PPM1A Phosphatase is Involved in Regulating Pregnane Xenobiotic Receptor Mediated Cytochrome P450 3A4 Gene Expression

by

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Abstract

The liver is the most important organ of drug metabolism and plays a major role in the detoxification of both endobiotics and xenobiotics. The process of drug metabolism is undertaken in three distinct phases. Cytochromes p450s (CYPs), enzymes belonging to the first phase of drug metabolism, are vital to drug metabolism. In particular, cytochrome p450 3A4 (CYP3A4), which metabolizes 60% of FDA approved drugs, plays a crucial role in drug metabolism. The human pregnane xenobiotic receptor (hPXR), a ligand-dependent orphan nuclear receptor, is a major transcription factor that regulates the expression of key drug-metabolizing enzymes, including CYP3A4. Variations in the expression of hPXR-mediated CYP3A4 in liver can alter therapeutic response to a variety of drugs and may lead to potential adverse drug interactions. However, molecular mechanisms of hPXR-mediated CYP3A4 expression are not fully understood. We sought to determine whether Mg^{2+}/Mn^{2+}-dependent phosphatase 1A (PPM1A) regulates hPXR-mediated CYP3A4 expression in liver hepatocytes. PPM1A was found to be coimmunoprecipitated with hPXR. Genetic or pharmacologic activation of PPM1A led to a significant increase in hPXR transactivation of CYP3A4 promoter activity. In contrast, knockdown of endogenous PPM1A not only attenuated hPXR transactivation, but also increased proliferation of HepG2 cells. This suggests that PPM1A expression levels regulate hPXR, and that PPM1A expression and hPXR activity are regulated in a proliferation-dependent manner. Indeed, PPM1A expression and hPXR transactivation were significantly reduced in sub-confluent HepG2 cells compared to confluent HepG2 cells, suggesting that both PPM1A expression and hPXR-mediated

CYP3A4 expression may be downregulated in proliferating livers. Elevated PPM1A levels led to attenuation of hPXR inhibition by tumor necrosis factor-alpha and cyclin-dependent kinase 2 which are known to be upregulated and essential during liver regeneration. In mice regenerating livers, similar to sub-confluent HepG2 cells, expression of both PPM1A and the mouse PXR target gene cyp3a11 (human ortholog of CYP3A4) was found to be downregulated. Together, our results show that PPM1A is essential for PXR function and can positively regulate PXR activity by counteracting PXR inhibitory signaling pathways that play a major role in liver regeneration. These results implicate a novel role for PPM1A in regulating hPXR-mediated CYP3A4 expression in liver hepatocytes, and may explain a mechanism for CYP3A repression in regenerating livers.

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List of Abbreviations

ABC	ATP-Binding Cassette Transporters
ADI	Adverse Drug Interactions
AF-1	Transactivation Function-1
AF-2	Transactivation Function-2
AhR	Aryl Hydrocarbon Receptor
BPA	Bisphenol A
CAR	Constitutive Androstane Receptor
cAMP	Cyclic Adenosine Monophosphate
CCl4	Carbon Tetrachloride
CDK	Cyclin Dependent Kinase
CK2	Casein Kinase II
COMT	Catechol O-Methyltransferase
COS7	Monkey Kidney Fibroblast Cells
CYP	Cytochrome p450
DBD	DNA-Binding Domain
Db-cAMP	Dibutyryl-cAMP
DMSO	Dimethyl Sulfoxide
EGF	Epidermal Growth Factor
EtOH	Ethanol

FMO	Flavin-containing Monoxygenases
FOXO-1	Forkhead Transcription Factor FKHR
GSK3	Glycogen Synthase Kinase 3
GST	Glutathione-S-Transferase
HGF	Hepatocyte Growth Factor
HRE	Hormone Response Element
HSC	Hepatic Stellate Cells
IL	Interleukin
Ki-67	Nuclear Protein Used as a Cell Proliferation Marker
LBD	Ligand-Binding Domain
LSEC	Liver Sinusoidal Epithelial Cells
MAPK	Mitogen Activated Protein Kinase
MDR	Multiple Drug Resistance Protein
MRP	Multidrug Resistance-Associated Protein
N-6-benz-cAMP	N6-Benzoyladenosine-cAMP
NAT	N-Acetyltransferase
NCoR1	Nuclear Receptor Co-repressor 1
NCoR2	Nuclear Receptor Co-repressor 2
NF-κB	Nuclear Factor Kappa B
NQO	Quinone Oxoreductase
NRIP1	Nuclear Receptor Interacting Protein 1

OATP	Organic Anion Transporting Protein
p70 S6K	70kDa form of Ribosomal Protein S6 Kinase
P-Gp	P-Glycoprotein
PGC-1	Peroxisome Proliferator-Activated Receptor Gamma Coactivator
PHX	Partial Hepatectomy
PKA	Protein Kinase A
PKC	Protein Kinase C
PMA	Phorbol-12-Myristate-13-Acetate
PPAR-γ	Peroxisome Proliferator-Activated Receptor γ
PPM1A	Protein Phosphatase $Mg^{2+/}Mn^{2+}$ Dependent 1A
PXR	Pregnane Xenobiotic Receptor
RIF	Rifampicin
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RXR	Retinoid X Receptor
shRNA	Small Hairpin RNA
SRC-1	Steroid Receptor Co-activator 1
STAT3	Signal Transducer and Activator of Transcription 3
SULT	Sulfotransferases
TAA	Thioacetamide
TGF-β	Transforming Growth Factor β
TMPT	Thiopurine S-Methyltransferase

TNFα	Tumor Necrosis Factor α
UGT	UDP Glucuronosyltransferases
uPA	Urokinase Plasminogen Activator
VDR	Vitamin D Receptor

Chapter I

Review of the Literature

Liver

One of the main functions of the liver is to detoxify endobiotics and xenobiotics, including therapeutic drugs. These detoxified materials are then excreted from the body. The liver is composed of specialized parenchymal and non-parenchymal cells. The hepatocytes of the liver parenchyma, make up around 60% of all liver cells and approximately 80% of liver mass. It is in these cells that the majority of drug metabolism in the body occurs [10-12]. Under normal conditions, hepatocytes are relatively quiescent. However, when the liver is injured, these cells are capable of extreme levels of replication. In studies using a partial hepatectomy, a common surgical procedure to study liver regeneration where 2/3 of the liver is resected, approximately 95% of the normally quiescent hepatocytes will undergo proliferation [13].

The non-parenchymal cells of liver include Kuppfer cells, hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells (LSECs). Kuppfer cells are the resident macrophages of the liver representing approximately 35% of non-parenchymal liver cells, and 80-90% of tissue macrophages in the body [14]. Kuppfer cells can be activated directly or indirectly causing the release of inflammatory signals, growth control modulators, and reactive oxygen species [15]. The Kuppfer cells also play a part in the regulation of drug metabolizing enzymes via the release of signaling molecules [16-19]. LSECs line the sinusoidal wall and endothelial linings of the liver forming fenestrae that act as sieve plates, allowing for an opening between the sinusoidal lumen and the space of Disse, where transport of fluids, solutes, and particles takes place [20, 21].

HSCs are perisinusoidal cells found within the space of Disse, and have a close interaction with both sinusoidal endothelial cells and hepatic epithelial cells [22]. These HSCs comprise about 5-8% of cells in the liver [23] and play roles in drug metabolism, retinoid metabolism, and lipid metabolism [24]. Damage to liver causes HSCs to transdifferentiate into cells similar to myofibroblasts, via signaling from injured immune cells and hepatocytes [24, 25]. The HSC, as the primary extracellular matrix producing cells in the liver, are responsible for creating a temporary scar at the site of damage, preventing further injury to the liver. Prolonged activation of these cells causes fibrosis of the liver [24]. During recovery of liver fibrosis, HSC activity is greatly reduced, via cellular senescence and apoptosis, or a return to the quiescent state [26, 27]. HSCs are also involved in much of the signaling that occurs during liver regeneration [24, 25].

Overview of Drug Metabolism

Metabolism is a major mechanism by which xenobiotics, including drugs, are eliminated from the body, and this process occurs primarily through drug metabolizing enzymes. Generally, these enzymes are a protective mechanism of the body against potentially harmful effects from endogenous and exogenous compounds, and can be found in various tissues and organs [28]. There are many sites of drug metabolism including the kidney, skeletal muscle, brain, heart, skin, gastrointestinal tract, and the lungs. However, it is the liver that is most metabolically active by weight [29, 30]. Normally, in the case of drug metabolism, a lipid soluble, non-polar compound is enzymatically converted into a polar, water soluble compound allowing for excretion from the body [29]. The progression of drug metabolism can be broken down into 3 distinct phases: two enzymatic phases and the final phase of drug transport out of the cell [31].

Phases of Drug Metabolism

Phase I Metabolism

Phase I metabolism enzymes refer to cytochromes p450 (CYPs), flavin-containing monooxygenases (FMOs), NADPH-P450 reductases, aldehyde and ketone reductases, epoxide hydrolases, esterases, NAD(P)H: quinone oxoreductases (NQO), sulfatases, peptidases, and β-glucoronidases [32]. Of these phase I metabolism enzymes, the CYPs are the most extensively studied due to the fact that the majority of drugs are metabolized by this group. CYP nomenclature is broken down into a number for a family, followed by a letter for sub-family, and finally another number for the specific isozyme. For example, CYP3A4 would be in the third family, sub-family A, and be the fourth isozyme in that subfamily. To date, 18 families of CYPs have been discovered, which are divided into 44 sub-families [33]. Of these, approximately a dozen enzymes from families 1-3 are responsible for the biotransformation of 70-80% of xenobiotics in the body. The most prominent of CYPs in the metabolism of clinically used drugs are CYP1A2, CYP2D6, CYP2C9, CYP2B6, CYP2C19, CYP2C8, CYP2A6, CYP2J2, CYP2E1, and CYP3A4/5. Due to the small number of enzymes responsible for metabolizing such a wide range of drugs increases the necessity of understanding the regulation of CYPs [33].

All CYPs are cysteinato-heme enzymes with a prosthetic group constituted of an iron (III) protophyrin-IX. This is covalently linked to the protein via a sulfur atom of a proximal cysteine ligand. This prosthetic group allows for a wide range of enzymatic reactions to take place to substrates, including, aliphatic and aromatic hydroxylation, oxidation, *N*- and *O*- dealkylation, and destaturation [34, 35]. These reactions allow for the unmasking or formation of functional groups (-OH, -NH2, -SH, etc.), leading to a more polar metabolite from a parent compound [35].

CYPs act not only on clinical drugs, but also on other xenobiotics, such as environmental chemicals, along with endogenous hormones [36]. For example, CYP3A4 acts on both testosterone and estrogen [37, 38]. Additionally, CYP3A4 is responsible for the metabolism of bisphenol A (BPA), an endocrine disruptor, along with 60% of clinically used drugs, making just this single enzyme a vital part of the body's defense mechanism [36, 39]. The CYP enzymes are capable of inactivating drugs as well as activating pro-drugs [40-42]. Due to the wide range of substrates knowledge of the regulation of CYPs becomes imperative, particularly in the field of pharmacokinetics.

Regulation of the CYPs is varied, and can occur via their substrates, genetics, and pathophysiology [33, 43, 44]. Due to the vast number of substrates that are acted on by such a small number of enzymes, modulation of any one of the CYP enzymes can lead to drastic effects on metabolism. Most notably are adverse drug interactions (ADIs) that can occur when two or more drugs are co-administered that are metabolized by the same enzyme. In chronic diseases that require several drugs to be administered simultaneously, there is a drastic increase in the likelihood of ADIs [45]. For most drugs, toxicity can occur when the key enzyme level is repressed by a substrate, to the extent that toxic levels of one or several drugs are in circulation in the body. The loss of drug efficacy can occur when an enzyme is induced; causing the drugs to be metabolized more quickly than it can have the desired effect. The notable exception of this is in the case of pro-drugs, which are activated upon metabolism. In these cases, induction of an enzyme may lead to drug toxicity, and repression may lead to reduced drug efficacy. An example of which is drug-induced toxicity via acetaminophen. It has been shown that increased CYP3A is capable of converting acetaminophen to its toxic intermediate metabolite, N-acetyl-p-benzoquinone imine [46, 47].

Phase II Metabolism

Major Phase II metabolizing enzymes are described as conjugation enzymes, and are mostly tansferases, which include: UDP-glucuronosyltransferases (UGTs), N-acetyltransferases (NATs), sulfotransferases (SULTs), glutathione S-transferases (GSTs), and methyltransferases such as thiopurine S-methytransferase (TMPT) and catechol O-methyl transferase (COMT). Phase II metabolizing enzymes have garnered less attraction than their phase I counterparts due to the relative rarity of ADIs as a consequence of the large capacity of the conjugation system. [35]. Despite the rarity of ADIs, a drastic repression or induction of these enzymes can lead to clinically significant alterations of normal pharmacokinetics [48]. Like CYPs, phase II metabolizing enzymes are capable of acting on endogenous compounds and xenobiotics [35].

Phase II enzymes often require the availability of oxygen, nitrogen, or sulfur groups for conjugation to occur. These open sites will act as acceptors for a hydrophilic moiety, such as glutathione, acetyl group, sulfate, or glucuronic acid. Although phase II metabolism enzymes often build off the reactions in phase I metabolism, it is not always necessary for phase I to occur first. After the conjugation reaction has taken place, the remaining metabolite will have an increased molecular weight and polarity, flagging it for excretion in the urine or bile [49]. The largest group of clinically relevant phase II metabolizing enzymes is that of the UGTs, followed by the SULTs and GSTs. NATs comprise a small portion of clinical relevance, while TMPTs represent the smallest clinically relevant family [50].

Phase III Metabolism

Phase III proteins can be found in the liver, kidney, brain, and intestine where they are responsible for the uptake and excretion of drugs [51, 52]. Examples of key phase III enzymes include P-glycoprotein (P-gp; a.k.a. multidrug resistance protein 1 [MDR1]), multidrug resistance associated proteins (MRPs), and organic anion transporting polypeptides (OATPs) [53-55]. Both MDRs and MRPs are considered ATP-binding cassette transporters (ABCs) due to the use of energy from ATP hydrolysis for transport of the substrates across the membrane [56]. Additionally, ABCs have a huge range of substrates, allowing for a wide range of endobiotics and xenobiotics to be transported during phase III metabolism. OATPs are members of the solute carrier family, and mediate ATP and sodium independent transport of organic anions, some organic cations, conjugated steroids, and bilirubin, allowing for bidirectional transport across cellular membranes [57-59].

Phase I Metabolizing Enzyme CYP3A4

CYP3A4 is a phase I drug metabolizing enzyme and is the most abundant CYP in both the liver and the intestine, constituting approximately 40% of the total CYP in the body [1, 60]. In the liver alone, CYP3A makes up 35% of the total CYP amount (**Fig. 1**). Furthermore, it has been shown that of CYP3A in the liver, 95% is of the isoform CYP3A4 [1, 5]. CYP3A4 is responsible for metabolizing approximately 60% of all clinically used drugs [61]. Additionally, CYP3A4 is capable of metabolizing not only endobiotics such as sex steroid hormones, leukotrienes and prostaglandins, but also other xenobiotics including pesticides, herbicides and environmental chemicals [38, 41, 62-67]. Due to the wide substrate specificity, particularly in regards to clinically used drugs, elucidation of CYP3A4 regulation is a critical area of research.

CYP3A4 has the broadest substrate specificity known to date, and is capable of acting on molecules as small as acetaminophen (*Mr* =151) to the large cyclosporine A (*Mr* =1201) [68, 69]. CYP3A4 has been shown to bind to ligands with non-Michaelis-Menton type kinetics, making accurate enzymatic predications difficult. Moreover, due to the low specificity of CYP3A4 and the large binding pocket, two or more ligand molecules are capable of binding to the ligand site (heme pocket) simultaneously [70, 71]. In some cases, such as with metyrapone and progesterone, the bound conformation of CYP3A4 is very similar to the unliganded version [72]. In other cases, such as with ketoconazole and erythromycin, drastic changes in CYP3A4 conformation take place, including an increase in active site volume by >80% [73]. The pocket around the heme iron in CYP3A4 is significantly larger compared to other CYPs [74]. The heme iron itself is ligated to the protein by a cysteine and interacts with several side chains. Additional characteristics of CYP3A4 such as hydrophobic loops, phenylalanine clusters, and unique short helices, allow for this enzyme to have diverse substrate specificity [72].

It is known that CYP3A4 levels are dynamic, not static. That is, the gene expression of CYP3A4 is dependent on many different factors including environmental exposures, genetics, pathologic states, and physiologic status [33, 75]. Due to the huge range of substrates acted upon by CYP3A4, it is vital to understand the regulation of this enzyme, particularly to avoid ADIs. For example, some substrates, such as the anti-tuberculosis drug rifampicin, are inducers of CYP3A4. It has been shown that induction of CYP3A4 caused by rifampicin will cause increased metabolism of other substrates of CYP3A4, such as estradiol when co-administered, leading to reduced efficacy of the drug [76]. In contrast to this, repression of CYP3A4 can cause decreased metabolism, an example of which is metformin, a drug used to treat type-2 diabetes [77]. In such a scenario, the repression of CYP3A4 due to metformin may lead to toxic levels of drugs co-administered in the

body. The ADIs caused by aforementioned scenarios can potentially lead to death [78]. It is for this reason that the regulation of CYP3A4 must be studied further to help prevent ADIs.

The Pregnane Xenobiotic Receptor

The enzymes and proteins of drug metabolism are mainly regulated by ligand activated xenobiotic receptors [79]. These receptors often times have overlapping ligand specificity [80]. The most common xenobiotic receptors are aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptor, constitutive androstane receptor (CAR), and the pregnane xenobiotic receptor (PXR) [81-84]. The pregnane xenobiotic receptor is an orphan nuclear hormone receptor with promiscuous ligand specificity, and is a major player in the regulation of genes involved in the metabolism of both endobiotics and xenobiotics [85]. PXR can be considered as a major xenosensor of the body, due to its ability recognize and regulate xenobiotics and endobiotics, keeping the body in homeostasis [86]. PXR activation has been shown to have species to species variation [87]. This receptor is abundantly expressed in the liver, but also is largely expressed in the intestine and to a lesser extent in other tissues such as kidney [88].

PXR is structurally a typical nuclear receptor in that it has a DNA-binding domain (DBD) located on the N-terminus, and a ligand-binding domain (LBD) that lies on the C-terminus **(Fig. 2)**. Between the DBD and LBD lies a flexible hinge region. At the very end of the C-terminus lies a ligand-dependent transactivation function (AF-2), but unlike other steroid nuclear receptors, PXR lacks a ligand independent transactivation function (AF-1) which would normally be found at the N-terminus [89]. When PXR is unbound by an activating ligand, a co-repressor complex, such as nuclear receptor co-repressor 1 (NCoR1), NCoR2, or small heterodimer partner (SHP/NC0B2) will associate with PXR, reducing it to basal activity via the recruitment of histone deacetylases

[89-91]. When in the presence of an activating ligand, PXR undergoes a conformational change, the co-repressor complex is removed and a co-activator, like the steroid receptor co-activator 1 (SRC-1), SRC2, nuclear receptor interacting protein 1 (NRIP1), or the peroxisome proliferator-activated receptor gamma co-activator (PGC-1) interacts with PXR [89, 92]. PXR must heterodimerize with retinoid X receptor (RXR) in order to bind with the target hormone response element (HRE), a short DNA-motif that regulates gene transcription [93].

The protein structure of PXR allows for a large binding pocket necessary for the wide range of ligands that act as activators. The binding pocket itself is thought to be approximately 1300$\dot{A}^3$, and has the ability to change shape to accommodate ligands of various sizes. Within the pocket itself, there are twenty-eight amino acid side chains, of which, eight are polar and capable of forming hydrogen bonds [92]. Although the ligand binding pocket of PXR is largely uncharged and hydrophobic, evenly spaced polar residues critical to binding have been discovered within the binding pocket. [94].

PXR is capable of both inducing and repressing multiple target genes allowing for coordinate gene regulation [95]. PXR is considered the master regulator of CYP3A4, as well as playing a part in the regulation of CYP1A1, CYP1A2, CYP2A4, CYP2A6, CYP2B6, CYP2B10, CYP2C8, CYP2C9, CYP2C19, CYP2E1, and CYP4As, all of which contribute to 77-81% of drug metabolism in the liver [96-102]. The most clinically relevant phase II enzymes; UGTs, GSTs, and SULTs, all are transcriptionally activated via PXR [103, 104]. Finally, key phase III enzymes such as MDR1 and MRP2 are also under transcriptional regulation via PXR activation [105, 106].

In addition to its role in metabolism, it has also been shown that PXR plays a role in hepatocyte proliferation. Partial hepatectomy of PXR-null mice showed a decrease of 17% liver mass at the

estimated end-point of liver growth (Day 10) [7]. Hepatic steatosis was markedly decreased in mouse models lacking PXR [7]. PXR also plays a role in diabetes and obesity, both of which occurs due to cross talk between various hormone responsive transcription factors such as fork head box 01 , fork head box A2 , PPARγ coactivator 1α (PGC-1α), and cAMP-responsive element-binding protein, decreasing energy metabolism via downregulation of gluconeogenesis. PXR-knockout in mice models protects against diet-induced obesity [107]. PXR also plays a role in the regulation of genes involved in lipid homeostasis [107]. The ability of PXR to regulate the biosynthesis and transport of bile salts may allow this receptor to become a therapeutic target in combating cholesterol gallstone disease [108].

PXR Regulation

The regulation of PXR is critical to maintaining homeostasis via the transcriptionally regulation of phase I and phase II metabolizing enzymes, such as CYP3A4 and UGTs, alongside phase III transporter proteins, like MDR1 [80]. PXR activity is regulated by endobiotics, such as bile acids and xenobiotics including therapeutic drugs, dietary components, herbal supplements and environmental chemicals [79, 109-114].

The regulation of PXR gene expression is not well understood. However, it is known that certain endobiotics and xenobiotics are capable of modulating PXR gene expression. For instance, litholic acid and dexamethasone have been shown to increase both PXR and its heterodimer RXR mRNA expression in human hepatocytes [115, 116]. Additionally, clofibrate, isoniazid, perflurodecanoic acid, and troleandomycin have been shown to modulate the levels of PXR mRNA [88]. Xenobiotics alter PXR levels and/or activity by directly binding to PXR or by indirectly acting through cellular signaling molecules. For example, clofibrate and perflurodecanoic acid increase

rPXR mRNA levels despite a lack of PXR ligand binding [88]. In fact, a study of 170 different FDA approved drugs found that 54% of them modified PXR transactivation, however, not all of these compounds were ligands of PXR [117]. The finding that PXR is can be activated in cases without direct ligand binding, led to the discovery of PXR activation via signaling molecules.

Indeed, many signaling molecules have been shown to modulate PXR activity, resulting in altered PXR target gene expression. An example of which is the decreased levels of PXR-mediated CYP3A4 gene expression due to the inhibitory effect of lipopolysaccharide and tumor necrosis factor (TNFα) induced NF-κB on the PXR-RXR dimerization in human hepatocytes [118, 119]. Conversely, when NF-κB activity is inhibited, PXR activity is enhanced [119]. Likewise, kinases such as PKA, PKC and CDK2 signaling pathways have also been shown to reduce the activity of PXR, most likely by altering the phosphorylation status of PXR [4, 91, 120]. Modulation of PXR activity by signaling molecules can occur through protein-protein interactions and/or posttranslational modifications [121-124]. An important example of a post-translational modification affecting PXR is that of phosphorylation, a reversible process using a phosphate group, that allows for regulation of PXR activity [9].

PXR Phosphorylation

In 1992, Fisher and Krebs were awarded the Nobel Prize for their discovery of reversible phosphorylation as a biological regulatory mechanism [125]. Phosphorylation of proteins, including transcription factors, is a reversible mechanism for the control of many biological functions [126]. This post-translational modification is characterized by the addition or removal of a phosphate group by a kinase or phosphatase respectively. The phosphorylation status of a

protein can have an effect on its conformation, expression, stability, protein-protein interactions, subcellular localization, etc. [127].

It is obvious that PXR regulation is dependent on several kinases, and therefore on phosphorylation. For instance, CDK2 phosphorylates PXR on the Ser^{114}, Thr^{153}, and Thr^{155} residues. The effects of phosphorylation on specific residues such as these can be studied using site directed mutagenesis. A phosphomimetic mutant imitates phosphorylation of a residue by replacing a serine or threonine with a negatively charged residue such as aspartic acid. Conversely, a phosphodeficient mutant, imitates dephosphorylation of a residue via a residue change to a small non-polar residue such as alanine. Using this method, it has been shown that when Ser^{114} is mutated to an aspartic acid, attenuation of PXR transcriptional activity was the greatest. Independent phosphomimetic mutations of Thr^{153} or Thr^{155} had a marginal effect, however, simultaneous phosphomimetic mutation of both Thr^{153} and Thr^{155} led to a significant inhibition of PXR activity [128].

A second group used a similar method of phosphomimetic and phosphodeficient mutations in 18 kinase recognition sequences, discovering six amino acid residues critical for PXR activity. Ser^{8}, Thr^{57}, Ser^{208}, Ser^{305}, Ser^{350}, and Thr^{408} all play a role in PXR activity. Thr^{57} was found to be important for PXR binding to promoter regions of target genes, while Ser^{8}, Ser^{305}, Ser^{350}, and Thr^{408} are critical for the heterodimerization of PXR with RXR. Ser^{208}, Ser^{305}, Ser^{350}, and Thr^{408} are implicated in interaction between PXR and its cofactors. Lastly Thr^{408} was found to play an additional role in PXR subcellular localization [129].

In another study using site directed mutagenesis, it has been shown that changing the 57^{th} residue, a threonine, to an aspartic acid residue significantly decreased PXR transcriptional activity.

Despite the lack of transcriptional activity there was no decrease in PXR protein levels and the mutation did not interfere with the SRC-1-PXR interaction. The mutation did however alter the nuclear distribution of PXR and decreased the DNA binding activity [130].

Investigators have found in HepG2 cells that flavonoids increased PXR gene activity without directly binding to PXR. These flavonoids were found to decrease CDK-5 and p35, a kinase and a subunit necessary for the activation of the kinase, respectively. This information suggests that flavonoids increase PXR activity by decreasing the inhibition from the protein kinase CDK-5 [131].

Protein kinase C (PKC) signaling is capable of drastically decreasing PXR activity in cultured hepatocytes and cell-based assays. PKC signaling can be activated by cytokines that are secreted during time of sepsis, inflammation, or liver regeneration [91]. An additional kinase, protein kinase A (PKA), has been shown to drastically reduce PXR activity in rat and human cells. Conversely, PKA activation in mouse hepatocytes increases PXR target gene transcription, displaying the species dependent specificity that is characteristic of PXR activation [87]. By activating PKA or PKC signaling, PXR activity can be effectively reduced, which in turn affects PXR target gene expression, such as CYP3A4. Other kinases that have been shown to modulate/phosphorylate PXR activity include CDK1, casein kinase II (CK2), a 70kDa form of ribosomal protein S6 kinase (p70 S6K), and glycogen synthase kinase 3 (GSK3) [130]. Regulation of PXR activity by these kinases demonstrates that PXR activity is regulated by reversible phosphorylation, and that phosphatases may also play a role in regulating PXR function.

Phosphatases

Phosphatases generally act in opposition of kinases, removing phosphate groups as part of reversible phosphorylation. Phosphatases are generally enriched in and sometimes located solely in the nucleus. Protein phosphatases have been implicated in many diverse biological functions within the nucleus that include: chromosome condensation, chromatin remodeling, ribosome biogenesis, DNA replication/repair, and signal transduction pathways [132, 133]. These protein phosphatases are classified into three families based on sequence, catalytic mechanism, and structure homology. The first of the protein families is that of the large phosphoprotein phosphatase (PPP) which contains the classic ser/thr phosphatases. The second family is the protein tyrosine phosphatase (PTP) family. The third family is the Asp-based phosphatases that have a distinctive DXDXT/V catalytic signature [134].

The PPP family of ser/thr phosphatases was originally subdivided into either type 1 (PP1) or type 2 (PP2) phosphatases, and then was further divided based on metal ion requirements. For example, PP2A has no metal requirements, while PP2B requires Ca^{+2} to be present, and that PPM (a.k.a. PP2C) requires active Mg^{2+}/Mn^{2+} ions [134]. The crystal structure of the PPM family active sites are very similar to that of the PPP family, although they share little sequence homology [135]. The PPM family is further separated from other protein phosphatases by their metal ion requirements, insensitivity to the phosphatase inhibitor okadoic acid, and due to their ability to function as monomers [126]. Many of the protein phosphatases of the PPM family have been noted to have significant restriction to the nucleus [136-138].

Many members of the PPM family have been shown to play critical roles in cellular functions. For example, one study found that PPM1A was capable of negatively regulating p38 and JNK mitogen-

activated protein kinase (MAPK) pathways by dephosphorylating MAPK kinase and p38 MAPK [139]. Another member of this family PPM1G, which resides almost exclusively in the nucleus, was found to target the stress-response kinase p38 MAPK [140, 141]. Additionally, PPM1A has been shown to be key in the dephosphorylation of SMAD, an essential step in the termination of transforming growth factor-β (TGF-β) signaling. PPM1A was shown to have a higher affinity for the phosphorylated SMAD 2 and SMAD 3 and was able to physically bind to both [142].

The PTP family was originally based on its catalytic signature. However, more recent data has shown that these proteins are more widespread than originally thought, and have been further classified into evolutionarily diverse groups [134, 143]. Most Asp-based phosphatases display the DXDX (T/V) catalytic signature and use a unique Asp catalytic mechanism for dephosphorylation [134, 144].

Because phosphorylation regulates PXR function, it is logical to speculate that phosphatases are directly or indirectly involved in regulating PXR function by inhibiting the kinase pathways. However, in comparison to the understanding of the role of kinase signaling, there is only a meager understanding of the extent to which PXR is regulated by phosphatase signaling. For instance, okadaic acid, a nonspecific phosphatase inhibitor, reduce PXR's transcriptional activity in cell-based gene reporter assays [91], suggesting that okadoic acid–sensitive protein phosphatases (i.e., PP1and PP2A) are involved in regulating PXR-mediated CYP3A4 gene expression, yet the exact mechanism remains unknown. It is important to more fully understand the contribution of phosphatases in regulating PXR function to comprehensively address the role of reversible phosphorylation in regulating PXR-mediated CYP3A4 expression.

Liver Regeneration

The liver has long been known for its extraordinary replicative properties. It has been shown many times throughout the years and in many different species that an injured or resectioned liver can return to normal size and function over the course of several weeks [145-147]. A human liver will return to functional size and metabolic state 2-3 weeks post operation, a significantly longer time period than smaller animal models such as the mouse, in which liver regeneration is completed after 7-10 days [148-150]. After an insult to the liver has occurred, three general stages occur (**Fig. 3)**. The first is the priming or initiation stage in which hepatocytes that are in proliferative quiescence, G0 phase, are prepared for proliferation. The second stage is that of the proliferation stage, at this point actual proliferation of the hepatocyte population takes place. Finally, a termination stage occurs where proliferation is halted as the liver reaches normal size and function. Much of the signaling among these phases overlaps, and distinctive end points to each phase are difficult to determine [151].

Two cell types of the liver are capable of activation to restore the liver normal conditions after injury. The primary cell type in liver regeneration is the hepatocyte. In studies using a partial hepatectomy (PHX) models, up to 90% of hepatocytes undergo at least one round of cell division [152]. Hepatocytes have been shown to have amazing replicative ability, in that up to 70 replications of a hepatocyte cell has been noted [123]. These parenchymal cells also have diploid, tetraploid, and octoploid nuclei, which may play a role in the replicative process [153]. Conversely, hepatoblasts, a common progenitor of bile duct epithelial cells and hepatocytes adds a second possibility for cell proliferation. It is thought that hepatocyte proliferation is relied on as the

primary form of regeneration, and only if this pathway is inhibited are hepatoblasts called upon [154].

There are many different models for studying the effect of liver regeneration. Cellular models include primary hepatocytes or cell lines such as HepG2 [155, 156]. Several different animal models have also been devised for use in studying liver regeneration. These can be broken down into three general categories: surgical, pharmacological (chemical), or pre-existing (genetic) models (**Fig. 4**) [8]. Surgical models are varied, however, the mouse partial hepatectomy model is the most commonly used due to its reproducibility, low mortality, and defined end-points [157]. This model is classically a 2/3 surgical resection of the mouse liver. Five lobes exist in the mouse liver; the caudate lobe, the left and right lateral lobe, and the left and right medial lobes. Of the five lobes, 2-3 lobes are removed depending on the protocol, leaving approximately 30% of liver mass remaining [157, 158]. Note that the return of liver mass is compensatory growth, not true regeneration. No lobes will re-emerge from the resected liver; instead overall liver mass will increase.

The pharmacological models use compounds that cause liver damage, thereby, allowing for study of the subsequent regeneration. Carbon tetrachloride (CCl4) is a hepatotoxin that causes centrilobular necrosis via the formation of a trichloromethyl radical [159]. *D*-galactosamin causes hepatic damage via a decrease in uridine metabolites [160]. Thioacetamide has been used as far back as 1959 in the study of liver regeneration [161]. Both acetaminophen and ethanol are commonly used and available compounds that are capable of causing liver damage [162, 163]. Pre-existing models in animals are varied. These range from Gunn rats expressing Crigler-Najjar

syndrome, the Wistar rat with hyperbilirubnemia, rabbits with heritable hyperlipidemia, and also Dalmatians with hyperuricemia as a model of gout [164-167].

In partial hepatectomy models, the priming or intiation phase of liver regeneration is brought about by several signaling molecules, most notably TNFα and IL-6 (**Fig.3**), both of which are secreted by Kuppfer cells and hepatocytes [147, 157, 168, 169]. These two signaling molecules, released in the first twelve hours after liver injury, activate transcription factors such signal transducer and activator of transcription 3 (STAT3), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and nuclear factor interlukin-6 (NFIL-6), which drive the hepatocyte past the G0/G1 checkpoint, priming them for proliferation [3, 170, 171]. The importance of TNFα and IL-6 has been studied in TNFα and IL-6 deficient mice. These models have been shown to mount a drastically decreased regenerative response, and in the case of IL-6, eventual hepatic failure after 2/3 PHX [170, 172]. NF-κB and STAT3, intermediaries of both TNFα and IL-6 signaling, have been shown to have increased activation just one hour after PHX, displaying the immediate response the liver is capable of mounting to acute damage [173-176]. Other important regulatory mechanisms in the initiation of liver regeneration are the Notch-1 intracellular domain (NICD) and β-catenin both of which increase 15-30 minutes after PHX, and when knocked down decrease the regenerative response [177-179]. The overall effect of these signaling molecules during liver regeneration leads to hepatocytes that have left a state of quiescence, to prepare for proliferation.

The proliferation phase is marked by many signaling molecules (**Fig. 3**). Key examples of which are hepatocyte growth factor (HGF), transforming growth factor α (TGFα), and epidermal growth factor (EGF), all of which activate and are secreted by HSCs in an autocrine manner [24, 25, 180, 181]. HGF can normally be found as an inactive single chain, however, in the presence of

urokinase plasminogen activator (uPA), which is released in response to wound healing, HGF is released as an active heterodimer [182]. This active heterodimer, now a potent mitogen, then binds to the c-Met receptor, allowing for proliferation of hepatocytes [183]. Another signaling molecule found in the proliferation phase of liver regeneration is EGF, which is perpetually barraging the liver. Normal, healthy livers are unaffected by the constant exposure to EGF, however, an injured liver does show a response to EGF. Whether this response is due to less hepatocyte targets, or increased sensitivity to the EGF receptor, is not known. In response to these signaling factors, mitogen-activated protein kinases (MAPKs), a family of kinases which play a critical role in the cell proliferation cycle, are activated. The MAPKs, especially p42/p44, play a significant role in driving the cell cycle past the G1 phase after activation via growth factors and cytokines [184]. These growth factors are capable of activating other protein kinase pathways, such as CDK2, which are essential for proliferation phase of liver regeneration [185-191].

The third and final phase of liver regeneration is that of termination (**Fig 3**). It is in this phase that the liver will return to normal size and function, further hepatocyte proliferation is inhibited, and signaling molecules involved in the priming and proliferation phase are no longer secreted. The liver has been deemed to have a “hepatostat”, that is, it is capable of regulating its own size and weight. This idea has come about due to the shrinkage of large livers to smaller recipients and vice versa [192]. The mechanism for these changes is not completely understood. However, signaling molecules released during the termination stage of liver regeneration offer an insight to this phenomenon. One such molecule is IL-1β, a cytokine released by Kuppfer cells that has been attributed to the decrease in proliferation of hepatocytes during the termination stage [193]. Increased levels of IL-1β mRNA are shown to occur during the time period appropriate for the termination stage of liver regeneration after PHX in rats. Furthermore, the exogenous addition of

IL-1β to regeneration livers attenuates the proliferative surge of hepatocytes in rats after PHX [194]. Possibly, the most important inhibitor of hepatocyte proliferation is TGF-β. This cytokine, secreted primarily by stellate cells, has been shown to inhibit proliferation of hepatocytes in culture, and decrease both the production and activation of HGF [195-198]. Another mito-inhibitor of hepatocytes is activin, which is also secreted by HSCs and belongs to TGF-β superfamily [24, 25, 199].

Hepatic CYP3A levels are altered in a variety of liver pathophysiologies, including liver regeneration triggered by liver injury. CYP3A levels are significantly reduced during liver regeneration [200-204]. It has been speculated that upregulation of PXR inhibitory signaling pathways or downregulation of PXR stimulatory signaling pathways contribute to the repression of CYP3A4 in proliferating hepatocytes of regenerating livers. However, the molecular mechanisms for this repression have yet to be clearly defined. Our results may explain a PXR-mediated mechanism for CYP3A repression in regenerating livers of human patients, including those that undergo therapeutic partial hepatectomy.

Conclusions and Objectives

The liver is the primary site of drug metabolism and contains many enzymes that are crucial for the metabolism of endobiotics and xenobiotics. As the metabolizer of 60% of clinically used drugs, changes in the phase I drug metabolizing enzyme CYP3A4 can lead to severe ADIs. The expression levels of CYP3A4 are primarily regulated by the orphan nuclear receptor PXR. The PXR-mediated CYP3A4 gene expression is regulated not only by endobiotics and xenobiotics, but also by cellular signaling pathways. In particular, kinases such as PKA, PKC, CDK2, p70 S6K and CaMK II were found to modulate PXR-activated CYP3A4 gene expression. While much is

known about kinases' regulation PXR-mediated CYP3A4 expression, very little is known regarding phosphatases. In the current study, we characterized PPM1A phosphatase regulation of PXR activity and PXR-mediated CYP3A4 expression in hepatocytes. The results of our study may provide a mechanism for CYP3A repression in regenerating livers.

Figures

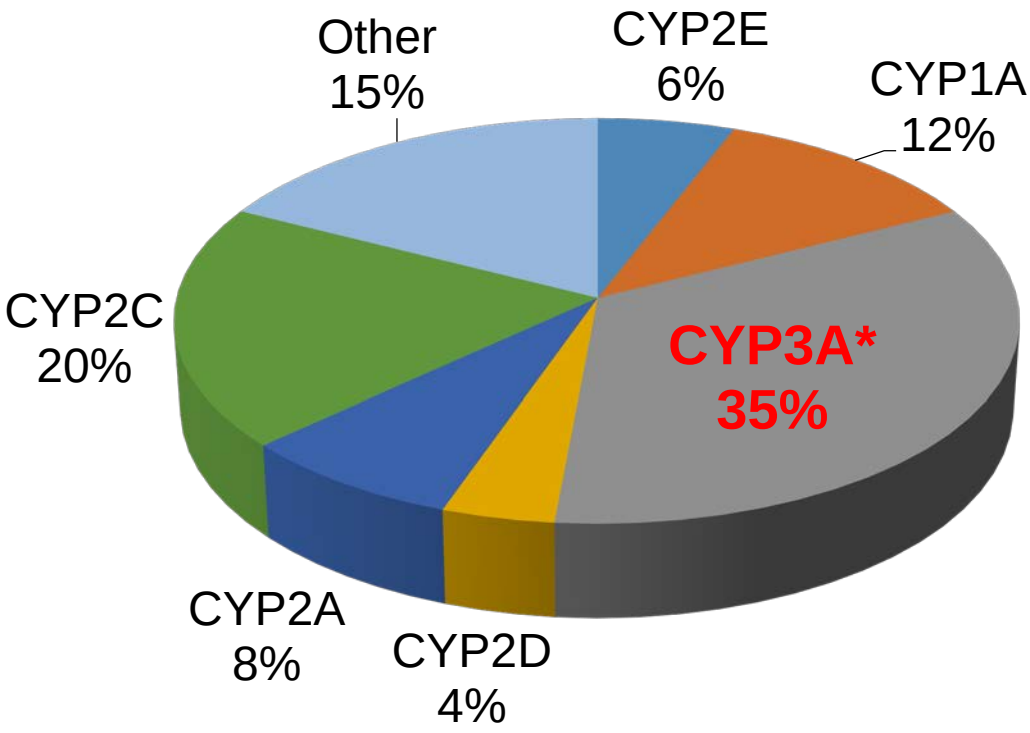
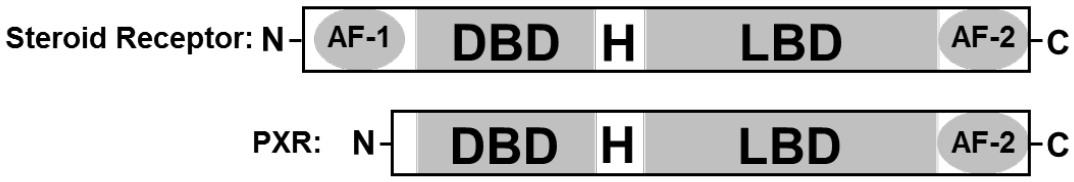


Figure 1: Relative amount of cytochrome P450s within the liver. CYP3A comprises 35% of all CYPs in the liver [1]. *95% of CYP3A consists of the CYP3A4 isoform [5].

A



B

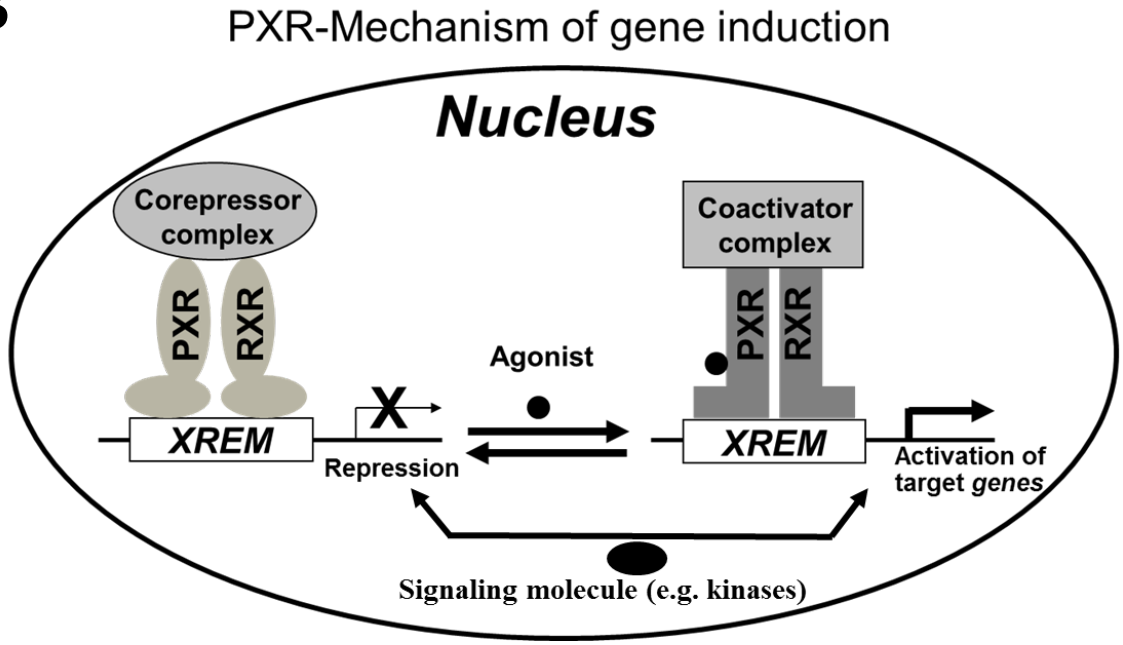


Figure 2: Schematic PXR structure and mechanism of target gene induction. (A) A schematic comparison of the domain structures of a steroid receptor and PXR. AF-1. Activation function 1. AF-2. Transactivation function 2. H. Hinge region. DBD. DNA-binding Domain. LBD. Ligand binding domain. (B) A current model of PXR-mediated gene regulation. PXR functions as heterodimer with retinoid X receptor (RXR). Agonist binding induces a dissociation of co-repressors, recruitment of co-activators and contributes to chromatin remodeling and transcriptional activation. Signaling molecules such as protein kinases contribute to the regulation of PXR. XREM, Xenobiotic responsive enhancer module [9].

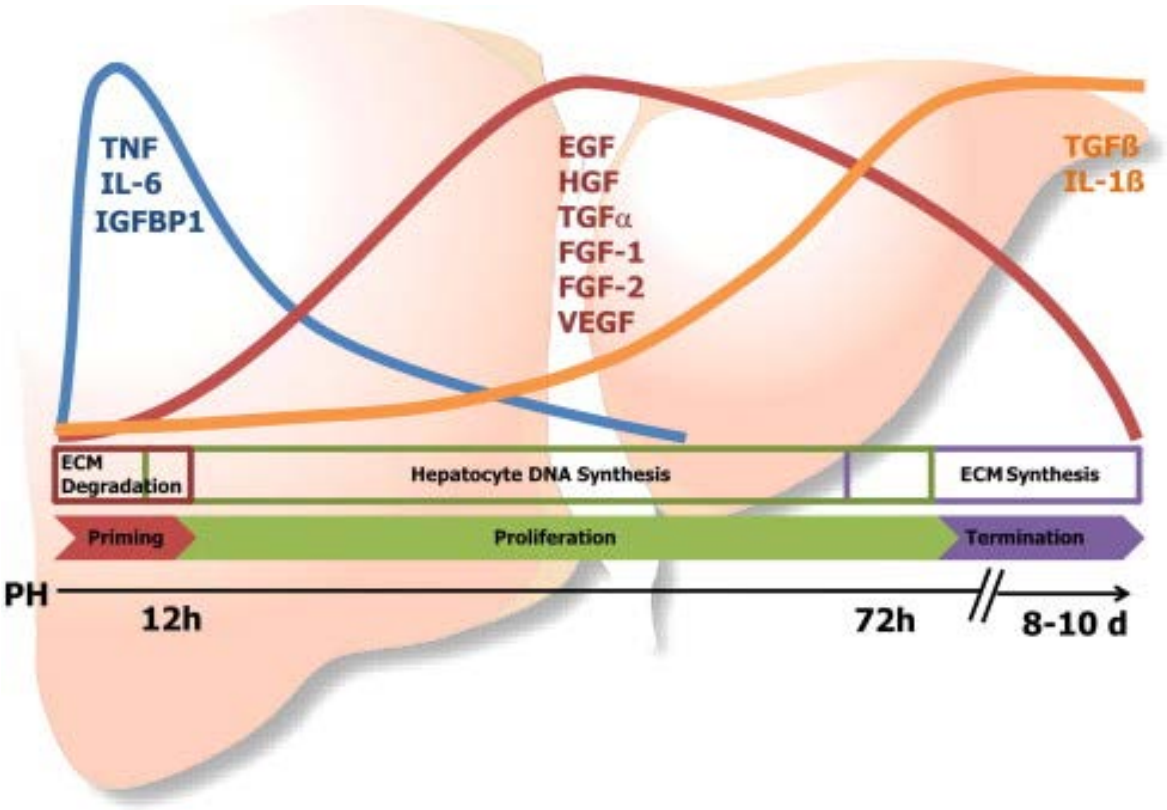


Figure 3: A schematic representation of the three primary phases of liver regeneration with important regulators of each phase [3].

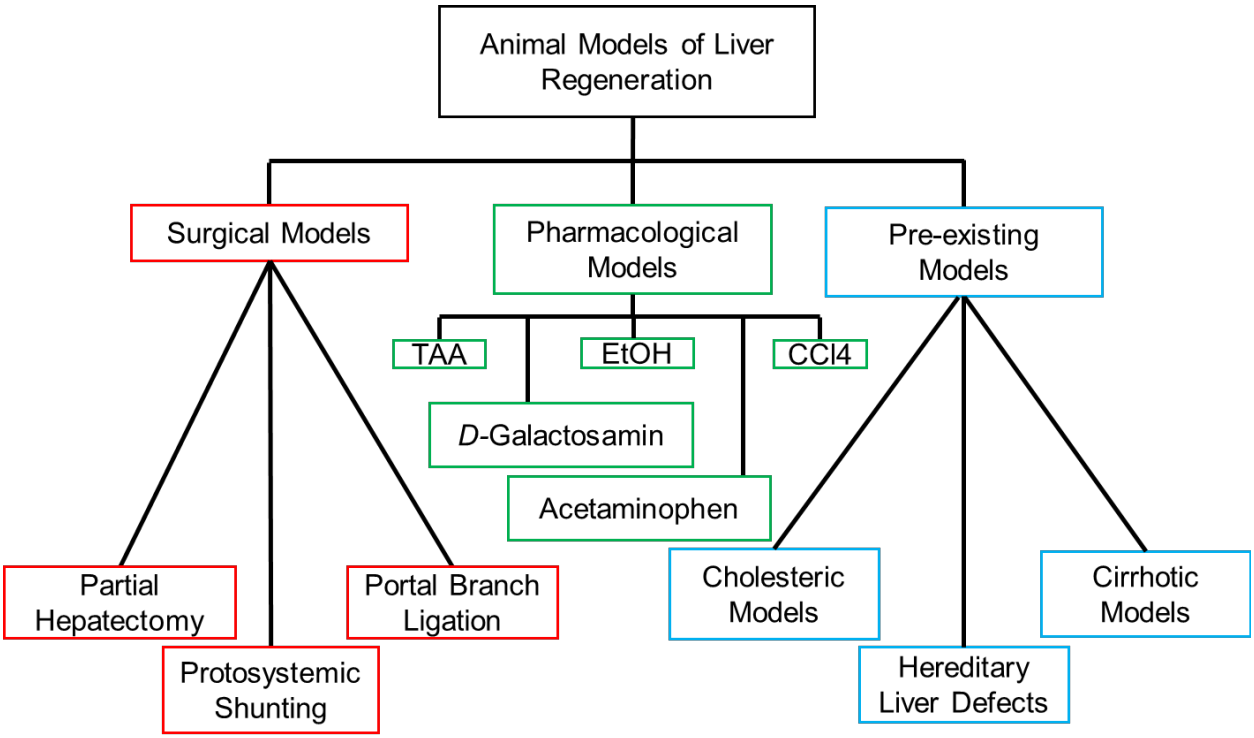


Figure 4: Various methods of studying liver regeneration in animal models. TAA, Thioacetamide. EtOH, Ethanol. CCL4, Carbon Tetrachloride. Adapted from [8]

Chapter II

PPM1A phosphatase is involved in regulating PXR-mediated CYP3A4 gene expression in liver hepatocytes

Abstract

Variations in the expression of human pregnane xenobiotic receptor (hPXR)-mediated cytochrome P450 3A4 (CYP3A4) in liver can alter therapeutic response to a variety of drugs and may lead to potential adverse drug interactions. However, molecular mechanisms of hPXR-mediated CYP3A4 expression are not fully understood. We sought to determine whether Mg^{2+}/Mn^{2+}-dependent phosphatase 1A (PPM1A) regulates hPXR-mediated CYP3A4 expression in liver hepatocytes. PPM1A could be coimmunoprecipitated with hPXR. Genetic or pharmacologic activation of PPM1A led to a significant increase in hPXR transactivation of CYP3A4 promoter activity. In contrast, knockdown of endogenous PPM1A not only attenuated hPXR transactivation, but also increased proliferation of HepG2 cells. This suggests that PPM1A expression levels regulate hPXR, and that PPM1A expression and hPXR activity are regulated in a proliferation-dependent manner. Indeed, PPM1A expression and hPXR transactivation were significantly reduced in sub-confluent HepG2 cells compared to confluent HepG2 cells, suggesting that both PPM1A expression and hPXR-mediated CYP3A4 expression may be downregulated in proliferating livers. Elevated PPM1A levels led to attenuation of hPXR inhibition by tumor necrosis factor-α and cyclin-dependent kinase 2 which are known to be upregulated and essential during liver regeneration. In mice regenerating livers, similar to sub-confluent HepG2 cells, expression of both PPM1A and the mouse PXR target gene cyp3a11 (human ortholog of CYP3A4) was found to be downregulated. Together, our results show that PPM1A is essential for PXR function and can positively regulate PXR activity by counteracting PXR inhibitory signaling pathways that play a major role in liver regeneration. These results implicate a novel role for PPM1A in regulating

hPXR-mediated CYP3A4 expression in liver hepatocytes, and may explain a mechanism for CYP3A repression in regenerating livers.

Introduction

CYP3A4 catalyzes the metabolism of more than 60% of clinically used drugs in humans [205, 206]. Therefore, altered expression of CYP3A4 can affect the therapeutic response to a broad spectrum of drugs and may lead to potential undesired drug interactions. hPXR, a member of the nuclear receptor superfamily, is a master regulator of CYP3A4 expression [207]. Altered hPXR activity could potentially lead to changes in CYP3A4 levels and activity.

The PXR target gene expression is regulated not only by endobiotics and xenobiotics [61, 93, 208], but also by cellular signaling pathways [9]. For instance, p53, FOXO and NF-kB interact with and regulate PXR function [118, 209-212]. Similarly, post-translational modifications including phosphorylation, ubiquitination, acetylation, and sumoylation regulate PXR activity [121, 122, 213-215]. In particular, kinases such as PKA, PKC, cyclin-dependent kinase 2 (CDK2), p70 S6 kinase and Ca^{2+}/calmodulin-dependent protein kinase II were found to modulate the activity of PXR [121, 128, 215]. While much is known about hPXR regulation by kinases, very little is known regarding phosphatases. We sought to determine whether Mg^{2+}/Mn^{2+}-dependent phosphatase 1A (PPM1A) regulates PXR activity.

PPM1A belongs to the family of metal ion-dependent Ser/Thr protein phosphatases (PPM). Phosphatases of the PPM family are structurally and functionally distinguished from other phosphatase families by their monomeric property, dependency on Mg^{2+}/Mn^{2+} for activity, and insensitivity to inhibition by okadoic acid or microcystin [216, 217]. While the PPM family currently contains 22 phosphatases, PPM1A is the most extensively characterized member of this family [216, 217]. Notably, PPM phosphatases, including PPM1A, have been recognized to regulate cell growth [2, 216, 218-221]. Signal cross-talk between Ser/Thr protein phosphatases

and nuclear receptors has been reported. For example, PP1 and PP2A physically associate with the vitamin D receptor (VDR) and PPM1B selectively modulates PPARγ activity [222, 223]. Similarly, PPM1A interacts with VDR, which belongs to the same subfamily of nuclear receptors as PXR does[224]. In the current study, we show that PPM1A interacts with and regulates the function of PXR.

Hepatic CYP3A levels are altered in a variety of liver pathophysiological conditions, including liver regeneration triggered by liver injury. CYP3A levels are significantly reduced during liver regeneration [200-204]. However, the molecular mechanisms of CYP3A repression are poorly understood. We show that PPM1A positively regulates hPXR-mediated CYP3A4 expression in HepG2 cells, and that PPM1A expression and PXR activity are regulated in a proliferation-dependent manner in HepG2 cells. We also show that the levels of PPM1A and Cyp3a11 are downregulated in mice regenerating livers after partial hepatectomy. This positive correlation between PPM1A and cyp3a11 expression provides *in vivo* biological relevance of PPM1A regulation of PXR-mediated CYP3A expression. Our results may explain a mechanism for CYP3A repression in regenerating livers of human patients, including those that undergo therapeutic partial hepatectomy.

Materials and Methods

Chemicals and plasmids: Dimethyl sulfoxide (DMSO), rifampicin (RIF), C_6-ceramide, dihydroC_6-ceramide, hepatocyte growth factor (HGF), dibutyryl-cAMP (db-cAMP), N6-Benzoyladenosine-cAMP (N-6-benz-cAMP), phorbol 12-myristate 13-acetate (PMA), and tumor necrosis factor alpha (TNFα) were purchased from Sigma-Aldrich (St. Louis, MO). The pcDNA3, FLAG-pcDNA3, pcDNA3-hPXR, FLAG-pcDNA3-hPXR, pGL3-CYP3A4-luc, V5-CDK2, V5-cyclin E and pGL3-CMV-Renilla luciferase plasmids were previously described [4, 130, 225]. The pcDNA3-PPM1A was a gift from Dr. Sara Lavi (Department of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel) [2]. The wild-type and mutant FLAG-pcDNA3-PPM1A plasmids were gift from Dr. Andrew Rice (Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas) [226].

Cell culture: HepG2 human liver carcinoma cells and COS-7 monkey kidney fibroblasts were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Allendale, NJ) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin and 100 µg/ml streptomycin (Cellgro, Swedesboro, NJ), 2 mM L-glutamine (Cellgro), and 1 mM sodium pyruvate (Cellgro). The cells were cultured in an incubator with a humidified atmosphere maintained at 5% CO2 and 95% air at 37ºC. The assay media included phenol red-free DMEM (Lonza) supplemented with 5% charcoal/dextran-treated FBS (HyClone) and the other additives. HepG2 cells stably expressing hPXR and CYP3A4-luc were maintained under the selection of G418 (Cellgro) [4].

Hepatocytes: Cryopreserved human primary hepatocytes (HU1488), the hepatocyte media and the culture plates were purchased from Invitrogen (Carlsbad, CA) and cultured by following the manufacturer's protocol. Briefly, the hepatocytes were maintained for 6 h in Williams' medium E without phenol red, supplemented with 5% fetal bovine serum and 500 nM dexamethasone, in collagen coated 24 well culture plates in an atmosphere of 5% CO2 at 37°C. The cells were then incubated in induction media (Williams' medium E without phenol red, supplemented 100 nM dexamethasone) for 12 h. The cells were then treated with the vehicle or drugs in induction medium for 48 h, unless otherwise stated, before harvesting total RNA for gene expression studies.

Transient transfection: Transient transfections were performed using FuGENE 6 (Promega). Briefly, transfections were performed in 6-well culture plates with 2.5 µg of total plasmid DNA per well. Cells were transfected with the following plasmids: hPXR (100 ng), PPM1A (400 ng), CDK2 (200 ng), cyclin E (200 ng), CMV-*Renilla* (100 ng), and pGL3-CYP3A4-luc (1500 ng). pcDNA3 was added to ensure that the total plasmid DNA in each transfection reaction was 2.5 µg.

Lentiviral shRNA transduction: HepG2 cells stably expressing hPXR and CYP3A4-luc were transduced with the lentiviral particles carrying either non-silencing control shRNA or PPM1A shRNA (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions. Stably-transduced cells were selected using puromycin (Cellgro) and maintained under the selection of puromycin. To minimize any insertion site bias, pooled populations of transduced cells were used for the assays. Human primary hepatocytes were also transduced with the control or PPM1A shRNA lentiviral particles for 48 h, followed by 24 h treatment with DMSO or rifampicin. Total RNA was then isolated for gene expression studies.

PXR transactivation assays: The cells were transfected with pcDNA3 or FLAG-pcDNA3, pcDNA3-hPXR or FLAG-pcDNA3-hPXR, CYP3A4-luc, pcDNA3-PPM1A, FLAG-pcDNA3-PPM1A, CMV-renilla luciferase plasmids using FuGENE 6 (Promega). Twenty-four hours after transfection in the growth media, about 10,000 live cells were plated in each well of a 96-well culture plate (PerkinElmer, Waltham, MA), and treated with the vehicle or drugs for an additional 20 or 24 h in the assay media. Next, forty-eight hours after transfection, a luciferase assay was performed to measure luminescence using the Dual-Glo luciferase assay system (Promega, Madison, WI) and FLUOstar Optima microplate reader (BMG Labtech, Cary, NC). The firefly luciferase activity was normalized to either the renilla luciferase activity or the number of liver cells. Cell viability was determined using the CellTiter-Glo luminescent cell viability assays (Promega). The normalized promoter activity was shown as relative luminescence units.

Cell proliferation assays: The lentiviral transduced and non-transduced cells were plated into 96-well culture plates (PerkinElmer) at a density of 10,000 cells per well in a final volume of 100 µl medium, and incubated for 48 h. The CellTiter-Glo luminescent cell viability assays (Promega) were performed to determine the number of viable cells by quantifying the ATP present, which indicates the presence of metabolically active cells. Luminescence was measured with a FLUOstar Optima microplate reader (BMG Labtech).

Cell-cycle analyses: Both the sub-confluent and confluent HepG2 cells in 6-well culture plates were trypsinized and processed for flow cytometry analysis [227]. Briefly, the cells were fixed in 70% cold ethanol and incubated with propidium iodide to stain DNA and with RNAse A to kill RNA. The cells were then analyzed for cell-cycle profile by flow cytometry (ACCURI C6). Cell-

cycle distribution was calculated using CFlow Plus software. The percentage of each population in each phase of the cell-cycle was determined [225, 227].

Animals, Surgeries and Tissue Harvesting: Partial hepatectomy (PHX) surgeries were performed on 2-3 month old male C57/BL6 mice as previously described [6]. Livers were collected at various time points between 0 to 14 days after PHX and processed as described before for gene and protein expression analyses [6].

RT-PCR analysis: Total RNA was extracted from the mice liver tissue, HepG2 cells or human primary hepatocytes by using the RNeasy Mini Kit (Qiagen; Valencia, CA). The quality and quantity of the total RNA were determined using NanoVue Plus Spectrophotometer (GE Healthcare). Reverse transcription was performed with the QuantiTect Reverse Transcription Kit (Qiagen) and quantitative PCR was performed by using the QuantiTect SYBR Green Kit (Qiagen) and iCycler iQ Real-Time PCR Detection System (Bio-Rad; Hercules, CA) according to the manufacturer's protocol. Transcripts of the 18S small subunit ribosomal RNA (18S rRNA) housekeeping gene and mouse PXR (mPXR), Cyp3a11, Ki-67, TNFα, CDK2, human PPM1A and CYP3A4 were amplified using gene-specific primers **(Table 1)**. The comparative Ct method was used for relative quantification for gene expression with the following formula: ΔCt = Ct (test gene) - Ct (*18S rRNA*); ΔΔCt (test gene) = ΔCt (test gene in treatment group) - ΔCt (test gene in vehicle control group); the fold changes of mRNA = $2^{-\Delta\Delta Ct}$, which indicated the relative mRNA level of the corresponding transcript to the control samples.

Western blotting analysis: The mice liver tissue, COS-7 or HepG2 total cell lysates were collected in RIPA buffer containing a cocktail of protease inhibitors, and protein concentration

was determined by Bio-Rad protein assay (Bio-Rad). Equal amounts of protein were separated on a SDS-polyacrylamide gel electrophoresis and were transferred on to nitrocellulose membranes. The membranes were then blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20, incubated with anti-PPM1A (Abcam), anti-FLAG (Sigma) or anti-actin (Santa Cruz) antibodies in blocking buffer, washed with Tris-buffered saline, and finally incubated with HRP-conjugated secondary antibodies (Santa Cruz) in blocking buffer. The proteins were visualized using HyGLO Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, Metuchen, NJ).

Coimmunoprecipitation assays: COS-7 cells were transiently cotransfected with FLAG-hPXR and PPM1A [2] or FLAG-pcDNA. FLAG-hPXR contains the FLAG epitope fused to the N terminus of hPXR. Fusion of FLAG tag to the N-terminal hPXR doesn't affect the transactivation function of hPXR in HepG2 or COS-7 cells [130]. 24h after transfection, cells were treated for additional 24 h with DMSO or 10 μM rifampicin. The cells were lysed in triton lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitor cocktail (Roche, Indianapolis, IN), and Halt phosphatase inhibitor cocktail (Pierce, Rockford, IL). Total protein concentration was determined by Bio-Rad protein assay (Bio-Rad). Immunoprecipitation was carried out on 1 mg of total protein using anti-FLAG M2 agarose beads (Sigma) for 2 hours at 4°C. The beads were washed twice each with lysis buffer and TBS and then boiled in sample loading buffer (Invitrogen) to release the bound proteins, followed by western blot analysis with an anti-PPM1A antibody. The same lysates were also probed with the anti-PPM1A antibody and a western blot was performed.

Statistical analysis: Data were analyzed with Student's *t* test by using GraphPad Prism 6 software. Differences were considered significant (*) for $P < 0.05$

Results and Discussion

PPM1A coimmunoprecipitates with hPXR. It is known that cellular signaling pathways mediated through phosphatases, regulate the function of several nuclear receptors, thereby modulating nuclear receptor-mediated gene expression [134, 223, 225, 228-230]. There is some evidence showing that cellular signaling pathways, through protein-protein interactions and post-translational modifications, regulate hPXR function and hPXR-mediated CYP3A4 expression in hepatocytes [9, 121-124, 128, 209-211]**.** While much is known about hPXR regulation by kinases [121, 128, 215], very little is known regarding phosphatases. To identify phosphatases that interact with hPXR, we sought a proteomics approach that would identify proteins that interact with hPXR in cell based assays. The rationale is that hPXR interacting proteins can be pulled by immunoprecipitation of transfected FLAG-hPXR, and that the coprecipitated proteins can be sequenced for their identity using mass spectrometry. FLAG-hPXR contains the FLAG epitope fused to the N terminus of hPXR, and fusion of the FLAG tag to the N-terminal hPXR doesn't affect the transactivation function of hPXR in HepG2 or COS-7 cells [130]. We identified PPM1A as one of the hPXR interacting proteins. Recently, phosphatases including PPM family members, such as PPM1A and PPM1B, have been shown to interact with nuclear receptors [222-224]. To confirm hPXR and PPM1A interaction, COS-7 cells were transiently co-transfected with FLAG-hPXR and PPM1A [2] or FLAG-pcDNA. 24 h after transfection, cells were treated for additional 24 h with DMSO or 10 μM rifampicin. PPM1A was coimmunoprecipitated with FLAG-hPXR in both basal (DMSO) and ligand (rifampicin)-stimulated conditions (**Figure 5**). This finding led us to hypothesize that PPM1A phosphatase may play a role in regulating hPXR activity.

Overexpression of PPM1A leads to increased rifampicin-induced hPXR-mediated CYP3A4 promoter activity. We used the induction of CYP3A4-luc to measure the activity of hPXR in HepG2 and COS-7 cells [130, 225]. Activation of PPM1A by overexpression led to a significant increase in both basal (DMSO) and rifampicin-induced hPXR activation in HepG2 and Cos7 cells (**Figures 6A & 6B)** [2, 226]. Similarly, pharmacological activation of PPM1A with C_6-ceramide also resulted in increased activation of hPXR (**Figure 7A**) [231]. On the other hand, dihydroC_6-ceramide, an inactive analog of C_6-ceramide, did not affect hPXR activity (**Figure 7B**) [232]. These results suggest that activation of PPM1A increases hPXR-mediated CYP3A4 activity.

Although we used C_6-ceramide to pharmacologically activate PPM1A in HepG2 cells, C_6-ceramide was also shown to inhibit PKCα [232]. PKA is shown to suppress hPXR activity [91]. It is not known whether C_6-ceramide binds to and activates hPXR. Therefore, it is possible that C_6-ceramide-increased hPXR activity could be a combinatorial effect of PPM1A activation, PKCα inhibition, and direct hPXR activation (serving as an agonist of hPXR). Nevertheless, PPM1A overexpression (genetic activation) studies confirm that activation of PPM1A leads to upregulation of hPXR activity (**Figures 6A & 6B**).

Knockdown of PPM1A RNA levels leads to impaired rifampicin-induced hPXR-mediated CYP3A4 promoter activity in HepG2 cells. Transactivation assays were carried out in HepG2 cells stably transfected with hPXR (pcDNA3-hPXR) and CYP3A4-luc, in which the expression of luciferase was controlled by the hPXR-regulated CYP3A4 promoter [4]. Knockdown of endogenous PPM1A in the HepG2 cells was achieved by transduction with lentiviral vectors carrying PPM1A shRNA (**Figure 8A**). While overexpression (activation) of PPM1A enhanced hPXR function (**Figure 6**), knockdown (inhibition) of PPM1A significantly impaired both basal

and rifampicin-induced transactivation function of hPXR (**Figure 8B**). Transduction with lentiviral vectors containing non-silencing shRNA (control shRNA) did not affect either PPM1A protein expression or rifampicin induced hPXR activity (**Figure 8A & 8B**).

To determine whether PPM1A regulates PXR-mediated CYP3A4 gene expression in human primary hepatocytes, PPM1A expression was knocked down using PPM1A shRNA (**Figure 9A**). Transduction with control shRNA did not affect PPM1A expression (**Figure 9A**). Notably, knockdown of PPM1A mRNA expression resulted in a significant reduction in rifampicin-induced CYP3A4 mRNA expression (**Figure 9B**). These results led us to speculate that PPM1A may play a significant role in the regulation of hPXR activity *in vivo.*

Knockdown of PPM1A promotes proliferation of hepatocytes. It is known that hPXR-activated CYP3A4 expression in hepatocytes is regulated in a proliferation-dependent manner [4, 213]. It is also known that PPM family phosphatases, including PPM1A, regulate cell growth [2, 216, 218-220]. A recent study using PPM1A gene knockout mice revealed that PPM1A plays a major role in regulating epithelial cell growth [221]. We examined whether PPM1A levels affect proliferation of HepG2 cells. Knockdown of PPM1A levels, using shRNA-mediated knockdown, significantly increased proliferation of HepG2 cells (**Figure 10**). On the other hand, control non-silencing shRNA did not affect proliferation of HepG2 cells (**Figure 10**). These results demonstrate that the knockdown PPM1A expression in HepG2 cells impairs hPXR activity but promotes proliferation.

PPM1A expression and hPXR activity are downregulated in sub-confluent HepG2 cells. Since PPM1A knockdown resulted in decreased hPXR activity and increased HepG2 proliferation, we determined whether PPM1A expression and hPXR activity are affected in sub-confluent HepG2 cells when compared to confluent HepG2 cells. Flow cytometry analysis was performed

on the sub-confluent and confluent HepG2 cells to determine distribution of the cells in different phases of the cell-cycle. Number of cells in the S phase of the cell-cycle was significantly higher in the sub-confluent cells (~17%) compared to the confluent cells (~4 %) (**Figure 11A**). Both PPM1A protein levels and hPXR transaction of CYP3A4 promoter activity were downregulated in the sub-confluent HepG2 cells compared to the confluent HepG2 cells (**Figure 11B & 11C**). These results confirm that PPM1A levels, similar to hPXR activity, are regulated in a proliferation-dependent manner, and suggest that PPM1A expression and hPXR activity may be regulated in proliferating livers [4, 213]. Additionally, similar to proliferating livers**,** mRNA levels of TNFα and CDK2 were upregulated in the sub-confluent HepG2 cells as compared to the confluent HepG2 cells (**Figure 11D**) [157, 233].

We next determined the biological relevance of PPM1A regulation of PXR-mediated CYP3A expression in HepG2 as well as in mice regenerating (proliferating) livers since PPM1A has been shown to regulate proliferation-dependent signaling pathways, such as CDK2, that modulate PXR activity [4, 118, 119, 213].

PPM1A counteracts inhibition of hPXR activity by TNFα, HGF and CDK2. Liver regeneration triggered by liver injury is a pathophysiological condition characterized by reduced CYP3A expression in proliferating hepatocytes [200-204]. Hepatocyte proliferation during liver regeneration after partial hepatectomy occurs in two phases; the priming phase and the proliferative phase [157]. The priming is induced by cytokines such as TNFα, primarily through NF-κB activation, whereas the proliferation is induced by growth factors such as HGF, and through activation of various signaling pathways, including CDK2 [185-188, 234, 235].

It is known that TNFα, HGF, and CDK2 signaling pathways, which are upregulated and essential for hepatocyte proliferation in regenerating livers, inhibit PXR-activated CYP3A expression[4, 118, 119, 128, 213, 236, 237]. While CDK2 phosphorylates PXR to inhibit PXR activity, TNFα induces NF-κB activation to inhibit PXR-RXRα interaction [4, 118, 128, 212]. As expected, activation of CDK2 (overexpression of CDK2 and cyclin E) (**Figure 12A**) or treatment with TNFα (**Figure 12B**) inhibited both basal and rifampicin-induced activation of hPXR [4].

PPM1A inhibits TNFα signaling by dephosphorylating and inactivating IKKβ, a central intermediate signaling molecule in TNFα-mediated activation of NF-κB [238]. PPM1A also inhibits CDK2 signaling by directly dephosphorylating and inactivating CDK2 [239, 240]. We therefore asked the question whether PPM1A activation attenuated TNFα and CDK2 inhibition of hPXR. Indeed, PPM1A activation attenuated inhibition of hPXR by both CDK2 and TNFα (**Figure 12A & B**), suggesting that PPM1A can safeguard the function of hPXR in hepatocytes by inhibiting or desensitizing TNFα and CDK2 signaling. Activated HGF has been shown to stimulate several intracellular kinases, including CDK2 [241-243]. We therefore tested whether HGF inhibits PXR activity, and whether PPM1A affects HGF inhibition of PXR. The results determined that while HGF inhibited PXR function, PPM1A counteracted the inhibition of HGF on PXR activity **(Figure 12C)**.

However, these three PXR inhibitory signaling pathways are required for hepatocyte proliferation during liver regeneration. If PPM1A expression is not downregulated in proliferating hepatocytes of regenerating livers, regeneration may be affected as a consequence of PPM1A desensitization or inhibition of HGF, TNFα and CDK2 signaling. It is therefore possible that PPM1A levels are downregulated in hepatocytes during liver regeneration. This logical speculation is further

supported by our findings of mutually negative regulation between PPM1A expression and HepG2 proliferation (**Figures 10 & 11**).

Cyp3a11 expression positively correlates with PPM1A expression in mice regenerating livers. It is well known that the expression of Cyp3a11 (the mouse ortholog of human CYP3A4) is significantly downregulated in proliferating hepatocytes of mice regenerating livers as compared to normal quiescent hepatocytes [200-204]. Since knockdown of PPM1A levels resulted in increased HepG2 proliferation and impaired hPXR-mediated CYP3A4 expression (**Figures 10 & 11**), we speculated that PPM1A levels could be downregulated in proliferating hepatocytes of regenerating livers, contributing to reduction of mouse PXR-mediated Cyp3a11 expression. In addition, PPM1A inhibition of both CDK2 and TNFα (**Figure 12A & 12B**), important factors in liver regeneration, corroborates the speculation of decreased PPM1A activity during liver regeneration.

To verify that the mouse livers were undergoing normal liver regeneration after PHX, we examined the mRNA levels of TNFα, CDK2, and Ki-67 in 0 h to 7d livers after PHX. As expected, mRNA levels of TNFα and CDK2 as well as cell proliferation marker Ki-67 were upregulated in livers after PHX [244]. We found that TNFα was increased significantly at 12 h, 24 h, and 48 h (**Figure 13A**). Both CDK2 and Ki-67 were increased significantly after 24 h until 5 d (**Figures 13B & 13C**).

We also examined the mRNA expression of Cyp3a11 and mPXR along with the protein levels PPM1A in 0 h to 14d regenerating livers of mice after PHX [6, 7, 245, 246]. As expected, Cyp3a11, an mPXR target gene, was significantly downregulated in regenerating livers (0h to 7d after PHX) (**Figure 14A**). Interestingly, PPM1A protein expression was also downregulated in mice

regenerating livers (0h to 5d after PHX) (**Figure 14B**). mPXR mRNA levels were either unaltered or higher after PHX (**Figure 14C**), indicating that Cyp3a11 repression in regenerating livers was not because of reduced mPXR levels. The positive correlation between PPM1A and Cyp3a11 expression in regenerating livers is consistent with downregulation of PPM1A expression and hPXR-mediated CYP3A4 promoter activity in the sub-confluent HepG2 cells (**Figure 11**). The mice liver regeneration studies provide an important *in vivo* biological relevance for PPM1A regulation of PXR activity and PXR-mediated CYP3A expression in liver hepatocytes. Taken together, these findings may provide a mechanism for repression of CYP3A in regenerating livers, where downregulation of PPM1A, which activates PXR, may lead to CYP3A repression in proliferating hepatocytes of regenerating livers.

Enzymatic activity of PPM1A is required for PPM1A upregulation of hPXR function. Overexpression of wild-type (catalytically-active) PPM1A, but not mutant (catalytically-inactive) PPM1A R174G, led to a significant upregulation of both basal (DMSO) and rifampicin-induced activation of hPXR (**Figure 15A & 15B**) [2, 226]. This result suggests that enzymatic (phosphatase or dephosphorylation) activity of PPM1A is essential for PPM1A to enhance hPXR function.

PPM1A activation counteracts inhibition of hPXR activity by kinase signaling. Several Ser/Thr kinases, including PKA**,** PKC, and CDK2 phosphorylate and inhibit hPXR function [4, 87, 91, 120, 128]. hPXR exists as a phosphoprotein *in vivo* and kinase signaling, such as PKA and CDK2, increase phosphorylation of Ser and Thr residues on hPXR *in vivo* to inhibit hPXR function [87, 128]. As expected, activation of PKA signaling by an activator of PKA, dibutyryl-cAMP (db-cAMP) or a specific activator of PKA, N6-Benzoyladenosine-cAMP (N-6-benz-cAMP), resulted

in inhibition of hPXR activity (**Figure 15**) [247, 248]. Likewise, activation of PKC signaling with phorbol 12-myristate 13-acetate (PMA) resulted in inhibition of hPXR activity (**Figure 16**).

Since PPM1A interacts with hPXR and the phosphatase activity is essential for PPM1A to enhance hPXR function **(Figure 15)**, we speculated that PPM1A may act as a major Ser/Thr phosphatase to dephosphorylate and enhance hPXR function. Indeed, PPM1A activation significantly attenuated hPXR inhibition by CDK2 (**Figure 12A**) as well as by PKA and PKC signaling (**Figures 16 & 17**). This suggests that PPM1A may desensitize these inhibitory signaling pathways possibly by dephosphorylating phosphoserine/phosphothreonine residues in PXR.

Phosphatases regulate the function of other proteins through their scaffolding and/or enzymatic activities. Currently, it is unknown whether PPM1A increases hPXR function by acting as a scaffolding protein to alter hPXR-coregulator interactions. Also, it remains to be determined whether PPM1A dephosphorylates hPXR/coregulators/other signaling molecules that affect hPXR activity. Additionally, other possible mechanisms, such as altered PXR expression, localization and DNA binding, cannot be ruled out. Future investigations are required to dissect the detailed mechanisms.

It is possible that HepG2 cells were driven into proliferation in response to PPM1A knockdown because PPM1A's normal inhibitory effect on CDK2 and TNFα was released or the activity of CDK2 and TNFα increased in response to reduced levels of PPM1A. However, it is unknown whether PPM1A attenuates TNFα inhibition of hPXR by desensitizing IKKβ phosphorylation. Similarly, it is unknown whether PPM1A attenuates CDK2 inhibition of hPXR by desensitization of CDK2 signaling and/or hPXR phosphorylation. Along the same lines, PPM1A attenuation of

PKA and PKC inhibition of PXR could be due to dephosphorylation of phosphorylated hPXR as there are no reports of direct PPM1A inactivation of PKA and PKC.

In summary, our results determine that PPM1A interacts with hPXR and positively regulates hPXR-mediated CYP3A4 expression by counteracting the inhibitory effect of TNFα, HGF, CDK2, PKA, and PKC signaling. Our results also show that PPM1A levels and hPXR-mediated CYP3A4 expression are downregulated in proliferating hepatocytes. Finally, our results show that PPM1A levels positively correlate with cyp3a11 levels in mice regenerating livers. These findings may provide a mechanism for reduced CYP3A levels in proliferating livers such as injured livers undergoing regeneration.

Our current model of PXR-mediated CYP3A4 regulation via PPM1A is based on previous studies and our current study results. Both TNFα and CDK2 signaling pathways are vital components of liver regeneration and inhibit PXR activity. PPM1A enhances PXR activity by counteracting the PXR inhibitory signaling pathways such as TNFα and CDK2 (**Figure 18A**). It is our current proposition that decreased levels of PPM1A in regenerating livers may in part contribute to repressed CYP3A4 levels in regenerating livers (**Figure 18B**).

Figures

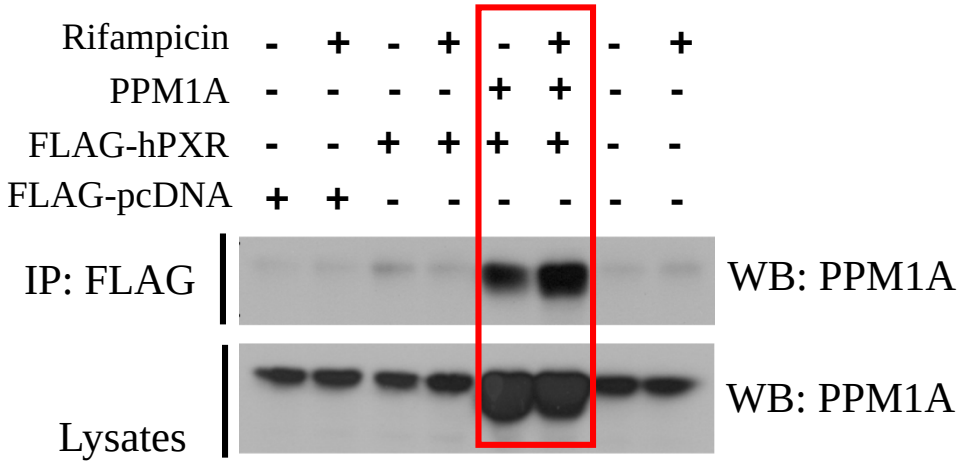


Figure 5: PPM1A coimmunoprecipitates with hPXR. COS-7 cells were transiently cotransfected with FLAG-hPXR and PPM1A [2] or FLAG-pcDNA. 24 h post transfection, the cells were treated with either DMSO or 10 µM rifampicin for 24 h. The cells were then lysed with triton lysis buffer. Immunoprecipitation was performed using anti-FLAG M2 antibody. The interaction of FLAG-hPXR with PPM1A was examined by immunoprecipitation with the anti-FLAG antibody followed by western blot analysis with an anti-PPM1A antibody. The same lysates also underwent a western blot and probed with the anti-PPM1A antibody. Data shown are from a representative experiment.

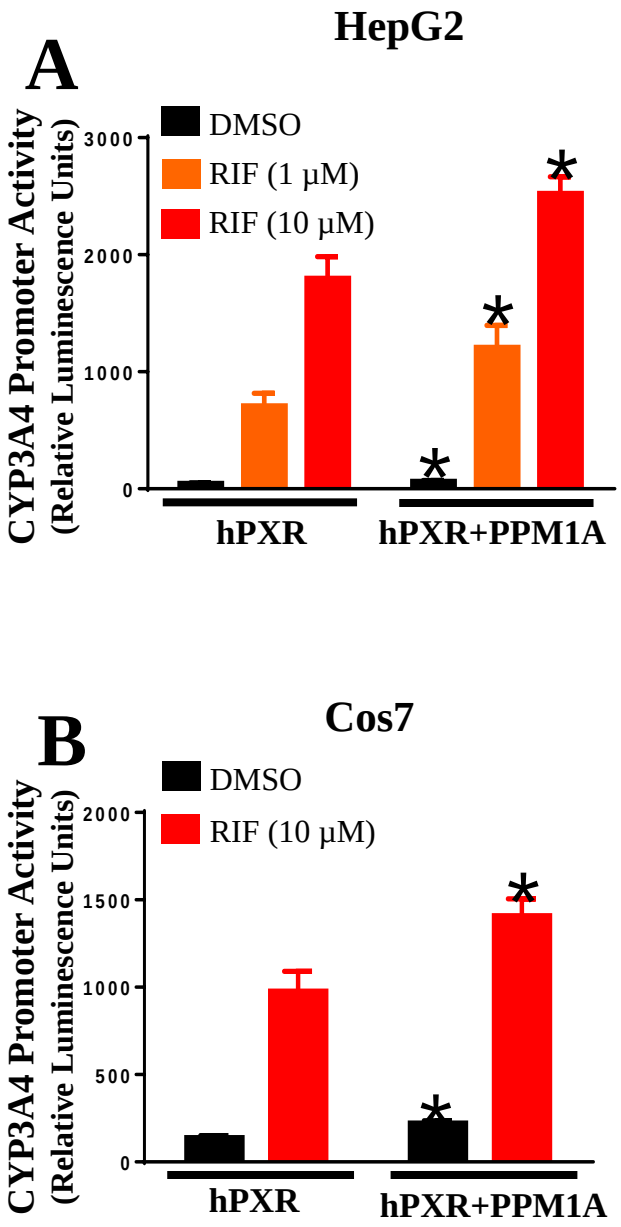


Figure 6: Genetic activation of PPM1A enhances the transactivation function of hPXR. HepG2 (A) and COS-7 (B) cells were cotransfected with hPXR, CYP3A4-luc, PPM1A and CMV-Renilla (transfection control). After 24 h transfections, the cells were treated with DMSO, 1 or 10 µM rifampicin (RIF) for 24 h before measuring the firefly and renilla luciferase activities. The relative luminescence units were determined by normalizing with the renilla luciferase control. Data represent mean ± SD from eight independent experiments. Statistical significance (*, $p < 0.05$) was determined using unpaired Students t test. PPM1A cotransfected samples were compared with hPXR transfected samples in each treatment group.

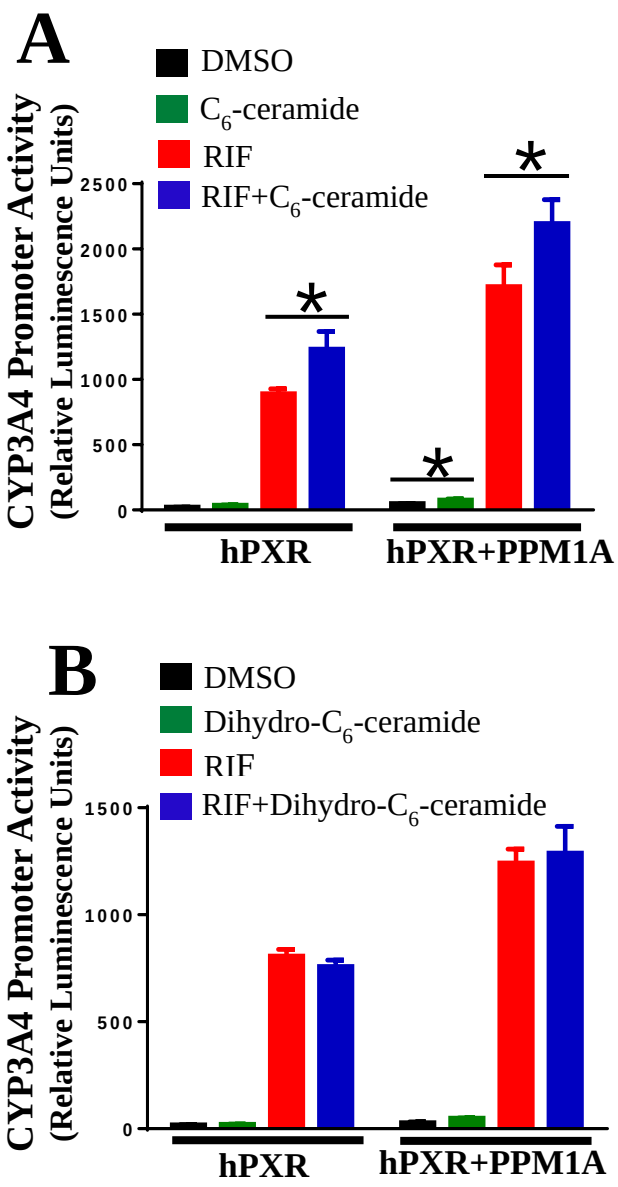


Figure 7: Pharmacological activation of PPM1A enhances the transactivation function of hPXR. HepG2 cells were cotransfected with hPXR, CYP3A4-luc, PPM1A and CMV-Renilla (transfection control). After 24 h transfections, the cells were treated with DMSO, 10 μM rifampicin (RIF), 10 μM C6-ceramide ± RIF or 10 μM dihydro-C6-ceramide ± RIF. The firefly and renilla luciferase activities were measured 24 h after the treatments. The relative luminescence units were determined by normalizing with the renilla luciferase control. Data represent mean ± SD from eight independent experiments. Statistical significance (*, $p < 0.05$) was determined using unpaired Students t test.

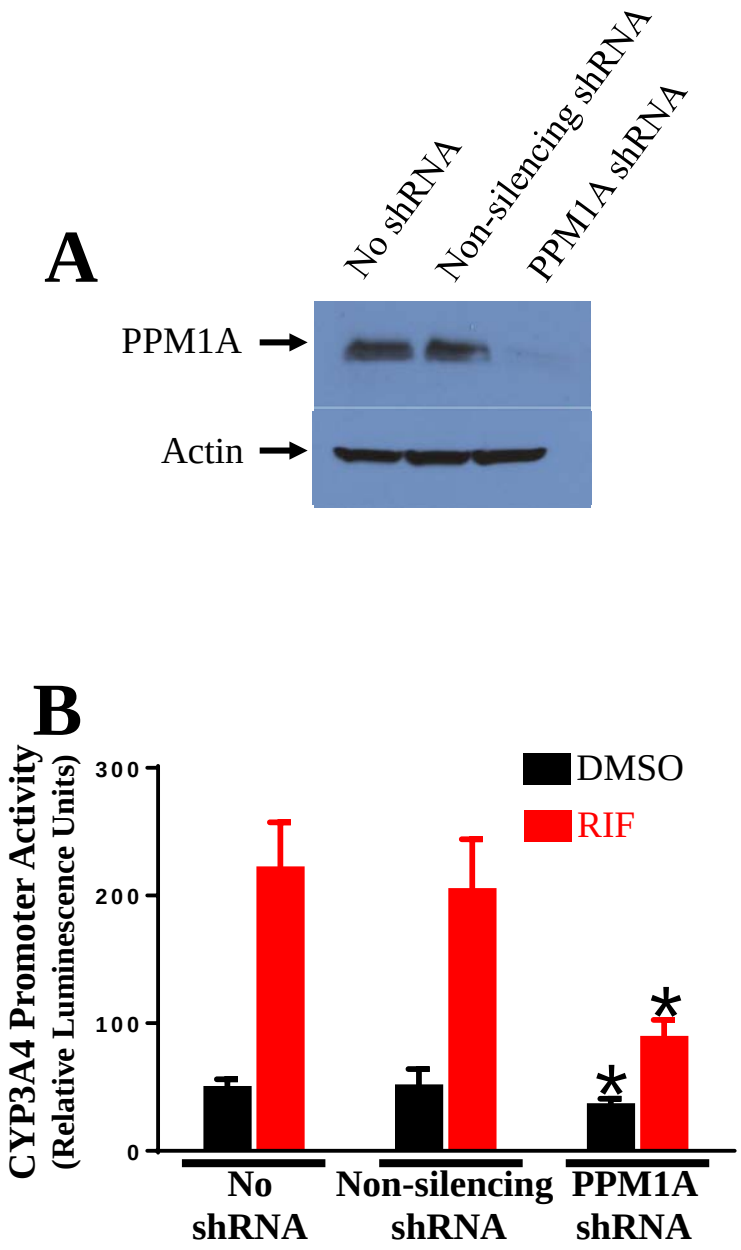


Figure 8: Knockdown of PPM1A RNA levels impairs hPXR activity in HepG2 Cells. (A) HepG2 cells stably expressing hPXR and CYP3A4-luc [4] were transduced with lentiviral vectors carrying control non-silencing shRNA or PPM1A shRNA. Whole-cell lysates were collected and subjected to western blot analysis using anti-PPM1A and anti-actin antibodies (as a loading control). Data shown are from a representative experiment. (B) Knockdown of PPM1A impairs hPXR transactivation of CYP3A4 promoter activity. The transduced and non-transduced cells were treated for 24 h with DMSO or 10 μM rifampicin (RIF) and firefly luciferase activity was measured and normalized with the total number of live cells (CellTiter-Glo cell viability assays). The relative luciferase activity is shown as the mean ± SD from six independent observations. Statistical significance (*, $p < 0.05$) was determined using unpaired Students *t* test by comparing PPM1A shRNA samples with no shRNA samples in each treatment group.

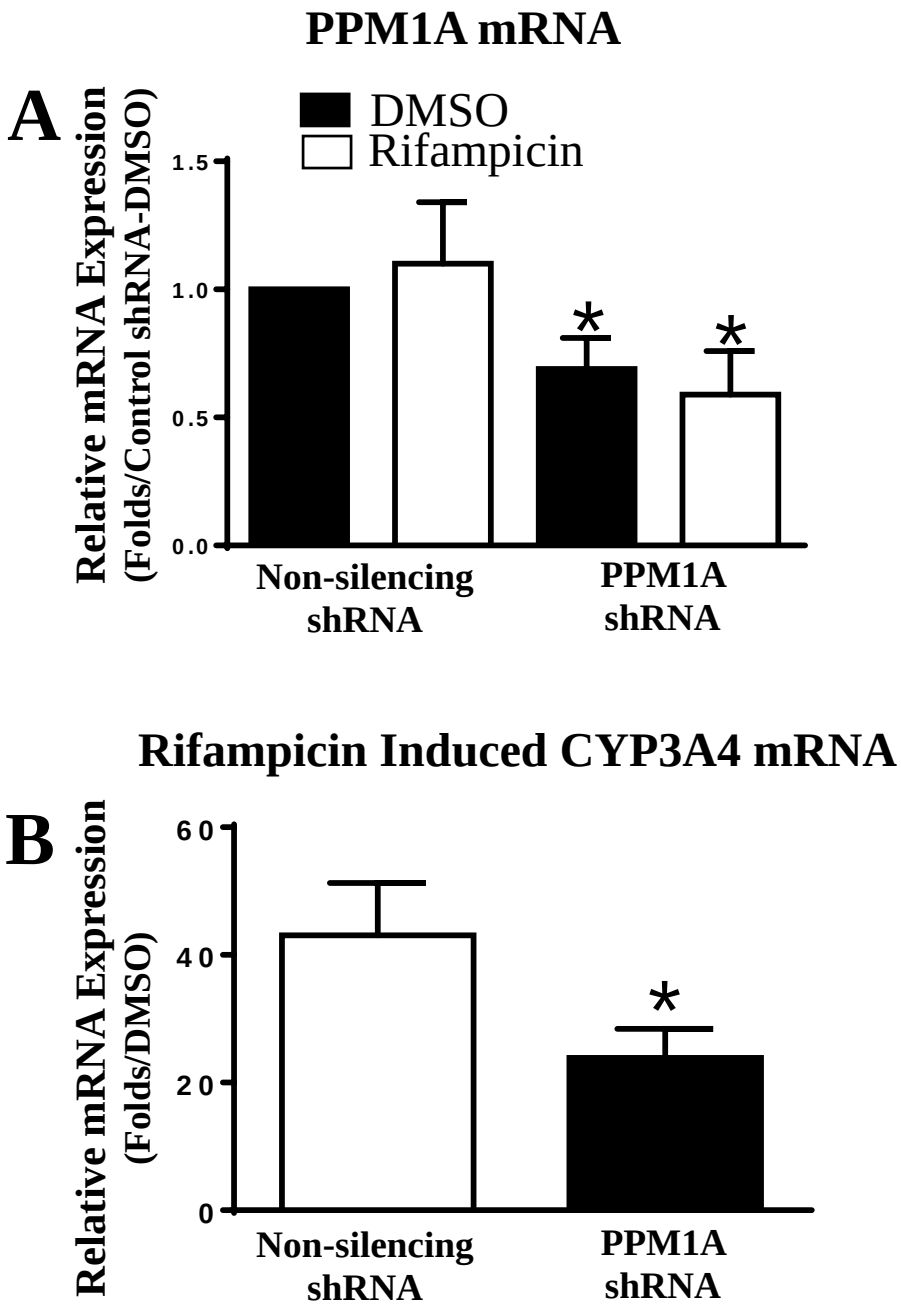


Figure 9: Knockdown of PPM1A RNA levels diminishes rifampicin induced CYP3A4 mRNA expression in primary human hepatocytes. (A) Primary human hepatocytes were transduced with lentiviral vectors carrying control non-silencing shRNA or PPM1A shRNA. 48 h after transduction, the cells were treated with DMSO or rifampicin (10 µM) for another 24 h before measuring PPM1A (A) and CYP3A4 (B) mRNA expression levels. Data represent mean ± SD from three independent experiments: *, $p < 0.05$; compared with control shRNA by unpaired Students *t* test.

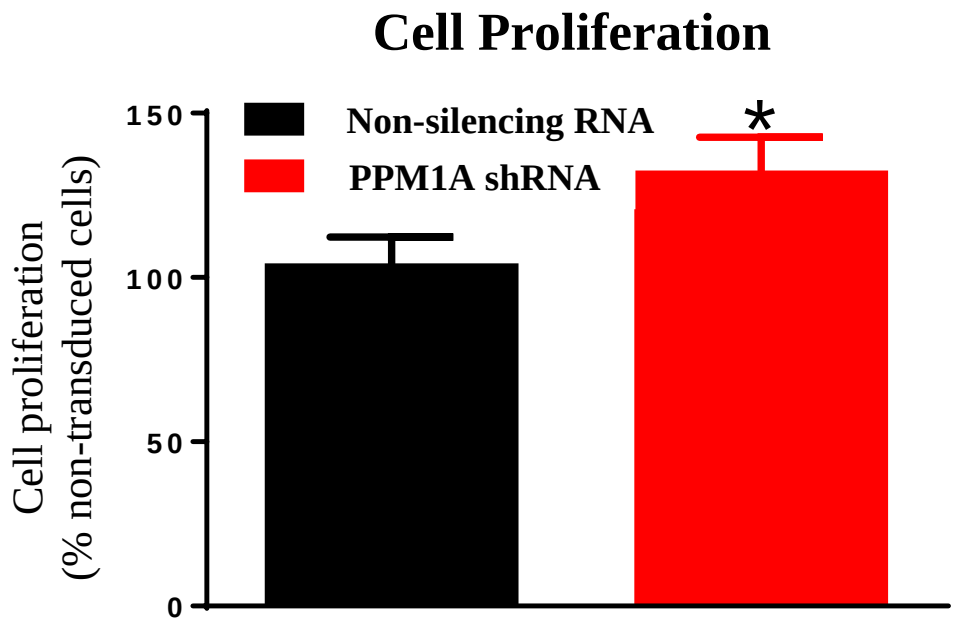


Figure 10: Knockdown of PPM1A RNA promotes proliferation of HepG2 cells. CellTiter-Glo luminescence assays were performed 48 h after plating 10,000 cells/well to monitor proliferation of non-transduced and transduced (control or PPM1A shRNA) HepG2 cells. The luminescence of transduced cells was normalized with the luminescence of non-transduced cells and shown as the mean ± SD of six independent samples. *, $p < 0.05$; compared with control shRNA by unpaired Students *t* test.

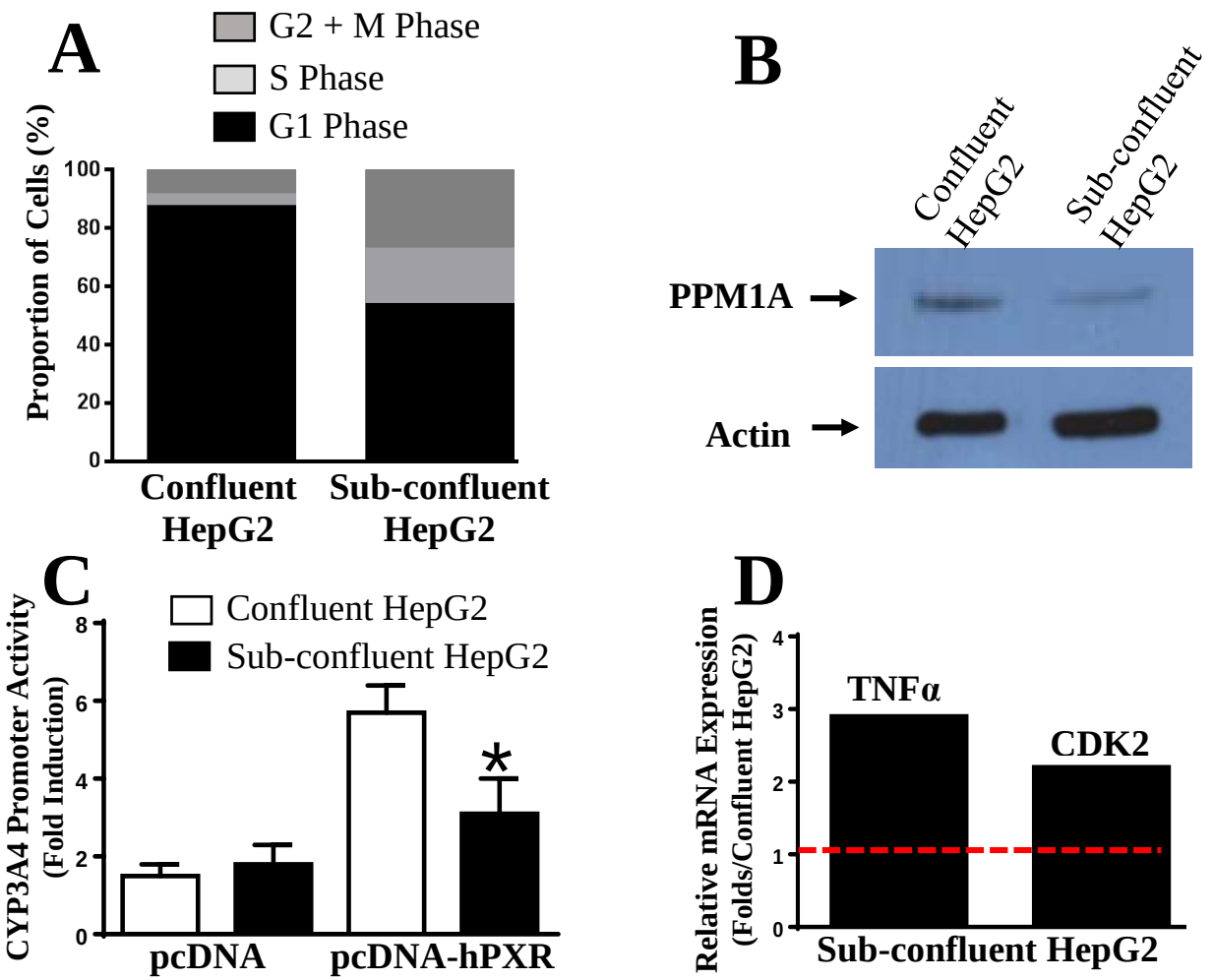


Figure 11: PPM1A protein levels (B) and hPXR activity (C) are downregulated in sub-confluent HepG2 cells while TNFα and CDK2 levels (D) are upregulated. (A) Both the confluent and sub-confluent HepG2 cells were analyzed for cell cycle distribution using Flow Cytometry as described in the methods. (B) Whole-cell lysates were collected from the confluent and sub-confluent HepG2 cells and subjected to western blot analysis using anti-PPM1A and anti-actin antibodies. Data shown are from a representative experiment. (C) hPXR transactivation of CYP3A4 promoter activity was determined in the confluent and sub-confluent HepG2 cells. The cells were transiently cotransfected with pGL3-CYP3A4-luc, CMV-Renilla luciferase (transfection control), and pcDNA3 or pcDNA3-hPXR plasmids. After 24 h transfection, the cells were treated with DMSO or rifampicin (10 μM) for another 24 h. The firefly and renilla luciferase activities were measured 24 h after the treatments using Dual-Glo luciferase assay system. CYP3A4 promoter activity was determined by normalizing the firefly luciferase activity with the renilla luciferase control. The results are presented as fold increase over DMSO. The values represent the mean ± SD of four experiments. *, $p < 0.05$; compared with the confluent HepG2 by unpaired Students *t* test. (D) mRNA expression of TNFα and CDK2 was analyzed by quantitative RT-PCR in the confluent and sub-confluent HepG2 cells. Results from sub-confluent HepG2 are presented as fold increase over confluent HepG2. Data represent the means of two independent experiments.

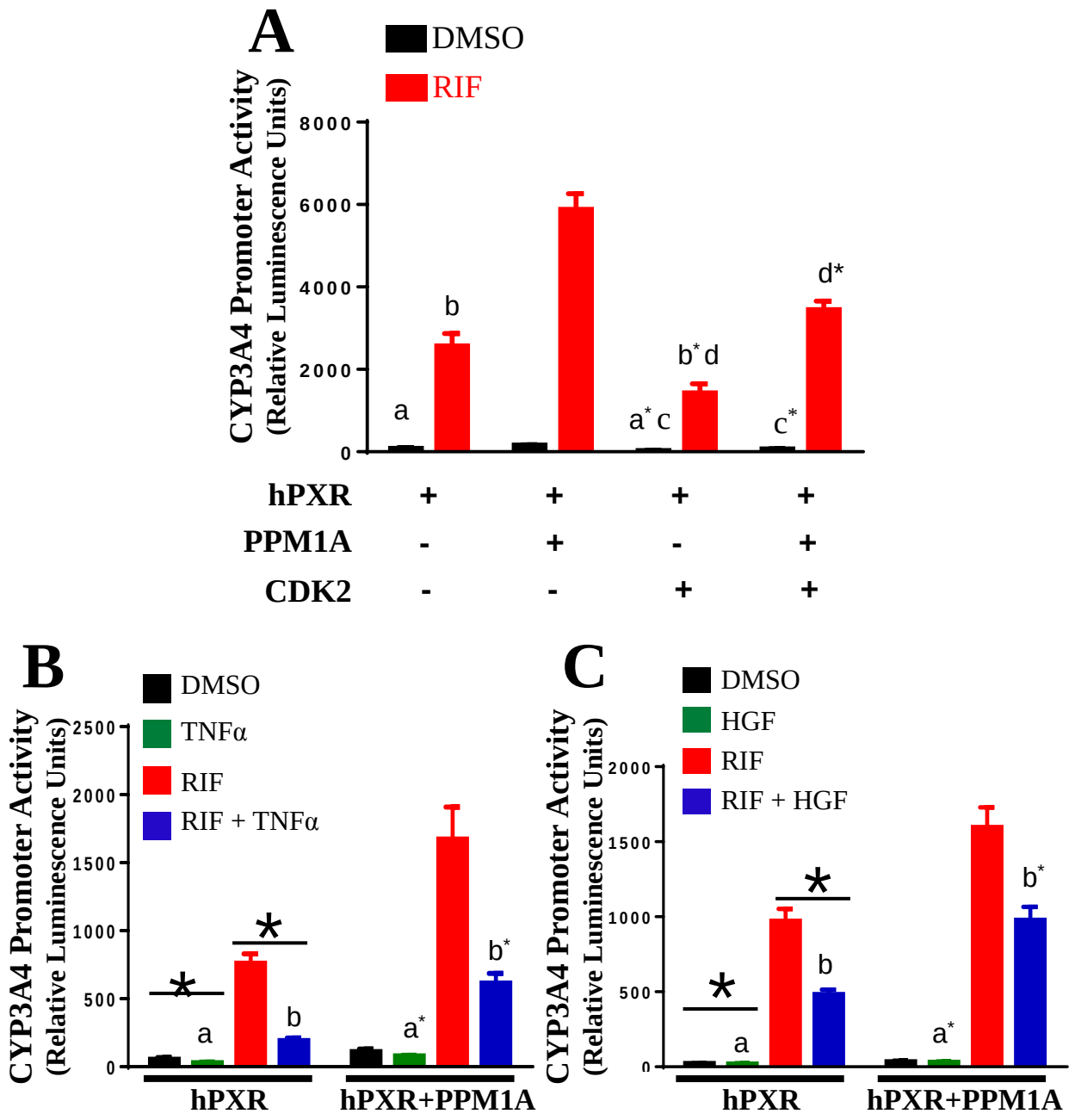


Figure 12: PPM1A activation attenuates hPXR inhibition by CDK2 (A), TNFα (B), and HGF (C). HepG2 cells were co-transfected with FLAG-hPXR, CYP3A4-luc, PPM1A, CDK2 (and cyclin E-not shown), and CMV-Renilla. The cells were treated with DMSO, 10 μM rifampicin (RIF), 20ng/ml HGF ± RIF, or 20 ng/ml TNFα ± RIF. The luciferase activities were measured 20 h after the treatments. The luciferase activities were measured 20 h after the treatments. The relative luminescence units were shown as the means ± SD of five to six experiments. *$p < 0.05$, determined by unpaired Students *t* test.

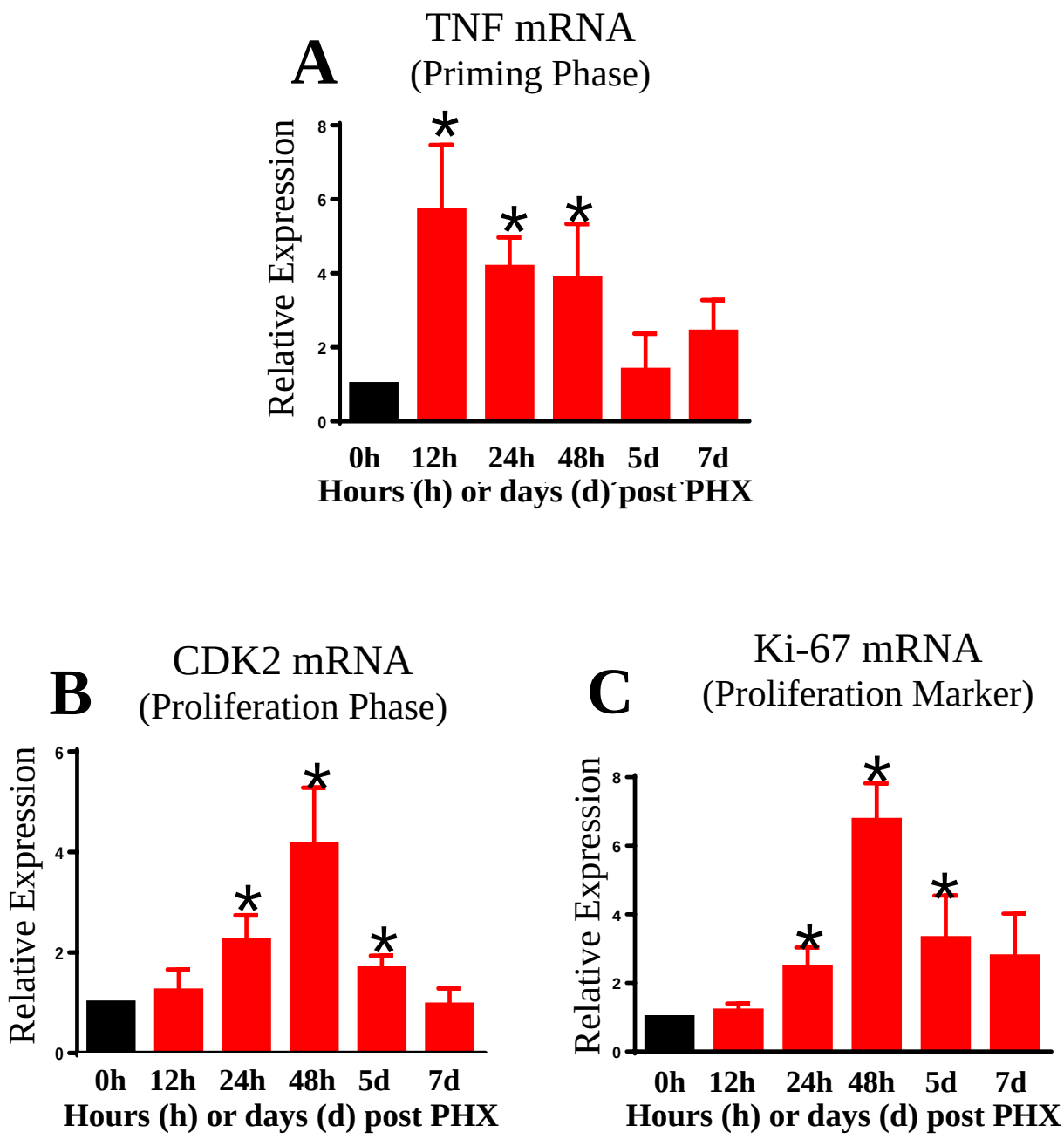


Figure 13: Regenerating mice livers have increased levels of signaling molecules attributed to priming and proliferation. mRNA levels of TNFα (A), CDK2 (B) and Ki-67 (C) were shown at different time points after PHX. mRNA was determined by using quantitative RT-PCR and by normalizing to 18 S mRNA level. Data represent mean ± SD. *, $p < 0.05$; compared with control (0 h) by unpaired Students *t* test.

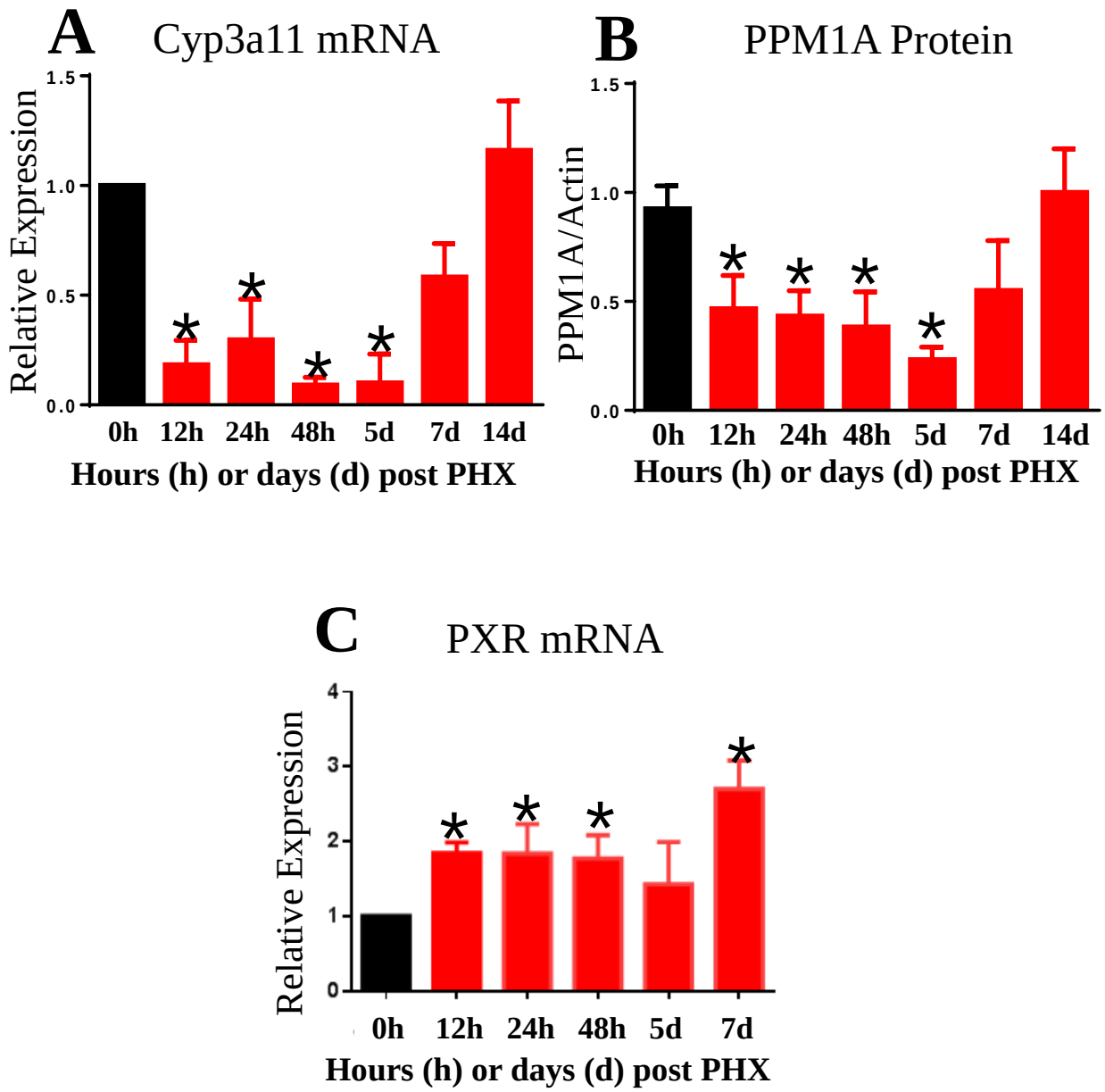


Figure 14: Cyp3a11 levels correlate positively with PPM1A levels in mice regenerating livers. mRNA levels of Cyp3a11 and mPXR (A & C) and protein levels of PPM1A (B) were shown at different time points after PHX. mRNA was determined by using quantitative RT-PCR and by normalizing to 18 S mRNA level. Protein was determined using western blot analysis and by normalizing to actin protein level. PHX was performed as described in materials and methods [6, 7]. At indicated time point post-PHX, liver tissues were removed and snap-frozen in liquid nitrogen, and stored at –80^{o}C until processed for RT-PCR (n=3) or western blot (n=3). Data represent mean ± SD. *, $p < 0.05$; compared with control (0 h) by unpaired Students *t* test.

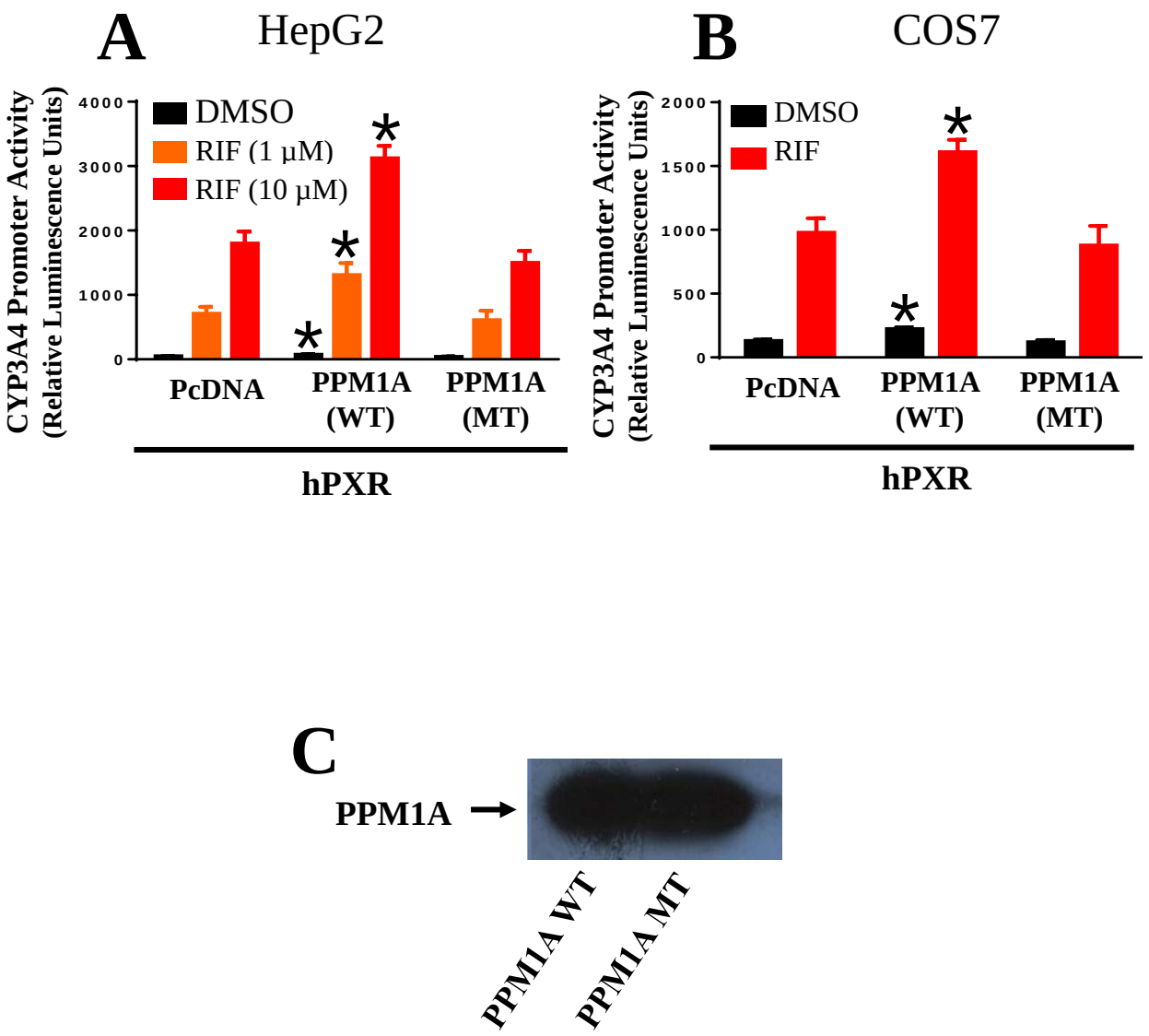


Figure 15: Enzymatic activity of PPM1A is necessary for enhancing hPXR mediated CYP3A4 promoter activity. HepG2 (A) and Cos7 (B) cells were transiently cotransfected with pGL3-CYP3A4-luc, CMV-Renilla luciferase (transfection control), and pcDNA3-hPXR, PPM1A wild type (WT), or PPM1A mutant (MT) plasmids. After 24 h transfection, the cells were treated with DMSO or rifampicin (10 µM) for another 24 h. The relative luciferase activity was determined by normalizing with the renilla luciferase control. Data represent mean ± SD from eight independent experiments. Statistical significance (*, $p < 0.05$) was determined using unpaired Students *t* test. (C) Western blot analysis showing expression of both WT and MT PPM1A proteins in HepG2 cells.

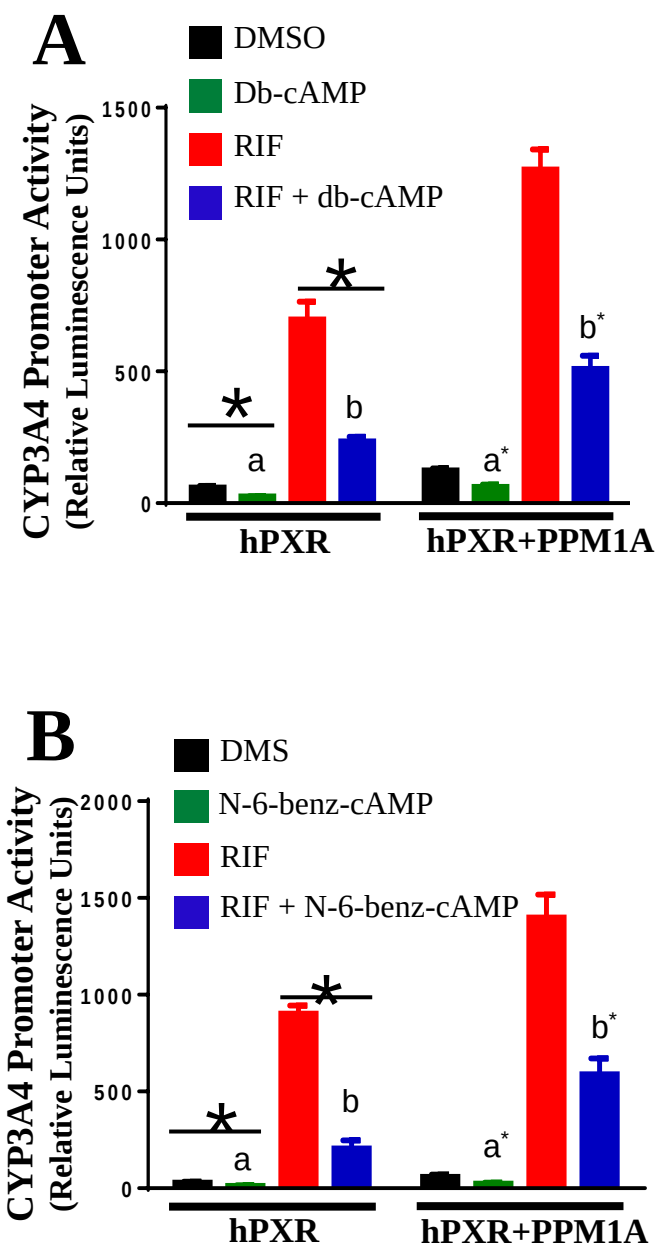


Figure 16: PPM1A attenuates inhibition of hPXR activity by PKA signaling. HepG2 cells were co-transfected with hPXR, CYP3A4-luc, PPM1A, and CMV-Renilla (transfection control). The cells were treated with DMSO, 10 µM rifampicin (RIF), 100 µM db-cAMP ± rifampicin or 100 µM N-6-benz-cAMP ± RIF. Luciferase activities were measured 15 h after treatments. The relative luminescence units were determined by normalizing the firefly luciferase activity with the renilla luciferase control. The values represent the means ± SD from six experiments. Statistical significance (*$p < 0.05$) was determined by unpaired Students *t* test.

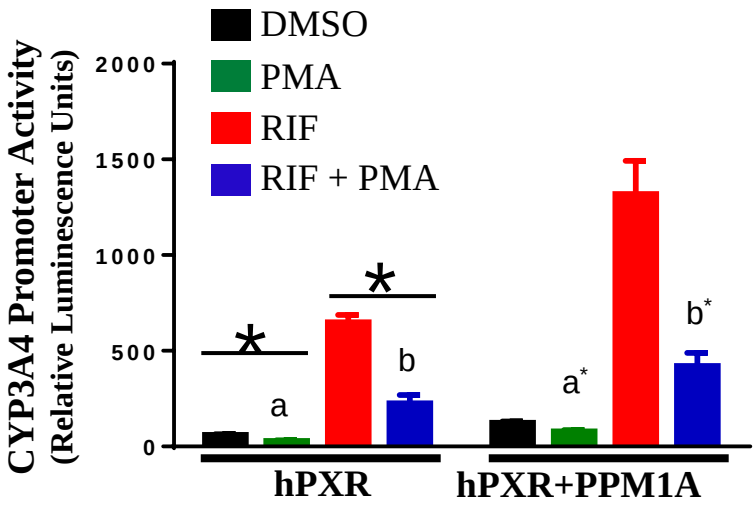


Figure 17: PPM1A attenuates inhibition of hPXR activity by PKC signaling. HepG2 cells were co-transfected with hPXR, CYP3A4-luc, PPM1A, and CMV-Renilla (transfection control). The cells were treated with DMSO, 10 μM rifampicin (RIF), or 100 nM phorbol-12-myristate-13-acetate (PMA) ± RIF. Luciferase activities were measured 15 h after treatments. The relative luminescence units were determined by normalizing the firefly luciferase activity with the Renilla luciferase control. The values represent the means ± SD from six experiments. Statistical significance (*$p < 0.05$) was determined by unpaired Students *t* test.

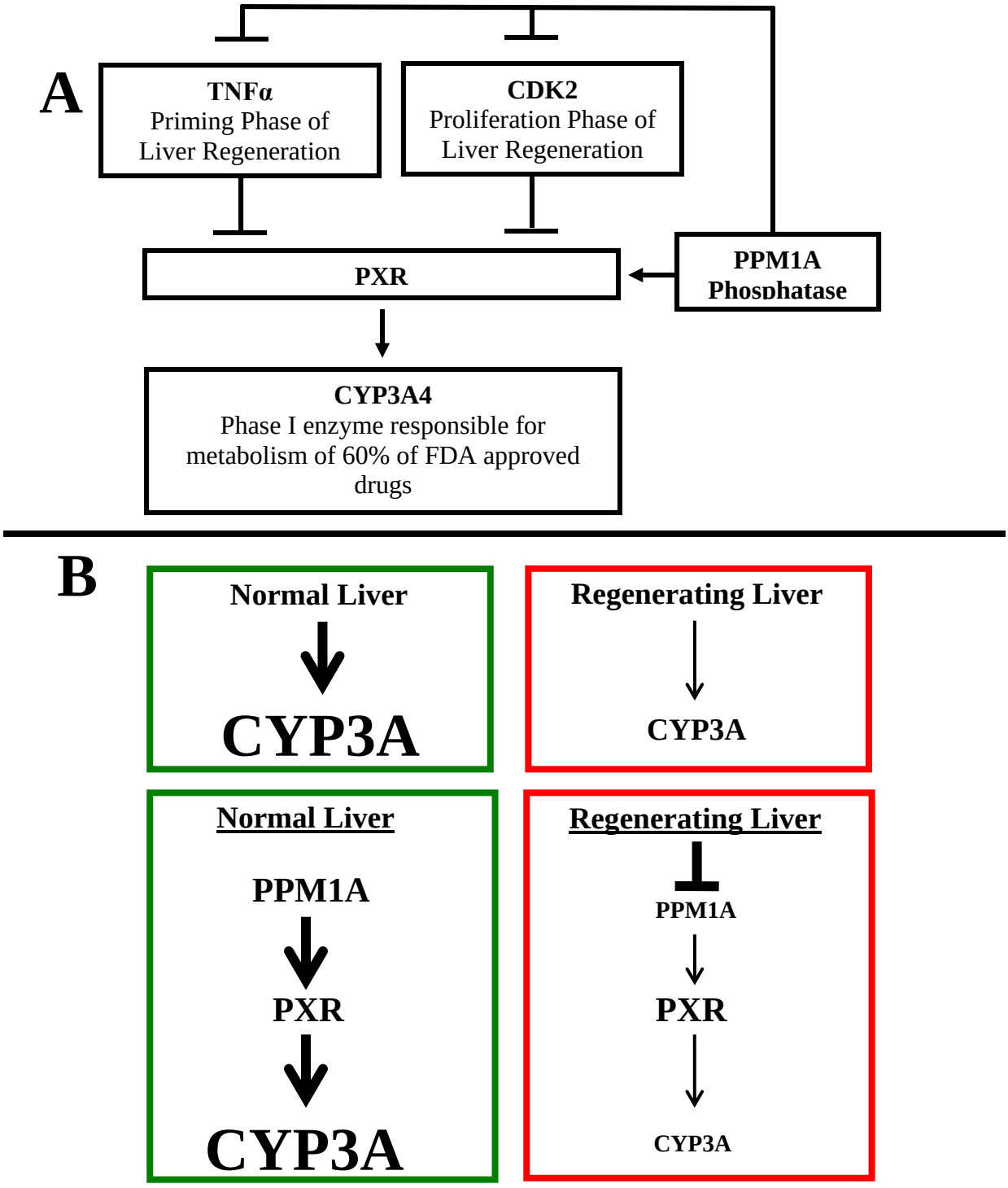


Figure 18: Proposed mechanism for PPM1A regulation of PXR-mediated CYP3A4 expression in liver. (A) The current model of PXR activation via PPM1A directly or indirectly by desensitizing PXR inhibitory signaling pathways that are essential for liver regeneration. (B) One of the proposed mechanisms of CYP3A repression during liver regeneration.

Gene/Primer sequence	Amplified segment (bp)	Gene Bank Accession no
18S rRNA F: 5'-GAGGTTCGAAGACGATCAGA-3' R: 5'- TCGCTCCACCAACTAAGAAC-3'	315	BK000964
mPXR F: 5'- GGGATAGGGTTACAGCACGA -3' R: 5'- CCACCGCCATAGTTCTCATC -3'	258	NM010936
mKi-67 F: 5'-TCTGGTGTTCAGGAGGTCTTC-3' R: 5'-CTTACTGCGAGGCACATTGA-3'	318	NM001081117.2
mTNFα F: 5'-GGCAGGTCTACTTTGGAGTCATTGC-3' R: 5'-ACATTCGAGGCTCCAGTGAATTCGG-3'	277	NM013693.3
mCDK2 F: 5'-CATTCCTCTTCCCCTCATCA-3' R: 5'-TTCCCCACAGCACTTAGCAT-3'	337	NM183417.3
Cyp3a11 F: 5′- CGCCTCTCCTTGCTGTCACA -3′ R: 5′- CTTTGCCTTCTGCCTCAAGT -3′	260	NM007818
CYP3A4 F: 5′- TTGGAAGTGGACCCAGAAAC -3′ R: 5′- CTGGTGTTCTCAGGCACAGA -3′	265	NM017460
hPPM1A F: 5′- AGGGGTGTGTGTGTGTGTGT-3′ R: 5′- AGAAGGGCACGGAACAGTAA-3′	278	NM021003.4

Table 1: Sequences of forward (F) and reverse (R) primers used for quantitative RT-PCR. 18S, rRNA, mPXR, mKi-67, mTNFα, mCDK2, Cyp3a11, CYP3A4, and hPPM1A

Chapter III

Conclusions

Changes in the expression of drug-metabolizing enzymes can lead to serious adverse drug interactions (ADIs). It is estimated that ADIs contribute to approximately 25% of adverse drug reactions [249], which occur in more than 2 million hospitalized patients costing more than 100,000 lives and $100 billion dollars annually in the US [250-252]. ADIs mediated through altered levels of drug-metabolizing enzymes are preventable adverse drug reactions [253].

It is known that altered levels of drug-metabolizing enzymes, including CYP3A4, contribute to ADIs [254-260]. It is also known that hPXR plays a major role in activating the expression of several drug-metabolizing enzymes, primarily CYP3A4 [261]. PXR is activated by various structurally diverse molecules, including therapeutic drugs [61, 93, 208, 262]. In recent years, an increased awareness has been given to cellular signaling pathways for potential induction of ADIs through modulation of PXR-regulated CYP3A4 expression. Because CYP3A4 contributes to the metabolism of more than 60% of clinically used drugs [205, 206], changes in the levels of CYP3A4 can tremendously affect the therapeutic response of a variety of therapeutic drugs, causing serious ADIs. Such undesired effects through PXR-mediated induction of CYP3A4 have garnered attention in clinical and preclinical studies [112]. In some preclinical/clinical studies, PXR-mediated induction of CYP3A4 has been shown to reduce the plasma concentrations of therapeutic drugs, such as cyclosporine A, indinavir, oral contraceptives, tacrolimus, warfarin, verapamil and fexofenadine [263, 264], leading to undertreatment and therapeutic failure. Besides decreasing drug efficacy, PXR also plays an important role in drug-induced toxicity. Recent studies showed that PXR activation enhanced acetaminophen-induced hepatotoxicity in mice, by inducing CYP3A

and hence converting acetaminophen to its toxic intermediate metabolite, N-acetyl-p-benzoquinone imine [46, 47].

The work cited above provides evidence that the altered levels of PXR-mediated CYP3A4 expression significantly contributes to ADIs. However, the molecular mechanisms of PXR-mediated CYP3A4 expression are not well defined. It is known that phosphatases regulate the function of nuclear receptors, thereby modulating nuclear receptor-mediated gene expression [134, 223, 225, 228-230]. Our results show for the first time that PPM1A interacts with hPXR and regulates hPXR-mediated CYP3A4 expression which may eventually help predict accurate dosage and combination of drugs to prevent the risk of ADIs. Additionally, our results show that increased levels of PPM1A enhance the function of hPXR and decreased levels of PPM1A attenuate this function. Future studies using PPM1A knockout mouse model would provide *in vivo* evidence of PPM1A regulation of PXR-mediated CYP3A4 expression. Furthermore, future studies are required to elucidate the mechanism of PPM1A activation of PXR-mediated CYP3A4 expression.

Since its discovery in 1998, the modulation of PXR activity, and in turn its gene targets such as CYP3A4, has been researched greatly. Despite PPM1A's similar date of discovery, this phosphatase is only now garnering more attention. The regulation of PPM1A levels and activity is a topic of great interest, especially due to its role in regulating cell growth and inflammation [2, 216, 218-221, 238-240, 265]. Therefore, PPM1A expression and activity should be regulated. External signals could increase or decrease PPM1A expression depending on the cellular context [2, 266-269]. PPM1A could also be regulated through its subcellular localization [2, 270]. Finally, PPM1A can be activated using pharmacological tools such as NPLC0393, a small

molecule activator of PPM1A [271, 272]. However, a detailed understanding of PPM1A regulatory mechanisms is lacking.

Some progress has been made on the mechanistic studies of the induction of CYP3A4. However, very little has been achieved on the mechanistic studies on the repression of CYP3A4. It has long been known that CYP3A4 expression is significantly repressed in proliferating hepatocytes. These observations were made under various proliferating conditions, most notably in regenerating livers [200-204]. The level of CYP3A repression in proliferating hepatocytes has been seen to vary in different studies. In a rat liver regeneration model, the level of the major forms of CYP3A was repressed by 80% [204]. In a chemically induced rat liver tumor model, CYP3A expression was repressed by 60% [202]. In patients with liver tumor, CPY3A4 expression decreased to varying extents among individuals [201]. A regenerative response can be triggered by the loss of liver mass because of chemical, traumatic, metabolic, alcoholic, infectious liver injuries, obesity, or liver cancer. Liver regeneration is achieved mainly through driving quiescent mature hepatocytes to proliferate [157, 273, 274].

It has been speculated that upregulation of PXR inhibitory signaling pathways or downregulation of PXR stimulatory signaling pathways contribute to the repression of CYP3A4 in proliferating hepatocytes of regenerating livers. However, the molecular mechanisms for this repression have yet to be clearly defined. All the investigators, that addressed the repression of CYP3A in hepatocytes of regenerating livers, emphasized that therapy should be carefully designed for patients with undergoing liver regeneration in order to avoid ADIs due to a reduced drug-metabolizing capacity. However, without the knowledge of the molecular mechanisms responsible

for the repression of CYP3A in proliferating hepatocytes, it is highly unlikely to effectively and safely design therapies.

Our results show that both PPM1A levels and hPXR-mediated CYP3A4 expression are repressed in proliferating hepatocytes. Additionally, our results show that elevated PPM1A levels lead to attenuation of hPXR inhibition by TNFα and CDK2 which are known to be upregulated and essential during liver regeneration. Most importantly, our results show that expression of both PPM1A and cyp3a11 is repressed in mice regenerating livers. Together, these findings may provide a mechanism for reduced levels of CYP3A4 in proliferating livers such as regenerating livers. Further studies using PPM1A knockout mouse model would provide *in vivo* significance of PPM1A in regulating liver proliferation and PXR-mediated CYP3A4 expression.

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