signaling) and anti-SUMO2/3 antibodies (18H8, Cell Signaling). The mouse monoclonal anti-acetylated lysine antibody cocktail consisted of Cat# 15G10 from Novus Biologicals, Cat# AKL5C1 from Santa Cruz, Cat # Ac-K-103 from Cell Signaling, and Cat # 1C6 from Thermo Scientific.

3.2.9 Liquid Chromatography-Tandem Mass Spectrometry Analysis

The LC-MS/MS analysis for identification of PXR post-translational modification was performed as described previously (Cui et al., 2015).

3.2.10 Statistical Analysis

The statistical analysis was executed wherever required. Statistical differences among one experimental group were determined using a one-way ANOVA followed by the Duncan's multiple range post hoc test. Moreover, statistical differences between experimental groups were determined using the Student's *t* test.

3.3 RESULTS

3.3.1 The Interface between PXR Acetylation and SUMOylation.

A study involving the farnesoid X receptor, a close relative of PXR, revealed the existence of a SUMO-acetyl 'switch' [20]. PXR has previously been identified as a likely substrate for the acetylation and SUMOylation signal transduction pathways [11, 25-27].

Furthermore, a recent study indicates that PXR physically associates with the lysine/histone de-acetylating enzyme HDAC3 in cell line-based assays, and it has been suggested that this enzyme can de-acetylate PXR [17]. In vivo, HDAC3 forms an obligate and stable complex with the well-known nuclear receptor co-repressor protein SMRT, and the SMRT-HDAC3 co-repressor complex exhibits strong lysine de-acetylase activity [28, 29]. An additional study indicates that the SMRT protein is the preferred co-repressor protein-partner of PXR [23]. Therefore, we sought to determine the extent to which potential de-acetylation of PXR by HDAC3-SMRT co-repressor complex affects the SUMOylation level of PXR in cells. The murine hepatoma cell line, Hepa1-6, can support high levels of PXR SUMO-modification [11]. Cultures were therefore transfected with expression plasmids encoding PXR, $(His)_6$-SUMO3, the SUMO-E3 ligase enzyme- PIAS1, HDAC3, and SMRT as indicated (**Figure 3-1A**). Forty-eight hours post-transfection, total SUMOylated proteins were gathered using strong denaturing conditions in an immobilized metal affinity pull-down assay [25]. The level of SUMOylated PXR was determined using western blotting analysis**.** Forced over-expression of the HDAC3-SMRT lysine de-acetylase enzyme complex strongly reduced the overall SUMOylation level of PXR.

To investigate whether increased acetylation of PXR alters its SUMOylation status we used TSA, a pharmacological inhibitor of the class I and II mammalian HDAC enzymes. Cultures of Hepa1-6 cells were co-transduced with adenoviral vectors encoding Ad-$(His)_6$-PXR, Ad-$(His)_6$-SUMO3, and Ad-PIAS1 (**Figure 3-1B**). Twenty-four hours post-transduction, cells were treated with vehicle (0.1% DMSO), 10 μM Rif, or 0.5 μM TSA for an additional 24 hours. SUMO-modified PXR proteins were enriched and the level of SUMO-modified PXR protein was determined using western blotting. Inhibition of lysine de-acetylase activity with TSA promoted the formation of high levels of SUMO-modified PXR, while both vehicle- and Rif-treated cells

exhibited comparatively low levels of SUMO-modified PXR. Taken together, the data in **Figure 3-1** suggests that acetylation and SUMOylation interface with each other at the level of the PXR protein and that acetylation of PXR likely promotes its subsequent SUMOylation in a cooperative and coincident manner.

Figure 3-1A.

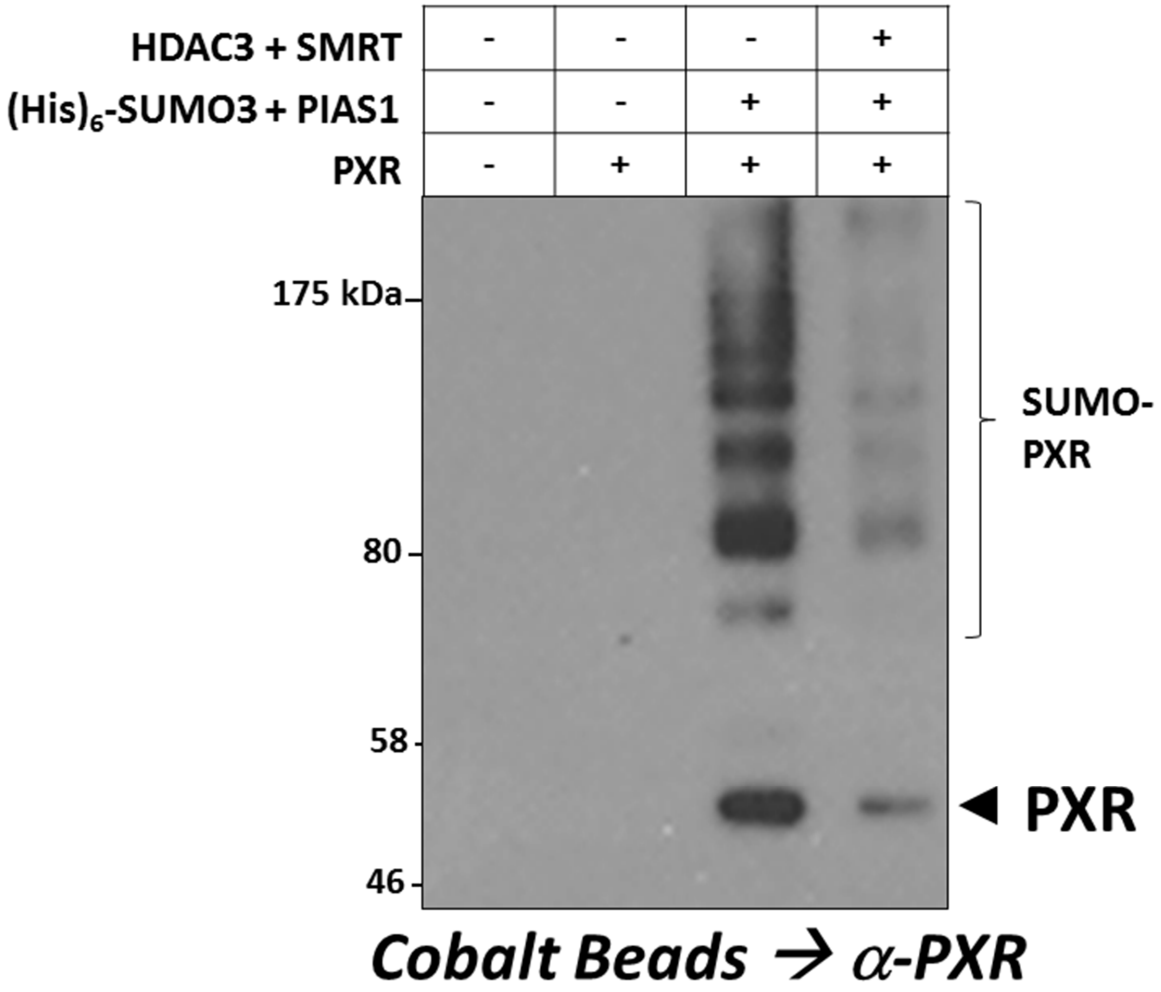

HDAC3 + SMRT
(His)6-SUMO3 + PIAS1
PXR
- - - +
- - + +
- + + +
175 kDa
80
58
46
SUMO-PXR
PXR
Cobalt Beads → α-PXR

Figure 3-1B.

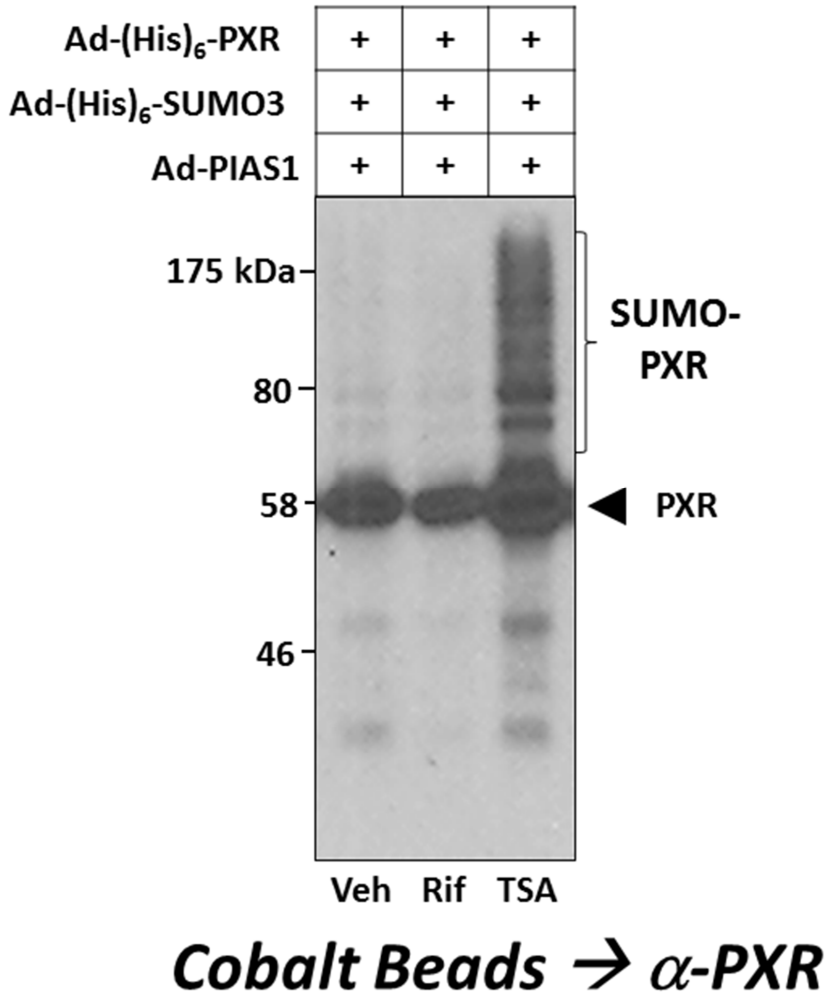


Figure 3-1. The Interface between Acetylation and SUMOylation of PXR. (A) Hepa1-6 cells were transfected with the indicated plasmid-based expression vectors. Cell lysates were produced using strong denaturing conditions to inhibit de-SUMOylation enzymes. SUMOylated proteins were enriched using cobalt beads and were washed sequentially using both guanidine-HCl and urea-based wash buffers. Proteins were eluted using 2X-Laemmli buffer and were then resolved using 10% SDS-PAGE. Western blot analysis was performed with an anti-PXR antibody that detects all modified forms of the protein (Santa Cruz, H-11 monoclonal Ab). **(B)** Hepa1-6 cells were transduced with the indicated adenoviral expression vectors. Twenty-four hr post-transduction, cells were treated with vehicle (0.1% DMSO) rifampicin (Rif, 10 μM) or Trichostatin A (TSA, 0.5 μM) for an additional eighteen hr. SUMOylated proteins were gathered as in (A) and western blot with the anti-PXR antibody was used to analyze the extent of SUMO-modification.

3.3.2 Acetylation of PXR is altered during the Trans-activation Process in Hepatocytes.

To examine the extent to which PXR acetylation status is altered in liver in response to ligand activation, we transduced hepatocytes isolated from wild type C57BL/6 mice with an adenoviral expression vector encoding a $(His)_6$-tagged form of human PXR [Ad-$(His)_6$-PXR]. Twenty-four hours post-transduction, hepatocytes were treated with vehicle (0.1% DMSO), 10 μM Rif, 0.5 μM TSA, or were co-treated with Rif and TSA together for an additional 24 hours. Acetylated proteins were immuno-purified from the respective cell extracts using a cocktail consisting of four commercially available anti-acetylated lysine monoclonal antibodies as described in Materials and Methods. A non-immune mouse IgG antibody was used as a negative control. Acetylated human PXR protein was detected in the acetylated lysine-enriched protein extracts by western blot analysis (**Figure 3-2A**). Note the slight decrease in electrophoretic mobility that is typical of acetylated nuclear receptor proteins. Treatment with TSA produced a significant increase in the level of PXR acetylation. In contrast, ligand activation of PXR by Rif tempered the TSA-induced acetylation of PXR when compared with the vehicle treated cells (**Figure 3-2B**). These results reveal that PXR is the molecular target of the acetylation signaling pathway in hepatocytes, and suggest that PXR trans-activation capacity is inversely correlated with this PTM.

Figure 3-2A.

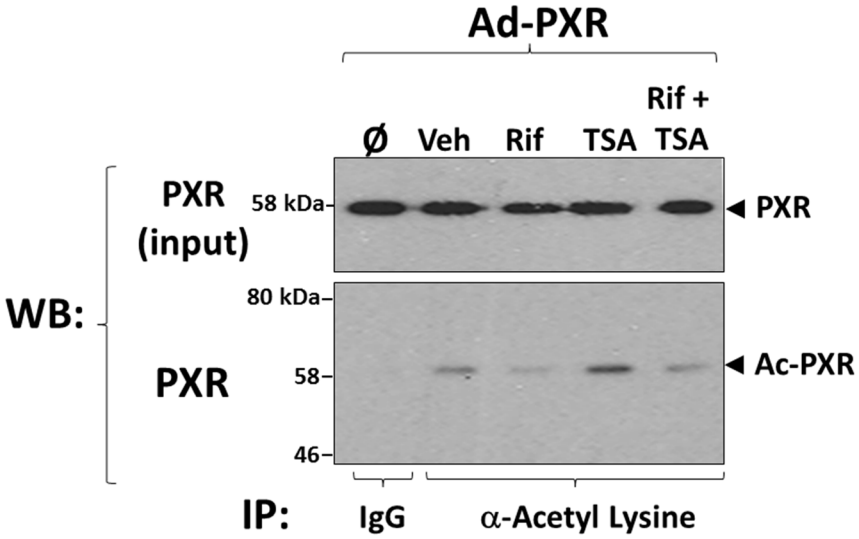


Figure 3-2B.

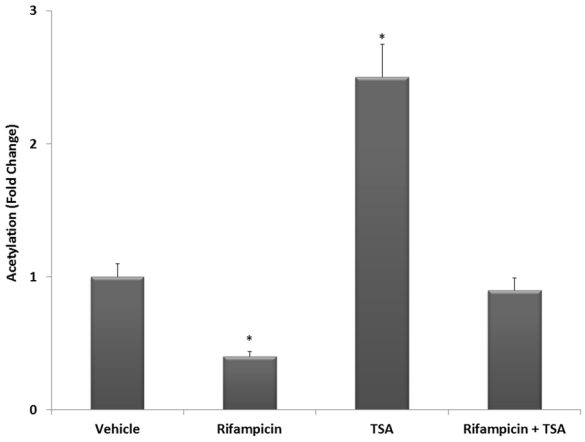


Figure 3-2. Acetylation of PXR is Altered during Trans-activation by Ligand. (A) Primary hepatocytes isolated from wild type C57BL/6 mice were isolated and transduced with an adenoviral expression vector encoding a FLAG-tagged form of human PXR (Left Panel). Total acetylated proteins were immunoprecipitated from cell extracts using a cocktail of four anti-acetylated lysine monoclonal antibodies as described in Materials and Methods. A non-immune antibody was also used as a negative control (IgG). Acetylated PXR was identified by western blotting with anti-PXR polyclonal antibody. **(B)** Western Blot images were quantitated by densitometric scanning of the X-ray films with the UVP Biodoc-It 220 image analysis system and 1D Gel Analysis Software. The numbers represent the relative densitometric image intensity of acetylated PXR divided by the image intensity of input levels of PXR, where vehicle treated control group was set to equal 1. Asterisks indicate a statistical difference from vehicle-treated samples ($n = 3$, and $P < 0.05$).

3.3.3 The Trans-activation Capacity of PXR is modified by the Acetylation Signaling Pathway in Hepatocytes.

We next sought to examine the functional consequence of increased acetylation upon drug-inducible PXR activity. Primary hepatocytes isolated from wild type C57BL/6 mice were cultured overnight. The cultures were then treated for 24 hours with either vehicle (0.1% DMSO), 10 μM, PCN, 0.5 μM TSA, or were co-treated with PCN and TSA. Total RNA was isolated and the expression of the prototypical PXR-target gene *Cyp3a11* was measured using real-time quantitative PCR analysis (**Figure 3-3**). As expected, significant induction of *Cyp3a11* was observed following treatment with PCN. Treatment with TSA by itself did not produce significant alterations in *Cyp3a11* gene expression. In contrast, the co-treatment of PCN together with TSA significantly diminished PCN-inducible *Cyp3a11* gene expression levels when compared with the PCN alone treatment group. These data indicate that the canonical PXR trans-activation capacity is suppressed by acetylation in cultures of primary hepatocytes.

Figure 3-3.

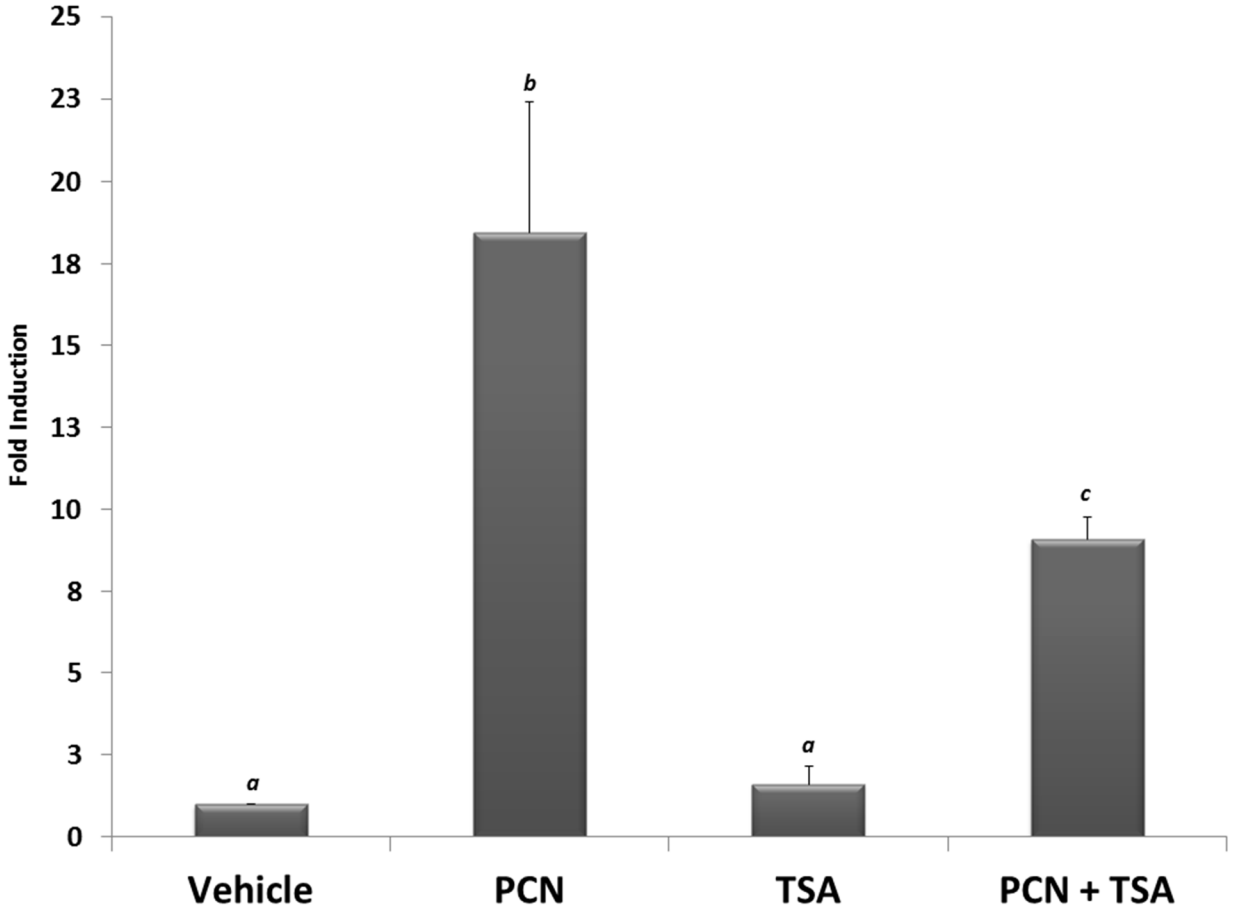


Figure 3-3. PXR Trans-activation Capacity is the Molecular Target of Acetylation in Hepatocytes. Primary hepatocytes isolated from C57BL/6 mice were cultured overnight. Following twenty-four hr treatment with pregnenolone 16α-carbonitrile (PCN, 10 μM), trichostatin A (TSA, 0.5 μM), or both together, total RNA was isolated and the relative expression level of the Cyp3a11 gene was determined. Data are normalized to β-actin levels and are presented as fold induction. Letters different from each other indicate a statistical difference between treatment groups (n=3, and $p<0.05$).

3.3.4 Acetylation Affects the Sub-cellular Localization of PXR in Hepatocytes.

Like other nuclear receptors, PXR can shuttle between the cytoplasmic and nuclear compartment to modulate its transcriptional activity in response to different cellular signaling pathways [30]. The translocation of PXR to the nucleus is thought to be tightly regulated by various PTMs [31]. We first determined whether the engineered fusion between the red fluorescent protein and PXR (RFP-PXR) is able to undergo SUMO-modification, and whether RFP-PXR SUMOylation is subject to regulation by the HDAC3-SMRT lysine de-acetylase co-repressor complex. Cultured hepatoma cells were transfected with expression vectors encoding RFP-PXR, $(His)_6$-SUMO3, PIAS1, HDAC3, and SMRT as indicated (**Figure 3-4A**). Forty-eight hours post-transfection, SUMOylated proteins were gathered and SUMOylated RFP-PXR proteins were detected by western blotting. The RFP-PXR fusion protein indeed retained its ability to be targeted by SUMO, and like wild type PXR, the HDAC3-SMRT co-repressor de-acetylation complex significantly inhibited SUMO-modification of RFP-PXR.

We next sought to investigate the extent to which acetylation might alter the sub-cellular localization of PXR. Primary hepatocytes were transfected with expression vectors that encode RFP-PXR and green fluorescent-tagged HDAC3 (HDAC3-GFP) proteins, respectively (**Figure 3-4B**). Twenty-four hours post-transfection, cultures were treated with vehicle (0.1% DMSO), 10 μM Rif, 0.5 μM TSA, or both together for an additional 24 hours. Fluorescence image analysis was performed as described in Materials and Methods. Hoechst 33342 (3μM) was used to visualize the nucleus by staining for 30 minutes immediately prior to imaging. Under vehicle-treated conditions, PXR was distributed roughly equally between the nuclear periphery and cytoplasmic compartments, and also exhibited strong co-localization with HDAC3-GFP. Treatment with Rif produced nearly complete translocation of RFP-PXR to the nucleus and also

produced much less co-localization with HDAC3-GFP, save for some low levels of punctate sub-nuclear localization. Treatment with produced a high level of RFP-PXR localization to the aforementioned punctate sub-nuclear architecture. Interestingly, additional co-localization of RFP-PXR and HDAC3-GFP was observed at the cell periphery following TSA treatment. Co-treatment with Rif and TSA together produced broad and diffuse RFP-PXR fluorescence in the nucleus with a somewhat diminished presence of the punctate nuclear pattern. In contrast to the Rif-treated hepatocytes, the co-treated cells exhibited a significant amount of PXR cytoplasmic co-localization with HDAC3-GFP. These data suggest that acetylation of PXR can likely affect its trans-activation capacity, in part, through altering its sub-nuclear and sub-cellular localization.

Figure 3-4A.

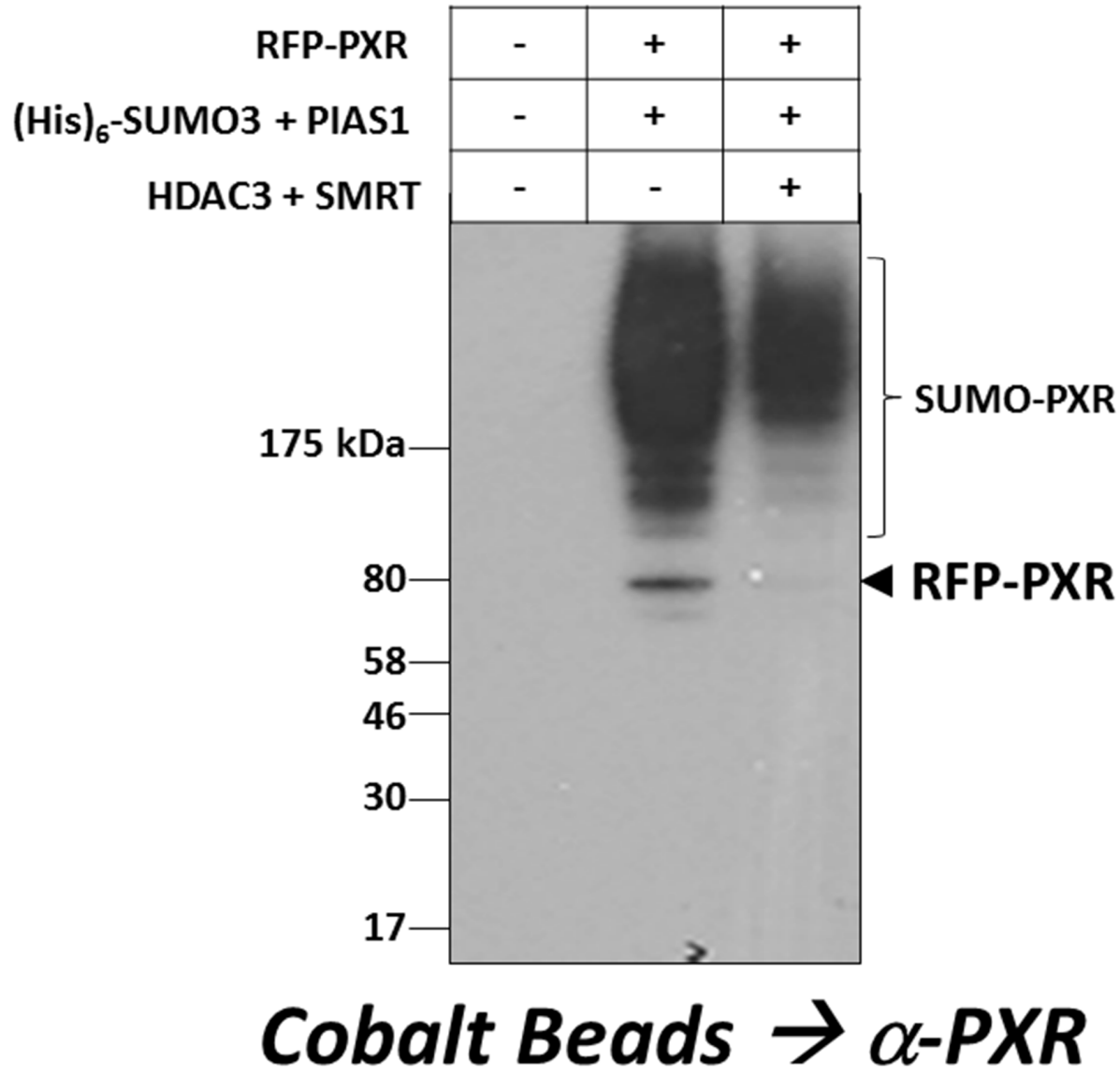

RFP-PXR
(His)6-SUMO3 + PIAS1
HDAC3 + SMRT
- + +
- + +
- - +
SUMO-PXR
175 kDa
80
RFP-PXR
58
46
30
17
Cobalt Beads → α-PXR

Figure 3-4B.

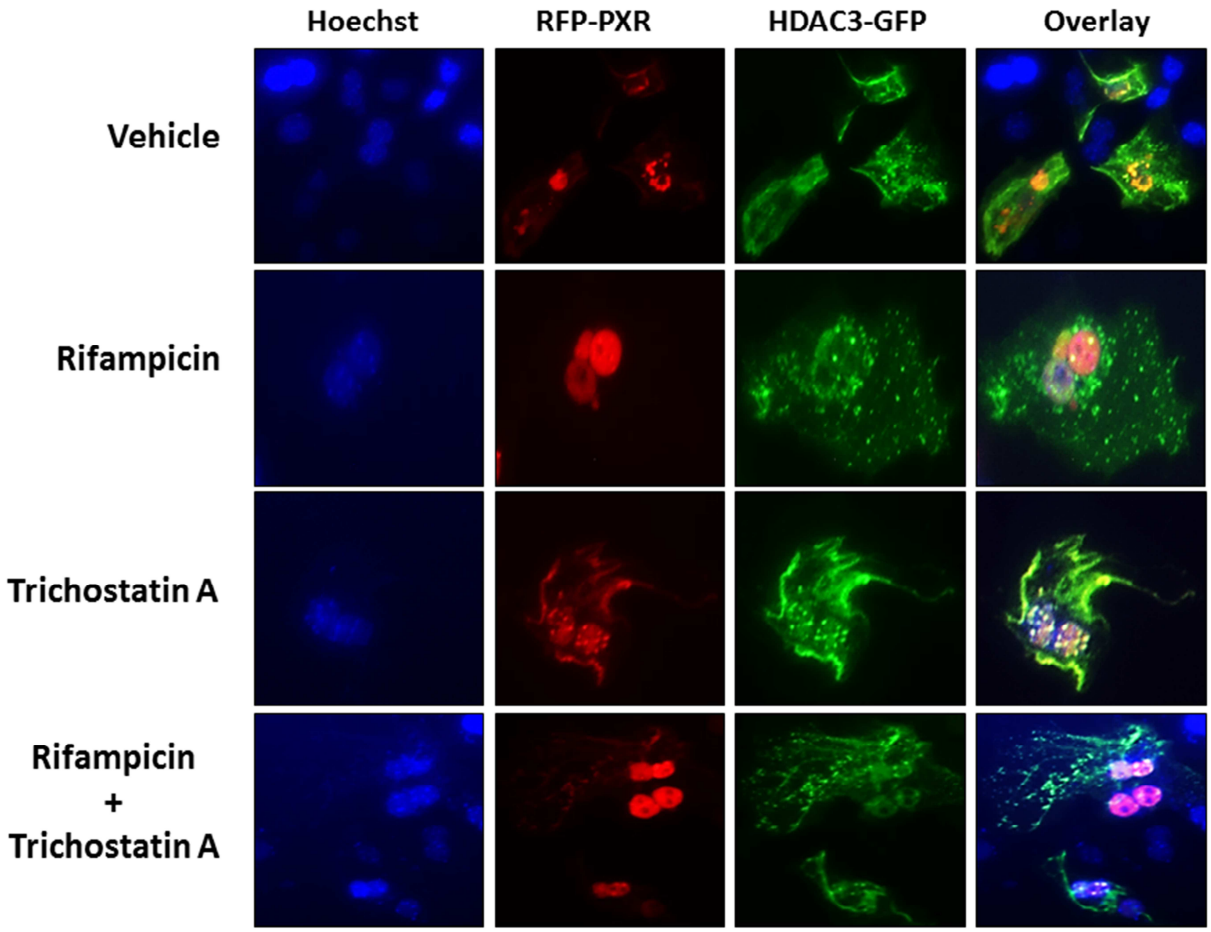


Figure 3-4. The HDAC3 Deacetylase Enzyme Affects PXR-SUMOylation and Co-localizes with PXR in Hepatocytes. (A) Hepa1-6 cells were transfected with the indicated plasmid expression vectors. SUMOylated proteins were captured using cobalt beads and the extent of RFP-PXR modification was analyzed using western blotting with an anti-PXR antibody. **(B)** Primary cultures of mouse hepatocytes were transfected with RFP-PXR and HDAC3-GFP. Twenty-four hr post-transfection, hepatocytes were treated with rifampicin (10 μM), trichostatin A (0.5 μM), or both for an additional 18 hr. Fluorescent cells were imaged as described under Materials and Methods. To facilitate the visualization of the nucleus Hoechst 33342 (3 μM) was added to the live cells thirty min prior to imaging.

3.3.5 Acetylated PXR interacts with HDAC3-SMRT Co-repressor Complex.

We next investigated the extent of PXR-HDAC3-SMRT protein-protein interactions using an immunoprecipitation approach. Cultures of Hepa1-6 cells were transfected with expression vectors encoding PXR, FLAG-HDAC3, SMRT, SUMO3, and PIAS1 as indicated (**Figure 3-5**). Twenty-four hours post-transfection, cells were treated with vehicle (0.1% DMSO), 10 μM Rif, 0.5 μM TSA, or both for an additional 24 hours. Immunoprecipitation was performed using a monoclonal anti-FLAG antibody that recognizes FLAG-tagged HDAC3. A non-immune mouse IgG antibody was used as negative control. The ability of PXR to associate with HDAC3 was determined using western blot analysis. The PXR protein interacts with HDAC3 in Hepa1-6 cell extracts (**Lane 3**), and over-expression of SMRT further enhanced this interaction (**Lane 4**). Interestingly, forced over-expression of the SUMO signaling machinery abolished interaction between PXR and the HDAC3-SMRT de-acetylation co-repressor complex (**Lanes 5-7**). In contrast, treatment of cells with TSA restored the interaction of PXR with the HDAC3-SMRT co-repressor complex (**Lane 8**). As expected, the interaction between PXR and the HDAC3-SMRT was reduced by addition of Rif (**Lane 9**). These data support the concept that acetylated PXR interacts with HDAC3-SMRT while SUMOylated PXR has a greatly diminished capacity to bind to this co-repressor complex.

Figure 3-5.

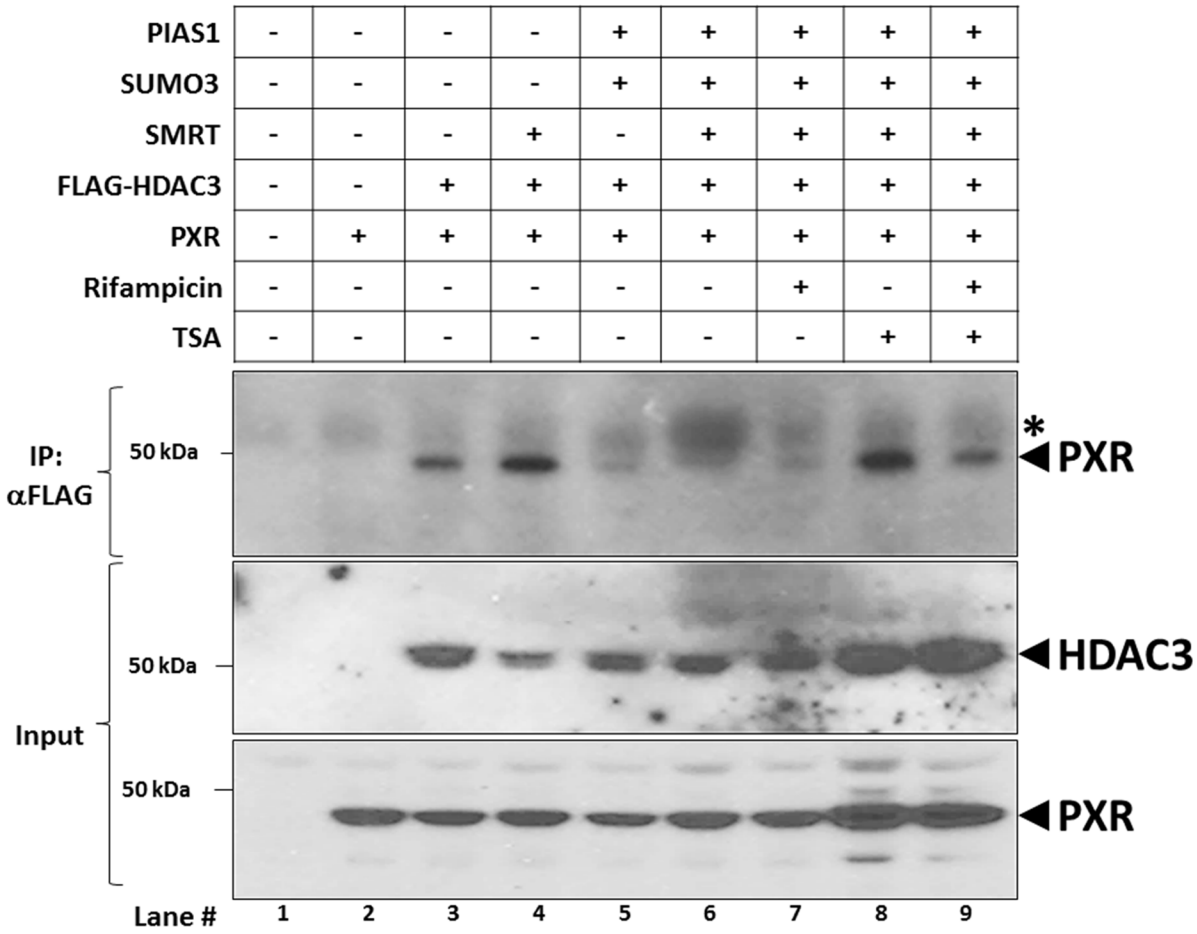


Figure 3-5. Acetylated PXR Interacts with HDAC3-SMRT Corepressor Complex. Hepa1-6 cells were transfected with the indicated expression vectors. Twenty-four hr post-transfection, cells were treated with rifampicin (10 μM), trichostatin A, or both for an additional 24 hr. Cell extracts were subjected to immunoprecipitation with an antibody that recognizes FLAG-HDAC3 (α-FLAG). A non-immune antibody was also used as a negative control (IgG). Western blot analysis was conducted using an anti-PXR polyclonal antibody to detect interaction between HDAC3-SMRT corepressor protein complex and PXR. The asterisk (*) indicates a non-specific background band.

3.3.6 Covalent Attachment of SUMO Represses Ligand-dependent Trans-activation Capacity.

It is widely held that SUMOylation of transcriptional factors leads to transcriptional repression via alteration of sub-nuclear localization, or perhaps by increasing the interactions with transcription co-repressor proteins [32]. However, a recent publication indicates that PXR activity is increased following SUMO-modification [15]. We have previously found that co-expression of the SUMO E3-ligase enzyme PIAS1 modestly increases drug-inducible *Cyp3a11* gene expression in hepatocytes [11]. However, in the same study we also found that PXR is required for PIAS1-dependent rifampicin-mediated suppression of pro-inflammatory cytokine gene expression. One strategy utilized to determine whether SUMOylation suppresses trans-activation capacity has been to utilize a gene fusion approach [33]. Thus, to more precisely determine how SUMO-modification of PXR affects its ability to activate gene expression we generated a series of expression vectors that encode a variety of linear PXR-SUMO fusion proteins as depicted in **Figure 3-6A**. The relative protein expression level of the five forms of PXR was roughly equal as verified using antibodies that recognize PXR, SUMO1, and SUMO3 in western blot analysis (**Figure 3-6B**). We next examined the effect of covalent attachment of SUMO on regulating drug-inducible PXR-dependent trans-activation capacity using a reporter gene derived from the CYP3A4 promoter to drive luciferase activity (XREM-Luc) in cell based assays [34]. Twenty-four hours post-transfection, CV-1 cells were treated with Rif for an additional 24 hours. Treatment of cells expressing wid type PXR (**construct number 1**) with Rif increased reporter gene activity by approximately 50-fold. To determine if covalently attaching additional protein sequences to the N-terminus or C-terminus of PXR had non-specific inhibitory effects upon its trans-activation capacity, we fused the six-histidine tag $(His)_6$ to the

N-terminus and the triple FLAG-tag [$(FLAG)_3$] to the C-terminus of PXR, respectively, as shown in **construct number 2**. Cells expressing this form of PXR did not significantly alter its Rif-inducible trans-activation capacity. In contrast, cells expressing either the PXR-SUMO1 (**construct number 3**), the PXR-SUMO3 (**construct number 4**), or the SUMO3-PXR (**construct number 5**) exhibited significantly diminished Rif-inducible reporter gene activity (**Figure 3-6C**). These data indicate that covalent attachment of SUMO to PXR, even in the absence of isopeptide linkage and irrespective of the location of SUMO at the N- or C-terminus, produces a strong repression of its drug-inducible trans-activation capacity.

Figure 3-6A.

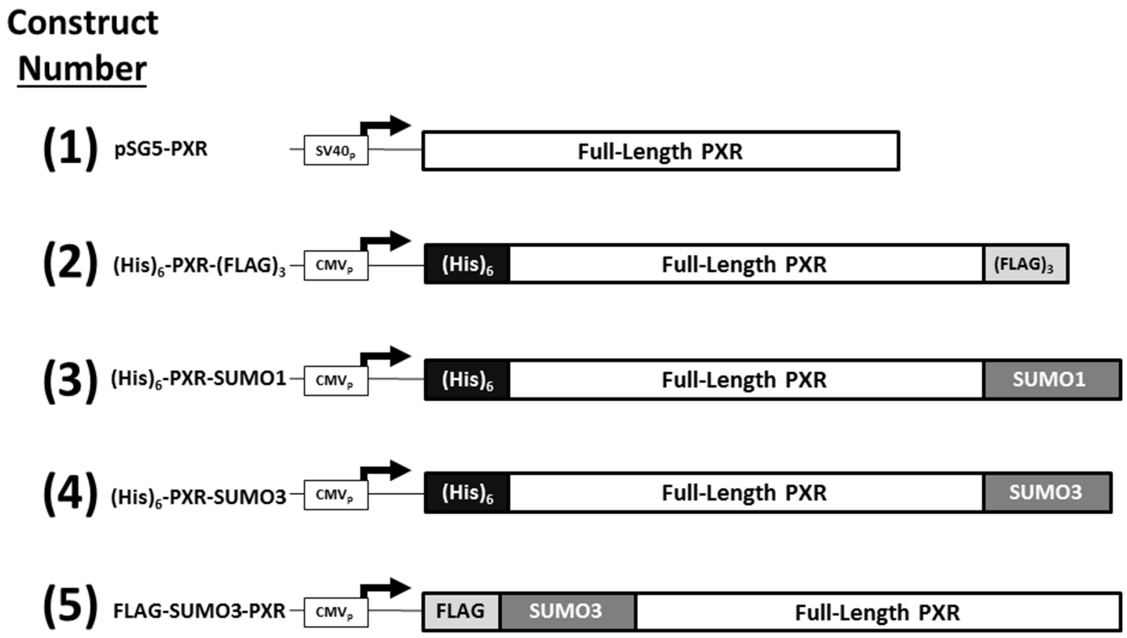


Figure 3-6B.

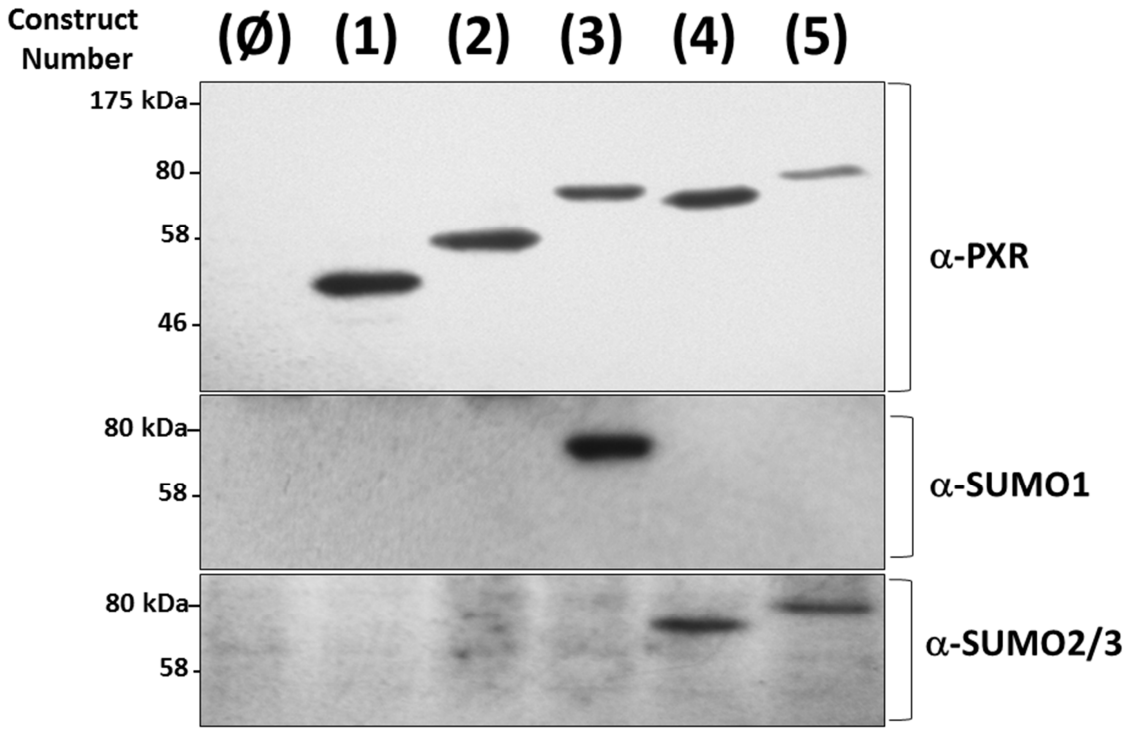


Figure 3-6C.

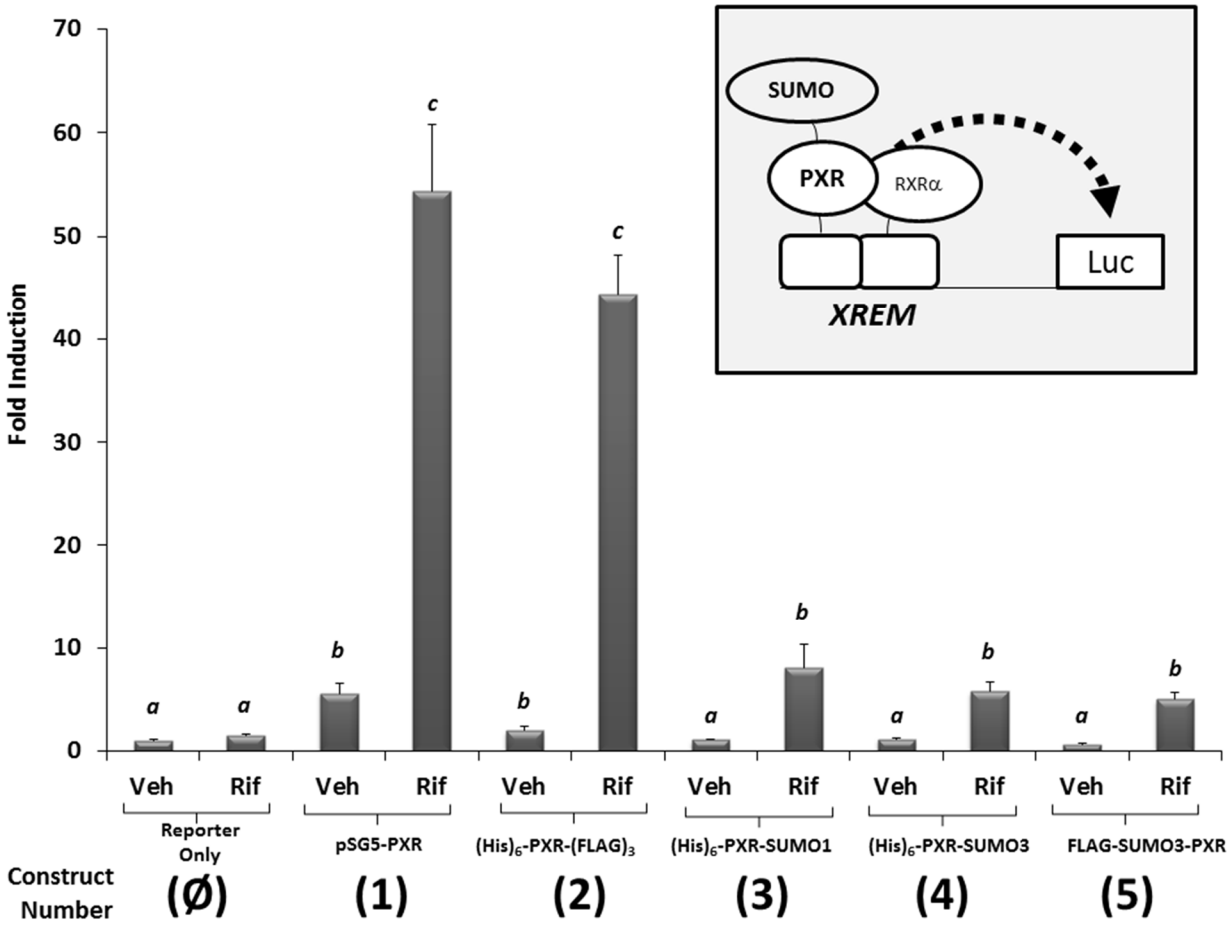


Figure 3-6. Linear SUMO-fusion Proteins are Deficient in Trans-activation Capacity. **(A)** A series of expression vectors were constructed (Left Panel) encoding various forms of PXR, with some fused to SUMO proteins, as described in Materials and Methods. **(B)** Western blot analysis was performed using the indicated antibodies that recognize PXR, SUMO1, and SUMO3 using cell extracts from CV-1 cells that were transfected with the plasmid-based expression vectors as indicated. **(C)** CV-1 cells were transfected with the XREM-Luc reporter gene together with the indicated construct. The reporter gene alone was used as a negative control (Reporter Only). Twenty-four hr post-transfection, cells were treated with either vehicle (0.1% DMSO) or rifampicin (Rif, 10 μM) for an additional 24 hours. Luciferase activity was normalized to β-galactosidase controls and data are presented as fold induction + SEM. Letters different from each other indicate statistically significant differences between relevant treatment groups ($p<0.05$).

3.3.7 SUMOylation of PXR is Dominant over HDAC3-mediated Inhibition of PXR Activity.

Under normal conditions, the co-repressor protein SMRT is required for the lysine-deacetylase activity of HDAC3 in vivo [28, 29]. The SMRT co-repressor also serves as a molecular scaffold for additional regulatory proteins to modulate metabolic and inflammatory processes in liver [35]. Using a multimerized PXR-response element driving the luciferase reporter gene [$(ER6)_3$-Luc] we sought to determine the extent to which HDAC3-SMRT co-repressor complex contributes to the repression of PXR trans-activation capacity following SUMO-modification. We chose the multimerized PXR-response element reporter gene because it lacks the additional complex and over-lapping enhancer elements associated with the CYP3A4-derived XREM-Luc reporter gene [34], and it likely represents a more direct readout of PXR trans-activation capacity in cell line-based assays versus the XREM-Luc reporter gene. Treatment of cells with Rif that were transfected with either PXR alone, PXR together with HDAC3, or PXR together with HDAC3 and SMRT produced approximately 3-4-fold increase in reporter gene activity (**Figure 3-7**). Treatment with TSA also produced very modest levels of reporter gene activity in these three experimental groups (3-4-fold induction). In contrast, co-treatment of PXR-transfected cells with Rif and TSA together produced an approximately 16-fold increase in reporter gene activity. The addition of HDAC3 dramatically increased reporter gene activity to approximately 36-fold, while the addition of SMRT significantly reduced the reporter gene activity to approximately 24-fold. Similar experiments performed in the absence of PXR did not produce synergistic reporter gene activity in the absence of the receptor (data not shown). These data confirm that HDAC3 and SMRT have a profound impact upon ligand-dependent PXR trans-activation capacity such that pharmacological inhibition of HDAC3-SMRT

co-repressor complex activity with TSA produces a synergistic activation of this PXR-dependent reporter gene. When the same experimental groups were used in the presence of expression vectors encoding SUMO3 and PIAS1, the overall ligand-dependent synergistic trans-activation capacity produced by co-treatment with Rif and TSA was strongly diminished, independent of the presence of HDAC3 and SMRT. These data indicate that SUMOylation likely represses PXR trans-activation capacity in a manner that is distinct from that mediated by the HDAC3-SMRT co-repressor complex.

Figure 3-7.

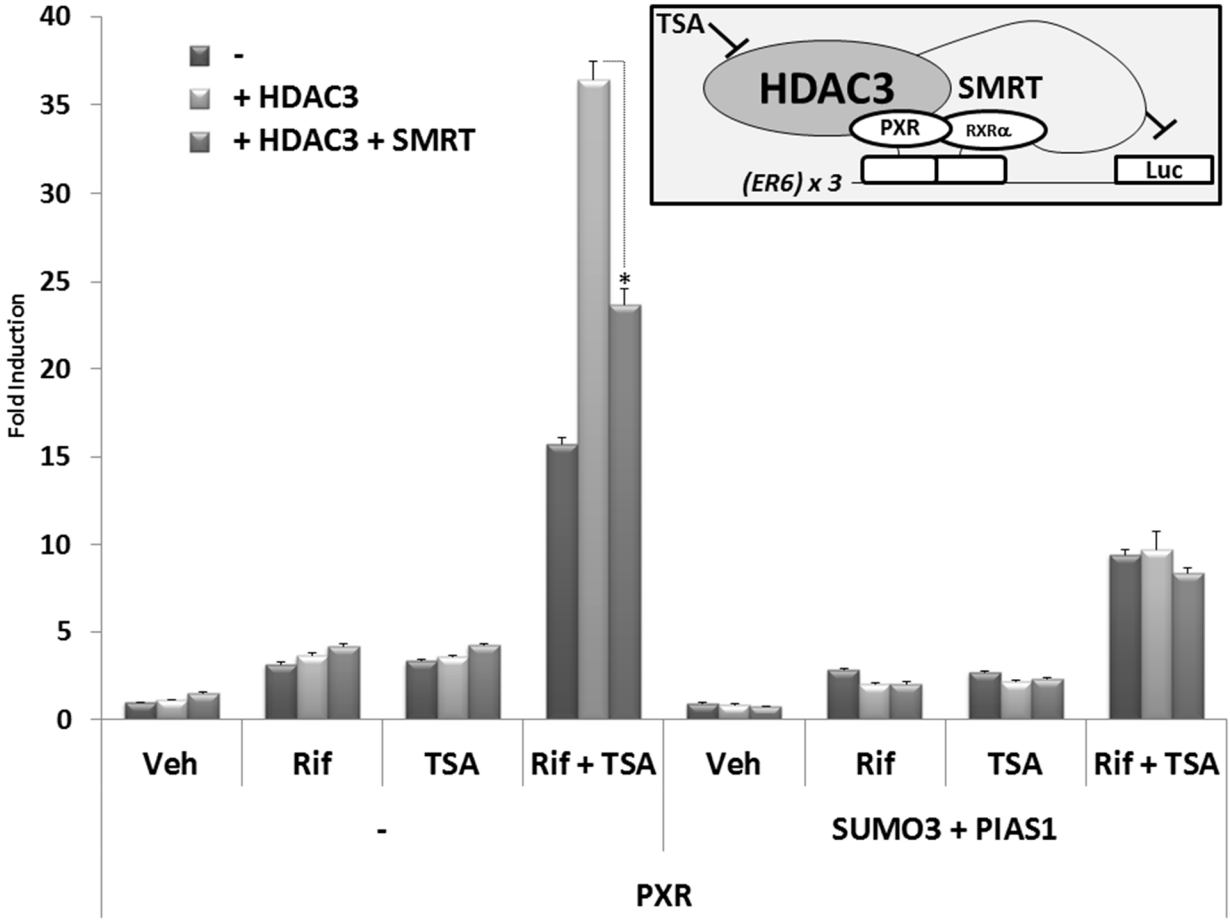


Figure 3-7. SUMOylation of PXR Represses PXR Trans-activation. CV-1 cells were transfected with the (ER6)x3-Luc reporter gene together with the indicated expression vectors. Twenty-four hr post-transfection, cells were treated with either vehicle (Veh, 0.1% DMSO), rifampicin (Rif, 10 μM), trichostatin A (TSA, 0.5 μM), or both together for an additional twenty-four hours. Luciferase activity was normalized to β-galactosidase controls and data are presented as fold induction above vehicle control + SEM. Asterisks indicate statistically significant differences between relevant treatment groups ($p<0.05$).

3.3.8 Ubiquitination of PXR on Lysine 109 in Hepatocytes is TSA-Dependent.

Many laboratories have identified potential PTMs in PXR through an *in vitro* approach using either LC-MS/MS, or by using a biochemical approach with purified components [12, 13, 17]. These experimental approaches are encumbered by the relative absence of meaningful biology when performed purely *in vitro*. In an effort to identify a specific site of acetylation *in vivo*, we took advantage of our adenoviral system to express and purify the $(His)_6$-tagged form of PXR from primary cultures of rat hepatocytes following treatment with either vehicle (0.1% DMSO) or TSA (0.5 μM). Using this experimental approach we routinely achieve high expression and robust purification of the Ad-PXR protein from cultured hepatocytes as demonstrated using coomassie blue staining (**Figure 3-8A**). Our overall coverage of the Ad-PXR protein following digestion with trypsin using LC-MS/MS methods was approximately 60%, to include fifteen out of twenty-eight total lysine residues contained within the human PXR protein. We failed to detect acetylation on observable lysine residues following trypsin digestion. However, we found that vehicle treated PXR is heavily multi-mono ubiquitinated on K109, K160, K170, K198, and K226 (**Figure 3-8B-F**). Following treatment with TSA, both K160 and K170 were still heavily ubiquitinated. In contrast, ubiquitin modification at K198 and K226 was absent. Moreover, the PTM-status at the K109 position was ambiguous due to a lack of coverage in the spectra of this particular lysine residue within the TSA-treated experimental group. The reason for a lack of coverage at K109 specifically in the TSA-treated group is unknown at present.

Figure 3-8A.

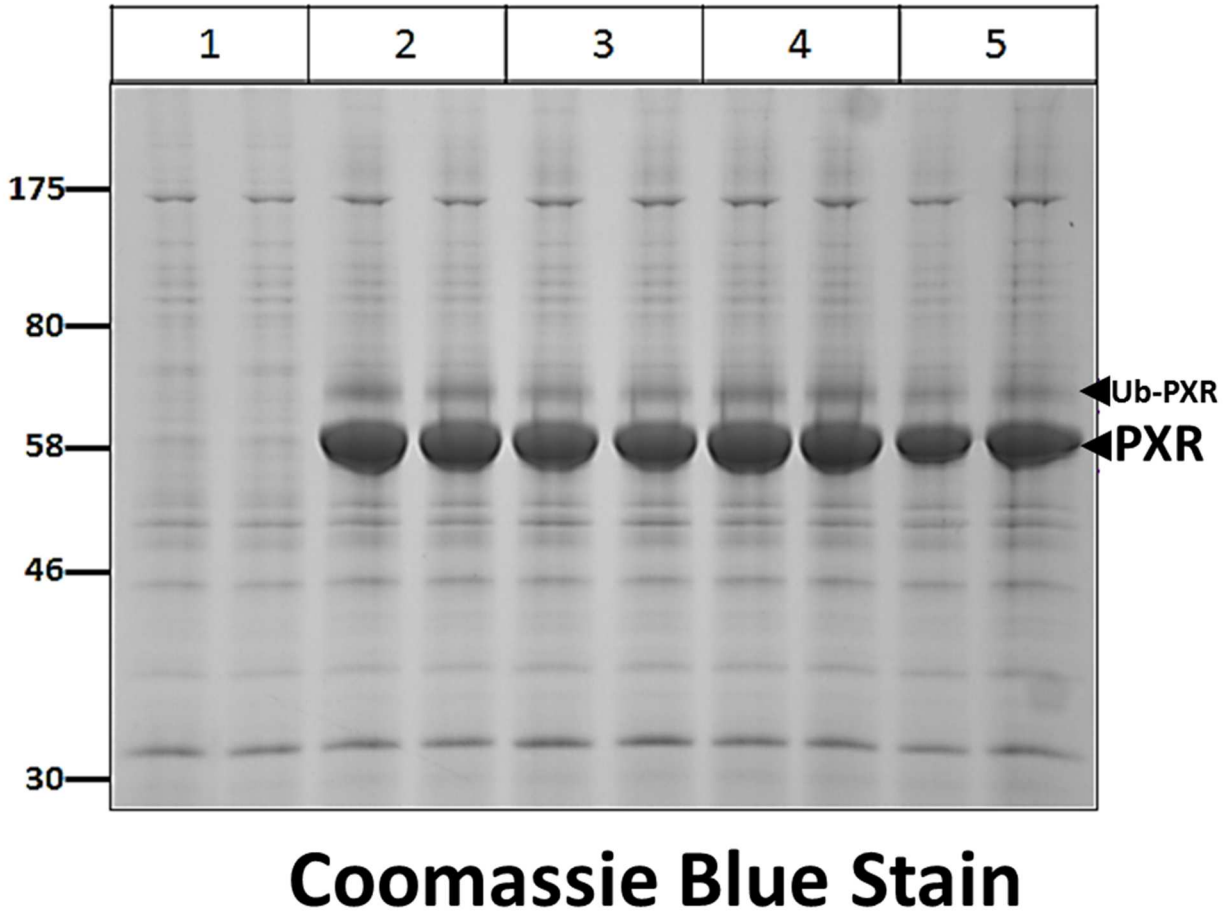
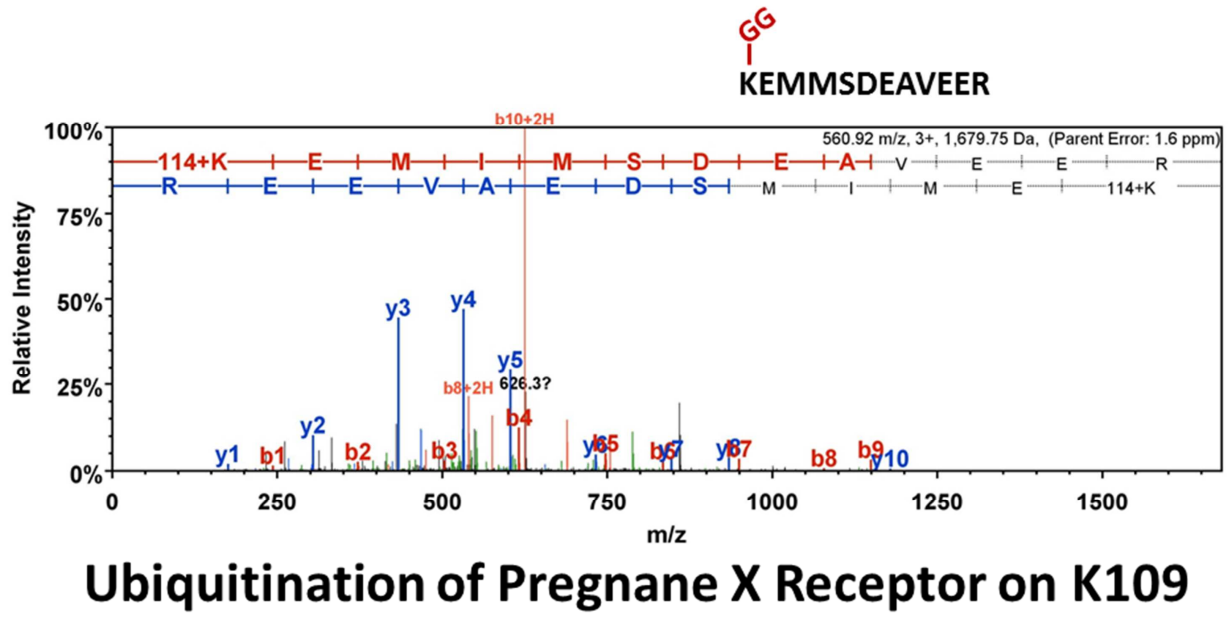
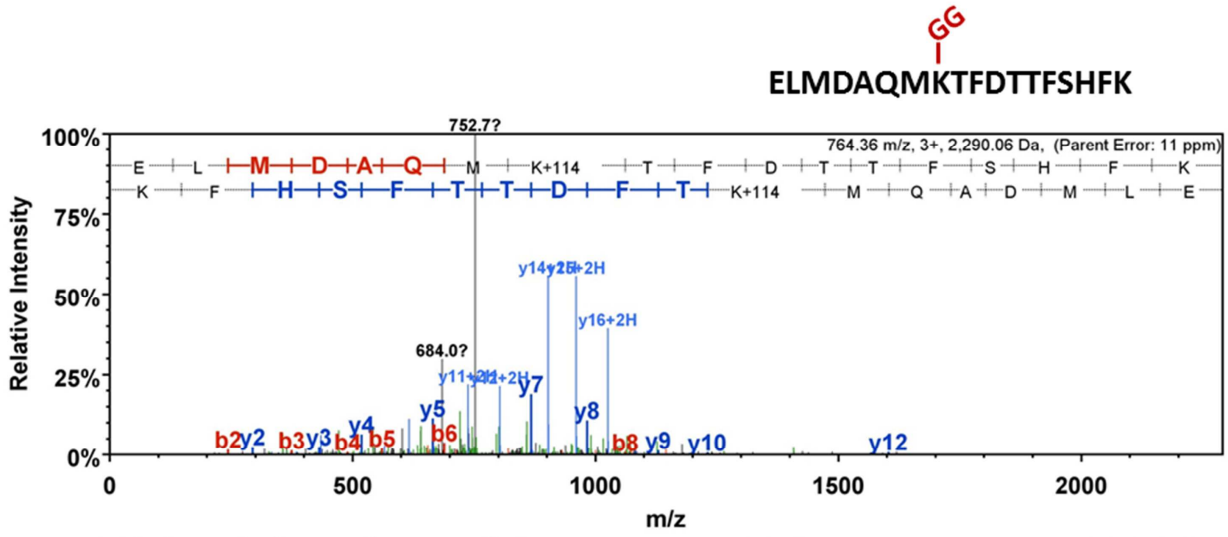


Figure 3-8B.



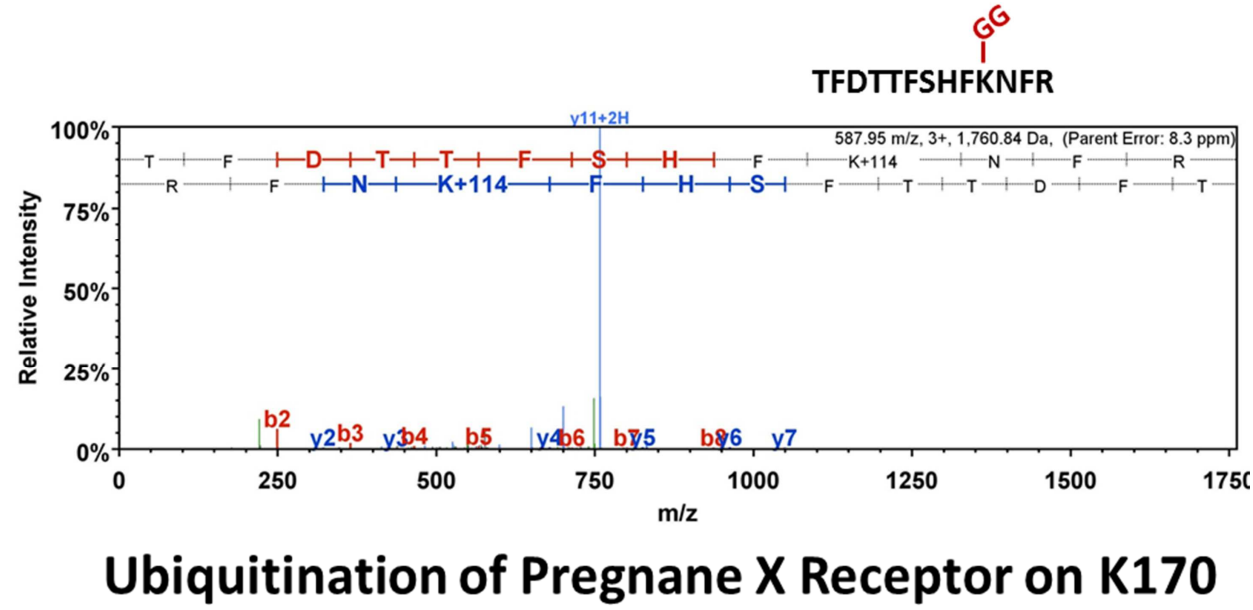
Ubiquitination of Pregnane X Receptor on K109

Figure 3-8C.



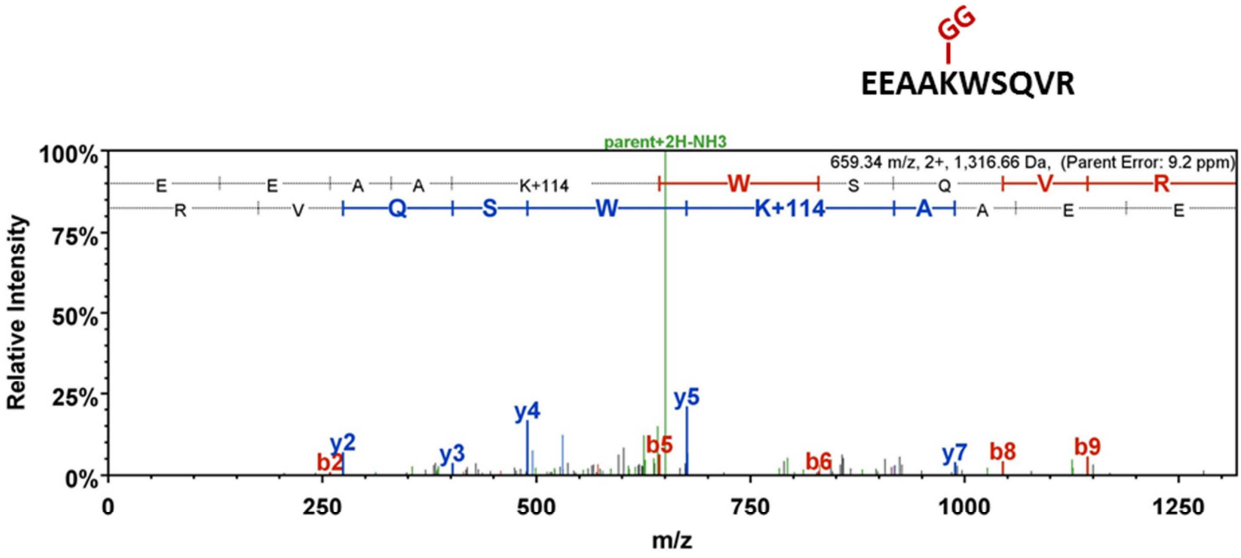
Ubiquitination of Pregnane X Receptor on K160

Figure 3-8D.



Ubiquitination of Pregnane X Receptor on K170

Figure 3-8E.



Ubiquitination of Pregnane X Receptor on K198

Figure 3-8F.

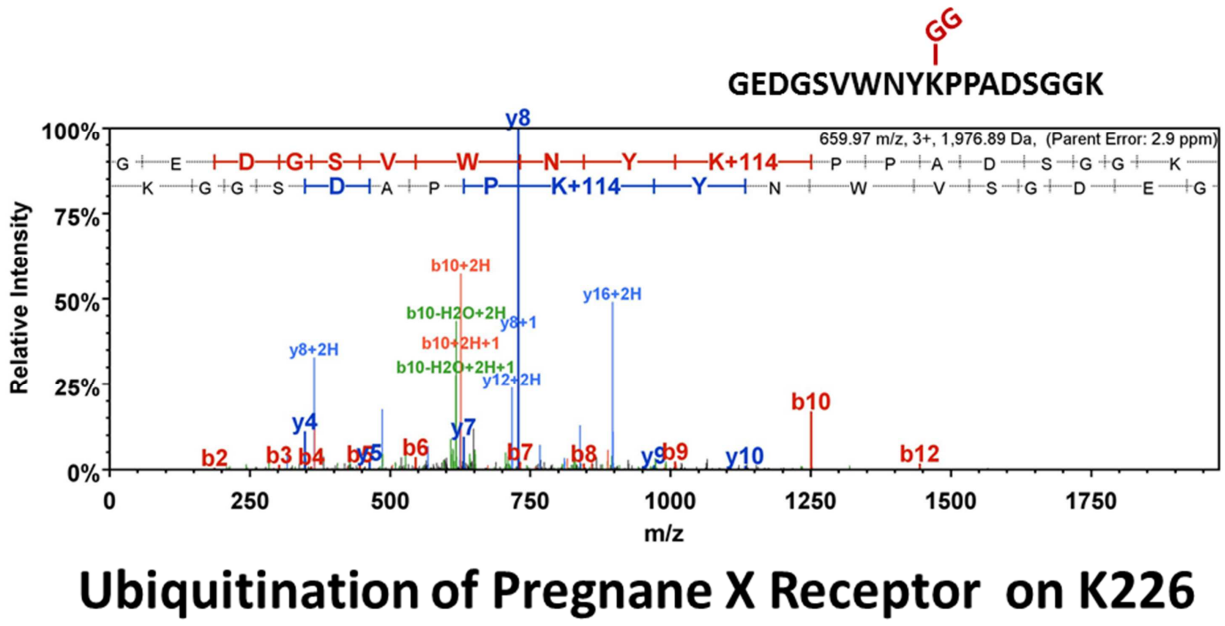


Figure 3-8. Identification of Ubiquitin-Modified Peptides of PXR Isolated from Hepatocytes using LC-MS/MS. (A) Primary hepatocytes were isolated from a male 14 week old rat and were cultured overnight in ten separate 15 cM dishes. The adenoviral expression vector encoding the six-histidine-tagged form of PXR [Ad-(His)6-PXR] was added to experimental groups 2 through 5 on the morning of day 2. On day 3, cultures were treated with vehicle (Groups 2 and 3) or 0.5 □M TSA (Groups 4 and 5) for an additional 24 hours. Following IMAC-enrichment under denaturing conditions (1 lane per 15 cM dish), the bands corresponding to PXR and Ub-PXR were excised and in-gel trypsin digestion was performed. **(B-F)** ESI-CID-MS/MS analysis of in-gel digested PXR resulted in a number of spectra that were assigned to tryptic peptides carrying covalently bound ubiquitin residues of Gly-Gly (ubiquitin di-glycine remnant post-trypsin digestion). The tryptic peptides of ubiquitin-modified PXR were identified with a mass addition of 114 at the lysine residues K109, K160, K170, K198, and K226 based on the assignment of multiple product ions (y and b ions) as indicated in the MS/MS scan.

3.4 DISCUSSION

The notion that lysine-directed PTMs including SUMOylation, ubiquitination, and acetylation interact with each other within the context of a given transcriptional regulatory complex is gaining wide acceptance [8]. The class I lysine de-acetylase HDAC3 regulates metabolism through multiple signalling pathways in the liver, and liver-specific deletion of HDAC3 disrupts normal hepatic metabolic homeostasis [36]. A recent article focused on farnesoid x receptor acetylation postulates that targeting acetylation of this receptor and its transcriptional cofactors may provide a new molecular strategy for development of pharmacological agents to treat metabolic disorders [37]. An additional study from the same group indicates that a dysregulated SUMO-acetyl switch on farnesoid x receptor that occurs during morbid obesity may serve as a general mechanism for diminished anti-inflammatory response observed in these patients [20]. Moreover, this group has shown that acetylation of farnesoid x receptor is normally regulated by the acetyl transferase E1A binding protein p300 and the class III NAD-dependent deacetylase Sirtuin-1 [38]. Similarly, the de-acetylation of PXR has been previously suggested to be mediated by both Sirtuin-1 and a TSA-dependent lysine de-acetylase [26].

Previous studies conducted in our laboratory have revealed that SUMOylation of PXR alters its ubiquitination, and likely regulates two distinct facets of PXR biology. The SUMOylation of PXR increases its stability, apparently through protection of this nuclear receptor from the proteasome-mediated degradation pathway [11]. Another likely function of SUMO-modified PXR is the active suppression of the expression of pro-inflammatory cytokines in hepatocytes as well as participation in the resolution of inflammation [27, 39]. While PXR SUMOylation is critical for maintaining cell integrity in response to inflammatory stress, the role

of PXR acetylation in this process is not clear. Two studies have independently detected acetylation of PXR by using either biochemical over-expression methods in cultured cell lines [26], or by using a western blotting approach in whole-cell protein extracts from rodent liver [20]. How acetylation of PXR potentially affects its SUMOylation or ubiquitination was not addressed in these studies. However, it was suggested that in addition to Sirtuin 1, other as yet to be identified lysine de-acetylase enzymes likely play an important role in enhancing or assisting with de-acetylation upon PXR activation [26], and our current study suggests that HDAC3 is intimately involved in PXR de-acetylation.

Taken together, the data obtained in our current study put forward a working model of the role of acetylation, SUMOylation, and ubiquitination in regulating PXR biology (**Figure 9**). We favor the notion that acetylation and SUMOylation are mutually exclusive events, but that acetylation is likely a prerequisite for its subsequent SUMO-modification. Moreover, it appears that ubiquitin migrates to various lysine residues within PXR, likely depending upon the repertoire of other PTMs that co-exist on the protein and its state of ligand activation. The ligand-dependent ubiquitination of PXR likely promotes its degradation through the proteasome-mediated pathway during the canonical response, in the absence of concurrent pathological conditions such as inflammatory-related disease states. In the presence of a pathological stimulus, such as inflammation, the acetyl group is removed and a PXR-mediated gene activation program is supplanted by active repression of select PXR-target genes by SUMO-modified and stabilized PXR. Our hypothesis agrees with the previous observation that poly-ubiquitination of transcription factors on a single lysine residue authorizes their trans-activation capacity by linking gene activation to their subsequent destruction [40]. Moreover, mono- or multi-mono-ubiquitination confers distinct biological outcomes when compared with poly-ubiquitination

through the formation of ubiquitin chains [41]. Our data show that PXR is heavily multi-mono-ubiquitinated in a silent state, and that the formation of poly-ubiquitinated PXR at K160 and K170 is stimulated by a more heavily acetylated receptor. We have previously shown that SUMOylation of PXR focuses the formation of K48-linked ubiquitin chains at K170 to support degradation of ligand-activated PXR [11]; and that this event is likely required in order for additional rounds of transcription to proceed [42]. It therefore appears that acetylation of PXR also affects its ubiquitin modification.

It is worth noting that while acetyl-lysine modification of PXR was readily detected in this study using an immunological approach, we were not successful in identifying any acetylated lysine residues in PXR using mass spectrometry analysis. This is likely due to the relatively low stoichiometry of the acetyl-modification [43], or perhaps due to the large size of the SUMO- and acetyl-modified target peptide(s), or the inherent limitations of the LC-MS/MS approach. Further refinement of our experimental conditions through the use of proteases other than trypsin, or by the addition of a resolving column pre-LC-MS/MS may improve our detection capabilities.

Figure 3-9.

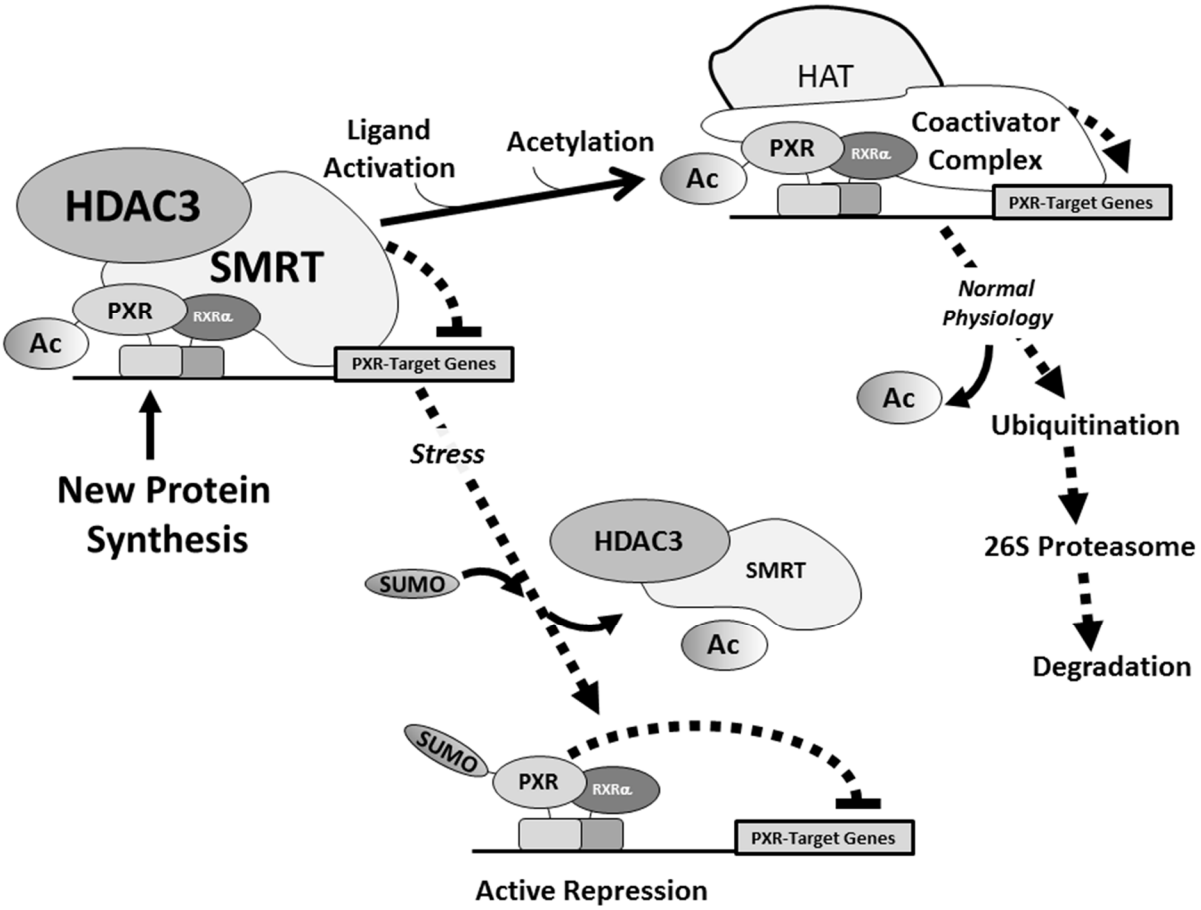


Figure 3-9. Working model of the Role of the Acetyl-SUMO Switch in PXR Biology. Newly synthesized PXR protein is acetylated and poised on canonical PXR-target genes in a complex with the HDAC3-SMRT co-repressor multi-protein complex and is transcriptionally silent. Ligand activation promotes hyper-acetylation of the genomic locus, likely through the action of canonical histone/lysine acetyltransferase enzymes belonging to the E1A binding protein p300/CREB-binding protein coactivator family. Following one round of transcription the PXR-associated multi-protein complex is degraded by the 26S proteasome in an ubiquitin-dependent manner, and the promoter is thus cleared and poised to receive another round of transcriptional machinery. In the presence of specific signals, such an inflammatory stress or potentially other extra-cellular stimuli, PXR is de-acetylated and the HDAC3-SMRT co-repressor multi-protein complex is disassociated. The resulting signal-dependent action of a SUMO E3 ligase enzyme, such as PIAS1, promotes PXR-SUMOylation to inhibit PXR-target gene expression in an acetylation-dependent manner.

3.5 CONCLUSIONS

In conclusion, the acetylation, SUMOylation, and ubiquitination of PXR interface with each other to alter PXR biolgical activity. We show here that PXR is the molecular target of the acetylation signal transduction pathway and that the HDAC3-SMRT multi-protein corepressor complex is a key component of the PXR de-acetylation pathway. The promotion of PXR acetylation enhances its SUMO-modification, while de-acetylation of PXR by HDAC3-SMRT tends to inhibit its SUMOylation in cell line-based assays. Ligand-mediated activation with rifampicin fuels de-acetylation of PXR, while inhibition of HDAC3 activity using pharmacological methods mildly suppresses the PXR-mediated gene activation program in hepatocytes. Acetylated PXR interacts with HDAC3 and forced over-expression of SMRT further increases PXR-HDAC3 interactions. The SUMOylation of PXR likely forms the molecular basis of PXR-mediated active gene repression during pathological disease states. However, further research in this area should determine the extent to which these and other signaling pathways contribute to this effect. What is abundantly clear is that the regulation of PXR by various PTMs is highly interactive and in a state of constant fluidity. Giving the fact that PXR is a pivatol regultor of drug metabolism and disposition and is heavily involved in the pathogenesis of important human diseases, unrevling the molecular and biochemical details of how PTMs determine PXR biology should remian an important thust of future research efforts. The studies presented here utilize cell line-based and biochemical methods as well as primary cultures of rodent hepatocytes; future experiments should include the use of transgenic mouse models. Specifically, mouse models that express mutant forms of the PXR protein refractory to modification with SUMO and acetyl groups should greatly aid in furthering the knowledge base regarding the effect these signaling pathways have on PXR biology in vivo.

3.6 REFERENCES

[1] S.A. Kliewer, J.T. Moore, L. Wade, J.L. Staudinger, M.A. Watson, S.A. Jones, D.D. McKee, B.B. Oliver, T.M. Willson, R.H. Zetterstrom, T. Perlmann, J.M. Lehmann, An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway, Cell, 92 (1998) 73-82.
[2] J.M. Lehmann, D.D. McKee, M.A. Watson, T.M. Willson, J.T. Moore, S.A. Kliewer, The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions, The Journal of clinical investigation, 102 (1998) 1016-1023.
[3] S. Gotoh, M. Negishi, Statin-activated nuclear receptor PXR promotes SGK2 dephosphorylation by scaffolding PP2C to induce hepatic gluconeogenesis, Scientific reports, 5 (2015) 14076.
[4] J. Gao, W. Xie, Pregnane X receptor and constitutive androstane receptor at the crossroads of drug metabolism and energy metabolism, Drug metabolism and disposition: the biological fate of chemicals, 38 (2010) 2091-2095.
[5] D. Robbins, T. Chen, Tissue-specific regulation of pregnane X receptor in cancer development and therapy, Cell & bioscience, 4 (2014) 17.
[6] J. Cheng, Y.M. Shah, F.J. Gonzalez, Pregnane X receptor as a target for treatment of inflammatory bowel disorders, Trends in pharmacological sciences, 33 (2012) 323-330.
[7] M. Venkatesh, S. Mukherjee, H. Wang, H. Li, K. Sun, A.P. Benechet, Z. Qiu, L. Maher, M.R. Redinbo, R.S. Phillips, J.C. Fleet, S. Kortagere, P. Mukherjee, A. Fasano, J. Le Ven, J.K. Nicholson, M.E. Dumas, K.M. Khanna, S. Mani, Symbiotic bacterial metabolites regulate gastrointestinal barrier function via the xenobiotic sensor PXR and Toll-like receptor 4, Immunity, 41 (2014) 296-310.
[8] X.J. Yang, E. Seto, Lysine acetylation: codified crosstalk with other posttranslational modifications, Molecular cell, 31 (2008) 449-461.
[9] T. Smutny, S. Mani, P. Pavek, Post-translational and post-transcriptional modifications of pregnane X receptor (PXR) in regulation of the cytochrome P450 superfamily, Current drug metabolism, 14 (2013) 1059-1069.
[10] J.L. Staudinger, K. Lichti, Cell signaling and nuclear receptors: new opportunities for molecular pharmaceuticals in liver disease, Molecular pharmaceutics, 5 (2008) 17-34.
[11] W. Cui, M. Sun, N. Galeva, T.D. Williams, Y. Azuma, J.L. Staudinger, SUMOylation and Ubiquitylation Circuitry Controls Pregnane X Receptor Biology in Hepatocytes, Drug metabolism and disposition: the biological fate of chemicals, 43 (2015) 1316-1325.
[12] A. Elias, A.A. High, A. Mishra, S.S. Ong, J. Wu, J. Peng, T. Chen, Identification and characterization of phosphorylation sites within the pregnane X receptor protein, Biochemical pharmacology, 87 (2014) 360-370.
[13] K. Lichti-Kaiser, D. Brobst, C. Xu, J.L. Staudinger, A systematic analysis of predicted phosphorylation sites within the human pregnane X receptor protein, The Journal of pharmacology and experimental therapeutics, 331 (2009) 65-76.
[14] K. Lichti-Kaiser, C. Xu, J.L. Staudinger, Cyclic AMP-dependent protein kinase signaling modulates pregnane x receptor activity in a species-specific manner, The Journal of biological chemistry, 284 (2009) 6639-6649.
[15] Priyanka, D. Kotiya, M. Rana, N. Subbarao, N. Puri, R.K. Tyagi, Transcription regulation of nuclear receptor PXR: Role of SUMO-1 modification and NDSM in receptor function, Molecular and cellular endocrinology, (2015).

[16] R. Rana, S. Coulter, H. Kinyamu, J.A. Goldstein, RBCK1, an E3 ubiquitin ligase, interacts with and ubiquinates the human pregnane X receptor, Drug metabolism and disposition: the biological fate of chemicals, 41 (2013) 398-405.
[17] J. Sugatani, Y. Hattori, Y. Noguchi, M. Yamaguchi, Y. Yamazaki, A. Ikari, Threonine-290 regulates nuclear translocation of the human pregnane X receptor through its phosphorylation/dephosphorylation by Ca2+/calmodulin-dependent protein kinase II and protein phosphatase 1, Drug metabolism and disposition: the biological fate of chemicals, 42 (2014) 1708-1718.
[18] J. Sugatani, Y. Noguchi, Y. Hattori, M. Yamaguchi, Y. Yamazaki, A. Ikari, Threonine-408 Regulates the Stability of the Human Pregnane X Receptor Through its Phosphorylation and the CHIP/Chaperone-Autophagy Pathway, Drug metabolism and disposition: the biological fate of chemicals, (2015).
[19] W.W. Blakeslee, C.L. Wysoczynski, K.S. Fritz, J.K. Nyborg, M.E. Churchill, T.A. McKinsey, Class I HDAC inhibition stimulates cardiac protein SUMOylation through a post-translational mechanism, Cellular signalling, 26 (2014) 2912-2920.
[20] D.H. Kim, Z. Xiao, S. Kwon, X.X. Sun, D. Ryerson, D. Tkac, P. Ma, S.Y. Wu, C.M. Chiang, E. Zhou, H.E. Xu, J.J. Palvimo, L.F. Chen, B. Kemper, J.K. Kemper, A dysregulated acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity, Embo J, 34 (2015) 184-199.
[21] S. Emiliani, W. Fischle, C. Van Lint, Y. Al-Abed, E. Verdin, Characterization of a human RPD3 ortholog, HDAC3, Proceedings of the National Academy of Sciences of the United States of America, 95 (1998) 2795-2800.
[22] R.L. Montgomery, M.J. Potthoff, M. Haberland, X. Qi, S. Matsuzaki, K.M. Humphries, J.A. Richardson, R. Bassel-Duby, E.N. Olson, Maintenance of cardiac energy metabolism by histone deacetylase 3 in mice, The Journal of clinical investigation, 118 (2008) 3588-3597.
[23] D.R. Johnson, C.W. Li, L.Y. Chen, J.C. Ghosh, J.D. Chen, Regulation and binding of pregnane X receptor by nuclear receptor corepressor silencing mediator of retinoid and thyroid hormone receptors (SMRT), Molecular pharmacology, 69 (2006) 99-108.
[24] P.G. Shaw, R. Chaerkady, Z. Zhang, N.E. Davidson, A. Pandey, Monoclonal antibody cocktail as an enrichment tool for acetylome analysis, Analytical chemistry, 83 (2011) 3623-3626.
[25] J.L. Staudinger, C. Xu, A. Biswas, S. Mani, Post-translational modification of pregnane x receptor, Pharmacological research, 64 (2011) 4-10.
[26] A. Biswas, D. Pasquel, R.K. Tyagi, S. Mani, Acetylation of pregnane X receptor protein determines selective function independent of ligand activation, Biochemical and biophysical research communications, 406 (2011) 371-376.
[27] G. Hu, C. Xu, J.L. Staudinger, Pregnane X receptor is SUMOylated to repress the inflammatory response, The Journal of pharmacology and experimental therapeutics, 335 (2010) 342-350.
[28] M.G. Guenther, O. Barak, M.A. Lazar, The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3, Molecular and cellular biology, 21 (2001) 6091-6101.
[29] S.H. You, H.W. Lim, Z. Sun, M. Broache, K.J. Won, M.A. Lazar, Nuclear receptor co-repressors are required for the histone-deacetylase activity of HDAC3 in vivo, Nature structural & molecular biology, 20 (2013) 182-187.

[30] K. Kawana, T. Ikuta, Y. Kobayashi, O. Gotoh, K. Takeda, K. Kawajiri, Molecular mechanism of nuclear translocation of an orphan nuclear receptor, SXR, Molecular pharmacology, 63 (2003) 524-531.
[31] Y.E. Timsit, M. Negishi, CAR and PXR: the xenobiotic-sensing receptors, Steroids, 72 (2007) 231-246.
[32] S.H. Yang, A.D. Sharrocks, SUMO promotes HDAC-mediated transcriptional repression, Molecular cell, 13 (2004) 611-617.
[33] S. Ross, J.L. Best, L.I. Zon, G. Gill, SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization, Molecular cell, 10 (2002) 831-842.
[34] B. Goodwin, E. Hodgson, C. Liddle, The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module, Molecular pharmacology, 56 (1999) 1329-1339.
[35] A. Mottis, L. Mouchiroud, J. Auwerx, Emerging roles of the corepressors NCoR1 and SMRT in homeostasis, Genes & development, 27 (2013) 819-835.
[36] S.K. Knutson, B.J. Chyla, J.M. Amann, S. Bhaskara, S.S. Huppert, S.W. Hiebert, Liver-specific deletion of histone deacetylase 3 disrupts metabolic transcriptional networks, Embo J, 27 (2008) 1017-1028.
[37] J.K. Kemper, Regulation of FXR transcriptional activity in health and disease: Emerging roles of FXR cofactors and post-translational modifications, Bba-Mol Basis Dis, 1812 (2011) 842-850.
[38] J.K. Kemper, Z. Xiao, B. Ponugoti, J. Miao, S. Fang, D. Kanamaluru, S. Tsang, S.Y. Wu, C.M. Chiang, T.D. Veenstra, FXR Acetylation Is Normally Dynamically Regulated by p300 and SIRT1 but Constitutively Elevated in Metabolic Disease States, Cell Metab, 10 (2009) 392-404.
[39] M. Sun, W. Cui, S.K. Woody, J.L. Staudinger, Pregnane X receptor modulates the inflammatory response in primary cultures of hepatocytes, Drug metabolism and disposition: the biological fate of chemicals, 43 (2015) 335-343.
[40] S.E. Salghetti, A.A. Caudy, J.G. Chenoweth, W.P. Tansey, Regulation of transcriptional activation domain function by ubiquitin, Science, 293 (2001) 1651-1653.
[41] M. Sadowski, R. Suryadinata, A.R. Tan, S.N. Roesley, B. Sarcevic, Protein monoubiquitination and polyubiquitination generate structural diversity to control distinct biological processes, IUBMB life, 64 (2012) 136-142.
[42] A.P. Dennis, B.W. O'Malley, Rush hour at the promoter: how the ubiquitin-proteasome pathway polices the traffic flow of nuclear receptor-dependent transcription, The Journal of steroid biochemistry and molecular biology, 93 (2005) 139-151.
[43] C. Choudhary, B.T. Weinert, Y. Nishida, E. Verdin, M. Mann, The growing landscape of lysine acetylation links metabolism and cell signalling, Nature reviews. Molecular cell biology, 15 (2014) 536-550.

Chapter 4: Identification and Mechanistic Analyses of PXR Phosphorylation

ABSTRACT

The nuclear receptor Pregnane X Receptor (PXR, NR1I2) is a DNA-binding transcription factor that primarily regulates gene expression of enzymes involved in xenobiotic metabolism and transport in the liver and intestines. Upon ligand binding, PXR undergoes a conformational change to dissociate from the corepressor protein complex and to simultaneously recruit the coactivator protein complex. Many mechanistic investigations have revealed that post-translational modifications (PTMs) play a critical role in modulating PXR biological activity. In the current study, we report that phosphorylation regulates the circulation of coregulator protein complex at the level of PXR. We detected PXR phosphorylation at T135 and S221 in primary hepatocytes using a novel LC-MS/MS-based proteomic approach. To investigate the biological outcome of PXR phosphorylation at the identified sites, we constructed both phosphomimetic and phosphodeficient mutants of PXR. Phosphorylation at either T135 or S221 suppresses the trans-activation capacity of PXR. We next employed a mammalian two-hybrid system to examine whether phosphorylation affects the PXR-coregulator protein-protein interaction. Constitutive phosphorylation at either T135 or S221 inhibits PXR heterodimerization with retinoid x receptor alpha (RXRα). Moreover, S221 phosphorylation dramatically induces PXR interaction with the nuclear receptor corepressor 1 (NCoR1) while suppressing its interaction with coactivator proteins including steroid receptor coactivator 1 (SRC1), glucocorticoid receptor-interacting protein 1 (GRIP1), and peroxisome proliferator-activated receptor (PPAR)-binding protein (PBP). Interestingly, T135 phosphorylation shows no effect on PXR-NCoR1 interaction. Mutations at T135 inhibit PXR association with the coactivator proteins SRC1, PBP,

and corepressor NCoR1 in a phosphorylation independent manner. Furthermore, we demonstrate that neither T135 nor S221 phosphorylation affects the ubiquitination of PXR. In conclusion, our results suggest site-specific phosphorylation determines the coordination of coregulator protein complexes at the level of PXR.

4.1 INTRODUCTION

Pregnane X Receptor (PXR, NR1I2) belongs to the nuclear receptor superfamily and is enriched in liver and intestine. The nuclear receptor family members are evolutionarily conserved transcription factors that share a similar functional structure comprised of an N-terminal DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD) and a flexible hinge region between DBD and LBD. Unlike other classic nuclear receptors, PXR LBD possesses a large volume and flexible binding cavity that enables the accommodation of a broad spectrum of compounds to trigger PXR transcriptional activity. Such compounds known as PXR ligands are comprised of steroid hormones, bile acids, environmental toxins, and most xenobiotic chemicals. In response to ligand activation, PXR attaches to a DNA promoter region to facilitate the activation of genes involved in the regulation of drug metabolism and efflux. The prototypical target genes of PXR include Cytochrome P450 family 3 subfamily A member 4 (CYP3A4) and multi-drug resistance gene 1 (MDR1). Studies have shown that CYP3A4 is responsible for metabolizing more than 50% of prescribed drugs for humans(1-3), while MDR1, encoding p-glycoprotein 1 (P-gp 1), serves as an ATP-dependent efflux pump for xenobiotic substances(4,5). Accumulating evidence indicates that PXR plays a critical role in mediating adverse drug-drug interaction, drug toxicity, and drug resistance to cancer chemotherapy in humans through direct activation of CYP450 enzymes and MDR1(6-11). Several lines of

evidence show that PXR serves as a negative regulator of the inflammatory response in the intestines and liver(12-14). The clinical observation of patients suffering from inflammatory bowel disease (IBD) have impaired drug detoxification response suggests a mutual suppression between PXR and NF-κB. PXR is implicated in numerous human diseases include chronic inflammatory liver disease, IBD, diabetes, and many cancer types(14-21). Therefore, targeting PXR has become an attractive therapeutic strategy to combat the chronic inflammatory diseases in the liver and intestines.

PXR-mediated gene activation requires PXR coordination with coregulator proteins. PXR initially binds to the corepressor protein complex in the silent state. Ligand-activated PXR protein undergoes a conformational change to dissociate from the corepressor protein complex followed by the recruitment of the coactivator protein complex to initiate a full activation of its target genes. The major coactivator proteins involved in PXR-mediated trans-activation are ligand-dependent steroid receptor coactivator (SRC) family members SRC1, SRC2 (also known as glucocorticoid receptor-interacting protein 1, GRIP1), and transcription mediator PPAR-interacting protein (PBP). The nuclear corepressor protein 1 (NCoR1) and the silencing mediator for retinoid or thyroid hormone receptors (SMRT) are the two essential corepressor proteins for PXR-mediated gene silencing. Understanding the precise circulation of the coregulator protein exchange in a temporal and spatial manner in response to xenobiotic stimulus and pathogenic challenges is critical for unraveling the mechanism of PXR-initiated drug-drug interaction and drug resistance.

To the present day, the mechanism by which PXR suppresses the inflammatory response in the liver remains unclear. Intensive research has been conducted to examine the involvement of post-translational modifications (PTMs) in regulating PXR biological activity in the liver(22-

30). Such PTMs include phosphorylation, acetylation, ubiquitination, and SUMOylation. Previous studies from our laboratory have demonstrated that cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC)-mediated phosphorylation of PXR at predicted sites inhibits its trans-activation capacity through modulating PXR heterodimerization with RXRα and its association with coregulator proteins(22,31,32). To date, the specific phosphorylation sites on PXR protein in primary hepatocytes remain unknown. In the current study, we utilized a novel LC-MS/MS-based proteomic approach established by our laboratory to identify the phosphorylation sites of PXR protein in primary mouse hepatocytes(29). We identified that PXR is phosphorylated at Threonine 135 (T135) and Serine 221 (S221). To examine the biological outcome of the site-specific phosphorylation of PXR, we constructed the phosphomimetic mutants T135D and S221D, and the phosphodeficient mutant T135A. Our results reveal that phosphorylation of PXR at both identified sites indeed impairs PXR-mediated transactivity. The heterodimerization of PXR-RXRα, which is considered a critical step for gene activation, is also inhibited by PXR phosphorylation at both T135 and S221. Additionally, S221 phosphorylation results in a decreased association with all three of the tested coactivator proteins and a significant induction regarding association with corepressor proteins NCoR1 and SMRT. However, T135 phosphorylation results in reduced association with coactivator GRIP1 and corepressor NCoR1. In summary, we identified novel phosphorylation sites of PXR in primary cultures of mouse hepatocytes and our further investigation suggested a site-specific phosphorylation event that inhibits PXR transactivation capacity through governing the coregulator protein circulation. Our findings provide novel insights into the mechanism of phosphorylation in the regulation of PXR-mediated gene repression in the liver.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals and Plasmids.

Rifampicin and recombinant human TNFα were purchased from Sigma-Aldrich (557303) and Thermo Fisher (PHC3015), respectively. All other reagents including culture medium for primary hepatocytes and mammalian cell lines were purchased from standard sources. Human PXR wild type and mutant constructs were fused to the VP16 transcriptional activation domain by sub-cloning into the pVP16 expression vector (Clontech, Mountain View, CA) at EcoRI and BamHI restriction enzyme sites. GAL4-SRC1, GAL4-GRIP1, GAL4-PBP, GAL4-NCoR1, and GAL4-SMRT expression vectors were generously given by Dr. Barry Forman (City of Hope, CA). These expression vectors were constructed as previously described(33). To generate the GAL4- RXRα expression vector, the cDNA-encoding human RXRα was inserted into the GLA4 expression vector using BamHI and EcoRI restriction sites. The pFR-LUC reporter gene, which is responsive to GAL4-fusion proteins, is commercially available (BD Biosciences, San Jose, CA). The CYP3A4 reporter plasmid XREM-Luc was previously described(29). The adenoviral expression construct encoding human PXR (Ad-$(His)_6$-PXR) was described previously(29).

4.2.2 Site-directed Mutagenesis.

The identified phosphorylation sites of PXR (T135, S221) were either mutated to an aspartic acid as a phosphomimetic mutation or mutated to an alanine as a phosphodeficient mutation. The mutant pCMV-FLAG-hPXR expression vectors were generated by site-directed mutagenesis with the use of the QuikChange Mutagenesis system (Stratagene, La Jolla, CA). Primer sequences used for site-directed mutagenesis are shown in **Table 4-1**.

Table 4-1. PCR primers used in site-direct mutagenesis

Amino Acid	Oligos for Mutagenesis to A		Oligos for Mutagenesis to D	
T135	Left Primer	5’ tgaacggacaggggctcagccactggg 3’	Left Primer	5’ aagtgaacggacaggggatcagccactgggagtg 3’
	Right Primer	5’ cccagtggctgagcccctgtccgttca 3’	Right Primer	5’ cactcccagtggctgatcccctgtccgttcactt 3’
S221	Left Primer	5' ttgtagttccagacagcgccatcctccccccg 3'	Left Primer	5' tttgtagttccagacatcgccatcctccccccgc 3'
	Right Primer	5' cgggggggaggatggcgctgtctggaactacaa 3'	Right Primer	5' gcgggggggaggatggcgatgtctggaactacaaa 3'

4.2.3 Isolation and Culturing of Primary Hepatocytes.

Primary hepatocytes were isolated from C57BL6 mice at the age of 6-10 weeks with a standard collagenase perfusion procedure as previously described(34). Potential sex differences were evaluated and identical results were obtained from both male and female mice. The representative results were acquired from male mice.

4.2.4 Cell-Based Cobalt-bead Affinity Pull-Down Assay.

Post-translationally modified PXR was purified and enriched from either primary cultured mouse hepatocytes or hepatoma cell line Hepa1-6 cells as previously described(35).

4.2.5 Western Blot Analysis.

Western blot analysis was performed as described previously(36). Antibodies used include the mouse monoclonal anti-PXR antibody (Santa Cruz, H-11), anti-GLA4 (DNA Biding domain) monoclonal antibody (Upstate, 06-262), and an anti-actin monoclonal antibody (MP Biomedicals, 691002).

4.2.6 Liquid Chromatography-Tandem Mass Spectrometry Analysis.

The LC-MS/MS analysis for identification of PXR phosphorylation was performed as described previously(29).

4.2.7 Luciferase Reporter Gene Assay.

The luciferase reporter gene assay was performed as described previously(30).

4.2.8 Statistical Analysis.

Data are presented as mean ± standard error of the mean (SEM) and analyzed with Prism 7 software (GraphPad, La Jolla, CA). All data were analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA. Post hoc tests were conducted using Dunnett's or Turkey's multiple comparison test. To determine the differences between groups, a value of $p<0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Identification of PXR Phosphorylation Sites Using LC-MS/MS in Primary Hepatocytes.

It is a well-established notion that site-specific phosphorylation of PXR contributes to the PXR-mediated gene activation through modulating a broad range of its biological activities including heterodimerization, DNA-binding, and coregulator interactions(22,31,32,37-39). The reported kinases that facilitate such site-specific phosphorylation of PXR include 70-kDa ribosomal S6 kinase (p70 S6K)(32,39,40), PKA(22,32,37), PKC(31,32), cyclin-dependent kinase 2 (CDK2)(24,41-43), and CDK5(44). Many efforts have been made to detect the sites of PXR phosphorylation *in vitro* or in immortalized cell lines. To achieve a more relevant biological meaning, we sought to identify the sites of PXR phosphorylation in primary cultured mouse hepatocytes. A previously reported adenoviral expression system (Ad-$(His)_6$-PXR) was utilized

to achieve a robust protein expression and a convenient protein purification in primary mouse hepatocytes. In general, we transduced primary mouse hepatocytes with the adenoviral construct encoding human PXR (Ad-$(His)_6$-PXR) for 48 hours followed by the treatment of either 0.1% DMSO (Veh) or 10μM rifampicin (Rif) for an additional 24 hours(29). This approach has yielded abundant expression of human PXR protein in primary mouse hepatocytes as demonstrated using Coomassie blue staining **(Figure 4-1A)**. The corresponding western blot indicates the positions of immunoreactive PXR proteins using an anti-PXR monoclonal antibody. The overall coverage of the IMAC-enriched tryptic peptides of Ad-$(His)_6$-PXR in LC-MS/MS analysis is approximately 60%. We have identified two phosphorylation sites of human PXR protein, which are threonine 135 (T135) and serine 221 (S221) **(Figure 4-2B-C)**. The mass spectrometric parameters for the identification of PXR phosphorylation sites are listed in **Table 4-2**. It is worth noting that while T135 phosphorylation was only present in the lower band of PXR protein across treatment, S221 phosphorylation was detected in both bands of PXRs.

Table 4-2. Mass spectrometry analysis of PXR phosphorylation.

Identified Phosphorylation Site	**Peptide Sequence**	**Charge of Parent Ion**	***m/z***	**Parent Error (ppm)**	**Retention Time (Sec)**
T135	TG**T**QPLGVQGLTEEQR	2	897.44	11	1200
S221	GEDG**S**VWNYKPPADSGGK	2	972.42	11	1110

Figure 4-1A.

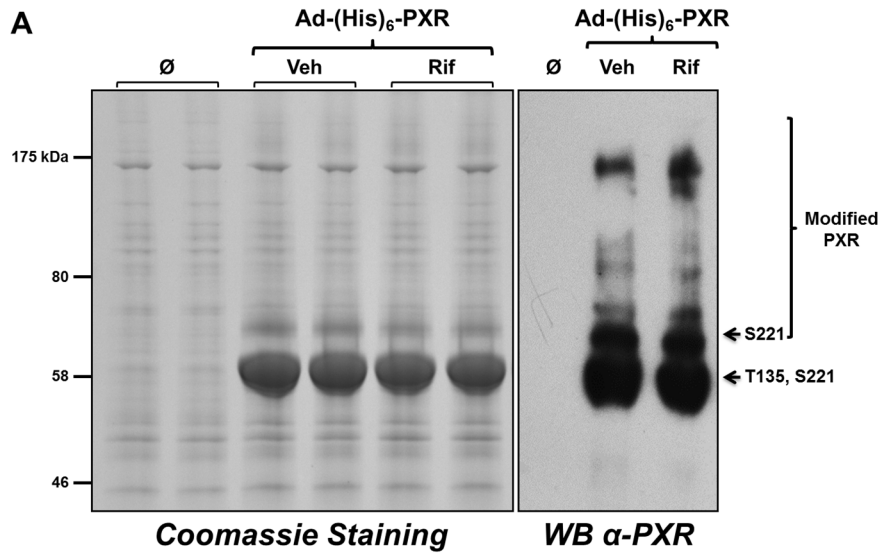


Figure 4-1B.

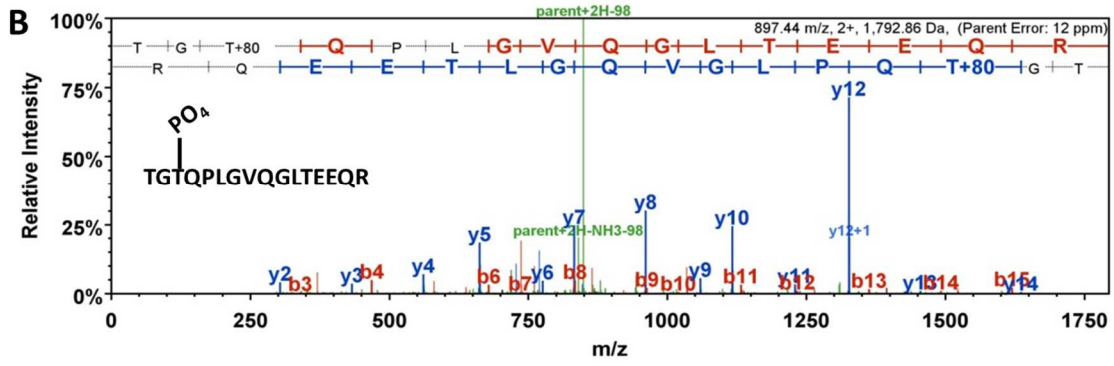


Figure 4-1C.

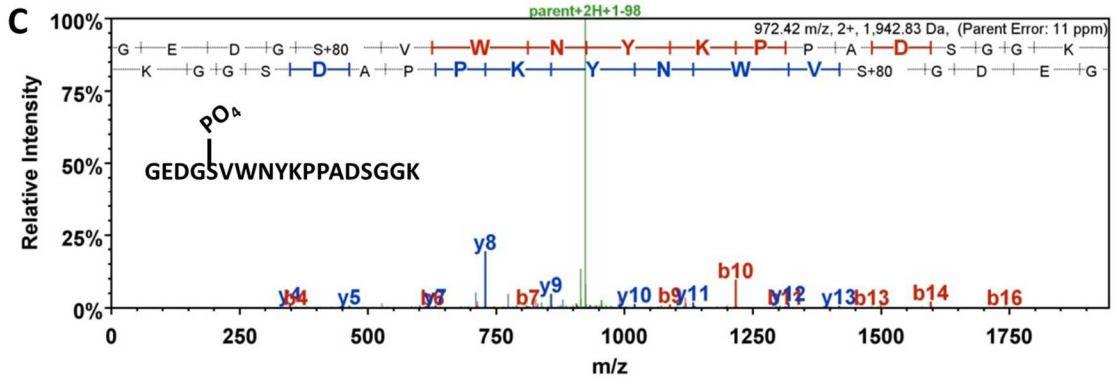


Figure 4-1. Identification of the Phosphorylation Sites of PXR using LC-MS/MS. (A) Primary hepatocytes were isolated from C57Bl6 mice and were subsequently left either non-transduced or transduced as indicated for 48 hours. Hepatocytes were treated with 0.1% DMSO or 10μM rifampicin 24 hours prior to harvest. Hepatocytes were lysed in a potent denaturing buffer as described in Materials and Methods. Cell lysates were subjected to enrichment and purification using cobalt-bead affinity pull-down assay. Captured unmodified and post-translational modified PXR protein were eluted using the 2X-Laemmli buffer and resolved in 10% SDS-PAGE. Western blot analysis was performed using a monoclonal anti-PXR antibody (Santa Cruz, H-11, sc-48340) that detects all modified forms of PXR protein. Arrow (←) represents detected phosphorylated PXR protein. An identical gel has proceeded to Coomassie blue staining. **(B)** Threonine 135 (T135) phosphorylation on human PXR was identified according to the assignment of multiple product ions (b and y ions) in the MS2 scan of the precursor ion at m/z 897.44 to the PXR tryptic peptide sequence, with a mass addition of 80 at the threonine residue (phosphate group). **(C)** Serine 221 (S221) phosphorylation on human PXR was identified according to the assignment of multiple product ions (b and y ions) in the MS2 scan of the precursor ion at m/z 972.42 to the PXR tryptic peptide sequence, with a mass addition of 80 at the threonine residue (phosphate group).

4.3.2 Constitutive Phosphorylation Inhibits the Trans-activation Capacity of PXR.

To examine the biological outcome of PXR phosphorylation at identified sites, we conducted site-direct mutagenesis to construct PXR phosphomimetic mutants T135D, S221D, and phosphodeficient mutant T135A in a pCMV-FLAG expression vector. Unfortunately, we were unable to generate the phosphodeficient mutation at S221 due to a technical difficulty to modify the guanine-enriched region on DNA. We first sought to determine whether phosphorylation at indicted site alters PXR basal or ligand-inducible transcriptional activity using a cell line-based reporter gene assay. An expression vector encoding wild type or mutant PXR was co-transfected with the PXR-dependent CYP3A4 reporter gene XREM-Luc. Twenty-four hours post-transfection, cells were treated with either vehicle (0.1% DMSO) or Rifampicin (10 μM) for an additional 24 hours. The transcription activities of XREM-Luc were examined using luciferase assay. Fold induction of each experimental group was compared to the vehicle-treated wild type PXR group. Both of the T135D and the S221D mutation on PXR significantly impair the ligand-mediated transcriptional activation ($p<0.0001$, $p<0.0001$, respectively). These results are along in line with previous findings that PXR phosphorylation leads mostly to a suppressive effect on its trans-activation capacity(31,32,40,45).

Figure 4-2.

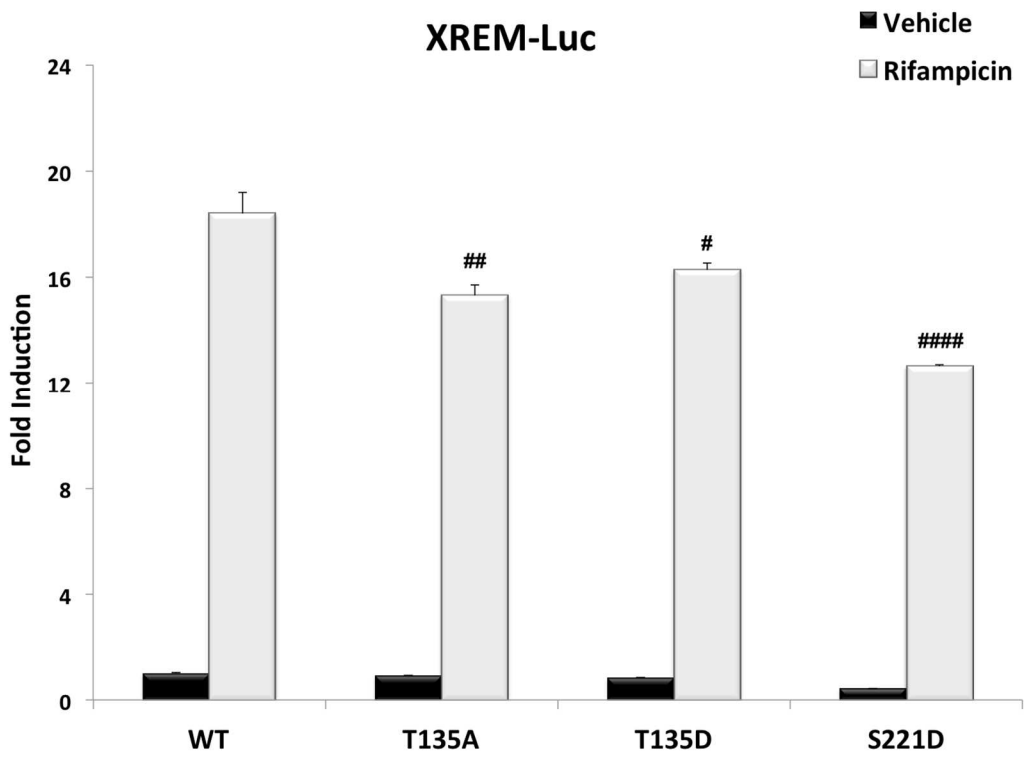


Figure 4-2. Constitutive Phosphorylation Inhibits the Trans-activation Capacity of PXR. CV-1 cells were transfected with a CYP3A4-derived reporter gene XREM-Luc and expression vector encoding FLAG tagged-PXR or its mutant as indicated. Twenty-four hours post-transfection, cells were treated with 0.1% DMSO or 10μM rifampicin for an additional 24 hours. Luciferase activity was normalized to β-gal readouts. Data is presented as fold induction ± SEM. Two-way ANOVA shows a significant effect of the T135A mutation {F (1, 12)=12.35, $p< 0.01$}, a significant effect of ligand-treatment {F (1, 12)=1248, $p<0.0001$}, and a significant interaction between ligand-treatment and phosphor-deficient mutation on PXR transactivation capacity {F (1, 12)=11.26. $p< 0.005$}. Tukey's multiple comparison test indicate n.s., n=4 compared with vehicle-treated WT-PXR group; ##, $p<0.001$, n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows a significant effect of the T135D mutation {F (1, 12)=7.265, $p< 0.05$}, a significant effect of ligand-treatment {F (1, 12)=1508, $p<0.0001$}, and a significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=5.425, $p<0.05$}. Tukey's multiple comparison test indicate n.s., n=4 compared with vehicle-treated WT-PXR group; #, $p<0.05$, n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows a significant effect of the S221D mutation {F (1, 12)=61, $p< 0.0001$}, a significant effect of ligand-treatment {F (1, 12)=1334, $p<0.0001$}, and a significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=41.23, $p<0.0001$}. Tukey's multiple comparison test indicate n.s., n=4 compared with vehicle-treated WT-PXR group; ####, $p<0.0001$, n=4 compared with rifampicin-treated WT-PXR group.

4.3.3 Validation of the Mammalian-Two-Hybrid System.

PXR-mediated transcriptional events require the recruitment of a variety of positive and negative regulatory proteins, namely coactivator proteins and corepressor proteins. The potential mechanism that controls the circulation of the coregulator protein complex at the level of PXR has been hypothesized and proven to be post-translational modifications. As the most studied post-translational modification of PXR, phosphorylation is considered as a negative regulator of PXR-mediated transcription activation. Several lines of evidence suggest that phosphorylation at certain sites ameliorates PXR heterodimerization with RXRα(23,32). However, whether phosphorylation contributes to the coregulator exchange on PXR is poorly studied. Therefore, we sought to utilize a mammalian two-hybrid system to assess the effect of site-specific phosphorylation on PXR-coregulator protein-protein interaction. We first constructed fusion genes by sub-cloning the PXR mutants into a VP16 vector. All of the VP16-PXR expression vectors are expressed in CV-1 cells as the immunoreactivity was detected using a monoclonal anti-PXR antibody **(Figure 4-3A)**. Next, a similar experiment was accomplished for the validation of expression vectors encoding GAL4-fused coregulator proteins. The protein expression levels were evaluated by western blot analysis using a monoclonal anti-GAL4-DBD antibody **(Figure 4-3B)**. At last, we conducted mammalian two-hybrid assay to examine the capability of VP16-PXR to interact with its dimerization partner RXRα, the coactivator proteins SRC1, GRIP1, PBP, and the corepressor proteins NCoR1 and SMRT. CV-1 cells were co-transfected with a GAL4-dependent reporter gene pFR-Luc, the expression vector encoding VP16-PXR protein, together with GAL4-coregulator fusion proteins as indicated. Twenty-four hours post-transfection, cells were treated either with 0.1% DMSO (Vehicle) or with the ligand of PXR Rifampicin (10μM) for an additional 24 hours. Luciferase activity was measured 48

hours post-transfection. The VP16-PXR fusion protein efficiently interacts with all tested coregulator proteins and its heterodimer partner RXRα. In addition, ligand activation results in a decreased heterodimerization of PXR-RXRα **(Figure 4-3C)**. As expected, ligand activation induces the interaction between PXR and coactivator proteins including SRC1, GRIP1, and PBP, while the interactions between PXR and co-repressor proteins namely NCoR1 and SMRT are inhibited **(Figure 4-3C)**.

Figure 4-3.

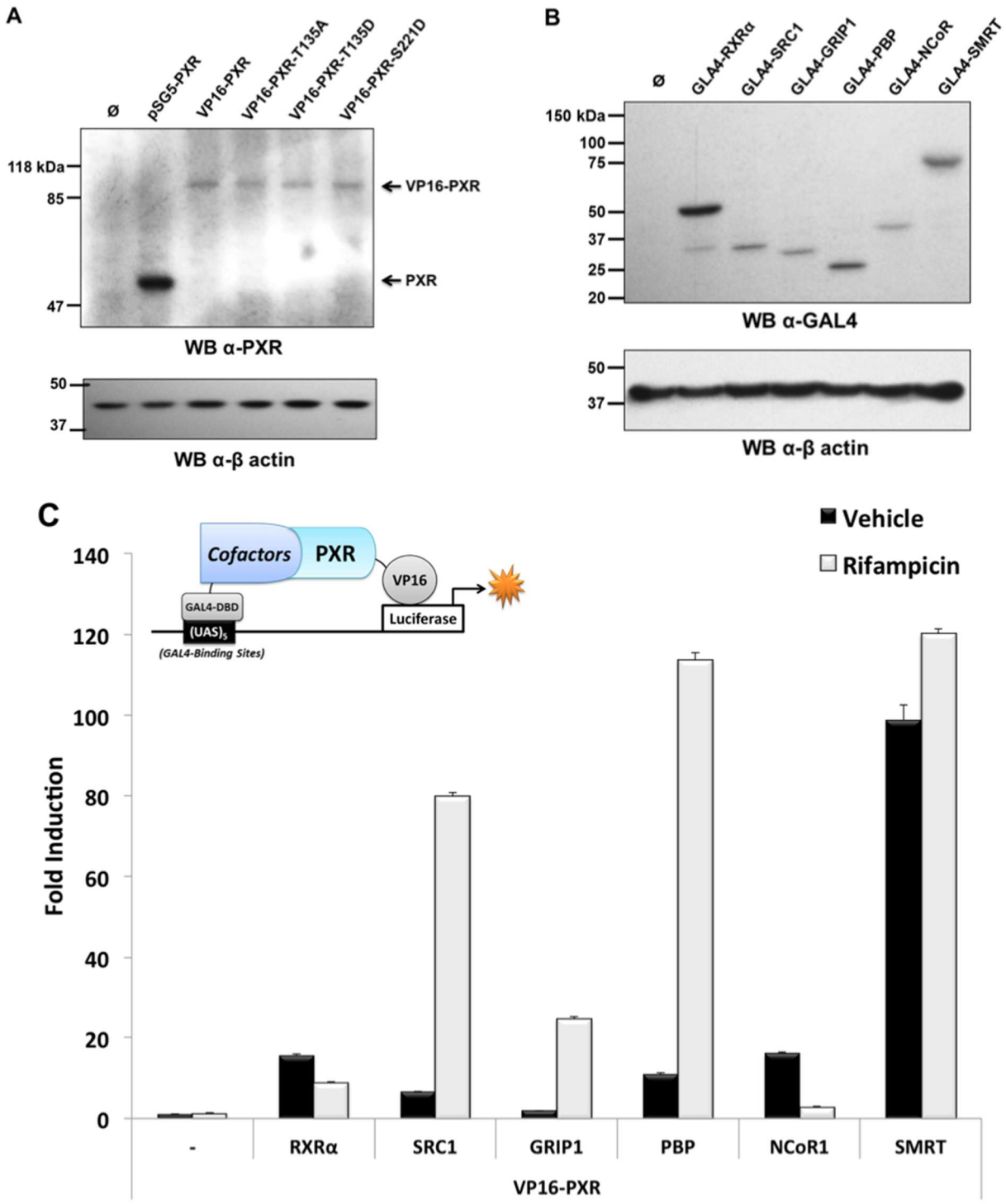

A
Ø
pSG5-PXR
VP16-PXR
VP16-PXR-T135A
VP16-PXR-T135D
VP16-PXR-S221D
118 kDa
85
47
VP16-PXR
PXR
WB α-PXR
50
37
WB α-β actin
B
Ø
GLA4-RXRα
GLA4-SRC1
GLA4-GRIP1
GLA4-PBP
GLA4-NCoR
GLA4-SMRT
150 kDa
100
75
50
37
25
20
WB α-GAL4
50
37
WB α-β actin
C
Cofactors
PXR
VP16
GAL4-DBD
(UAS)5
Luciferase
(GAL4-Binding Sites)
Vehicle
Rifampicin
Fold Induction
140
120
100
80
60
40
20
0
-
RXRα
SRC1
GRIP1
PBP
NCoR1
SMRT
VP16-PXR

Figure 4-3. Validation of the Mammalian Two-Hybrid System. (A-B) Indicated expression vectors were transfected into CV-1 cells. Forty-eight hours post-transfection, whole cell lysates were collected. **(A)** Expression constructs that encoding PXR proteins were subjected to 10% SDS-PAGE and subsequent western blot analysis using a monoclonal anti-PXR antibody (Santa Cruz, H-11, sc-48340). The arrows (←) indicate detected PXR proteins. The expression vector encoding pSG5-PXR protein (positive control) is approximately 52kDa in size (lower arrow). The expression vectors encoding VP16-PXR proteins are approximately 100kDa in size (upper arrow). **(B)** Expression vectors encoding GAL4-coregulator proteins were subjected to 4-20% gradient SDS-PAGE and subsequent western blot analysis using an anti-GLA4 (DNA Binding domain) monoclonal antibody (Upstate, 06-262). **(C)** CV-1 cells were co-transfected with a mammalian two-hybrid reporter gene pFR-Luc, expression vector encoding VP16-PXR, and the expression vectors encoding GAL4-RXRα, GAL4-SRC1, GAL4-GRIP1, GAL4-PBP, GLA4-NCoR1, or GAL4-SMRT as indicated. Twenty-four hours post-transfection, cells were treated with 0.1% DMSO or 10μM rifampicin for an additional 24 hours. Luciferase activity was normalized to β-gal readouts. Data is presented as fold induction ± SEM.

4.3.4 Phosphorylation Inhibits the PXR-RXRα Heterodimerization.

The heterodimerization of PXR and RXRα is the first wave of action for ligand-mediated transcriptional activation. While sensing the ligand stimulation, PXR and RXRα form a heterodimer and subsequently attach to the promoter region to initiate gene activation. A previous study in our laboratory demonstrated that phosphorylation at certain sites on PXR impairs the heterodimerization of PXR-RXRα(32). Therefore, we sought to determine whether the T135 and S221 phosphorylation on PXR weakens its heterodimerization with RXRα. CV-1 cells were co-transfected with the reporter gene pFR-Luc, the expression vector encoding the GAL4-RXRα fusion protein, and the wild type or mutant form of VP16-PXR fusion construct. Twenty-four hours post-transfection, cells were treated with 0.1% DMSO (Vehicle) or Rifampicin (10μM) for an additional 24 hours. The luciferase activity was measured to evaluate the interaction between PXR and RXRα. Both T135D and S221D mutations interrupt the ability of PXR to dimerization with RXRα **(Figure 4-4)**. Moreover, the phosphor-deficient mutant T135A abolishes the phosphorylation-reduced PXR- RXRα heterodimerization **(Figure 4-4)**.

Figure 4-4.

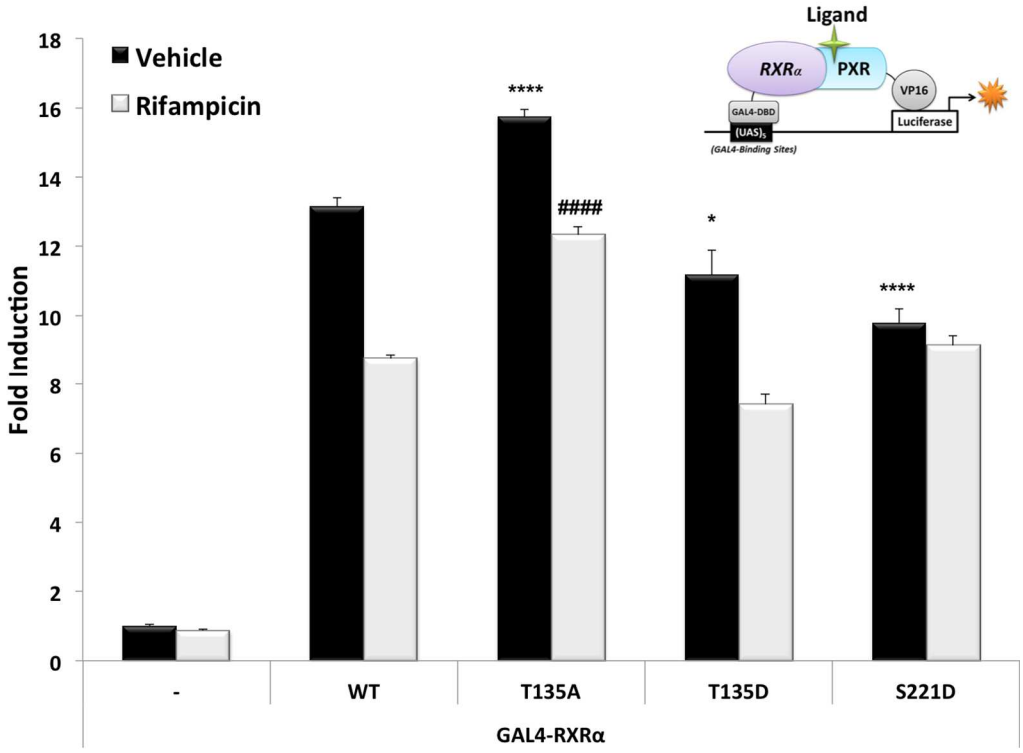


Figure 4-4. Phosphorylation Inhibits the Heterodimerization of PXR-RXRα. CV-1 cells were co-transfected with a mammalian two-hybrid reporter gene pFR-Luc, expression vector encoding GAL4-RXRα, and the expression vectors encoding VP16-PXR as indicated. Twenty-four hours post-transfection, cells were treated with 0.1% DMSO or 10μM rifampicin for an additional 24 hours. Luciferase activity was normalized to β-gal readouts. Data presentation is fold induction ± SEM. Two-way ANOVA shows a significant effect of the T135A mutation {F (1, 12)=228.6, p< 0.0001}, a significant effect of ligand-treatment {F (1, 12)=367.1, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-deficient mutation on PXR transactivation capacity {F (1, 12)=5.837, p<0.05}. Tukey's multiple comparison test indicate ****, p<0.0001, n=4 compared with vehicle-treated WT-PXR group; ####, p<0.0001, n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows a significant effect of the T135D mutation {F (1, 12)=16.61, p< 0.01}, a significant effect of ligand-treatment {F (1, 12)=101.3, p<0.0001}, and no significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=0.5963, p=0.4549}. Tukey's multiple comparison test indicate *, p<0.05, n=4 compared with vehicle-treated WT-PXR group; n.s., n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows a significant effect of the S221D mutation {F (1, 12)=26.37, p< 0.0005}, a significant effect of ligand-treatment {F (1, 12)=74.67, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=41.64, p<0.0001}. Tukey's multiple comparison test indicate ****, p<0.0001, n=4 compared with vehicle-treated WT-PXR group; n.s., n=4 compared with rifampicin-treated WT-PXR group.

4.3.5 Site-Specific Phosphorylation Determines PXR Cooperation with Corepressor Proteins.

To decipher the mechanism regarding phosphorylation-mediated suppression on PXR trans-activation capacity, we attempted to evaluate whether phosphorylation at identified sites alters PXR cooperation with its corepressor proteins. Standard mammalian two-hybrid assays were conducted and the luciferase activity was measured for quantitative analysis. Phosphodeficient mutation at T135 shows no effect on the PXR-NCoR1 protein-protein interaction, while the S221D significantly inducing PXR association with NCoR1 by 40 fold ($p<0.0001$) **(Figure 4-5A)**. It is interesting to note that the phosphomimetic mutant S221D exhibits an opposite effect on PXR-SMRT protein-protein interactions compared to wild type PXR. The phosphomimetic mutant T135D significantly reduces PXR interaction with SMRT ($p<0.0001$) while S221D shows no effect on the PXR-SMRT protein-protein interaction **(Figure 4-5B)**. Moreover, Ligand activation results in an induction of wild type PXR-SMRT protein-protein interaction, whereas PXR S221 phosphorylation reduces this ligand-induced PXR-SMRT protein-protein interaction as shown in **Figure 4-5B**. Interestingly, the PXR-SMRT protein-protein interaction is significantly inhibited by the phosphodeficient mutation T135A ($p < 0.0001$). One possible theory regarding this phosphorylation-independent suppression is that the mutation at T135 disrupts the architecture of PXR protein for the proper binding of corepressor protein SMRT.

Figure 4-5A.

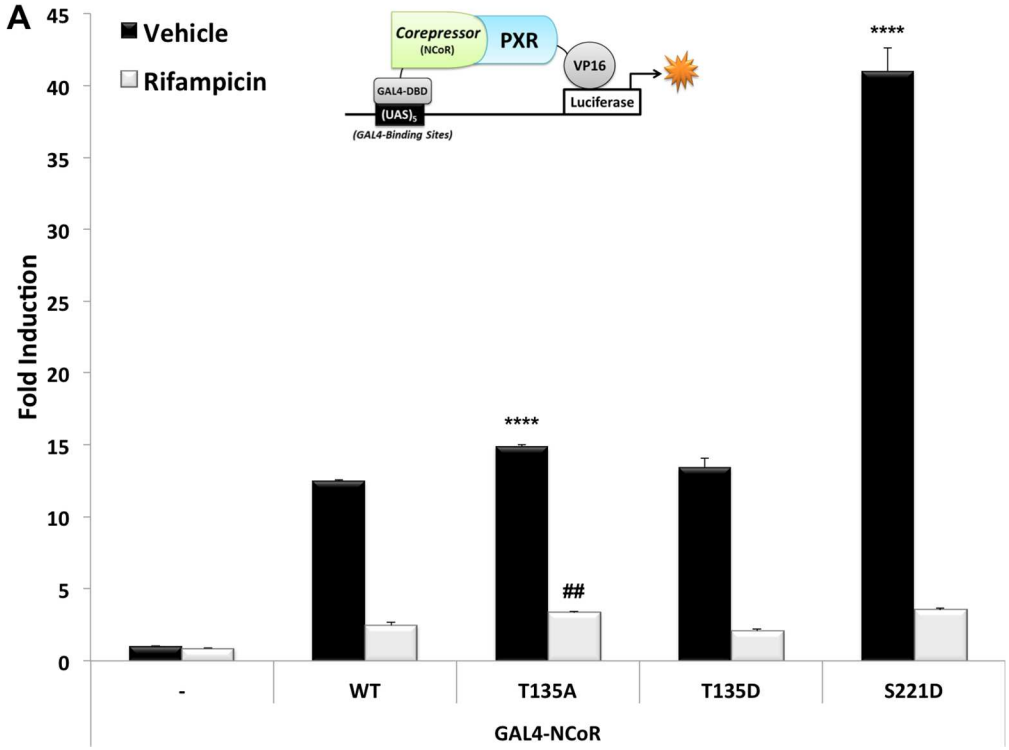


Figure 4-5B.

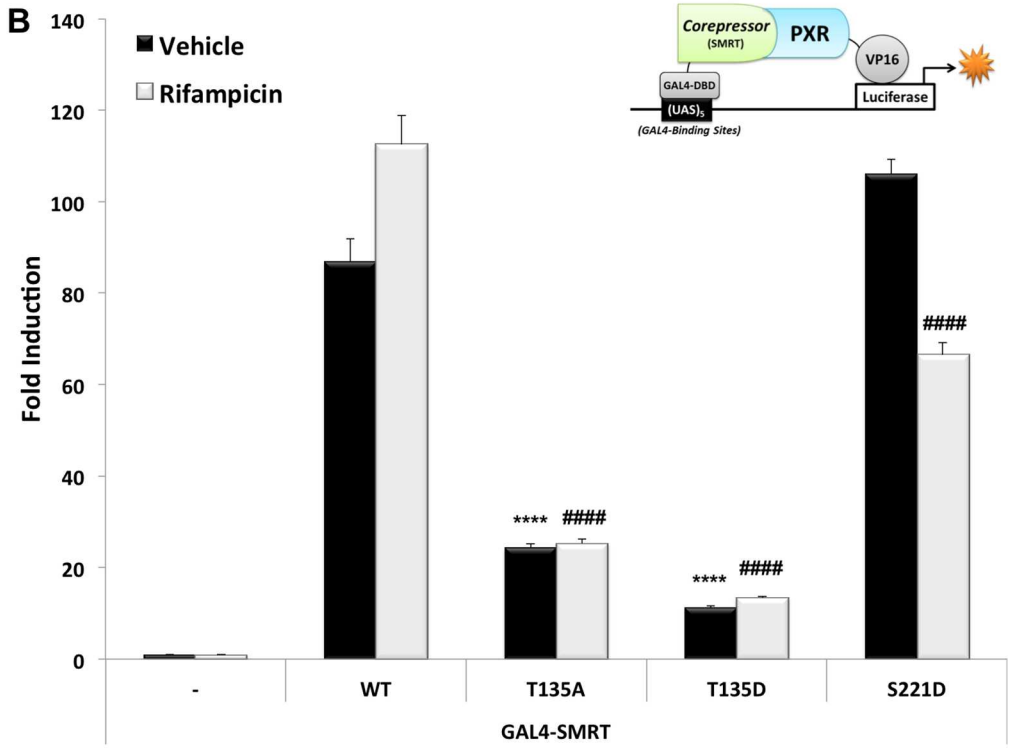


Figure 4-5. Phosphorylation Alters PXR Association with Different Corepressor Proteins. CV-1 cells were co-transfected with a mammalian two-hybrid reporter gene pFR-Luc, expression vector encoding VP16-PXR, and the expression vectors encoding GAL4 fused genes as indicated. Twenty-four hours post-transfection, cells were treated with 0.1% DMSO or 10μM rifampicin for an additional 24 hours. Luciferase activity was normalized to β-gal readouts. Data presentation is fold induction ± SEM. **(A)** Quantitative evaluation of the PXR-NCoR1 interaction using mammalian two-hybrid assay. Two-way ANOVA shows a significant effect of the T135A mutation {F (1, 12)=168.1, p< 0.0001}, a significant effect of ligand-treatment {F (1, 12)=7344, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-deficient mutation on PXR transactivation capacity {F (1, 12)=35.73, p<0.0001}. Tukey's multiple comparison test indicate ****, p<0.0001, n=4 compared with vehicle-treated WT-PXR group; ##, p<0.01, n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows no significant effect of the T135D mutation {F (1, 12)=0.5267, p=0.4819}, a significant effect of ligand-treatment {F (1, 12)=935.2, p<0.0001}, and no significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=3.38, p=0.0909}. Tukey's multiple comparison test indicate n.s., n=4 compared with vehicle-treated WT-PXR group; n.s., n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows a significant effect of the S221D mutation {F (1, 12)=321.5, p< 0.0001}, a significant effect of ligand-treatment {F (1, 12)=832.1, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=277.1, p<0.0001}. Tukey's multiple comparison test indicate ****, p<0.0001, n=4 compared with vehicle-treated WT-PXR group; n.s., n=4 compared with rifampicin-treated WT-PXR group. **(B)** Quantitative evaluation of the PXR-SMRT interaction using mammalian two-hybrid assay. Two-way ANOVA shows a significant effect of the T135A mutation {F (1, 12)=331.4, p< 0.0001}, a significant effect of ligand-treatment {F (1, 12)=10.29, p<0.01}, and a significant interaction between ligand-treatment and phosphor-deficient mutation on PXR transactivation capacity {F (1, 12)=9.044, p<0.05}. Tukey's multiple comparison test indicate ****, p<0.0001, n=4 compared with vehicle-treated WT-PXR group; ####, p<0.0001, n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows a significant effect of the T135D mutation {F (1, 12)=459.5, p<0.0001}, a significant effect of ligand-treatment {F (1, 12)=11.5, p<0.01}, and a significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=8.286, p<0.05}. Tukey's multiple comparison test indicate ****, p<0.0001, n=4 compared with vehicle-treated WT-PXR group; ####, p<0.0001, n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows a significant effect of the S221D mutation {F (1, 12)=8.521, p< 0.05}, no significant effect of ligand-treatment {F (1, 12)=2.356, p=0.1508}, and a significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=50.53, p<0.0001}. Tukey's multiple comparison test indicate n.s., n=4 compared with vehicle-treated WT-PXR group; ####, p<0.0001, n=4 compared with rifampicin-treated WT-PXR group.

4.3.6 Phosphorylation Compromises PXR Association with Coactivator Proteins.

Gene repression is a spatial and temporal event that is precisely carried out by the circulation of coregulator proteins. Opposite to ligand-mediated gene activation that is executed by the dissociation of corepressor protein complex along with the recruitment of coactivator protein complex, termination of gene expression commands the release of coactivator machinery accompanied by the physical interaction with corepressor protein complex. Therefore, we examined the extent to which phosphorylation-inhibited PXR transcription activity is carried out by accelerated coactivator protein dissociation. Standard mammalian two-hybrid assays were conducted for quantitative evaluation of the protein-protein interactions. Phosphomimetic mutant S221D significantly inhibits PXR interaction with coactivator proteins SRC1 ($p<0.0001$), GRIP1 ($p<0.05$), and PBP ($p<0.0001$) in the absence of ligand **(Figure 4-6)**. Furthermore, S221D decreases the ligand-induced PXR association with coactivator proteins SRC1 ($p<0.0001$), GRIP1 ($p<0.0001$), and PBP ($p<0.0001$). The phosphomimetic mutation at T135D hinders PXR ability to interact with coactivator protein SRC1 ($p<0.01$) and GRIP1 ($p<0.05$) in the presence of ligand. This T135 phosphorylation-mediated suppression on the ligand-induced PXR-coactivator interaction can be abolished by the phosphodeficient mutation. Notably, the constitutive phosphorylation at T135 was unable to interrupt the PXR association with the transcription mediator protein PBP (Figure 4-6C).

Figure 4-6A.

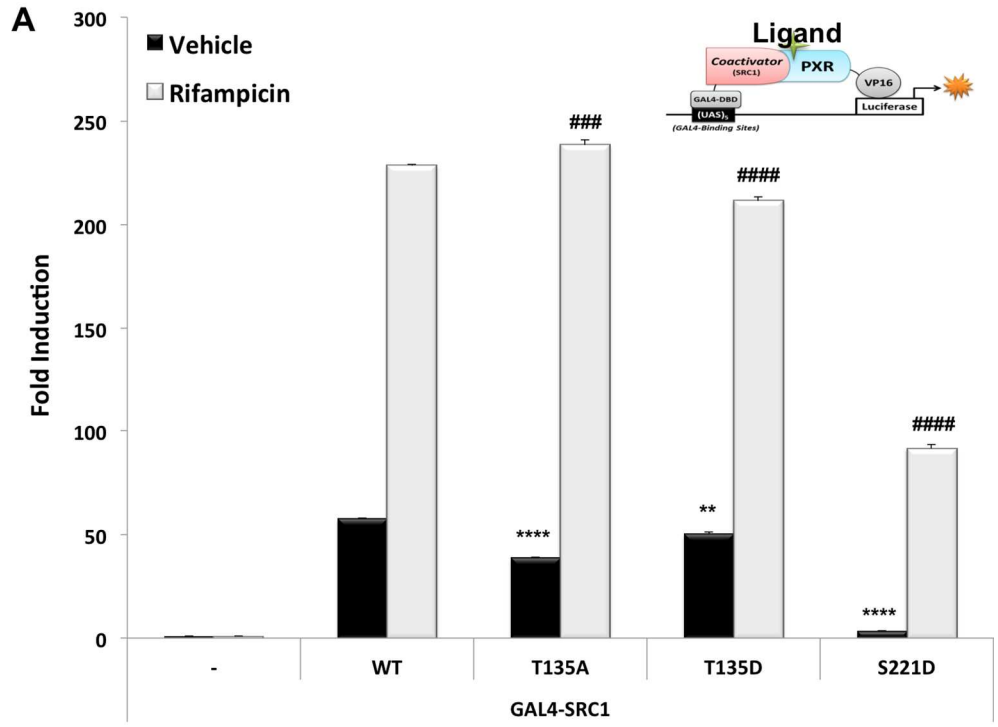


Figure 4-6B.

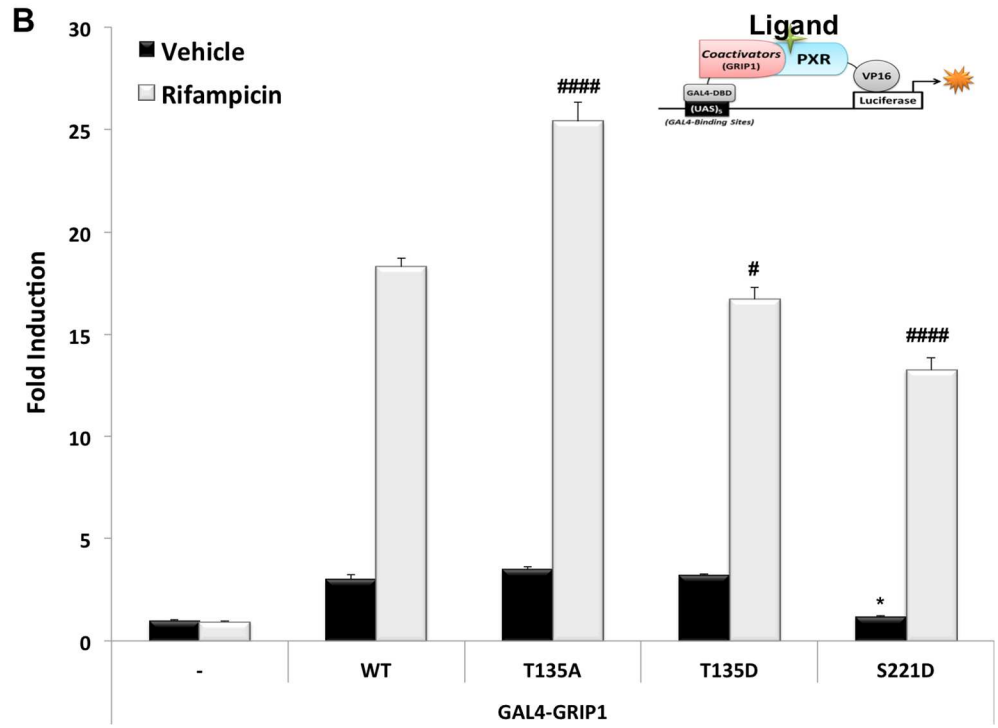


Figure 4-6C.

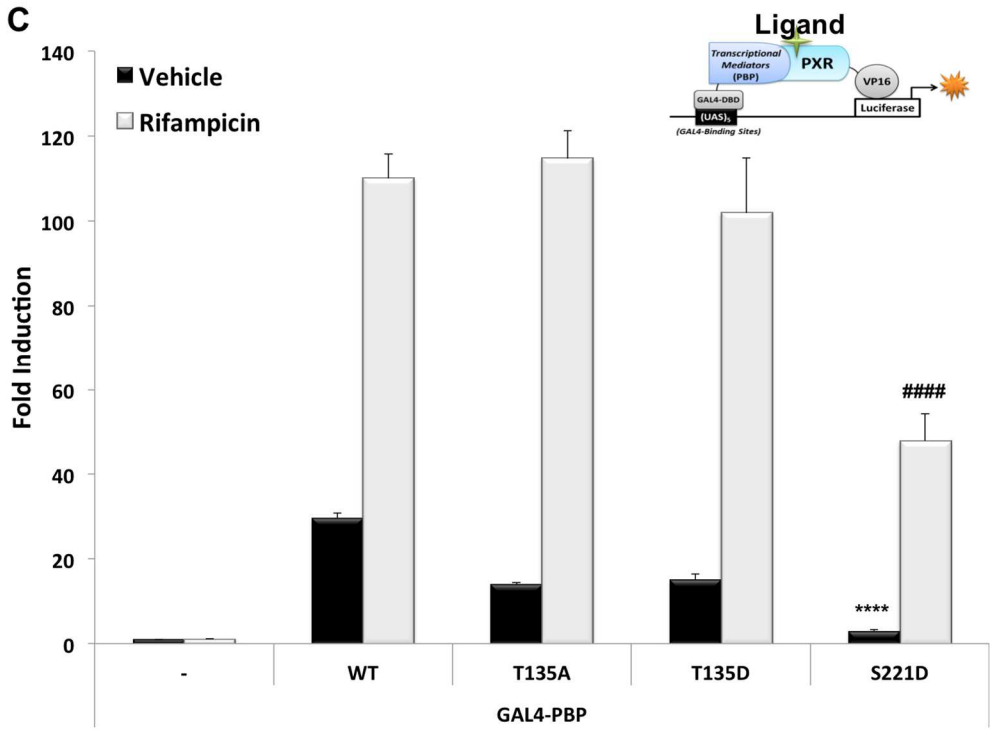


Figure 4-6. Phosphorylation Compromises PXR Association with Coactivator Proteins SRC1, GRIP1, and PBP. CV-1 cells were co-transfected with a mammalian two-hybrid reporter gene pFR-Luc, expression vector encoding VP16-PXR, and the expression vectors encoding GAL4 fused genes as indicated. Twenty-four hours post-transfection, cells were treated with 0.1% DMSO or 10μM rifampicin for an additional 24 hours. Luciferase activity was normalized to β-gal readouts. Data presentation is fold induction ± SEM. **(A)** Quantitative evaluation of the PXR-SRC1 interaction using mammalian two-hybrid assay. Two-way ANOVA shows a significant effect of the T135A mutation {F (1, 12)=14.02, p< 0.01}, a significant effect of ligand-treatment {F (1, 12)=23520, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-deficient mutation on PXR transactivation capacity {F (1, 12)=143.4, p<0.0001}. Tukey's multiple comparison test indicate ****, p<0.0001, n=4 compared with vehicle-treated WT-PXR group; ###, p<0.0005, n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows a significant effect of the T135D mutation {F (1, 12)=138, p<0.0001}, a significant effect of ligand-treatment {F (1, 12)=25364, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=21.77, p<0.005}. Tukey's multiple comparison test indicate **, p<0.01, n=4 compared with vehicle-treated WT-PXR group; ####, p<0.0001, n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows a significant effect of the S221D mutation {F (1, 12)=7487, p< 0.0001}, a significant effect of ligand-treatment {F (1, 12)=13695, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=1402, p<0.0001}. Tukey's multiple comparison test indicate ****, p<0.0001, n=4 compared with vehicle-treated WT-PXR group; ####, p<0.0001, n=4 compared with rifampicin-treated WT-PXR group. **(B)** Quantitative

evaluation of the PXR-GRIP1 interaction using mammalian two-hybrid assay. Two-way ANOVA shows a significant effect of the T135A mutation {F (1, 12)=51.61, p<0.0001}, a significant effect of ligand-treatment {F (1, 12)=1247, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-deficient mutation on PXR transactivation capacity {F (1, 12)=39.33, p<0.0001}. Tukey's multiple comparison test indicate n.s., n=4 compared with vehicle-treated WT-PXR group; ####, p<0.0001, n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows no significant effect of the T135D mutation {F (1, 12)=3.356, p=0.0919}, a significant effect of ligand-treatment {F (1, 12)=1479, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=5.674, p<0.05}. Tukey's multiple comparison test indicate n.s., n=4 compared with vehicle-treated WT-PXR group; #, p<0.05, n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows a significant effect of the S221D mutation {F (1, 12)=75.94, p< 0.0001}, a significant effect of ligand-treatment {F (1, 12)=1191, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=16.78, p<0.01}. Tukey's multiple comparison test indicate *, p<0.05, n=4 compared with vehicle-treated WT-PXR group; ####, p<0.0001, n=4 compared with rifampicin-treated WT-PXR group. **(C)** Quantitative evaluation of the PXR-PBP interaction using mammalian two-hybrid assay. Two-way ANOVA shows no significant effect of the T135A mutation {F (1, 12)=1.6, p=0.0387}, a significant effect of ligand-treatment {F (1, 12)=426.3, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-deficient mutation on PXR transactivation capacity {F (1, 12)=5.386, p<0.05}. Tukey's multiple comparison test indicate n.s., n=4 compared with vehicle-treated WT-PXR group; n.s., n=4 compared with rifampicin-treated WT-PXR group. Two-way ANOVA shows no significant effect of the T135D mutation {F (1, 12)=2.529, p=0.1377}, a significant effect of ligand-treatment {F (1, 12)=135, p<0.0001}, and no significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=0.1968, p=0.6652}. Two-way ANOVA shows a significant effect of the S221D mutation {F (1, 12)=105.2, p< 0.0001}, a significant effect of ligand-treatment {F (1, 12)=208.5, p<0.0001}, and a significant interaction between ligand-treatment and phosphor-mimetic mutation on PXR transactivation capacity {F (1, 12)=16.61, p<0.01}. Tukey's multiple comparison test indicate ****, p<0.05, n=4 compared with vehicle-treated WT-PXR group; ####, p<0.0001, n=4 compared with rifampicin-treated WT-PXR group.

4.3.7 The Effect of T135 and S221 Phosphorylation on the Ubiquitination of PXR.

In our previous studies, we have demonstrated that PXR protein is degraded through the ubiquitin-proteasomal pathway. Moreover, SUMO1-modification inhibits the ubiquitin-mediated proteasomal degradation of PXR. These findings suggest that post-translational modifications crosstalk at the level of PXR to determine its biological fate at the cellular level. Therefore, we sought to determine whether the phosphorylation-mediated repression on the PXR transactivity is carried out by accelerated ubiquitination-mediated degradation. We tested the ubiquitination status of these aforementioned PXR mutants in a mouse hepatoma cell line. Hepa1-6 cells were left non-transfected, or transfected with expression vector encoding $(His)_6$-tagged ubiquitin (His-Ub) and expression vector encoding wild type or phosphomimetic mutant PXR as indicated **(Figure 4-7)**. Forty-eight hours post-transfection, ubiquitinated PXR was enriched and purified using a Cobalt-bead based affinity pull down assay. The immunoreactivity of modified PXR was detected using a monoclonal anti-PXR antibody. Neither T135 phosphorylation nor S221 phosphorylation affects the levels of ubiquitination on PXR.

Figure 4-7.

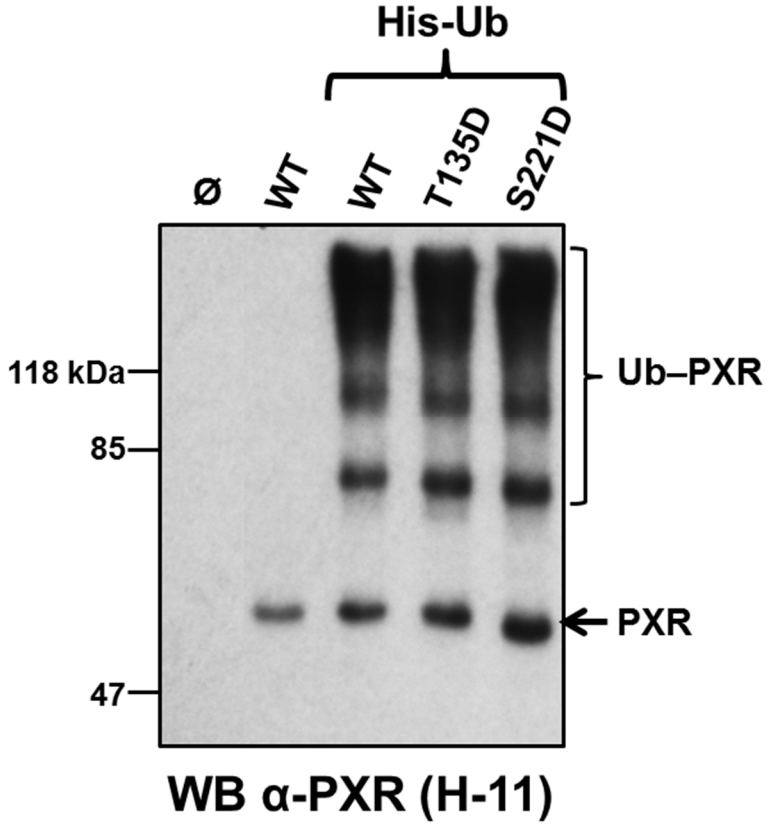


Figure 4-7. The Phosphomimetic Mutations Exhibit No Effect on Ubiquitination of PXR. Hepa1-6 cells were transfected with expression vectors as indicated. Ubiquitinated proteins were enriched using a cobalt bead based-affinity pull down procedure. Captured proteins were subjected to SDS-PAGE, and the blot was probed with a monoclonal anti-PXR antibody (Santa Cruz, H-11) for detecting the PXR immunoreactivity. Arrow (←) indicates the primary form of PXR, and braces indicated ubiquitin-modified PXR.

4.4 DISCUSSION

Utilizing LC-MS/MS-based proteomic approach is a trending strategy to monitor protein PTMs. To overcome the mass spectrometry detection challenge due to the low stoichiometry of PTMs, we have developed an innovative adenoviral expression construct to achieve the high expression of PXR in primary hepatocytes. This strategy allows us to detect PTMs of PXR in a biological environment, which is particularly beneficial for understanding the mechanism of PXR-mediated transcription events in the liver. We have successfully applied this approach to identify various types of PTMs at the level of PXR using LC-MS/MS in our laboratory. In the current study, we have identified two novel phosphorylation sites of PXR at T135 and S221. Structurally, these two phosphorylation sites show great potentials in regulating PXR transactivity in a site-specific fashion. On the one hand, T135 is buried in the hinge region that connects DBD and LBD of PXR. The hinge region controls the particular orientation of the DBD and LBD upon ligand binding which eventually leads to nuclear localization and activation(46,47). Several lines of evidence suggested that phosphorylation at the hinge region of many nuclear receptors contributes to their heterodimerization and transactivity(48,49). On the other hand, S221 locates within the LBD of PXR, which is considered a molecular switch by translating the ligand structure into the conformational change which transforms nuclear receptors into transcription activators or repressors(50). Unlike the LBD of a canonical nuclear receptor, which is composed of ten α helices and three β strands and forms a three-layer sandwich, LBD of PXR has two additional β1 and β1' strands, which are thought to adapt to bind a broad spectrum of ligands of PXR(51-53). The location of S221 is in the additional β1' strands of PXR LBD. Therefore, phosphorylation at this particular serine may be involved in the feedback response to terminate the ligand-activated PXR trans-activation.

Further investigation employing the phosphomimetic and phosphodeficient mutants of PXR indicates PXR phosphorylation at both sites impairs its trans-activation capacity. PXR-mediated transcription activity involves a series of protein-protein interaction events. Such protein-protein interaction events include the heterodimerization of PXR-RXRα and the circulation of coregulator proteins. PXR heterodimerization with RXRα is considered the first step in response to ligand binding and is essential for subsequent gene activation. The molecular basis for nuclear receptor-regulated transcription activation is ligand binding-triggered coregulator protein exchange. We utilized mammalian two-hybrid assays to examine the outcome of phosphorylation as PXR-specific protein-protein interactions. Our results showed phosphorylation at both identified sites is capable of inhibiting PXR-mediated CYP3A4 reporter gene activation. Regarding PXR association with coregulator proteins, S221 phosphorylation leads to a dissociation of tested coactivator proteins and increased interaction with the corepressor proteins NCoR1 and SMRT. However, phosphorylation at T135 significantly interrupts the PXR-SMRT protein-protein interaction while showing no effect on PXR association with NCoR1. Of note, our results show that phosphomimetic mutation at T135 hampers the ability of ligand-activated PXR to interact with the coactivator SRC1 and GRIP1, as well as the corepressor SMRT. Moreover, phosphorylation at T135 has no effect on the PXR interaction with PBP and NCoR1. Collectively, these finding suggests T135 phosphorylation-mediated inhibition of PXR transactivity may invloves alternative mechanism without the involvement of coregulator circulation.

It is now well accepted that phosphorylation suppresses PXR transactivity through interrupting its heterodimerization and interactions with coregulator proteins. To date, kinases that catalyze PXR phosphorylation include PKC, PKA, CDK2, CDK5, p70 S6K, glycogen

synthase kinase 3 (GSK3), and casein kinase II (CK2)(24,31,32,40,41,43). In response to specific cellular signals, PXR phosphorylation inhibits its transcription activity through the action of reducing the capability to form a heterodimer with RXRα, disrupting the interaction between PXR and coactivator proteins, and affecting the translocation of PXR into the nucleus. In our current study, we have observed a consistent outcome of PXR phosphorylation at the newly identified site S221. Using an "in silico" bioinformatic approach (GPS 3.0, http://gps.biocuckoo.org), the predicted kinase that catalyzes PXR phosphorylation at S221 is the mitogen-activated kinase (MAPK)-interacting serine/threonine-protein kinase MNK1/2. MNK is an effector protein kinase downstream of MAPK signaling that directly regulates many cellular events including differentiation, apoptosis, and the immune response to cytokine stimulation. Therefore, PXR phosphorylation at S221 may be the key step in inflammation-mediated inhibition of drug metabolism.

Post-translational modifications often regulate the biology of nuclear receptors in a collaborative fashion. Multiple individual studies have clearly observed that the crosstalk between PTMs at the level of PXR regulates many aspects of its biological activities. Our previous study demonstrated that SUMOylation of PXR suppresses the inflammatory gene activation and subsequently promotes the recognition by ubiquitin for proteasomal degradation(29). To examine the extent to which PXR phosphorylation-mediated trans-repression involves the cooperation of other PTMs, we monitored the ubiquitination of PXR using a biochemical approach. Phosphomimetic mutation at both identified sites does not affect the ubiquitination of PXR, which excludes the possibility of phosphorylation-stimulated protein degradation. In conclusion, our results provide evidence that detecting the onset of PXR phosphorylation in primary hepatocytes contributes to the inhibition of PXR transcription

activity through modulating the coregulator circulations. Though the specific kinase that catalyzes PXR phosphorylation at S221 remains unknown at the present stage, our findings may shed new light on developing novel therapeutic strategies to target PXR for the treatment of chronic inflammatory diseases in the liver and intestines.

4.5 REFERENCES

1. Jones, S. A., Moore, L. B., Shenk, J. L., Wisely, G. B., Hamilton, G. A., McKee, D. D., Tomkinson, N. C., LeCluyse, E. L., Lambert, M. H., Willson, T. M., Kliewer, S. A., and Moore, J. T. (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Molecular endocrinology* **14**, 27-39
2. Lehmann, J. M., McKee, D. D., Watson, M. A., Willson, T. M., Moore, J. T., and Kliewer, S. A. (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
3. Sinz, M., Wallace, G., and Sahi, J. (2008) Current industrial practices in assessing CYP450 enzyme induction: preclinical and clinical. *AAPS J* **10**, 391-400
4. 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
5. 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. *Molecular and cellular endocrinology* **231**, 75-85
6. Chen, Y., and Nie, D. (2009) Pregnane X receptor and its potential role in drug resistance in cancer treatment. *Recent Pat Anticancer Drug Discov* **4**, 19-27
7. Fichtenbaum, C. J., and Gerber, J. G. (2002) Interactions between antiretroviral drugs and drugs used for the therapy of the metabolic complications encountered during HIV infection. *Clin Pharmacokinet* **41**, 1195-1211
8. Niemi, M., Backman, J. T., Fromm, M. F., Neuvonen, P. J., and Kivisto, K. T. (2003) Pharmacokinetic interactions with rifampicin : clinical relevance. *Clin Pharmacokinet* **42**, 819-850
9. Plant, N. J., and Gibson, G. G. (2003) Evaluation of the toxicological relevance of CYP3A4 induction. *Curr Opin Drug Discov Devel* **6**, 50-56
10. Sinz, M. W. (2013) Evaluation of pregnane X receptor (PXR)-mediated CYP3A4 drug-drug interactions in drug development. *Drug Metab Rev* **45**, 3-14
11. Wolf, K. K., Wood, S. G., Hunt, J. A., Walton-Strong, B. W., Yasuda, K., Lan, L., Duan, S. X., Hao, Q., Wrighton, S. A., Jeffery, E. H., Evans, R. M., Szakacs, J. G., von Moltke, L. L., Greenblatt, D. J., Court, M. H., Schuetz, E. G., Sinclair, P. R., and Sinclair, J. F. (2005) Role of the nuclear receptor pregnane X receptor in acetaminophen hepatotoxicity. *Drug Metab Dispos* **33**, 1827-1836

12. Sun, M., Cui, W., Woody, S. K., and Staudinger, J. L. (2015) Pregnane X receptor modulates the inflammatory response in primary cultures of hepatocytes. *Drug Metab Dispos* **43**, 335-343
13. Shah, Y. M., Ma, X., Morimura, K., Kim, I., and Gonzalez, F. J. (2007) Pregnane X receptor activation ameliorates DSS-induced inflammatory bowel disease via inhibition of NF-kappaB target gene expression. *Am J Physiol Gastrointest Liver Physiol* **292**, G1114-1122
14. Zhou, C., Tabb, M. M., Nelson, E. L., Grun, F., Verma, S., Sadatrafiei, A., Lin, M., Mallick, S., Forman, B. M., Thummel, K. E., and Blumberg, B. (2006) Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest* **116**, 2280-2289
15. Wallace, K., Cowie, D. E., Konstantinou, D. K., Hill, S. J., Tjelle, T. E., Axon, A., Koruth, M., White, S. A., Carlsen, H., Mann, D. A., and Wright, M. C. (2010) The PXR is a drug target for chronic inflammatory liver disease. *J Steroid Biochem Mol Biol* **120**, 137-148
16. Dou, W., Zhang, J., Li, H., Kortagere, S., Sun, K., Ding, L., Ren, G., Wang, Z., and Mani, S. (2014) Plant flavonol isorhamnetin attenuates chemically induced inflammatory bowel disease via a PXR-dependent pathway. *J Nutr Biochem* **25**, 923-933
17. Hoekstra, M., Lammers, B., Out, R., Li, Z., Van Eck, M., and Van Berkel, T. J. (2009) Activation of the nuclear receptor PXR decreases plasma LDL-cholesterol levels and induces hepatic steatosis in LDL receptor knockout mice. *Mol Pharm* **6**, 182-189
18. Hukkanen, J., Hakkola, J., and Rysa, J. (2014) Pregnane X receptor (PXR)--a contributor to the diabetes epidemic? *Drug Metabol Drug Interact* **29**, 3-15
19. Lee, J. H., Zhou, J., and Xie, W. (2008) PXR and LXR in hepatic steatosis: a new dog and an old dog with new tricks. *Mol Pharm* **5**, 60-66
20. Xie, W., Radominska-Pandya, A., Shi, Y., Simon, C. M., Nelson, M. C., Ong, E. S., Waxman, D. J., and Evans, R. M. (2001) An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci U S A* **98**, 3375-3380
21. Zhou, C., Verma, S., and Blumberg, B. (2009) The steroid and xenobiotic receptor (SXR), beyond xenobiotic metabolism. *Nuclear receptor signaling* **7**, e001
22. Lichti-Kaiser, K., Xu, C., and Staudinger, J. L. (2009) Cyclic AMP-dependent protein kinase signaling modulates pregnane x receptor activity in a species-specific manner. *J Biol Chem* **284**, 6639-6649
23. Wang, Y. M., Chai, S. C., Lin, W., Chai, X., Elias, A., Wu, J., Ong, S. S., Pondugula, S. R., Beard, J. A., Schuetz, E. G., Zeng, S., Xie, W., and Chen, T. (2015) Serine 350 of human pregnane X receptor is crucial for its heterodimerization with retinoid X receptor alpha and trans-activation of target genes in vitro and in vivo. *Biochem Pharmacol* **96**, 357-368
24. Elias, A., High, A. A., Mishra, A., Ong, S. S., Wu, J., Peng, J., and Chen, T. (2014) Identification and characterization of phosphorylation sites within the pregnane X receptor protein. *Biochem Pharmacol* **87**, 360-370
25. Sugatani, J., Noguchi, Y., Hattori, Y., Yamaguchi, M., Yamazaki, Y., and Ikari, A. (2016) Threonine-408 Regulates the Stability of Human Pregnane X Receptor through Its Phosphorylation and the CHIP/Chaperone-Autophagy Pathway. *Drug Metab Dispos* **44**, 137-150

26. Pasquel, D., Doricakova, A., Li, H., Kortagere, S., Krasowski, M. D., Biswas, A., Walton, W. G., Redinbo, M. R., Dvorak, Z., and Mani, S. (2016) Acetylation of lysine 109 modulates pregnane X receptor DNA binding and transcriptional activity. *Biochim Biophys Acta* **1859**, 1155-1169
27. Biswas, A., Pasquel, D., Tyagi, R. K., and Mani, S. (2011) Acetylation of pregnane X receptor protein determines selective function independent of ligand activation. *Biochem Biophys Res Commun* **406**, 371-376
28. Hu, G., Xu, C., and Staudinger, J. L. (2010) Pregnane X receptor is SUMOylated to repress the inflammatory response. *J Pharmacol Exp Ther* **335**, 342-350
29. Cui, W., Sun, M., Galeva, N., Williams, T. D., Azuma, Y., and Staudinger, J. L. (2015) SUMOylation and Ubiquitylation Circuitry Controls Pregnane X Receptor Biology in Hepatocytes. *Drug Metab Dispos* **43**, 1316-1325
30. Cui, W., Sun, M., Zhang, S., Shen, X., Galeva, N., Williams, T. D., and Staudinger, J. L. (2016) A SUMO-acetyl switch in PXR biology. *Biochim Biophys Acta* **1859**, 1170-1182
31. Ding, X., and Staudinger, J. L. (2005) Repression of PXR-mediated induction of hepatic CYP3A gene expression by protein kinase C. *Biochem Pharmacol* **69**, 867-873
32. Lichti-Kaiser, K., Brobst, D., Xu, C., and Staudinger, J. L. (2009) A systematic analysis of predicted phosphorylation sites within the human pregnane X receptor protein. *J Pharmacol Exp Ther* **331**, 65-76
33. Synold, T. W., Dussault, I., and Forman, B. M. (2001) The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. *Nat Med* **7**, 584-590
34. Staudinger, J. L., Madan, A., Carol, K. M., and Parkinson, A. (2003) Regulation of drug transporter gene expression by nuclear receptors. *Drug Metab Dispos* **31**, 523-527
35. Staudinger, J. L., Xu, C., Biswas, A., and Mani, S. (2011) Post-translational modification of pregnane x receptor. *Pharmacol Res* **64**, 4-10
36. Xu, C., Wang, X., and Staudinger, J. L. (2009) Regulation of tissue-specific carboxylesterase expression by pregnane x receptor and constitutive androstane receptor. *Drug Metab Dispos* **37**, 1539-1547
37. Ding, X., and Staudinger, J. L. (2005) Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway. *J Pharmacol Exp Ther* **312**, 849-856
38. Doricakova, A., Novotna, A., Vrzal, R., Pavek, P., and Dvorak, Z. (2013) The role of residues T248, Y249 and T422 in the function of human pregnane X receptor. *Arch Toxicol* **87**, 291-301
39. Pondugula, S. R., Dong, H., and Chen, T. (2009) Phosphorylation and protein-protein interactions in PXR-mediated CYP3A repression. *Expert Opin Drug Metab Toxicol* **5**, 861-873
40. Pondugula, S. R., Brimer-Cline, C., Wu, J., Schuetz, E. G., Tyagi, R. K., and Chen, T. (2009) A phosphomimetic mutation at threonine-57 abolishes trans-activation activity and alters nuclear localization pattern of human pregnane x receptor. *Drug Metab Dispos* **37**, 719-730
41. Lin, W., Wu, J., Dong, H., Bouck, D., Zeng, F. Y., and Chen, T. (2008) Cyclin-dependent kinase 2 negatively regulates human pregnane X receptor-mediated CYP3A4 gene expression in HepG2 liver carcinoma cells. *J Biol Chem* **283**, 30650-30657
42. Sugatani, J., Osabe, M., Kurosawa, M., Kitamura, N., Ikari, A., and Miwa, M. (2010) Induction of UGT1A1 and CYP2B6 by an antimitogenic factor in HepG2 cells is

mediated through suppression of cyclin-dependent kinase 2 activity: cell cycle-dependent expression. *Drug Metab Dispos* **38**, 177-186
43. Sugatani, J., Uchida, T., Kurosawa, M., Yamaguchi, M., Yamazaki, Y., Ikari, A., and Miwa, M. (2012) Regulation of pregnane X receptor (PXR) function and UGT1A1 gene expression by posttranslational modification of PXR protein. *Drug Metab Dispos* **40**, 2031-2040
44. Dong, H., Lin, W., Wu, J., and Chen, T. (2010) Flavonoids activate pregnane x receptor-mediated CYP3A4 gene expression by inhibiting cyclin-dependent kinases in HepG2 liver carcinoma cells. *BMC Biochem* **11**, 23
45. Wang, Y. M., Ong, S. S., Chai, S. C., and Chen, T. (2012) Role of CAR and PXR in xenobiotic sensing and metabolism. *Expert Opin Drug Metab Toxicol* **8**, 803-817
46. McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) Nuclear receptor coregulators: cellular and molecular biology. *Endocrine reviews* **20**, 321-344
47. Nagy, L., and Schwabe, J. W. (2004) Mechanism of the nuclear receptor molecular switch. *Trends Biochem Sci* **29**, 317-324
48. Blanquart, C., Mansouri, R., Paumelle, R., Fruchart, J. C., Staels, B., and Glineur, C. (2004) The protein kinase C signaling pathway regulates a molecular switch between trans-activation and trans-repression activity of the peroxisome proliferator-activated receptor alpha. *Molecular endocrinology* **18**, 1906-1918
49. Delmotte, M. H., Tahayato, A., Formstecher, P., and Lefebvre, P. (1999) Serine 157, a retinoic acid receptor alpha residue phosphorylated by protein kinase C in vitro, is involved in RXR.RARalpha heterodimerization and transcriptional activity. *J Biol Chem* **274**, 38225-38231
50. Pawlak, M., Lefebvre, P., and Staels, B. (2012) General molecular biology and architecture of nuclear receptors. *Curr Top Med Chem* **12**, 486-504
51. Watkins, R. E., Wisely, G. B., Moore, L. B., Collins, J. L., Lambert, M. H., Williams, S. P., Willson, T. M., Kliewer, S. A., and Redinbo, M. R. (2001) The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science* **292**, 2329-2333
52. Xiao, L., Cui, X., Madison, V., White, R. E., and Cheng, K. C. (2002) Insights from a three-dimensional model into ligand binding to constitutive active receptor. *Drug Metab Dispos* **30**, 951-956
53. Iyer, M., Reschly, E. J., and Krasowski, M. D. (2006) Functional evolution of the pregnane X receptor. *Expert Opin Drug Metab Toxicol* **2**, 381-397

Chapter 5: Conclusions and Future Outlook

5.1 CONCLUSIONS AND CRITICAL ANALYSIS

In the past two decades, the physiological and pathophysiological roles of PXR in human have been intensively investigated. The canonical physiology of PXR is to regulate the expression of drug metabolizing enzymes and transporters at the transcription level. In this regard, PXR is defined as a master regulator of the hepatic detoxification pathway. From the pathophysiological perspective, PXR-induced drug metabolizing enzymes could increase the incidence of adverse drug-drug interaction by accelerating drug turnover or antagonizing the co-administrated drugs(1,2). Constitutive activation of PXR results in accumulation of drug metabolites, which is considered a driving force of hepatic steatosis(3). Moreover, PXR-induced MDR1 expression is the leading cause of drug resistance in numerous cancer types(4). The other aspect of PXR physiology is to transrepress the inflammatory response specifically in the liver and intestines. Multiple lines of evidence indicate PXR agonists significantly suppress the inflammatory response in mice and human, which suggest a potential application of the gut-specific PXR agonist, antibiotic drug Rifaximin (trade name as Xifaxan), in treating the inflammation-related bowel disease (IBD) (5,6). Despite the abundant evidence that indicates PXR governs the crosstalk between the xenobiotic response signaling and the inflammatory response signaling, the molecular mechanism regarding regulation of PXR is still ambiguous.

Post-translational modifications (PTMs) have emerged as a fundamental mechanism in the regulation of NRs in the past few years. Previous studies in our lab have shown that PXR is a molecular target of phosphorylation, ubiquitination, and SUMOylation. Ding and Lichti-Kaiser have revealed that phosphorylation of PXR inhibits the ligand-activated xenobiotic

response(7,8). Hu et.al have demonstrated that SUMOylation of PXR suppresses the inflammatory response(9). Moreover, results generated from Dr. Mani's group showed that PXR is acetylated in its basal repression state(10). Based on all of these discoveries, we proposed a hypothesis that PTMs fine-tune the PXR-mediated trans-repression of the inflammatory response in the liver **(Figure 1-2)**.

A working model in which SUMOylation cooperating with ubiquitination to regulate PXR biological activity in a context-specific fashion was first described in Chapter 2. SUMOylation is a recently well-accepted universal mechanism for NR-mediated trans-repression activity recently(11-17). Our results showed that both a ligand (rifampicin) and an inflammatory stimulus (TNFα) promoted PXR SUMOylation in primary mouse hepatocytes. We identified the E3 ligases (PIAS1 and PIASy) for PXR SUMOylation and SENPs (SENP2, 3 and 6) for PXR deSUMOylation. PIAS1-mediated SUMOylation of PXR repressed TNFα-induced expression of inflammatory genes while further increased rifampicin-induced gene expression of *Cyp3a11* in primary mouse hepatocytes. This context-specific difference lends confidence for future application of small molecule drugs that targeting PXR SUMOylation for the treatment of inflammation-related diseases in the liver and intestines. Further investigations suggested that SUMO(1)ylation facilitates PXR-mediated trans-repression, whereas SUMO(3)ylation promotes the ubiquitination-initiated PXR degradation through proteasomes. A growing body of evidence has revealed the existence of SUMO-targeted ubiquitin ligase (STUbL). Ring finger protein 4 (RNF4) is the typical STUbL enzyme that conserves the SUMO interacting motif (SIM). This SIM on the RNF4 enzyme permits its preferential binding to the poly-SUMOylation chain and promotes the subsequent ubiquitination-mediated protein degradation(18). It is well known that the basic function of ubiquitination is to regulate protein stability and homeostasis. In our

model, we suggest that STUbL initiates the ubiquitination of stress-induced PXR SUMOylation and thereby accelerates PXR protein turnover in response to xenobiotic or inflammatory stimuli. We have identified multiple ubiquitination sites on PXR protein using a proteomic approach. Unfortunately, we were unable to locate the site(s) of SUMOylation on PXR using the established proteomic approach due to the extremely low stoichiometry of SUMOylation. We utilized an alternative site-directed mutagenesis approach to identify the SUMOylation sites of PXR. Our results revealed that K108 is the primary site for both PXR SUMO(1)ylation and SUMO(3)ylation, and K128 is the second site for PXR SUMO(3)ylation. Collectively, we demonstrated SUMOylation and ubiquitination coordinately regulate PXR biological function in the liver in response to xenobiotic or inflammatory stress.

Subsequently, the interface between SUMOylation and acetylation in the context of PXR biology was discussed in Chapter 3. Acetylation contributes to the regulation of protein stability, functional activity, as well as capability to interact with other regulatory proteins(19). Lysine/histone acetyltransferase proteins (HATs) and lysine/histone deacetylase proteins (HDACs) coordinately regulate the protein acetylation. The histone deacetylase (HDAC) proteins are known to target a wide variety of non-histone proteins that are involved in the regulation of cell proliferation and apoptosis. Among all of the HDAC family members, HDAC3, in particular, regulates metabolism through many signaling pathways in the liver(20). Representative substrate proteins of HDACs are comprised of NRs, chaperone proteins, as well as cytoskeletal proteins(21). In the past decade, inhibition of HDACs has been an attractive therapeutic strategy for various human diseases. Therapeutic applications of HDAC inhibitors range from psychiatric disorders, neurodegenerative diseases, inflammatory diseases, to many cancer types. Several research groups proposed that the crosstalk of acetylation and

SUMOylation is critical for the regulation of the substrate protein. For instance, Blakeslee reported that inhibition of class I HDACs increases cardiac protein SUMOylation(22). Kim reported that acetylation of FXR disrupts its SUMOylation while inducing the inflammatory response. Thus we sought to examine the regulatory networking among acetylation, SUMOylation, and ubiquitination at the level of PXR in primary hepatocytes. Our results showed that PXR is acetylated at the transcriptional silent state and ligand-activation decreases the acetylation of PXR. Inhibition of class I/II HDACs by TSA treatment suppresses the ligand-mediated activation of *Cyp3a11* gene in mouse hepatocytes. This finding indicates that acetylation inhibits the PXR-mediated transcriptional activation, which is in line with previous discovery(10). Further investigations showed that HDAC3/SMRT corepressor protein complex diminished the SUMOylation of PXR while TSA treatment reversed such outcome. Furthermore, SUMOylation sufficiently disrupted the association of PXR and HDAC3/SMRT protein complex. Taken together, our results suggest that SUMOylation and acetylation exerts a synergistic effect on PXR-mediated trans-repression. These findings provide new insights on targeting HDACs in the treatment of inflammation-related liver diseases.

Finally, the site-specific phosphorylation in the regulation of PXR biology in the liver was discussed in Chapter 4. Phosphorylation is often considered a regulatory event towards PXR, which suppresses the PXR-originated trans-activation through multiple actions. Phosphorylation affects the heterodimerization of PXR-RXRα, modulates the coregulator exchange, and alters the subcellular localization of PXR(7,8,23,24). In the current study, we have identified two novel phosphorylation sites of PXR at threonine 135 (T135) and serine 221 (S221), respectively. Phosphorylation at either site suppresses the ligand-induced trans-activation capacity of PXR. Further investigations suggest that the phosphorylation-mediated

transcriptional repression on CYP3A4 reporter gene may through site-specific mechanisms. Precisely, S221 phosphorylation inhibits the ligand-induced PXR trans-activation capacity by 1) inhibiting the heterodimerization with RXR, 2) increasing the interaction with corepressor protein, and 3) decreasing the interaction with coactivator proteins. However, T135 phosphorylation-mediated trans-repression may involve additional mechanisms such as crosstalk with other PTMs at the level of PXR. Yet the kinase that phosphorylates PXR at identified sites is still unknown at the current stage. Reported kinases for PXR phosphorylation include cytokine-activated PKC, glycogen and lipid metabolism-activated PKA, and cell cycle regulator CDKs. These serine/threonine protein kinases are commonly activated by cellular stress. Therefore, PXR phosphorylation is a potential mechanism to reduce cellular stress through balancing the activation of the xenobiotic response and the inhibition of inflammatory response. Future efforts should focus on discovering the protein kinase that phosphorylates PXR at T135 and S221 for a better understanding of the physiological and pathophysiological functions of PXR phosphorylation.

In summary, PTMs are involved in the regulation of many aspects of PXR biology in the liver. They coordinately interact with each other to fine-tune the PXR-mediated transcription events in a signal-specific manner. A summary diagram is shown in **Figure 5-1**. In the silent state, PXR is acetylated and associated with corepressor complex. Upon ligand (rifampicin) binding, PXR is deacetylated and detached from the corepressor complex while recruiting the coactivator complex. Liganded PXR forms a heterodimer with its partner RXRα and binds to the DNA response element to regulate the transcription activation of genes encoding drug-metabolizing enzymes. In response to inflammatory stimulus (TNFα), a portion of PXR protein is SUMO(1)ylated to transrepress the expression of NFκB-activated inflammatory genes. In this

particular scenario, SUMOylation disrupts PXR association to HDAC3/SMRT corepressor protein complex and dominantly suppresses the inflammatory gene expression. Another portion of PXR protein is phosphorylated to suppress the expression of genes encoding drug-metabolizing enzymes. At the end of the PXR-mediated transcription events, SUMO(3)ylation and ubiquitination coordinately regulate the degradation of PXR. Specifically, liganded PXR or SUMO(1)ylated PXR is first targeted by the SUMO(3)ylation. The STUbL enzyme RNF4 recognizes the poly- SUMO(3)ylation chain and accelerates the ubiquitination of PXR. Ubiquitin primarily forms a K48-linked poly-ubiquitination chain on PXR then escorts PXR to the proteasome for degradation. This diagram suggests that the well-known PXR-mediated mutual repression between the xenobiotic response signaling and the inflammatory response signaling is regulated by PTMs in the liver.

Figure 5-1.

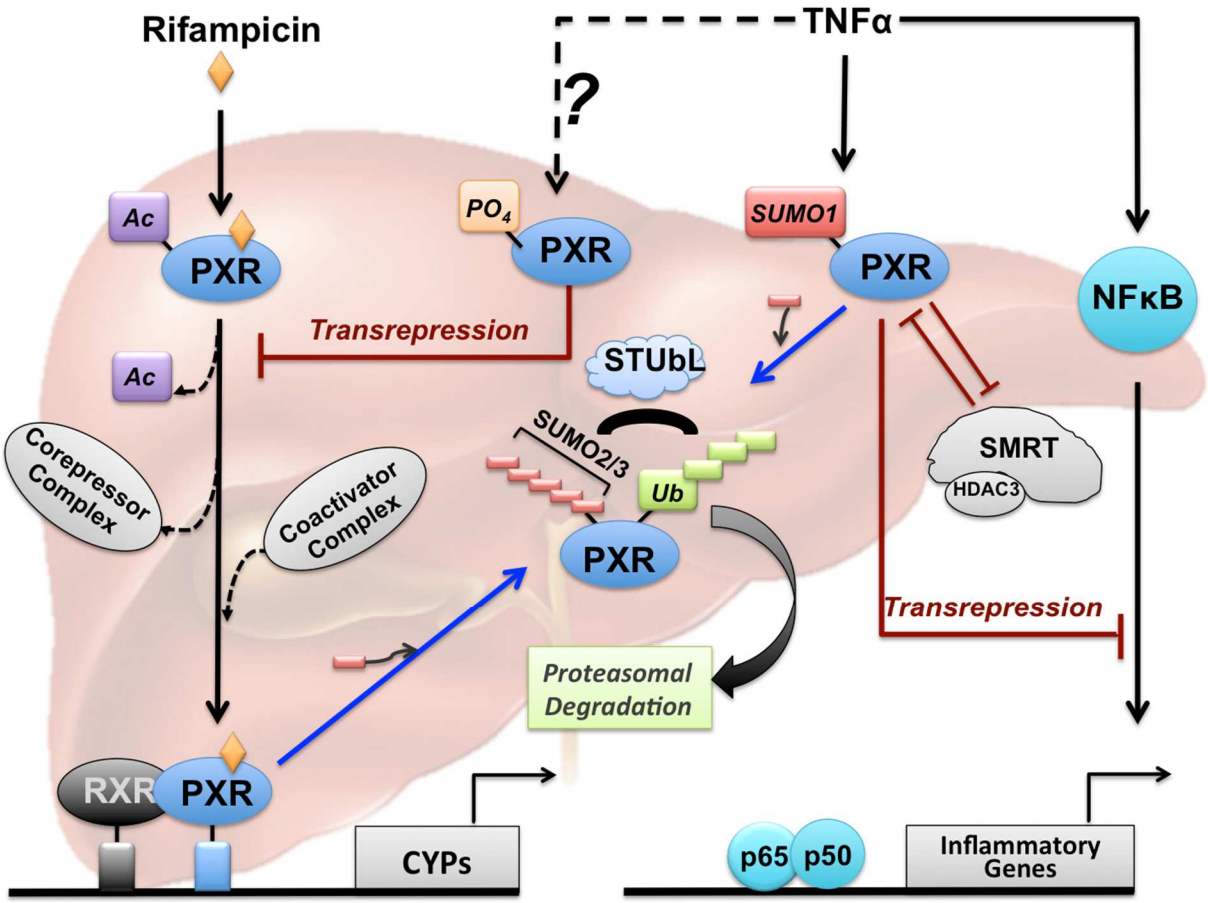


Figure 5-1. Concluding diagram of PTMs in the regulation of PXR biology in the liver. **(1)** PXR is acetylated and associated with corepressor proteins in the silent state. Upon ligand (rifampicin) binding, activated PXR forms a heterodimer with RXR to induce the expression of the cytochrome P450 genes. This transcriptional activation is regulated by dissociation from corepressor protein complex and recruitment of coactivator protein complex. **(2)** Inflammation stimulus TNFα promotes the SUMO(1)ylation of PXR to transrepress the NFκB-mediated activation of the inflammatory response. **(3)** Both rifampicin and TNFα increase the SUMO(3)ylation of PXR. STUbL enzymes recognize the SUMO3-mediated poly-SUMOylation on PXR and accelerate the ubiquitination-mediated proteasomal degradation of PXR protein. **(4)** Inflammation stimulus likely induces the phosphorylation of PXR at S221. Phosphorylation suppresses the PXR-mediated transactivation of CYPs through disrupting PXR interacts with coactivators while enhancing PXR association with corepressors. Moreover, phosphorylation interrupts the heterodimerization of PXR and RXR.

5.2 FUTURE OUTLOOK

5.2.1 Targeting PXR SUMOylation to Suppress Inflammation

Recently, targeting the SUMOylation signaling has been an attractive strategy for the treatment of many human diseases, which include cardiovascular diseases, neurodegenerative diseases, and cancer. In the context of PXR, we showed that SUMO1-modification is the critical step for PXR to facilitate trans-repression on inflammatory genes. Moreover, our data indicates that PIAS1-mediated SUMOylation of PXR suppresses the expression of inflammatory genes without affecting the expression of canonical PXR target genes. Therefore, targeting the SUMOylation of PXR could selectively suppress inflammation while maintaining normal physiology in the regulation of xenobiotic response. Furthermore, we have determined that PXR is SUMOylated at lysine 108 (K108). Future studies could focus on examining the therapeutic potential of manipulating PXR SUMOylation in the treatment of inflammation-related diseases. A gene knock-in mouse model that expresses the SUMO-deficient PXR (PXR-K108R) should be generated and employed for the phenotype characterization regarding PXR-mediated repression of inflammation. The model of PXR-K108R knock-in mouse is expected to lose its ability to transrepress the inflammatory response in the liver. The E3 ligases that promote PXR SUMOylation include PIAS1 and PIASy, whereas SENP2 possesses the most efficient deSUMOylation activity on PXR. Small molecule compounds that specifically inhibit the SENP2 enzymatic activities, or promote PIAS1 enzymatic activities should be screened for decreasing PXR SUMOylation in the liver. Finally, the ideal small-molecule drug should either selectively decrease the deSUMOylation of PXR or selectively increase the SUMOylation of PXR while affecting minimum numbers of other proteins involved in the inflammatory response signaling.

5.2.2 Targeting the SUMOylation-Ubiquitination Circuitry to Conquer Drug Resistance in Chemotherapy

Our results revealed a novel SUMO(3)ylation-promoted ubiquitination in primary mouse hepatocytes. MDR1-mediated drug resistance is primarily due to the chemotherapeutic agents-caused constitutive activation of PXR in specific tissues. Therefore, targeting the SUMO(3)ylation of PXR to accelerate its degradation could be a novel strategy to reduce MDR1-mediated drug resistance in chemotherapy. Our data showed that SENP3 and SNEP6 sufficiently remove the poly-SUMOylation chains that attached to PXR. Inhibition of SENP3 and SNEP6 by small molecule drugs is expected to retain the SUMOylation chain on PXR, which results in eventual ubiquitination-mediated proteasomal degradation of PXR. An alternative approach to modulate PXR stability could be promoting the expression or enzymatic activity of RNF4. While PXR activation increases the drug resistance in prostate cancer, it prevents the progression of breast cancer. Therefore, inhibition of RNF4-mediated PXR degradation could exert beneficial effects in the treatment of breast cancer.

5.2.3 Targeting PXR Phosphorylation To Enhance Xenobiotic Metabolism in Inflammation-Related Liver Diseases

In the United States, the incidence of the inflammation-related liver disease is dramatically growing in the recent years. These inflammation-related liver diseases, include non-alcohol fatty liver disease, non-alcoholic steatohepatitis, and chronic hepatitis B/C infection, are considered the driving forces of hepatocellular carcinoma. Accumulating evidence indicates PXR as the master regulator of hepatic detoxification pathway contributes to the pathogenesis of the inflammation-related liver diseases. Our results demonstrate that site-specific phosphorylation of PXR inhibits the ligand-induced xenobiotic response. Given the fact that

impaired xenobiotic response increases the cytotoxicity, inhibition of PXR S221 phosphorylation should reduce the cytotoxicity in the inflamed liver by improving the hepatic detoxification pathway. In this regard, small molecule inhibitors that selectively target the kinase, which phosphorylates PXR at S221, would be a novel therapeutic strategy for the inflammation-related liver diseases.

5.2.4 Concluding Remarks

The physiological functions of PXR have been intensively studied in the past twenty years. It is a well-accepted notion that PXR is not only a master regulator of the xenobiotic response but also a negative regulator of the inflammatory response in the liver and intestines. The mutual repression between the xenobiotic detoxification signaling and the inflammatory signaling in the liver is the foundation of this dissertation research. The data presented here revealed potential molecular mechanisms regarding PXR-mediated regulation of both signaling pathways in the liver. The knowledge we obtained from this study provides a new perspective to develop novel therapeutic strategies to target the PTMs at the level of PXR for the treatment of inflammation-related diseases in liver and intestines.

5.3 REFERENCES

1. Fichtenbaum, C. J., and Gerber, J. G. (2002) Interactions between antiretroviral drugs and drugs used for the therapy of the metabolic complications encountered during HIV infection. *Clin Pharmacokinet* **41**, 1195-1211
2. Lehmann, J. M., McKee, D. D., Watson, M. A., Willson, T. M., Moore, J. T., and Kliewer, S. A. (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
3. Zhou, C., Tabb, M. M., Nelson, E. L., Grun, F., Verma, S., Sadatrafiei, A., Lin, M., Mallick, S., Forman, B. M., Thummel, K. E., and Blumberg, B. (2006) Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest* **116**, 2280-2289

4. Chen, Y., and Nie, D. (2009) Pregnane X receptor and its potential role in drug resistance in cancer treatment. *Recent Pat Anticancer Drug Discov* **4**, 19-27
5. Cheng, J., Shah, Y. M., Ma, X., Pang, X., Tanaka, T., Kodama, T., Krausz, K. W., and Gonzalez, F. J. (2010) Therapeutic role of rifaximin in inflammatory bowel disease: clinical implication of human pregnane X receptor activation. *J Pharmacol Exp Ther* **335**, 32-41
6. Shah, Y. M., Ma, X., Morimura, K., Kim, I., and Gonzalez, F. J. (2007) Pregnane X receptor activation ameliorates DSS-induced inflammatory bowel disease via inhibition of NF-kappaB target gene expression. *Am J Physiol Gastrointest Liver Physiol* **292**, G1114-1122
7. Ding, X., and Staudinger, J. L. (2005) Repression of PXR-mediated induction of hepatic CYP3A gene expression by protein kinase C. *Biochem Pharmacol* **69**, 867-873
8. Lichti-Kaiser, K., Brobst, D., Xu, C., and Staudinger, J. L. (2009) A systematic analysis of predicted phosphorylation sites within the human pregnane X receptor protein. *J Pharmacol Exp Ther* **331**, 65-76
9. Hu, G., Xu, C., and Staudinger, J. L. (2010) Pregnane X receptor is SUMOylated to repress the inflammatory response. *J Pharmacol Exp Ther* **335**, 342-350
10. Biswas, A., Pasquel, D., Tyagi, R. K., and Mani, S. (2011) Acetylation of pregnane X receptor protein determines selective function independent of ligand activation. *Biochem Biophys Res Commun* **406**, 371-376
11. Hua, G., Ganti, K. P., and Chambon, P. (2016) Glucocorticoid-induced tethered trans-repression requires SUMOylation of GR and formation of a SUMO-SMRT/NCoR1-HDAC3 repressing complex. *Proc Natl Acad Sci U S A* **113**, E635-643
12. Hua, G., Paulen, L., and Chambon, P. (2016) GR SUMOylation and formation of an SUMO-SMRT/NCoR1-HDAC3 repressing complex is mandatory for GC-induced IR nGRE-mediated trans-repression. *Proc Natl Acad Sci U S A* **113**, E626-634
13. Lee, W. P., Jena, S., Rodriguez, E. P., O'Donovan, S. P., Wagner, C., Jurutka, P. W., and Thompson, P. D. (2015) Distinct functional modes of SUMOylation for retinoid X receptor alpha. *Biochem Biophys Res Commun* **464**, 195-200
14. Burrage, P. S., Schmucker, A. C., Ren, Y., Sporn, M. B., and Brinckerhoff, C. E. (2008) Retinoid X receptor and peroxisome proliferator-activated receptor-gamma agonists cooperate to inhibit matrix metalloproteinase gene expression. *Arthritis Res Ther* **10**, R139
15. Kim, D. H., Xiao, Z., Kwon, S., Sun, X., Ryerson, D., Tkac, D., Ma, P., Wu, S. Y., Chiang, C. M., Zhou, E., Xu, H. E., Palvimo, J. J., Chen, L. F., Kemper, B., and Kemper, J. K. (2015) A dysregulated acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity. *EMBO J* **34**, 184-199
16. Venteclef, N., Jakobsson, T., Ehrlund, A., Damdimopoulos, A., Mikkonen, L., Ellis, E., Nilsson, L. M., Parini, P., Janne, O. A., Gustafsson, J. A., Steffensen, K. R., and Treuter, E. (2010) GPS2-dependent corepressor/SUMO pathways govern anti-inflammatory actions of LRH-1 and LXRbeta in the hepatic acute phase response. *Genes Dev* **24**, 381-395
17. Zhou, W., Hannoun, Z., Jaffray, E., Medine, C. N., Black, J. R., Greenhough, S., Zhu, L., Ross, J. A., Forbes, S., Wilmut, I., Iredale, J. P., Hay, R. T., and Hay, D. C. (2012) SUMOylation of HNF4alpha regulates protein stability and hepatocyte function. *J Cell Sci* **125**, 3630-3635

18. Tatham, M. H., Geoffroy, M. C., Shen, L., Plechanovova, A., Hattersley, N., Jaffray, E. G., Palvimo, J. J., and Hay, R. T. (2008) RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. *Nat Cell Biol* **10**, 538-546
19. Glozak, M. A., Sengupta, N., Zhang, X., and Seto, E. (2005) Acetylation and deacetylation of non-histone proteins. *Gene* **363**, 15-23
20. Knutson, S. K., Chyla, B. J., Amann, J. M., Bhaskara, S., Huppert, S. S., and Hiebert, S. W. (2008) Liver-specific deletion of histone deacetylase 3 disrupts metabolic transcriptional networks. *EMBO J* **27**, 1017-1028
21. Xu, W. S., Parmigiani, R. B., and Marks, P. A. (2007) Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene* **26**, 5541-5552
22. Blakeslee, W. W., Wysoczynski, C. L., Fritz, K. S., Nyborg, J. K., Churchill, M. E., and McKinsey, T. A. (2014) Class I HDAC inhibition stimulates cardiac protein SUMOylation through a post-translational mechanism. *Cellular signalling* **26**, 2912-2920
23. Lin, W., Wu, J., Dong, H., Bouck, D., Zeng, F. Y., and Chen, T. (2008) Cyclin-dependent kinase 2 negatively regulates human pregnane X receptor-mediated CYP3A4 gene expression in HepG2 liver carcinoma cells. *J Biol Chem* **283**, 30650-30657
24. Wang, Y. M., Chai, S. C., Lin, W., Chai, X., Elias, A., Wu, J., Ong, S. S., Pondugula, S. R., Beard, J. A., Schuetz, E. G., Zeng, S., Xie, W., and Chen, T. (2015) Serine 350 of human pregnane X receptor is crucial for its heterodimerization with retinoid X receptor alpha and trans-activation of target genes in vitro and in vivo. *Biochem Pharmacol* **96**, 357-368

Appendix: Increased SUMO-Signaling Attenuates Interaction Between Heat Shock Protein 70 (HSP70) and Bcl2-Associated Athanogene 3 (BAG3)

ABSTRACT

Covalent modification of protein substrates by the SUMO is an important regulator of pivotal biochemical processes. Using novel expression tools in primary cultures of hepatocytes coupled with a mass spectrometry-based proteomic approach we identified the SUMOylation pattern of a heat-shock protein (HSP) 70-associated protein called Bcl2-associated athanogene 3 (BAG3). The BAG3 protein functions as a co-chaperone with HSP70 to regulate major physiological and pathophysiological processes. Our data reveal for the first time that the BAG3 is the molecular target of the SUMO-signaling pathway in hepatocytes, and identify enzymes capable of both SUMOylating and de-SUMOylating this key regulator of HSP70 biology. Our data further reveal that one likely outcome of increased SUMO-signaling with respect to BAG3 function is to attenuate its interaction with HSP70. Collectively, these data provide a new understanding and innovative framework that may provide novel strategies to develop new drugs that seek to control disease progression through modulation of the BAG3-HSP70 protein-protein interaction.

INTRODUCTION

The SUMO post-translational modification is known to regulate fundamental biological processes including DNA repair and cell division(1). SUMOylation is a highly regulated three-step enzymatic process that responds to cellular stimuli or pathogenic challenges, whereas de-SUMOylation is regulated by a group of enzyme known as Sentrin proteases (SENPs)(2). Of

note, the SUMO-signaling pathway is tightly associated with carcinogenesis such as cell growth, differentiation, senescence, oxidative stress, and apoptosis. In general, activation of the SUMO-signaling pathway is thought of as a pro-survival signal that cells adopt under stress. Another pro-survival signal used by cells is the heat shock response.

The BAG family of proteins performs diverse functions in normal cells and in cancerous cells in diverse tissue types. BAG family members are distinguished by an evolutionarily conserved region known as the BAG domain. This approximately 45 amino acid domain is exclusively found in this family of key protein modulators of HSP70(3). BAG proteins cooperate and interact with HSP70 and other molecular co-chaperones to mediate the proper folding of proteins, as well as the re-folding of certain protein aggregates. The BAG family members also help in mediating the degradation of protein aggregates through either lysosomal- or proteasomal- degradation pathways. The signal-dependent lysosomal-mediated degradation pathway process is called macroautophagy. Entry into the macroautophagy pathway is largely mediated through the HSP70-BAG3 protein-protein interaction module. This pathway is induced in numerous physiological conditions including the regulation of energy homeostasis, neuronal survival, and transcription. The macroautophagy pathway also operates in many pathophysiological conditions including in the development of certain neurodegenerative diseases and in the development and progression of many types of cancer.

While enrichment strategies for SUMOylated peptides have been developed for immortalized cell lines, usually stably transfected HeLa cells(4,5); there has been no survey of the protein targets of SUMOylation in cultured primary cells. This is primarily due to the lack of a facile method for readily detecting this post-translational modification in its target substrate proteins in primary cells. Here, we report a method for selective enrichment of PIAS1-inducible

SUMO(3)ylated peptides from complex cellular proteomes following enrichment from total cell extracts isolated from cultured primary cell types. Our method utilizes two adenoviral expression vectors. One vector drives the expression of the SUMO E3 ligase enzyme PIAS1 (Ad-PIAS1), while the other vector drives the expression of a genetically modified form of SUMO3 we call Ad-SUMO3(Q87R).

Using primary cultures of hepatocytes and our adenoviral-based LC-MS/MS experimental approach, we identify the SUMOylation of a subset of biochemically linked SUMO-substrate proteins. Five of the twelve potential SUMOylation substrate proteins identified in our screening function to coordinately regulate the macroautophagy pathway. Using cell-based and biochemical methods, we further characterize and validate the SUMOylation of the BAG3 co-chaperone protein in primary cultured hepatocytes and a hepatoma-derived cell line. We define the specific enzymes involved in generating the SUMOylation pattern of BAG3, and provide a working hypothesis of the mechanistic outcome of SUMO-modified BAG3-HSP70 protein-protein interaction.

MATERIALS AND METHODS

Chemicals and antibodies. N-Ethylmaleimide was purchased from Sigma-Aldrich (E1271). TALON® Metal Affinity Resin (Cobalt beads) was purchased from Clontech (635502). The antibodies that used in this study are: monoclonal anti-Xpress antibody (Thermo Fisher Scientific, R910-25), polyclonal anti-BAG3 antibody (Abcam, 47124), polyclonal anti-SUMO1 antibody (Cell signaling, C9H1), polyclonal anti-SUMO2/3 (Cell Signaling, 18H8), monoclonal anti-β-actin antibody (Chemicon, 1501), monoclonal anti-GFP antibody (Clontech, 632569), monoclonal anti-FLAG antibody (Agilent Technologies, 200473). Goat anti-rat IgG-

HRP (sc-2032), goat anti-mouse IgG-HRP (sc-2005), and goat anti-rabbit IgG-HRP (sc-2004) were all purchased from Santa Cruz.

Plasmids. The expression vector encoding human BAG3 (pcDNA3-BAG3) was a generous gift from Dr. Michael Sherman(6). To construct the FLAG-tagged human BAG3 expression vector, the cDNA encoding human BAG3 was excised from the pcDNA3-BAG3 expression vector using BamHI and XhoI restriction enzyme recognition sites and inserted into pCMV-Tag2B (Agilent) using BamHI and XhoI sites. The RFP-BAG3 expression vector was constructed by using BamHI and XhoI sites to excise the cDNA encoding human BAG3 and inserted into pmCherry-C1 expression vector using BglII and SalI sites. The expression vector encoding $(His)_6$-tagged SUMO3 was a generous gift from Dr. Ronald T. Hay(7). Expression vectors encoding PIAS proteins were kind gifts from Dr. Ke Shuai(8) and obtained from Addgene (plasmid #: FLAG-PIAS1, 15206; FLAG-PIASxα, 15209; FLAG-PIASxβ, 15210; FLAG-PIAS3, 15207; FLAG-PIASy, 15208). The expression vectors encoding the SENPs and the catalytically deficient mutant SENP2 were kind gifts from Dr. Ed Yeh(9) and obtained from Addgene (plasmid #: FLAG-SENP1, 17357; FLAG-SENP2, 18047; FLAG-SEMP2m, 18713; RGS-SENP3, 18048; RGS-SENP5, 18053; FLAG-SENP6, 18065; 3xFLAG-SENP7, 42886). Expression vector encoding pEGFP-hsp70 was a kind gift from Dr. Lois Greene (Zeng et al., 2004, PubMed 15367583) and obtained from Addgene (plasmid # 15215).

Isolation and culturing primary hepatocytes. Primary hepatocytes were isolated from either C57Bl6 mouse (aged 6-10 weeks) or Sprague Dawley rats (purchased from Charles River, aged 6 weeks) following a standard collagenase perfusion protocol as described previously(10). Hepatocytes isolated from either male or female mice/rats were used throughout the study for

detecting potential sex differences. Identical results were obtained from both sexes. The representative results were acquired from male mice/rats.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. LC-MS/MS analysis was carried out as described previously(11). The modification of lysine by QQTGG (+454, this molecular weight was calculated based on the cyclization of N-terminal glutamine, Q to pyroE), a tryptic remnant of SUMO(Q87R) attachment to lysine, was included in the searching parameters.

Immunoprecipitation assay. Generally, the immunoprecipitation assay was performed as previously described(10). For detecting SUMOylated BAG3 in primary mouse hepatocytes, a condensed immunoprecipitation assay was utilized. Briefly, primary culture of mouse hepatocytes were harvested in 1 mL of lysis buffer, which contains150 mM NaCl, 50 mM Tris-Cl (pH 8.0), 1% Triton X-100, 20 mM NEM, and 1% Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific). Cells were disrupted through sonication and subsequently centrifuged at 18,000 ×g for 10 min to remove the insoluble substances. A fraction of the supernatant (5%) was saved as a loading control for Western blotting, while the rest of sample was subjected to pre-clearing with 5% protein A/G sepharose beads at 4 °C. The pre-cleared cell lysates were separated from beads through centrifugation. Subsequently, pre-cleared cell lysates were incubated with the mixture of 5 vol.% of protein A/G sepharose beads and 4 μg polyclonal anti-BAG3 antibody for immunoprecipitation of BAG3 protein for 4 hours at 4 °C with rotating.

Immobilized metal affinity chromatography (IMAC). SUMO-modified ($(His)_6$-tagged) proteins were enriched and purified with cobalt affinity beads as described previously(10).

Western blotting. Western blot analysis was performed as described previously (Xu et al., 2009).

Subcellular co-localization analysis. The subcellular co-localization of BAG3 and HSP70 were detected using fluorescent microscopy. Primary cultures of mouse hepatocytes were transfected using Lipofectamine 2000 (Invitrogen) and maintained in William's E Media prior to image analysis. Twenty-four hours post-transfection, hepatocytes were washed once with 1x PBS and then stained with Hoechst 33432 for an additional 30 minutes. For fluorescent protein imaging, mouse hepatocytes were washed three times with 1x PBS and subsequently maintained in Opti-MEM® I Reduced Serum Media. Fluorescent proteins were imaged using an Olympus IX81 inverted epifluorescence microscope with 40x air objective, and excited at either 485 nm (GFP) or 561 nm (RFP). The co-localization of two proteins in fluorescent images was measured by using Slidebook 6 software, and Pearson's co-efficient (r) was measured as described previously(12).

Statistical analysis. The statistical analysis was performed wherever appropriate. Statistical differences between experimental groups were examined using paired two-sample Student's *t* test.

RESULTS

Strategy for Identification of PIAS1-Inducible SUMO-Substrate Proteins.

The experimental strategy we developed for use in primary cultures is shown in **Figure A1-A**. The addition of a hexa-histidine $(His)_6$-tag followed by the Xpress epitope (Invitrogen) at the N-terminus of SUMO3 supports the convenient purification and detection of SUMO-substrate proteins using an immobilized metal affinity chromatography (IMAC) strategy and Western blotting approach. The substitution of the glutamine at position 87 in SUMO3 with an arginine (Q87R) shortens the SUMO-derived peptide (-QQTGG) that is generated, thereby

allowing for detection of branched SUMO-substrate peptides post-trypsin digestion by liquid chromatography tandem mass spectrometry (LC-MS/MS). This modified form of SUMO2/3 has previously been shown to incorporate into endogenous protein substrates when expressed stably in HeLa cells similar to that observed with the wild type form of SUMO2/3(5). The co-transduction of an empirically defined mixture of these two adenoviral expression vectors facilitated the efficient SUMOylation of substrates by SUMO3(Q87R) in primary cultures of rat hepatocytes **(Figure A1-B)**.

Figure A1A.

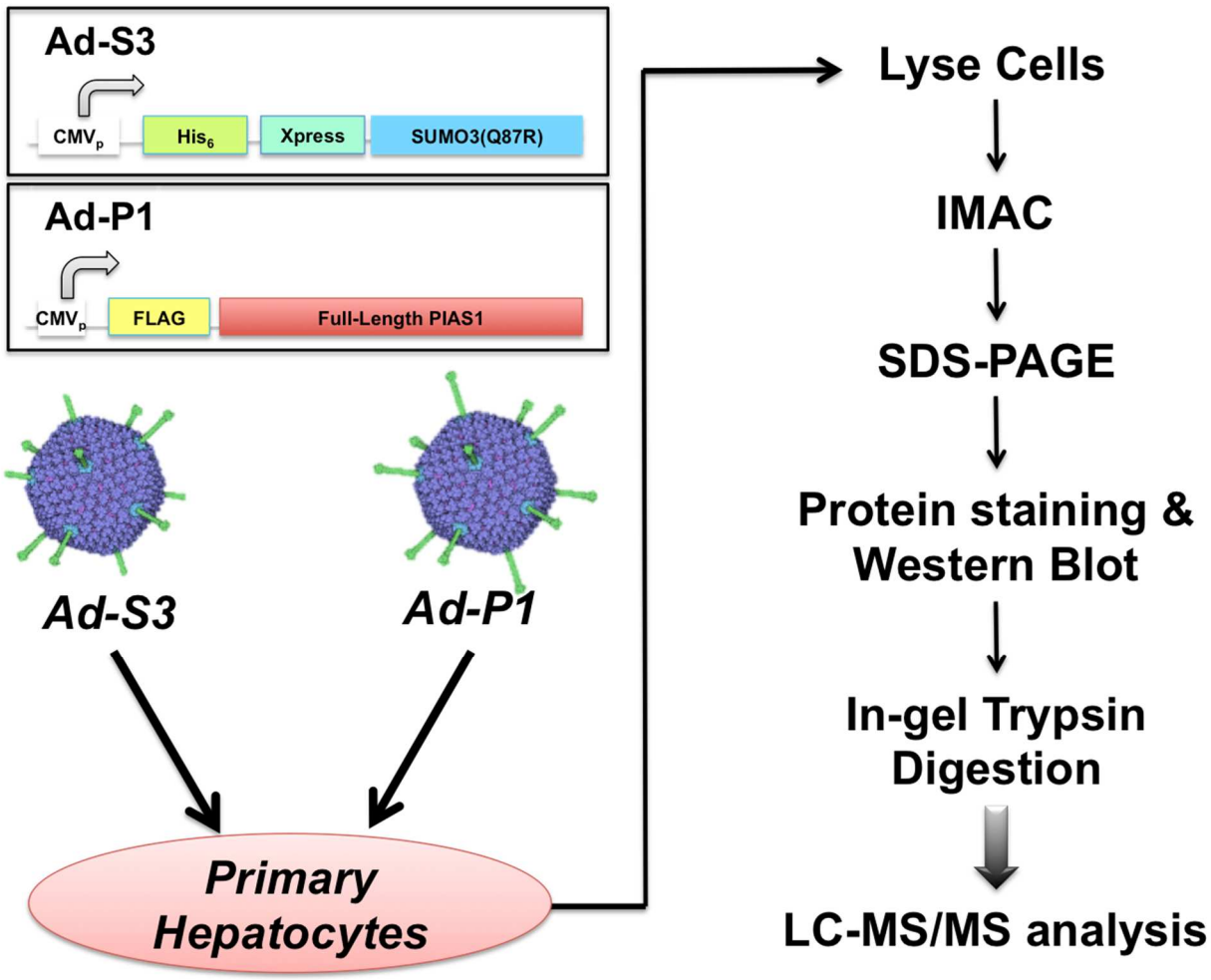

Ad-S3
CMVp
His6
Xpress
SUMO3(Q87R)
Ad-P1
CMVp
FLAG
Full-Length PIAS1
Ad-S3
Ad-P1
Primary Hepatocytes
Lyse Cells
IMAC
SDS-PAGE
Protein staining & Western Blot
In-gel Trypsin Digestion
LC-MS/MS analysis

Figure A1B.

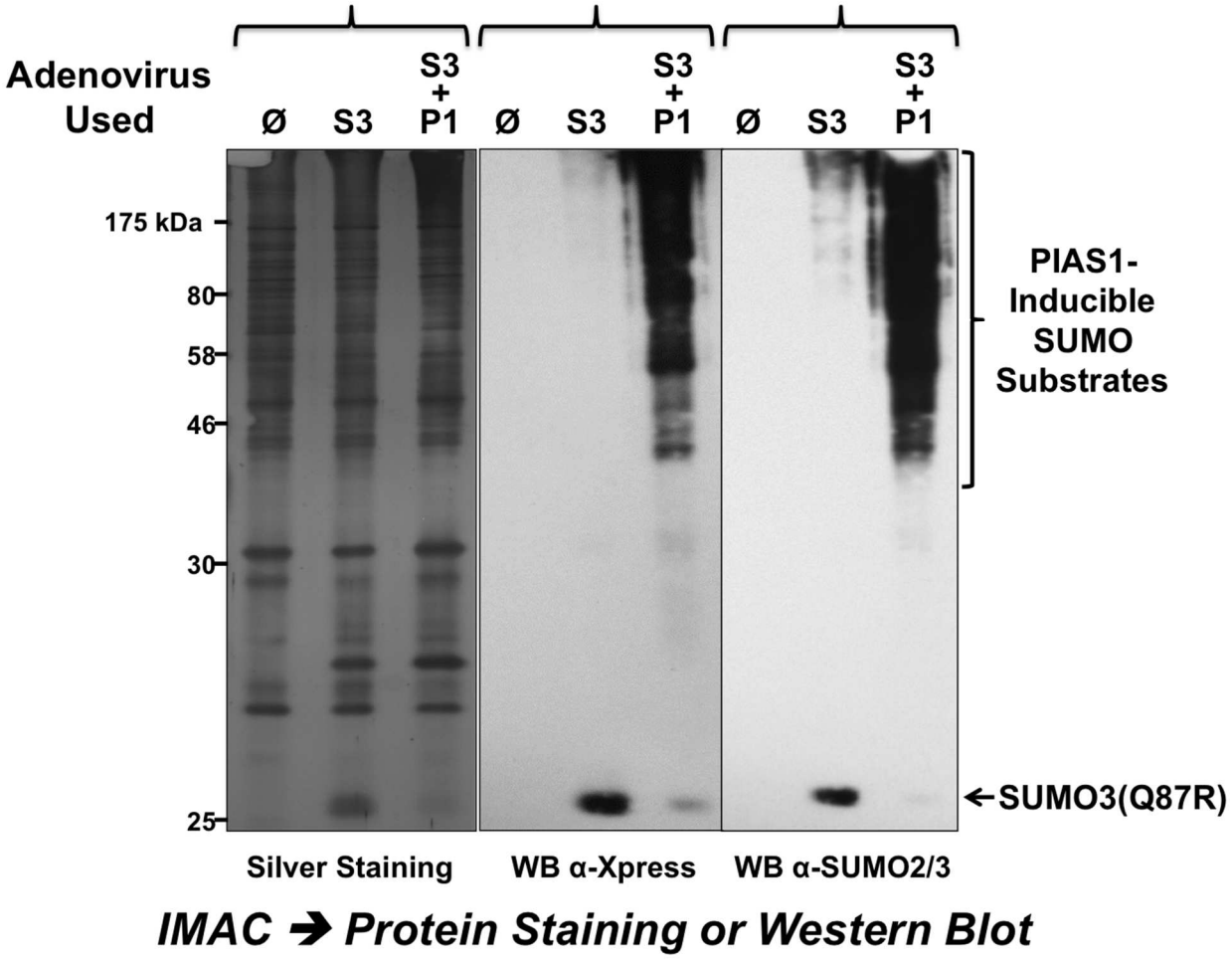


Figure A1. Validation of the Use of the Adenoviral-mediated Experimental Approach to Drive SUMOylation in Primary Hepatocytes. (A) Scheme depicting the adenoviral expression constructs (inset) and the steps used (flow chart) to identify PIAS1-driven SUMO-substrate proteins in primary hepatocytes. **(B)** Three fifteen centimeter plates per experimental group of primary hepatocytes were used to enrich total protein extracts using IMAC as described in *Materials and Methods*. The three experimental groups were blank adenovirus (Ø), Ad-$(His)_6$-SUMO3(Q87)R (S3), and Ad-$(His)_6$-SUMO3(Q87)R together with Ad-PIAS1 (S3 + P1). Following resolution of captured proteins using SDS-PAGE, three identical gels were applied to either silver-staining method (*left panel*) or Western blot analysis using antibodies that detect the expression of the Ad-S3-specific Xpress epitope (*middle panel*) or SUMO2/3(*right panel*).

IMAC Methods Capture Functionally Linked SUMO-Substrate Proteins.

Parallel mass spectrometry analysis of in-gel trypsin digests of protein isolates was performed following resolution of IMAC-captured proteins using conventional SDS-PAGE. Three experimental groups included in this analysis isolated from primary hepatocytes were (1) blank virus-transduced, (2) Ad-SUMO3(Q87R) (Ad-S3) transduced, and (3) Ad-PIAS1 (Ad-P1)/Ad-S3 co-transduced cell extracts. This analysis identified twelve proteins that were exclusive to the Ad-P1/Ad-S3 co-transduced samples and are listed in **Table A1**. Five previously reported SUMO-substrate proteins were identified in our analysis including RanGap(13,14), the two histone proteins H2B and H4(15,16), the transcriptional intermediary factor 1-β(17), and importantly, the SUMO3 protein itself(12). Additional enzymes involved in the SUMOylation pathway were identified including PIAS1 and the ubiquitin-like 1-activating enzyme E1B, also known as SAE2 or UBA2(18). Several important heat shock proteins known to regulate the response to environmental stress including HSP105, HSP40, and HSP70 were also identified exclusively in our experimental group. Two proteins that interface with the heat shock response to regulate the macroautophagy pathway were identified including BAG3, and sequestersome-1, also known as p62. BAG3 is a well-known master regulator of HSP70 biology that functions in part through its ability to physically associate with HSP70 and other important signaling proteins(19,20).

Table A1.

Protein Name	Identified Site(s)	Previously Reported	Reported Site(s)
RanGap1	N.A.	Yes	Lys 526
Histone H2B	Lys 12	Yes	N.A.
Histone H4	N.A.	Yes	Lys 14
Transcription intermediary factor 1 beta (TIF1β)	N.A.	Yes	Lys 750, 779
SUMO3	Lys 11	Yes	Lys 11
PIAS1	Lys 152, 315	No	N.A.
Uba2	N.A.	No	N.A.
BAG3	N.A.	No	N.A.
HSP70	N.A.	No	N.A.
HSP105	N.A.	No	N.A.
DnaJ (HSP40)	N.A.	No	N.A.
Sequestosome1 (p62)	N.A.	No	N.A.

Table A1. Identification of PIAS1-inducible SUMO-substrate Proteins in Primary Hepatocytes. IMAC was performed under strong denaturing conditions using primary rat hepatocytes as described in *Materials and Methods*. Briefly, forty-eight hours prior to generating protein isolates, hepatocytes were transduced with either an empty adenovirus alone as a control group, or co-transduced with Ad-S3 and Ad-P1 as experimental group as shown in **Figure A1**. The protein isolates were subsequently eluted from beads with SDS-sample buffer and were then resolved on a 10% SDS-PAGE gel. In-gel digested tryptic peptides of a total of ten gel-slices ranging in size from 30 kDa to the top of the gel were subjected to LC-MS/MS analysis followed by a MASCOT database search to identify potential SUMO-substrate proteins. Reported here are the unambiguously identified proteins that appeared exclusively in the experimental group. The white-shaded proteins represent previously identified SUMO-substrate protein. The light orange-highlighted proteins including SUMO3, PIAS1, and Uba2 are the proteins involved in SUMOylation pathway. The deep orange-highlighted proteins BAG3, HSP70, HSP105, HSP40, and p62 are all associated with the regulation of the heat shock response and macroautophagy.

The Endogenous BAG3 Protein is the Target of the SUMO-Signaling Pathway.

To determine whether endogenous BAG3 is the target of the SUMO-signaling pathway, we performed immunoprecipitation experiments. Equal amounts of whole cell extract isolated from six independent primary cultures of mouse hepatocytes were subjected to immuno-enrichment with an antibody that recognizes BAG3 **(Figure A2-A)**. A non-immune IgG was used as a negative control. Subsequent Western blot analysis with α-BAG3, α-SUMO1, and α-SUMO2/3 antibodies was used to examine the potential for SUMO-modified forms of BAG3 **(Figure A2-B)**. The BAG antibody detects an approximately 80 kDa un-modified form of BAG3 (*arrow*) as well as lower immuno-reactive levels of an approximately 100 kDa modified form of BAG3 (*asterisk*). Western blotting of identical electrophoretic transfers with either SUMO1- or SUMO2/3-specific antibodies reveals that the endogenous BAG3 protein indeed exists in a SUMO-modified form in primary cultured hepatocytes at approximately 100 kDa in size. It is worth noting here that the overall level and immunoreactivity of the SUMO-modified form of the BAG3 protein is relatively low when compared with the non-modified form, a well-known stoichiometry hallmark of the most SUMO-modified proteins.

Figure A2A.

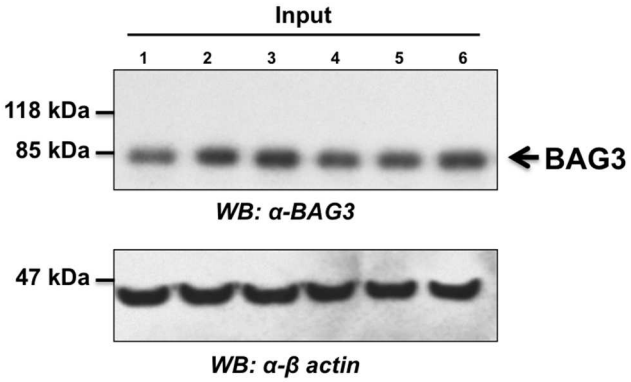


Figure A2B.

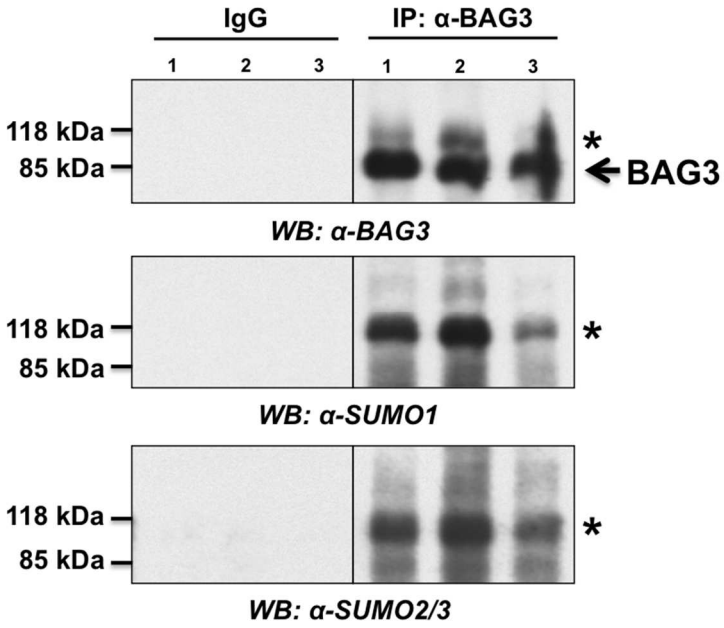


Figure A2. BAG3 Is Expressed in Primary Hepatocytes and Exists in a SUMOylation Form. (A) Primary hepatocytes were isolated from C57Bl/6 mice (n=6). The extent of equivalence of the total protein levels were determined by resolving an aliquot of total protein on an SDS-PAGE gel followed by Western blot analysis to detect expression of BAG3 and β-actin. **(B)** The BAG3 protein was immunoprecipitated from cell extracts using a polyclonal antibody (Abcam, ab47124) as described in *Materials and Methods*. A non-immune antibody was also used as a negative control (IgG). The immunoprecipitates were resolved using SDS-PAGE gel and Western blot analysis was performed with antibodies that recognize BAG3, SUMO1, and SUMO2/3, respectively (n=3). The unmodified BAG3 (*arrow*) and SUMO-modified forms of BAG3 are indicated (*asterisks*).

Identification of the Molecular Machinery that Regulates SUMOylation of BAG3.

To further examine the molecular machinery that drives the SUMOylation of BAG3 in cells, we constructed a FLAG-tagged form of BAG3 for plasmid-based co-expression experiments using the Hepa1-6 mouse hepatoma-derived cell line **(Figure A3)**. Co-transfection of BAG3 together with the $(His)_6$-tagged form of SUMO3 and the respective members of the PIAS family allows for IMAC-enrichment of SUMO-modified BAG3. The PIAS1 and PIASxα SUMO E3-ligase proteins catalyzed high levels of SUMO3ylated BAG3 protein **(Figure A3-A)**. A much lower but detectable level of SUMO-modified BAG3 was detected when PIASxβ, PIAS3, and PIASy were used.

We next examined which members of the SENP family of de-SUMOylation enzymes could catalyze the removal of SUMO3 from BAG3. The SENP1 and SENP2 de-SUMOylating enzymes catalyze the most efficient removal of SUMO3, while SENP3, SENP5, SENP6, and SENP7 do not function as effectively by comparison **(Figure A3-B)**. The catalytically deficient form of SENP2 (mutSENP2) was used to further confirm that the immunoreactivity detected using the BAG3 antibody represents a SUMO-modified form of the BAG3 protein. Indeed, the expression of mutSENP2 restores SUMO-modification of BAG3 **(Figure A3-C)**.

Figure A3A.

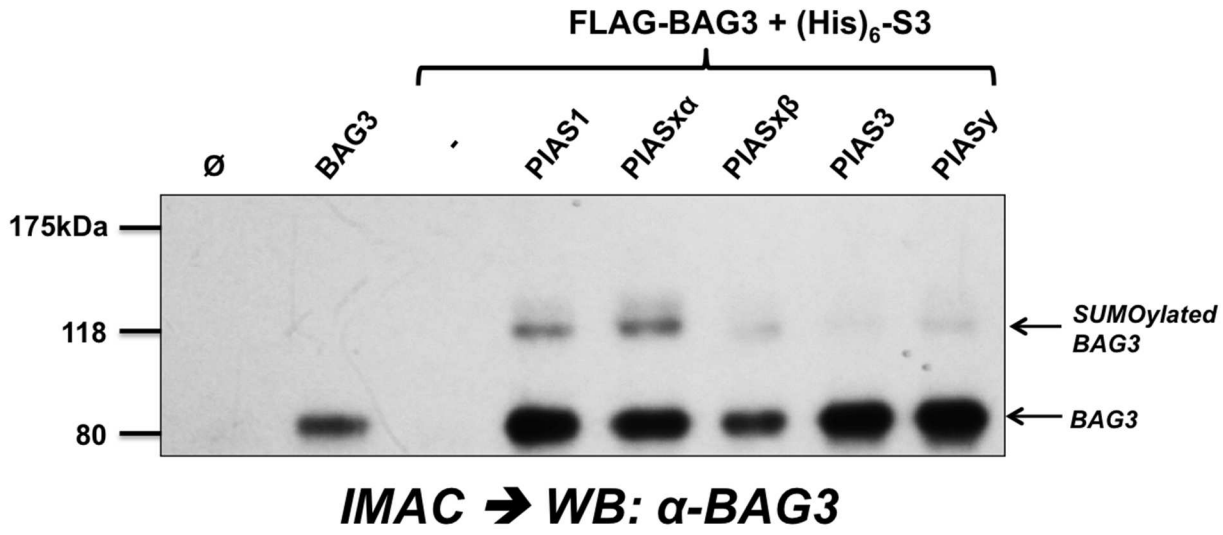


Figure A3B.

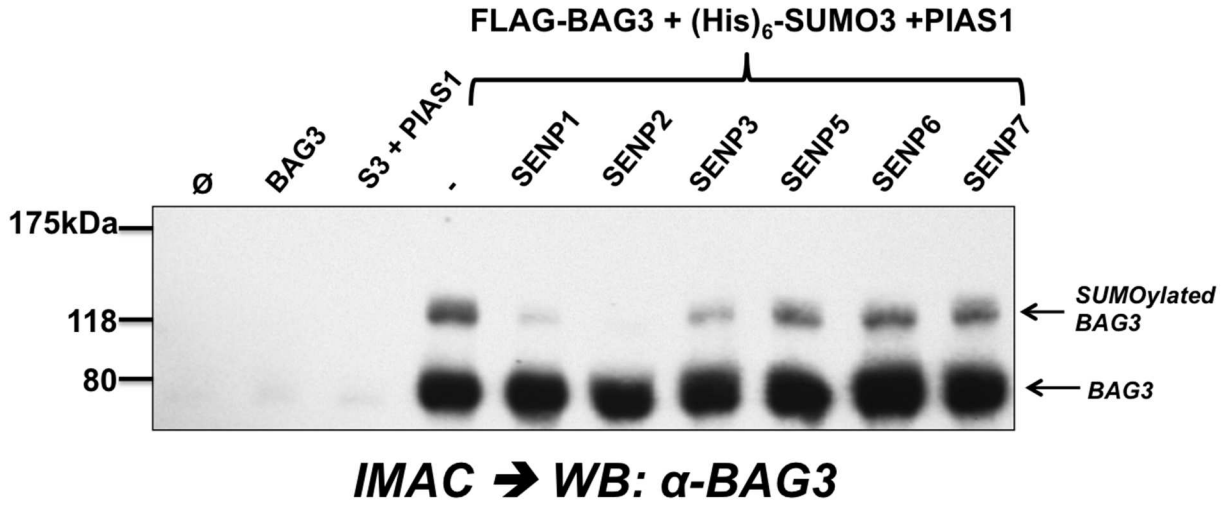


Figure A3C.

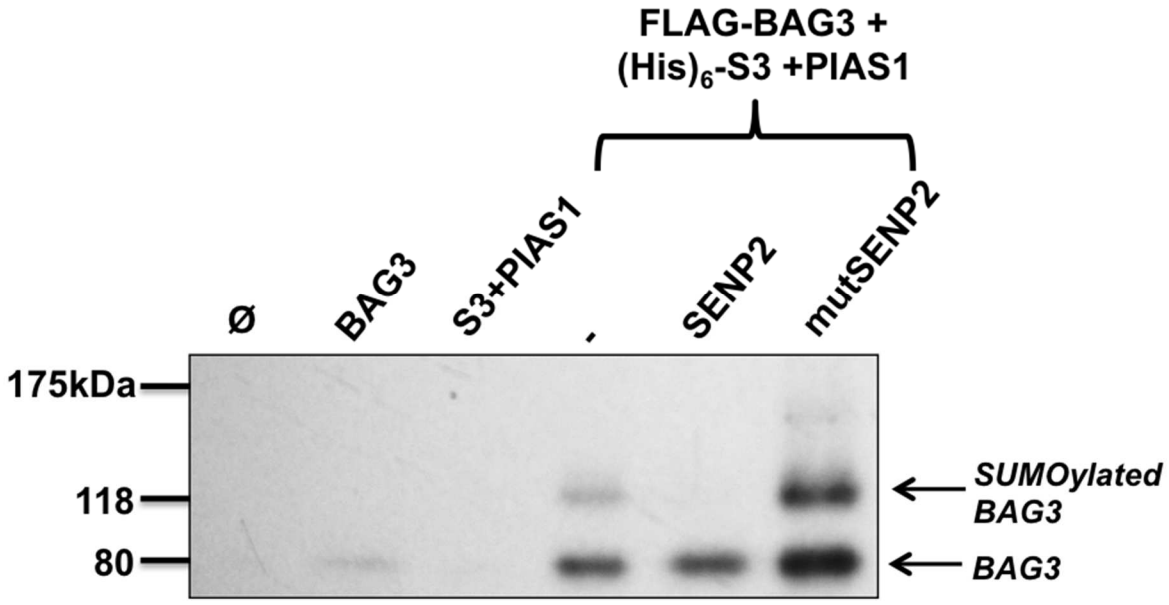


Figure A3. The PIAS Family of E3 SUMO Ligase and Sentrin Protease Enzymes Differentially Mediate the SUMO-Modification of the BAG3 Protein. As indicated in the figure, Hepa1-6 cells were transfected with plasmid-based expression vectors encoding **(A)** individual PIAS family members, **(B)** the individual SENP de-SUMOylation enzymes, and **(C)** the catalytically deficient mutant SENP2 (R577L, K578M) together with $(His)_6$-tagged SUMO3 and PIAS1. SUMOylated proteins were captured using IMAC and the extent of BAG3 modification was analyzed using Western blotting with an anti-BAG3 antibody.

Increased SUMO-Signaling Attenuates the HSP70-BAG3 Protein-Protein Interaction.

Much of the biology of BAG3 is mediated through its interaction with the heat shock response, and specifically through its interaction with HSP70. We next sought to examine the extent to which increased SUMO-signaling could alter the HSP70-BAG3 interaction in cultured cells. Triplicate cultures were transfected as indicated with expression vectors encoding the GFP-tagged form of HSP70 (GFP-HSP70) and the FLAG-tagged form of BAG3. Immunoprecipitation of either BAG3 or HSP70 was accomplished using FLAG or GFP immuno-reactive antibodies, respectively **(Figure A4-A and A4-C)**. The subsequent level of HSP70-BAG3 interaction was analyzed using either FLAG or GFP antibodies in parallel Western blotting experiments. A non-immune IgG was used as a negative control in parallel experiments. Densitometry image analysis of triplicate experiments reveals that co-expression of SUMO3 and PIAS1 significantly decreases the level of HSP70-BAG3 interaction **(Figure A4-B and A4-D)**. These data suggest that SUMO-modification of BAG3 likely interferes with or diminishes the HSP70-BAG3 protein-protein interaction.

We next examined the potential alteration of the sub-cellular location of BAG3 and HSP70 in primary cultures of mouse hepatocytes using the GFP-HSP70 and RFP-BAG3. Co-transfection of these expression vectors allows the quantitative examination of co-localization using biological microscopy(12). Healthy cultures (Hoechst staining) of GFP-HSP70 and RFP-BAG3 co-transfected mouse hepatocytes were visualized analyzed using Slidebook 6 software, and Pearson's co-efficient (r) was measured **(Figure A5)**. High levels of HSP70 and BAG3 were detected visually **(Figure A5-A)** and were highly co-localized with a correlation coefficient of approximately 0.65 **(Figure A5-B)**. In contrast, co-expression of SUMO3 and PIAS1 reduced the amount of visual co-localization **(Figure A5-C)** and the correlation coefficient to 0.48

(Figure A5-D). These data support the notion that increased SUMO-modification of BAG3 deceases its ability to interact with the HSP70 protein.

Figure A4A.

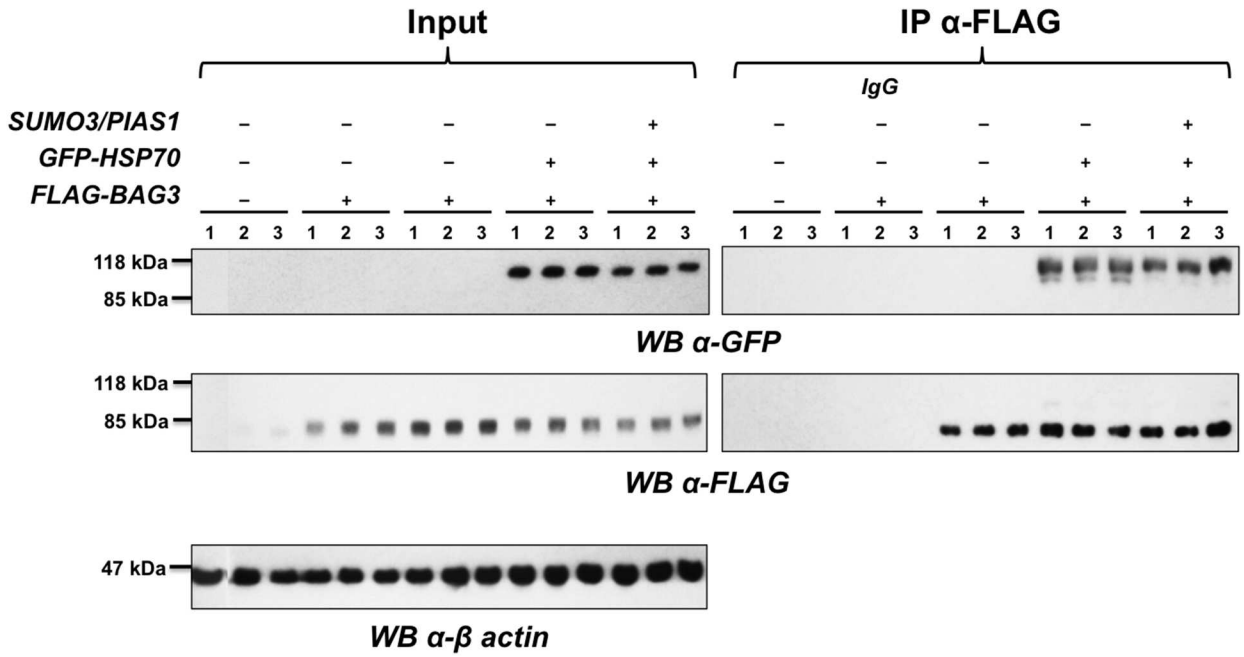


Figure A4B.

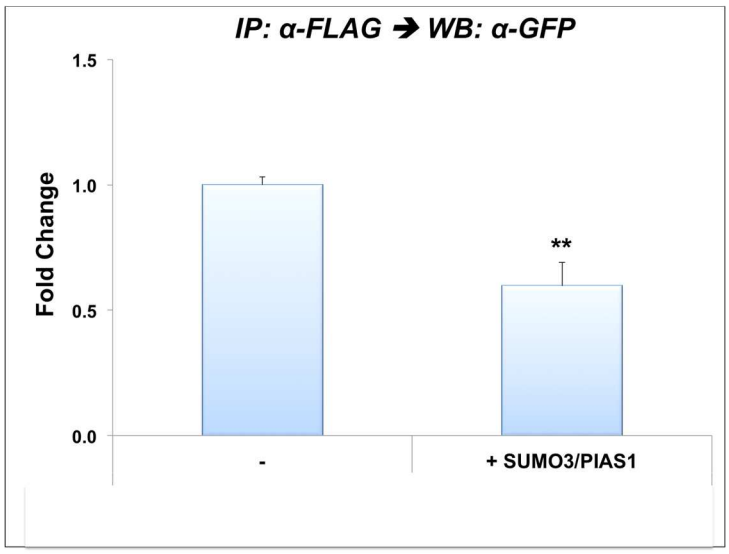


Figure A4C.

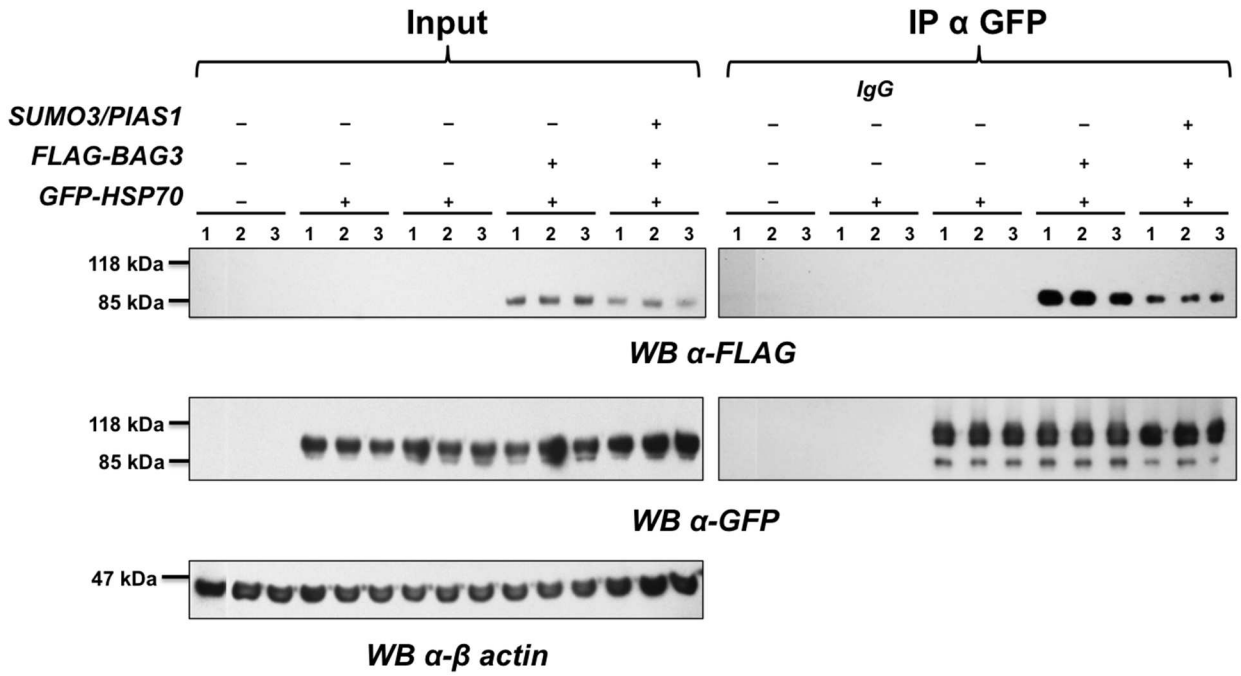


Figure A4D.

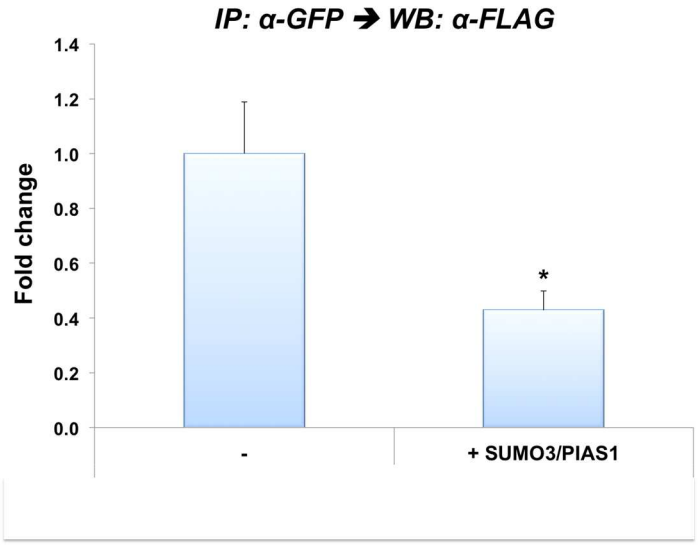


Figure A4. Increased SUMO Signaling Interferes with BAG3-HSP70 Protein-Protein Interaction. Hepa1-6 cells were transfected with the indicated plasmid-based expression vectors encoding SUMO3, PIAS1, GFP-HSP70, and FLAG-BAG3. **(A and C)** The relative equivalence of total protein input and the expression levels of transiently transfected plasmids were confirmed through Western blot analysis using antibodies that recognize GFP-HSP70 (ClonTech, anti-GFP, 632569), FLAG-BAG3 (Sigma-Aldrich, anti-FLAG M2, F3165), and β-actin (*left panel*). Immunoprecipitates from total cell lysates were gathered using either the anti-FLAG antibody to enrich for the BAG3 protein, or the anti-GFP to enrich for the HSP70 protein. A non-immune antibody (IgG) was used as a negative control. These samples were subsequently analyzed for the presence of HSP70 and BAG3 using western blotting methods. **(B and D)** Western blot images were quantitated by densitometric scanning of the X-ray films and analyzed by the ImageJ Software. The numbers represent the relative densitometric image intensity of the immunoprecipitated protein divided by the intensity of he transfected protein input levels. Data are subsequently normalized to β-actin expression. The non-SUMO-PIAS1 transfected result was set to equal 1. Asterisk indicates a statistical difference from non-SUMO-PIAS1 transfected samples (n=3, where * = $p < 0.05$ and ** = $p < 0.01$).

Figure A5A.

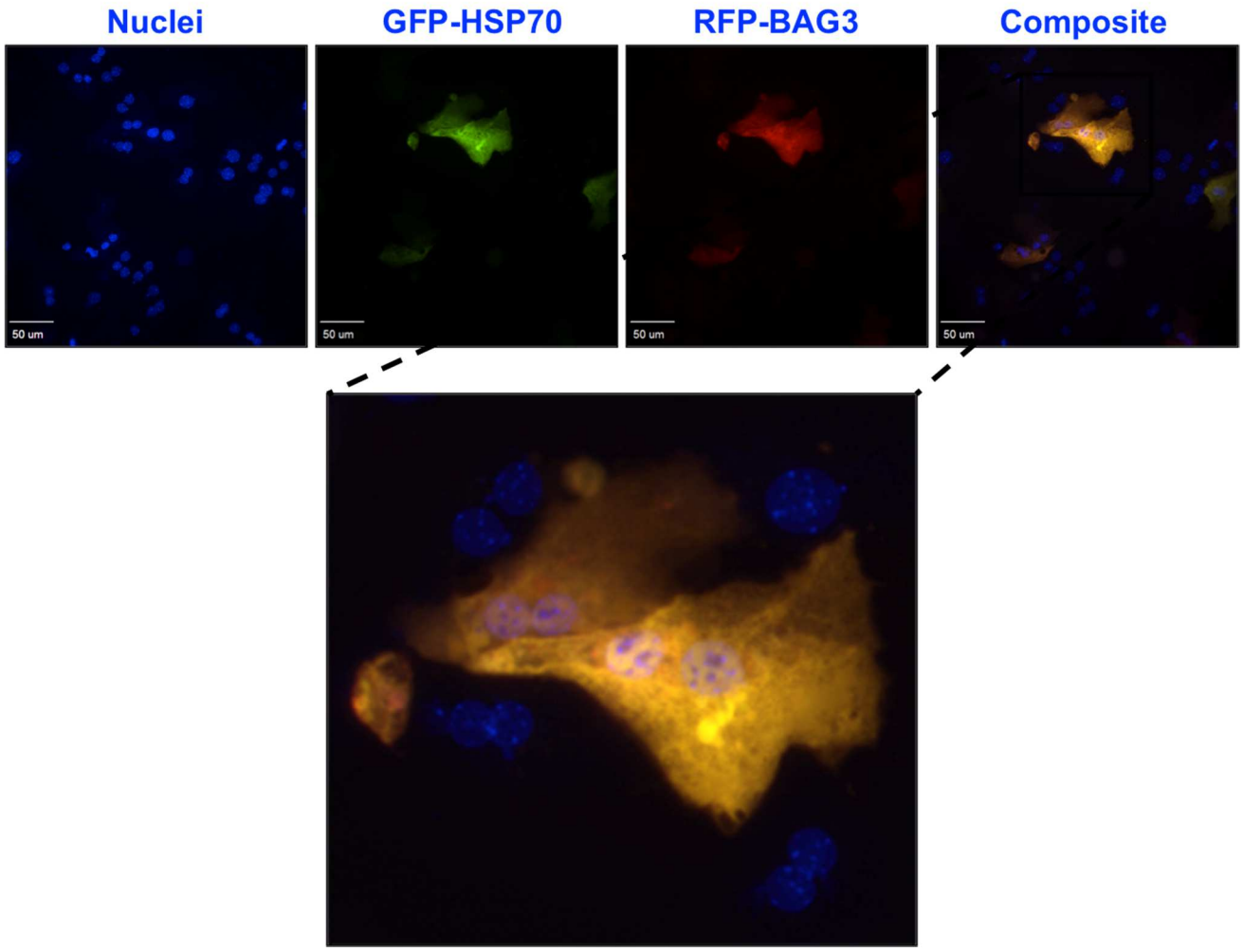


Figure A5B.

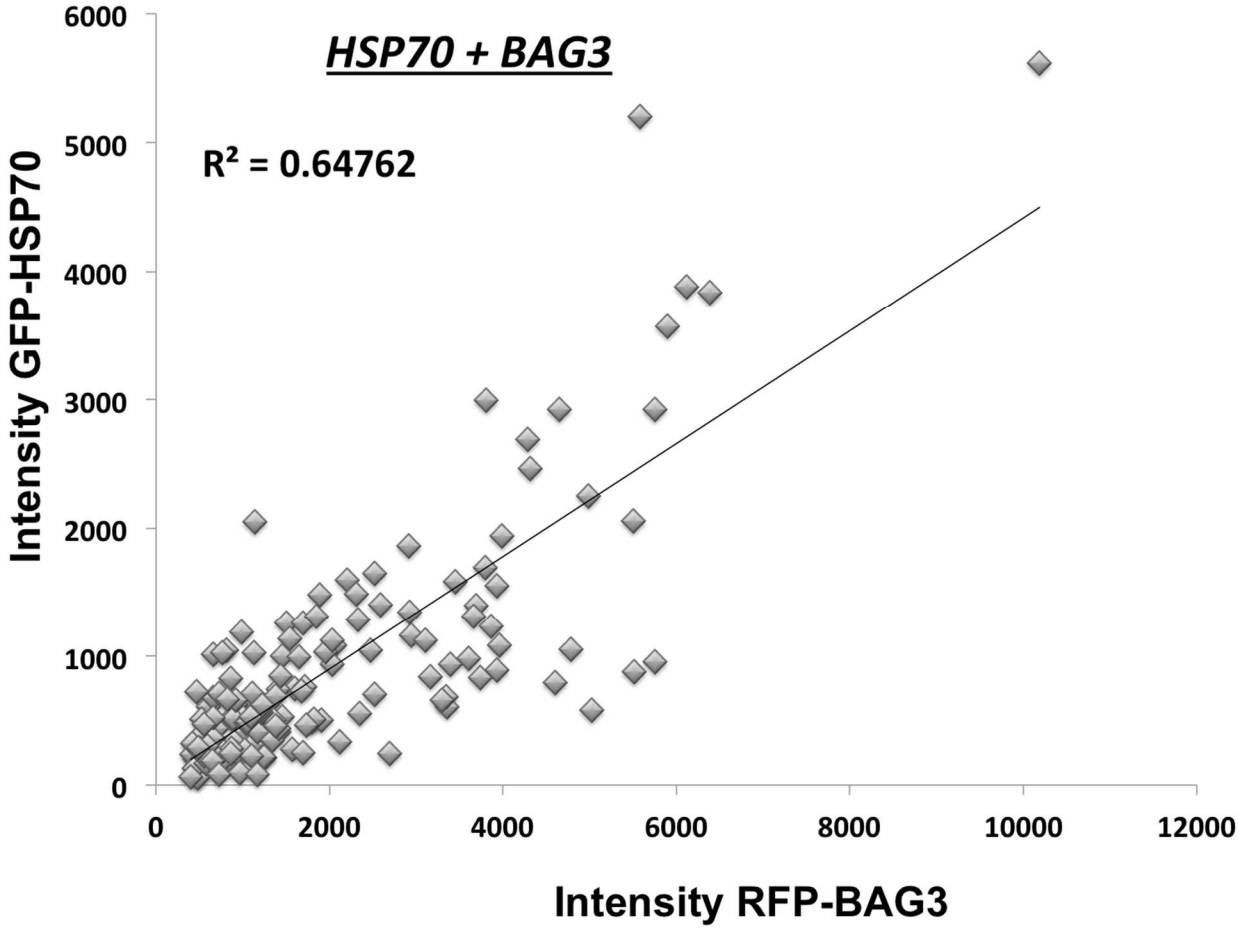

HSP70 + BAG3
R² = 0.64762
Intensity GFP-HSP70
Intensity RFP-BAG3
0
1000
2000
3000
4000
5000
6000
0
2000
4000
6000
8000
10000
12000

Figure A5C.

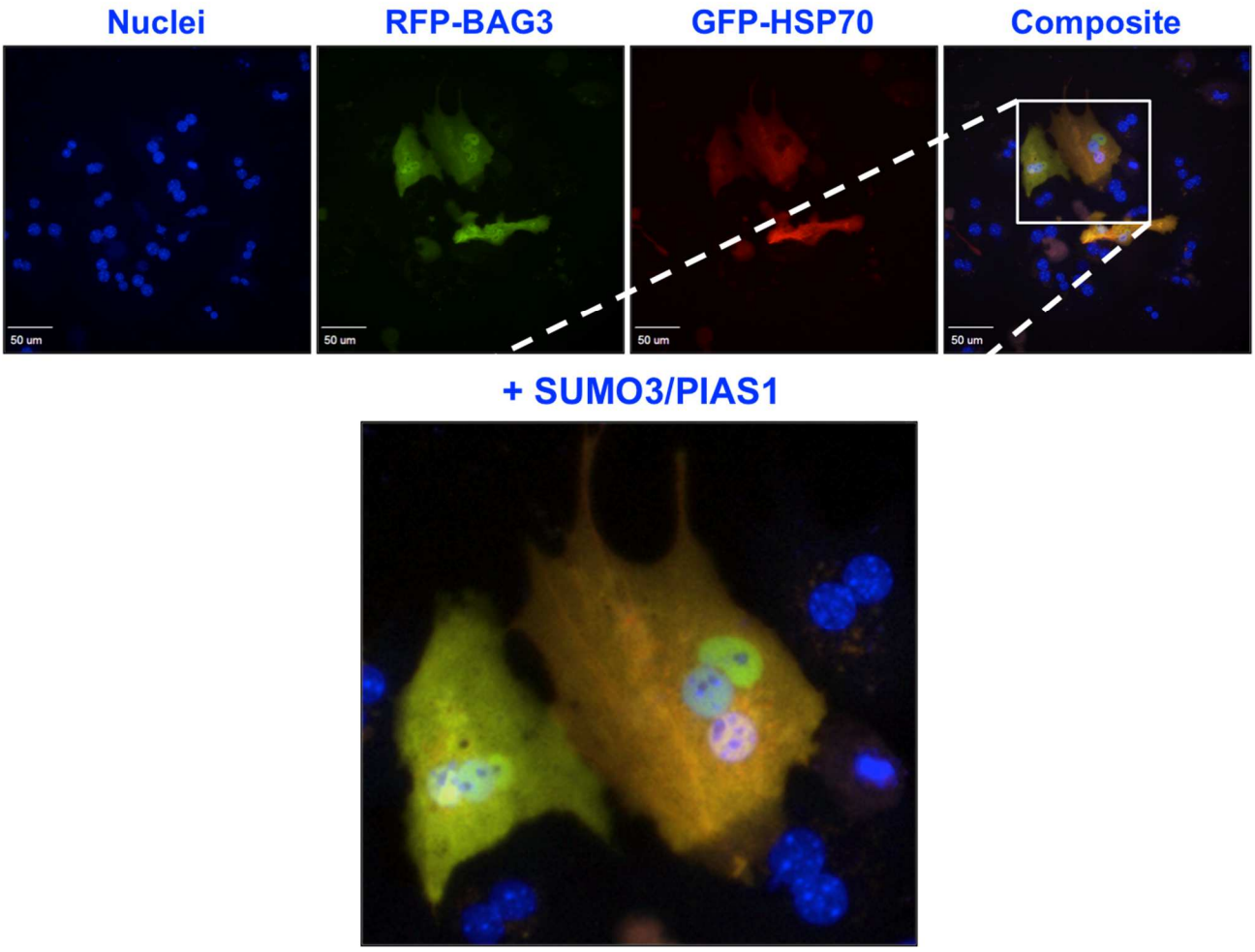

Nuclei
RFP-BAG3
GFP-HSP70
Composite
50 um
50 um
50 um
50 um
+ SUMO3/PIAS1

Figure A5D.

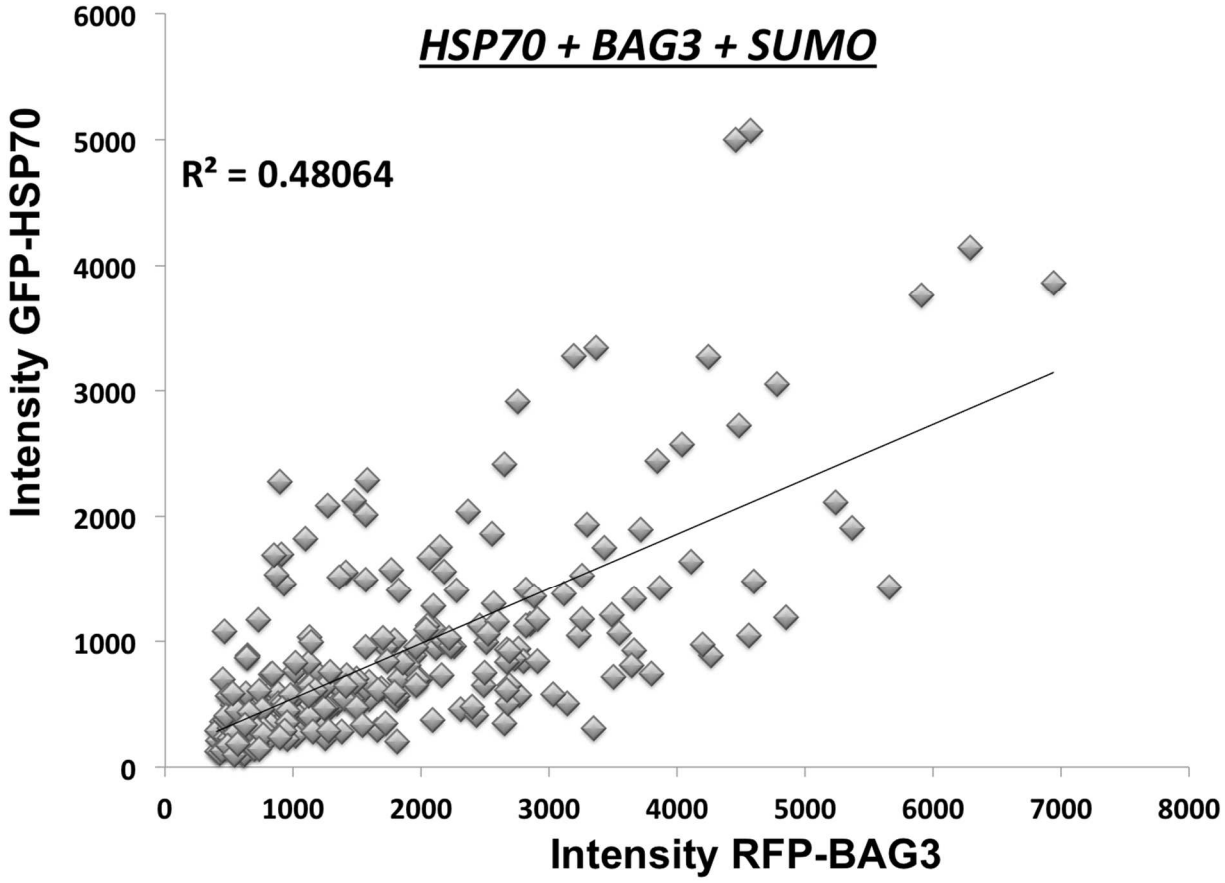


Figure A5. Primary Hepatocytes with Increased SUMO Signaling Exhibit Reduced BAG3-HSP70 Co-localization. Primary cultures of mouse hepatocytes were transfected with the indicated plasmid-based expression vectors encoding SUMO3, PIAS1, GFP-HSP70, and RFP-BAG3. **(A and C)** Fluorescent proteins were imaged using an Olympus IX81 inverted epifluorescence microscope with 40x air objective. Cells were excited at either 485nm (GFP) or 561nm (RFP). **(B and D)** The co-localization of two proteins in fluorescent images was analyzed by using Slidebook 6 Software, and Pearson's co-efficient (*r*) was measured.

DISCUSSION

The SUMO-signaling pathway enables the stress-inducible cellular regulation of biomedical processes such as targeted protein turnover, the repair of damaged DNA, as well as the appropriate response to heat shock, oxidative stress, and chemical insult(1,2,21). The precise and differential role of the four specific PIAS-family members is a current focus of intense investigation. Among the other OUAS protein family members, PIAS1-mediated SUMOylation is particularly essential for DNA repair, and elevated PIAS1 expression has been associated with cancer initiation in diverse tissue types(8,20,22-25).

Technical improvements in utilizing an LC-MS/MS-based approach to examine the global dynamics of protein phosphorylation have provided a wealth of new understanding into the formation of cellular signal transduction networks(13,14). While similar methods for examining SUMO-modification have been experimentally challenging, recent improvement have made it possible to detect SUMO-substrates and sites of SUMO-modification on a global scale(18,26-28). However, these analyses have typically been performed using HeLa cells or *in vitro* experimental model systems. To our knowledge, current studies and methods used to examine global SUMO-signaling networks in primary cell lines have yet to be described.

Our novel experimental strategy overcomes the problem of the extremely low stoichiometry of SUMOylation by increasing the PIAS1-dependent SUMOylation of specific protein substrates in primary hepatocytes **(Figure A1-B)**. Given the broad infectivity profile of the adenoviral system, we believe that these tools should be applied to other primary cultures systems. For example, our adenoviral approach could be used together with neuronal or enteric primary cultures to aid in the identification of SUMO-substrate proteins in those cell types. The successful isolation of well-known SUMO-substrate proteins in our screening including

RanGap1, select histone proteins, and a prominent transcriptional corepressor protein lends confidence to the validity of our novel adenoviral-mediated methods. The isolation of the biochemically linked SUMO-substrate HSP70, Sequestosome 1, DnaJ, HSP105, and BAG3 provides evidence of a likely widespread biochemical interface between PIAS1-mediated SUMO-signaling in the regulation of macroautophagy and the biology of HSP70. Our novel methodology enables us to identify endogenous SUMO-substrate proteins purified directly from primary cells and has yielded important insight into the likely role of PIAS1-mediated increases in SUMO-signaling in regulating the formation of the HSP70-BAG3 protein-protein interaction module in hepatocytes.

The six BAG family members (BAG1-BAG6) all share the conserved BAG protein domain from which the family name arises. All six BAG proteins bind to HSP70 through this domain, thereby acting as co-chaperones that regulate the activity of this key heat shock protein under divergent cellular conditions. Collectively, the BAG family proteins have both a pro-survival and an anti-apoptotic function. The interaction between HSP70 and the different BAG proteins play both overlapping and distinctive roles in regulating cellular protein homeostasis(7,29,30). Under normal physiological conditions, protein turnover via proteasomal degradation is driven by the HSP70-BAG1 protein-protein interaction(7,29). Importantly, in response to conditions of heat shock, oxidative stress, or cell aging the expression of BAG3 is highly induced and the resulting HSP70-BAG3 protein-protein interaction enables the macroautophagy pathway(31-33). This functional switch from BAG1-mediated proteasomal degradation to BAG3-induced macroautophagy has been characterized as an adaptive response to proteasomal impairment and overloading in a pathophysiological milieu(32,34).

Recent investigations suggest the BAG3 protein likely plays a critical role in driving tumorigenesis, cancer metastases, cardiac and skeletal muscle myopathies, and neurodegenerative diseases(6,35-37). A key observation is that elevated BAG3 expression is detected in many primary tumors and tumorigenic cell types including leukemia(38), thyroid tumors(39), neuroblastoma(40), prostate carcinomas(41,42), pancreatic tumors(43,44), ovarian cancer(45), glioblastoma(46,47), and aggressive forms of liver cancer(48). The BAG3 protein is comprised of a series of modular protein domains that allow the formation of diverse interactions with other signaling proteins. At the molecular level, the HSP70-BAG3 protein module is a broad-acting regulator of cancer cell signaling which functions by modulating the activity of transcription factors NF-κB, forkhead box M1 (FoxM1), hypoxia inducible factor 1 alpha (HIF1α), the translation regulator HuR, the cell cycle regulators p21, surviving, and Src signaling(6,36). Because the HSP70-BAG3 protein-protein interaction plays a prominent role in regulating macroautophagy and apoptosis, this signaling module has recently emerged as an attractive and viable drug target(6,36,37,49). Recent efforts have focused on the identification of small molecule inhibitors of the HSP70-BAG3 module to provide a novel means to selectively target the function of this cancer-associated molecular chaperone complex(6,36,37).

Liver cancer, or hepatocellular carcinoma (HCC), is a highly vascular and invasive tumor. HCC is the third leading cause of cancer deaths worldwide and the second leading cause of cancer deaths in China(50). Elevated expression of PIAS1 protein has been associated with non-responding chronic hepatitis C patients, who have a much greater risk of developing HCC(19). The primary driver of HCC formation and progression is un-checked inflammation in liver cells. Important for this study, the levels of BAG3(48), HSP70(51), and the enzymes involved in the SUMO-signaling pathway(52-54) are all elevated in aggressive forms of human

HCC. Overall, our results provide novel insights into the negative regulatory role of SUMO-signaling in HSP70-BAG3 signaling complex, and may offer another appealing biochemical target in HSP70-BAG3-driven cancers.

Our experimental approach, which demonstrates unequivocally that the endogenous BAG3 protein is a SUMO-substrate, is presented in **Figure A2**. This approach utilizes primary hepatocytes and immuno-enrichment (IP with anti-BAG3 antibodies) followed by immuno-detection (anti-SUMO antibodies). Merely using Western blotting for BAG3 using whole cell extracts consistently fails to detect the SUMO-modified form, or indeed any modified form of BAG3 **(Figure A2-A, *top panel*)**. Similarly, in **Figure A3**, we used a transfected cell line and a $(His)_6$-tagged form of SUMO3 followed by IMAC to specifically enrich the SUMO-modified form of BAG3. In each case, some sort of an enrichment step is required to visualize SUMO-modified BAG3. This is due to the extremely low stoichiometry of the SUMO-modification in cells. It is therefore not currently clear if the SUMOylation event is disrupting the HSP70-BAG3 interaction directly, or alternatively, the SUMOylation of BAG3 is a signal that delivers itself to the macroautophagy pathway. Deciphering between these mechanisms represent interesting issues for the future. The BAG3 protein was analyzed for the presence of the consensus SUMOylation sequence as defined by an online SUMOplot server (http://www.abgent.com/tools/SUMOplot). This type of bioinformatic analysis identifies nine potential sites for SUMOylation, three of which are predicted as "high probability" SUMOylation sites and six others that are predicted as "low probability" SUMOylation sites **(Figure A6)**. The highest probability consensus SUMOylation sites lie within the N-terminal WW domain and C-terminal BAG domain, respectively. When analyzed using the Clustal Multiple Sequence Alignment tool, the BAG4 family member also contains a conserved lysine

residue within its ABG domain at that position, while the other family members do not. We therefore feel that it is likely that BAG3 is modified by SUMO potentially at several positions. Additional site-direct mutagenesis and *in vitro* mapping approaches should address the effect of increased BAG3 SUMOylation on interaction with HSP70 and other proteins that are known to bind to BAG3 through its WW and PxxP domains.

Taken together, our data suggest that modulation of the HSP70-BAG3-SUMO3-PIAS1 interaction may be involved in sustaining HCC formation, and likely plays a role in driving tumor migration and invasion. The methods described here should yield further insight into the specific function of the PIAS1 SUMO E3 ligase enzyme in driving HCC. Future efforts should also be focused on determining the extent to which the SUMOylation of BAG3 alters its anti-apoptotic activity in cancerous cells. Additionally, future studies should examine the crosstalk between acetylation, phosphorylation and SUMO-signaling pathways that likely converge at the level of ABG3 protein.

Figure A6.

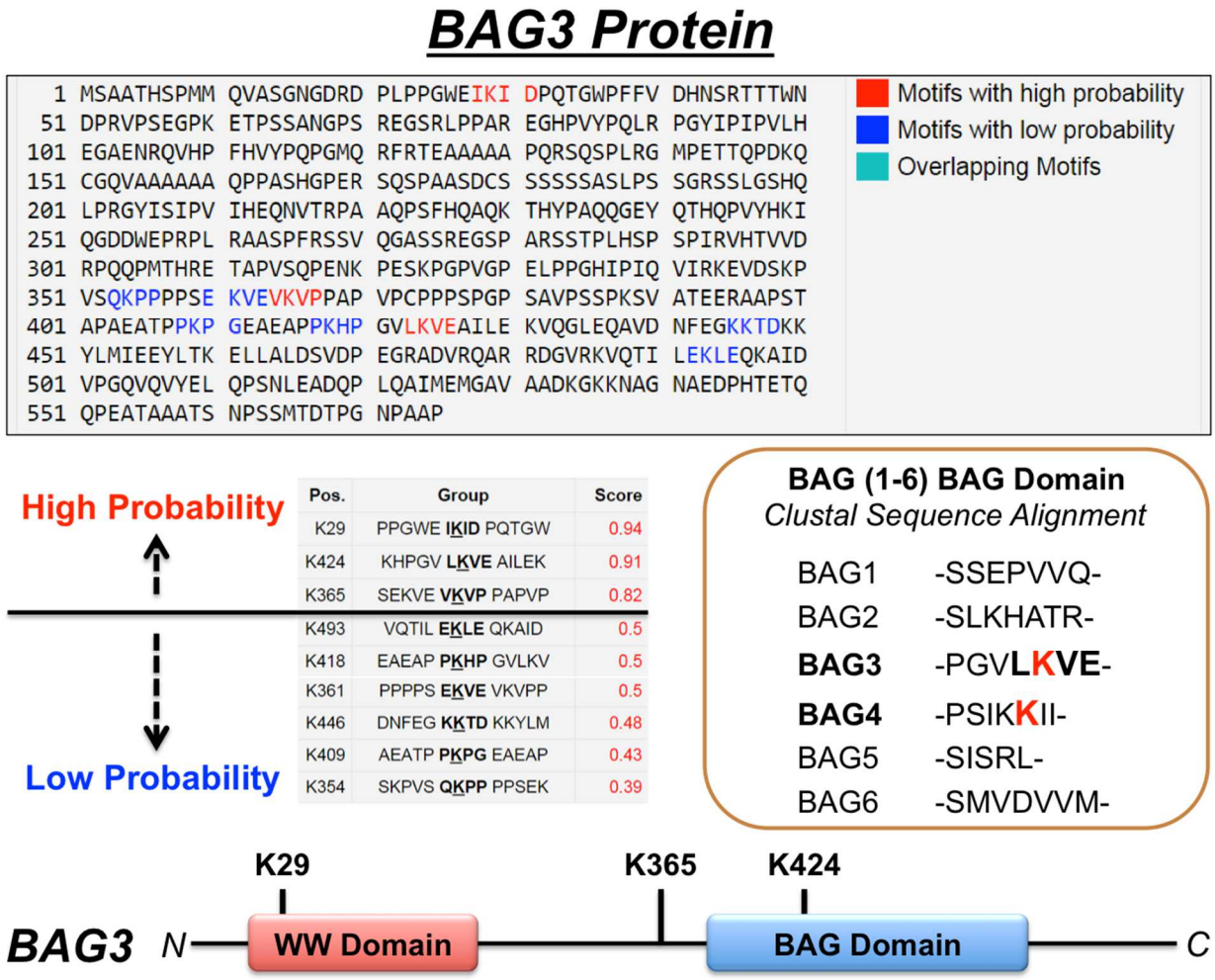


Figure A6. Bioinformatic Characterization of the Potential SUMOylation Sites within the BAG3 protein. The human BAG3 protein was analyzed for the presence of the consensus SUMOylation sequence as defined by an online SUMOPlot server (http://www.abgent.com/tools/SUMOPlot). This type of bioinformatic analysis identifies three potential "high probability" sites for SUMOylation and six others that are predicted as "low probability" SUMOylation sites. To provide additional context, a diagram depicts the high probability sites within the context of the whole protein amino acid sequence, while a linear cartoon depicts the presence of the three "high probability" sites. A Clustal analysis of the BAG domains within BAG1-BAG6 shows that BAG4 possesses a conserved lysine residue within a weak SUMOylation consensus site at that location as well, while the other BAG family members do not. Of note, a "high probability" consensus SUMOylation site (K29) occurs within the WW domain near the N-terminal region, and one occurs within the BAG domain (K424) at theBAG3 C-terminus.

REFERENCES

1. Hannoun, Z., Greenhough, S., Jaffray, E., Hay, R. T., and Hay, D. C. (2010) Post-translational modification by SUMO. *Toxicology* **278**, 288-293
2. Hay, R. T. (2007) SUMO-specific proteases: a twist in the tail. *Trends Cell Biol* **17**, 370-376
3. Doong, H., Vrailas, A., and Kohn, E. C. (2002) What's in the 'BAG'?--A functional domain analysis of the BAG-family proteins. *Cancer Lett* **188**, 25-32
4. Blomster, H. A., Imanishi, S. Y., Siimes, J., Kastu, J., Morrice, N. A., Eriksson, J. E., and Sistonen, L. (2010) In vivo identification of sumoylation sites by a signature tag and cysteine-targeted affinity purification. *J Biol Chem* **285**, 19324-19329
5. Matic, I., Schimmel, J., Hendriks, I. A., van Santen, M. A., van de Rijke, F., van Dam, H., Gnad, F., Mann, M., and Vertegaal, A. C. (2010) Site-specific identification of SUMO-2 targets in cells reveals an inverted SUMOylation motif and a hydrophobic cluster SUMOylation motif. *Mol Cell* **39**, 641-652
6. Colvin, T. A., Gabai, V. L., Gong, J., Calderwood, S. K., Li, H., Gummuluru, S., Matchuk, O. N., Smirnova, S. G., Orlova, N. V., Zamulaeva, I. A., Garcia-Marcos, M., Li, X., Young, Z. T., Rauch, J. N., Gestwicki, J. E., Takayama, S., and Sherman, M. Y. (2014) Hsp70-Bag3 interactions regulate cancer-related signaling networks. *Cancer Res* **74**, 4731-4740
7. Bukau, B., Weissman, J., and Horwich, A. (2006) Molecular chaperones and protein quality control. *Cell* **125**, 443-451
8. Driscoll, J. J., Pelluru, D., Lefkimmiatis, K., Fulciniti, M., Prabhala, R. H., Greipp, P. R., Barlogie, B., Tai, Y. T., Anderson, K. C., Shaughnessy, J. D., Jr., Annunziata, C. M., and Munshi, N. C. (2010) The sumoylation pathway is dysregulated in multiple myeloma and is associated with adverse patient outcome. *Blood* **115**, 2827-2834
9. Cheng, J., Kang, X., Zhang, S., and Yeh, E. T. (2007) SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. *Cell* **131**, 584-595
10. Cui, W., Sun, M., Zhang, S., Shen, X., Galeva, N., Williams, T. D., and Staudinger, J. L. (2016) A SUMO-acetyl switch in PXR biology. *Biochim Biophys Acta* **1859**, 1170-1182
11. Cui, W., Sun, M., Galeva, N., Williams, T. D., Azuma, Y., and Staudinger, J. L. (2015) SUMOylation and Ubiquitylation Circuitry Controls Pregnane X Receptor Biology in Hepatocytes. *Drug Metab Dispos* **43**, 1316-1325
12. Dunn, K. W., Kamocka, M. M., and McDonald, J. H. (2011) A practical guide to evaluating colocalization in biological microscopy. *Am J Physiol Cell Physiol* **300**, C723-742
13. Daub, H., Olsen, J. V., Bairlein, M., Gnad, F., Oppermann, F. S., Korner, R., Greff, Z., Keri, G., Stemmann, O., and Mann, M. (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Mol Cell* **31**, 438-448
14. Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* **127**, 635-648
15. Nathan, D., Ingvarsdottir, K., Sterner, D. E., Bylebyl, G. R., Dokmanovic, M., Dorsey, J. A., Whelan, K. A., Krsmanovic, M., Lane, W. S., Meluh, P. B., Johnson, E. S., and Berger, S. L. (2006) Histone sumoylation is a negative regulator in Saccharomyces

cerevisiae and shows dynamic interplay with positive-acting histone modifications. *Genes Dev* **20**, 966-976
16. Nathan, D., Sterner, D. E., and Berger, S. L. (2003) Histone modifications: Now summoning sumoylation. *Proc Natl Acad Sci U S A* **100**, 13118-13120
17. Mascle, X. H., Germain-Desprez, D., Huynh, P., Estephan, P., and Aubry, M. (2007) Sumoylation of the transcriptional intermediary factor 1beta (TIF1beta), the Co-repressor of the KRAB Multifinger proteins, is required for its transcriptional activity and is modulated by the KRAB domain. *J Biol Chem* **282**, 10190-10202
18. Ma, L., Aslanian, A., Sun, H., Jin, M., Shi, Y., Yates, J. R., 3rd, and Hunter, T. (2014) Identification of small ubiquitin-like modifier substrates with diverse functions using the Xenopus egg extract system. *Mol Cell Proteomics* **13**, 1659-1675
19. El-Saadany, S., Ziada, D. H., El Bassat, H., Farrag, W., El-Serogy, H., Eid, M., Abdallah, M., Ghazy, M., and Salem, H. A. (2013) The role of hepatic expression of STAT1, SOCS3 and PIAS1 in the response of chronic hepatitis C patients to therapy. *Can J Gastroenterol* **27**, e13-17
20. Li, R., Wei, J., Jiang, C., Liu, D., Deng, L., Zhang, K., and Wang, P. (2013) Akt SUMOylation regulates cell proliferation and tumorigenesis. *Cancer Res* **73**, 5742-5753
21. Bettermann, K., Benesch, M., Weis, S., and Haybaeck, J. (2012) SUMOylation in carcinogenesis. *Cancer Lett* **316**, 113-125
22. Galanty, Y., Belotserkovskaya, R., Coates, J., Polo, S., Miller, K. M., and Jackson, S. P. (2009) Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. *Nature* **462**, 935-939
23. Liu, B., Tahk, S., Yee, K. M., Yang, R., Yang, Y., Mackie, R., Hsu, C., Chernishof, V., O'Brien, N., Jin, Y., Fan, G., Lane, T. F., Rao, J., Slamon, D., and Shuai, K. (2014) PIAS1 regulates breast tumorigenesis through selective epigenetic gene silencing. *PLoS One* **9**, e89464
24. Puhr, M., Hoefer, J., Eigentler, A., Dietrich, D., van Leenders, G., Uhl, B., Hoogland, M., Handle, F., Schlick, B., Neuwirt, H., Sailer, V., Kristiansen, G., Klocker, H., and Culig, Z. (2016) PIAS1 is a determinant of poor survival and acts as a positive feedback regulator of AR signaling through enhanced AR stabilization in prostate cancer. *Oncogene* **35**, 2322-2332
25. Shima, H., Suzuki, H., Sun, J., Kono, K., Shi, L., Kinomura, A., Horikoshi, Y., Ikura, T., Ikura, M., Kanaar, R., Igarashi, K., Saitoh, H., Kurumizaka, H., and Tashiro, S. (2013) Activation of the SUMO modification system is required for the accumulation of RAD51 at sites of DNA damage. *J Cell Sci* **126**, 5284-5292
26. Hendriks, I. A., D'Souza, R. C., Chang, J. G., Mann, M., and Vertegaal, A. C. (2015) System-wide identification of wid type SUMO-2 conjugation sites. *Nat Commun* **6**, 7289
27. Tammsalu, T., Matic, I., Jaffray, E. G., Ibrahim, A. F., Tatham, M. H., and Hay, R. T. (2014) Proteome-wide identification of SUMO2 modification sites. *Sci Signal* **7**, rs2
28. Zhu, B., Farris, T. R., Milligan, S. L., Chen, H., Zhu, R., Hong, A., Zhou, X., Gao, X., and McBride, J. W. (2016) Rapid identification of ubiquitination and SUMOylation target sites by microfluidic peptide array. *Biochem Biophys Rep* **5**, 430-438
29. Hipp, M. S., Park, S. H., and Hartl, F. U. (2014) Proteostasis impairment in protein-misfolding and -aggregation diseases. *Trends Cell Biol* **24**, 506-514
30. Saibil, H. (2013) Chaperone machines for protein folding, unfolding and disaggregation. *Nat Rev Mol Cell Biol* **14**, 630-642

31. Behl, C. (2011) BAG3 and friends: co-chaperones in selective autophagy during aging and disease. *Autophagy* **7**, 795-798
32. Gamerdinger, M., Hajieva, P., Kaya, A. M., Wolfrum, U., Hartl, F. U., and Behl, C. (2009) Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. *EMBO J* **28**, 889-901
33. Minoia, M., Boncoraglio, A., Vinet, J., Morelli, F. F., Brunsting, J. F., Poletti, A., Krom, S., Reits, E., Kampinga, H. H., and Carra, S. (2014) BAG3 induces the sequestration of proteasomal clients into cytoplasmic puncta: implications for a proteasome-to-autophagy switch. *Autophagy* **10**, 1603-1621
34. Gamerdinger, M., Kaya, A. M., Wolfrum, U., Clement, A. M., and Behl, C. (2011) BAG3 mediates chaperone-based aggresome-targeting and selective autophagy of misfolded proteins. *EMBO Rep* **12**, 149-156
35. Behl, C. (2016) Breaking BAG: The Co-Chaperone BAG3 in Health and Disease. *Trends Pharmacol Sci* **37**, 672-688
36. Li, X., Colvin, T., Rauch, J. N., Acosta-Alvear, D., Kampmann, M., Dunyak, B., Hann, B., Aftab, B. T., Murnane, M., Cho, M., Walter, P., Weissman, J. S., Sherman, M. Y., and Gestwicki, J. E. (2015) Validation of the Hsp70-Bag3 protein-protein interaction as a potential therapeutic target in cancer. *Mol Cancer Ther* **14**, 642-648
37. Sherman, M. Y., and Gabai, V. L. (2015) Hsp70 in cancer: back to the future. *Oncogene* **34**, 4153-4161
38. Valdez, B. C., Murray, D., Ramdas, L., de Lima, M., Jones, R., Kornblau, S., Betancourt, D., Li, Y., Champlin, R. E., and Andersson, B. S. (2008) Altered gene expression in busulfan-resistant human myeloid leukemia. *Leuk Res* **32**, 1684-1697
39. Chiappetta, G., Ammirante, M., Basile, A., Rosati, A., Festa, M., Monaco, M., Vuttariello, E., Pasquinelli, R., Arra, C., Zerilli, M., Todaro, M., Stassi, G., Pezzullo, L., Gentilella, A., Tosco, A., Pascale, M., Marzullo, L., Belisario, M. A., Turco, M. C., and Leone, A. (2007) The antiapoptotic protein BAG3 is expressed in thyroid carcinomas and modulates apoptosis mediated by tumor necrosis factor-related apoptosis-inducing ligand. *J Clin Endocrinol Metab* **92**, 1159-1163
40. Brendel, A., Felzen, V., Morawe, T., Manthey, D., and Behl, C. (2013) Differential regulation of apoptosis-associated genes by estrogen receptor alpha in human neuroblastoma cells. *Restor Neurol Neurosci* **31**, 199-211
41. Ammirante, M., De Laurenzi, V., Graziano, V., Turco, M. C., and Rosati, A. (2011) BAG3 is required for IKKalpha nuclear translocation and emergence of castration resistant prostate cancer. *Cell Death Dis* **2**, e139
42. Iwasaki, M., Homma, S., Hishiya, A., Dolezal, S. J., Reed, J. C., and Takayama, S. (2007) BAG3 regulates motility and adhesion of epithelial cancer cells. *Cancer Res* **67**, 10252-10259
43. Liao, Q., Ozawa, F., Friess, H., Zimmermann, A., Takayama, S., Reed, J. C., Kleeff, J., and Buchler, M. W. (2001) The anti-apoptotic protein BAG-3 is overexpressed in pancreatic cancer and induced by heat stress in pancreatic cancer cell lines. *FEBS Lett* **503**, 151-157
44. Rosati, A., Basile, A., D'Auria, R., d'Avenia, M., De Marco, M., Falco, A., Festa, M., Guerriero, L., Iorio, V., Parente, R., Pascale, M., Marzullo, L., Franco, R., Arra, C., Barbieri, A., Rea, D., Menichini, G., Hahne, M., Bijlsma, M., Barcaroli, D., Sala, G., di Mola, F. F., di Sebastiano, P., Todoric, J., Antonucci, L., Corvest, V., Jawhari, A., Firpo,

M. A., Tuveson, D. A., Capunzo, M., Karin, M., De Laurenzi, V., and Turco, M. C. (2015) BAG3 promotes pancreatic ductal adenocarcinoma growth by activating stromal macrophages. *Nat Commun* **6**, 8695

45. Sugio, A., Iwasaki, M., Habata, S., Mariya, T., Suzuki, M., Osogami, H., Tamate, M., Tanaka, R., and Saito, T. (2014) BAG3 upregulates Mcl-1 through downregulation of miR-29b to induce anticancer drug resistance in ovarian cancer. *Gynecol Oncol* **134**, 615-623
46. Festa, M., Del Valle, L., Khalili, K., Franco, R., Scognamiglio, G., Graziano, V., De Laurenzi, V., Turco, M. C., and Rosati, A. (2011) BAG3 protein is overexpressed in human glioblastoma and is a potential target for therapy. *Am J Pathol* **178**, 2504-2512
47. Gentilella, A., and Khalili, K. (2011) BAG3 expression in glioblastoma cells promotes accumulation of ubiquitinated clients in an Hsp70-dependent manner. *J Biol Chem* **286**, 9205-9215
48. Xiao, H., Cheng, S., Tong, R., Lv, Z., Ding, C., Du, C., Xie, H., Zhou, L., Wu, J., and Zheng, S. (2014) BAG3 regulates epithelial-mesenchymal transition and angiogenesis in human hepatocellular carcinoma. *Lab Invest* **94**, 252-261
49. Kabir, M. A., Uddin, W., Narayanan, A., Reddy, P. K., Jairajpuri, M. A., Sherman, F., and Ahmad, Z. (2011) Functional Subunits of Eukaryotic Chaperonin CCT/TRiC in Protein Folding. *J Amino Acids* **2011**, 843206
50. He, J., Gu, D., Wu, X., Reynolds, K., Duan, X., Yao, C., Wang, J., Chen, C. S., Chen, J., Wildman, R. P., Klag, M. J., and Whelton, P. K. (2005) Major causes of death among men and women in China. *N Engl J Med* **353**, 1124-1134
51. Chuma, M., Sakamoto, M., Yamazaki, K., Ohta, T., Ohki, M., Asaka, M., and Hirohashi, S. (2003) Expression profiling in multistage hepatocarcinogenesis: identification of HSP70 as a molecular marker of early hepatocellular carcinoma. *Hepatology* **37**, 198-207
52. Lee, J. S., and Thorgeirsson, S. S. (2004) Genome-scale profiling of gene expression in hepatocellular carcinoma: classification, survival prediction, and identification of therapeutic targets. *Gastroenterology* **127**, S51-55
53. Qin, Y., Bao, H., Pan, Y., Yin, M., Liu, Y., Wu, S., and Li, H. (2014) SUMOylation alterations are associated with multidrug resistance in hepatocellular carcinoma. *Mol Med Rep* **9**, 877-881
54. Tomasi, M. L., Tomasi, I., Ramani, K., Pascale, R. M., Xu, J., Giordano, P., Mato, J. M., and Lu, S. C. (2012) S-adenosyl methionine regulates ubiquitin-conjugating enzyme 9 protein expression and sumoylation in murine liver and human cancers. *Hepatology* **56**, 982-993