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Tumor suppressor protein p53 negatively regulates human pregnane X receptor activity

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Running title: p53 regulates PXR function

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Abbreviations:

ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris buffered saline

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ABSTRACT

The human pregnane X receptor (PXR) regulates genes involved in drug metabolism and disposition. PXR associates with multiple corepressors that attenuate, and coactivators that enhance its activity. PXR plays a vital role in the drug metabolism pathway, and a comprehensive examination of PXR-associated proteins will provide greater insight into the regulation of the receptor and possible therapeutic implications. We performed a mass spectrometric screen to identify PXR-associated proteins. Here we report that the tumor suppressor protein p53 can associate with PXR and downregulate its activity. A loss-of-function p53 mutant (R175H) interacts with PXR but does not repress its activity. Mutant p53 can relieve the suppressive effect of wild-type p53 by competing with its interaction with PXR, suggesting that protein-protein interaction is required but not sufficient for p53 to repress PXR activity. Interestingly, a PXR variant with a naturally occurring deletion of a conserved, unique sequence in the ligand binding domain (PXRΔ174-210) did not interact with p53, indicating that the PXR-p53 interaction is specific. Using a chromatin immunoprecipitation assay, we showed that p53 inhibits the binding of PXR to the *CYP3A4* promoter. The loss of p53 function in tumor cells leads to aberrant cell proliferation, apoptosis, carcinogenesis, and altered sensitivity to chemotherapeutic drugs, while PXR contributes to chemoresistance in many cancer cells. Our findings show for the first time that wild-type p53 can negatively regulate PXR by physically associating with it. Thus, PXR and p53 appear to play important yet opposing roles in the sensitivity of tumor cells to chemotherapy.

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INTRODUCTION

Pregnane X receptor (PXR) is a xenobiotic receptor of the nuclear receptor (NR) family. PXR functions mainly in the liver and is activated by a wide range of ligands, which are usually toxic byproducts derived from the endogenous metabolism or exogenous chemicals (Blumberg et al., 1998a; Blumberg et al., 1998b).

The flexible ligand binding domain (LBD) of PXR, from amino acid 142 to 434, can expand to accommodate structurally and pharmacologically diverse ligands, thus contributing to its ability to function as an efficient xenobiotic sensor. Once activated, PXR forms a heterodimer with the retinoid X receptor to engage specific response elements in the promoter region of its target genes to mediate the transcription of a variety of drug metabolizing enzymes and drug transporters (Wang et al., 2012). A major class of proteins that is regulated by PXR is the 3A sub-family of cytochrome p450 (*CYP3A4*).

Human PXR has several alternatively spliced isoforms, of which PXR.1 (434 amino acids) is the most abundant and can be transcriptionally activated by ligands (Lamba et al., 2004) (we use “PXR” and “PXR.1” synonymously throughout the text). The second most abundant isoform, PXR.2 (397 amino acids; also referred to as [PXRΔ174-210]), constitutes 7% of the mRNA transcript and has a 37 amino acid deletion in the LBD, resulting in attenuated ligand binding capacity and reduced ligand- mediated transcriptional activity, although there is no loss of association with the PXR response elements in the genome (Dotzlaw et al., 1999; Lin et al., 2009). The physiological role of PXR.2 has not been fully defined.

PXR is regulated by a variety of protein-protein interactions and post-translational modifications. Protein-protein interactions are dynamic processes that, depending on the associated protein, can result in the modulation of activity, sub-cellular localization, substrate specificity, and stability of the target protein. PXR associates with a variety of protein coregulators that can act as either coactivators or corepressors. Corepressors such as the silencing mediator for retinoid and thyroid receptors (SMRT) and nuclear receptor corepressor (NCoR) attenuate gene transcription through their association with unliganded PXR (Ding and Staudinger, 2005; Johnson et al., 2006). Upon ligand binding, PXR activity is

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enhanced by the dissociation of corepressors and association instead with coactivators such as forkhead transcription factor and SRC-1 (Kodama et al., 2004; Watkins et al., 2003). To this end, we wanted to identify and expand upon the protein partners that associate with PXR and can contribute to its regulation using mass spectrometric analysis. Our study identified tumor suppressor p53 that can bind directly to PXR.

Wild-type p53, once activated by a variety of stressors that threaten the genomic integrity of cells, can induce cell cycle arrest, DNA repair, senescence, or apoptosis, depending on the severity of the damage. p53 is mutated or inactivated in almost all forms of cancer (Hainaut and Hollstein, 2000; Hollstein et al., 1991). As a transcription factor, p53 regulates the expression or function of a large array of proteins through transcriptional control or protein-protein interactions (Levine et al., 2006).

p53 regulates the activity of several NRs, such as glucocorticoid receptor (GR), androgen receptor (AR), estrogen receptor (ER), liver-X receptor, and HNF4α1 (Alimirah et al., 2007; Angeloni et al., 2004; Iwano et al., 2006; Maeda et al., 2002; Yu et al, 1997). For AR, p53 not only modulates its transcriptional activity but negatively regulates its expression (Alimirah et al., 2007; Shenk et al., 2001). p53 interacts directly with GR and downregulates its activity, and this downregulation depends on the activity of the wild-type p53 (Yu et al., 1997). p53 is known to enhance the recruitment of ERα to the proximal ER promoter in response to doxorubicin treatment (Angeloni et al., 2004; Shirley et al., 2009). We report here that wild-type p53 can attenuate PXR activity by inhibiting the binding of PXR to its target promoter. Furthermore, we show that the p53-PXR association is specific for PXR.1 and not for PXR.2, which lacks a 37 amino acid sequence unique for PXR from various species, indicating the specificity of the p53-PXR interaction.

PXR is a master regulator of drug metabolism that contributes to enhanced cellular chemoresistance (Chen et al., 2007; Raynal et al., 2010). On the other hand, cells harboring wild-type p53 are more chemosensitive than cells where p53 is inactivated (Fan et al., 1994; Fan et al., 1995). Our findings, for the first time, cast p53 in a regulatory role for PXR, a fact that can be exploited for

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therapeutic purposes, given the important roles that both p53 and PXR play in regulating chemosensitivity.

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MATERIALS AND METHODS

Materials. $p53^{-/-}$ HCT116 cells were obtained from the Genetic Resources Core Facility at Johns Hopkins University School of Medicine; U2OS cells stably expressing GFP-PXR were obtained from Thermo Scientific (Ashville, NC); 293T and HepG2 cells were obtained from ATCC (Manassas, VA). Anti-p53 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); rifampicin, anti-Flag M2 antibody, EZview Red anti-Flag M2 affinity gel, and Flag peptide were obtained from Sigma-Aldrich (St. Louis, MO); GFP-Trap agarose beads were obtained from ChromoTek (Planegg-Martinsried, Germany); charcoal-dextran treated FBS was obtained from HyClone; blocking buffer, anti-mouse, and anti-rabbit IRDye secondary antibodies were from LI-COR Biosciences (Lincoln, NE). Mouse IgG used as control in chromatin immunoprecipitation (ChIP) assays was from Cell Signaling Technology, Inc. (Danvers, MA), and protein-G agarose beads were obtained from Sigma-Aldrich.

Cell Culture, Plasmids, and Transfection. All cells were maintained in a humidified environment at 37°C with 5% CO_2. HCT116 cells were maintained in McCoy's 5A media supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. U2OS, HepG2, and 293T cells were maintained in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Additionally, U2OS stable cells were maintained in media containing 0.4 mg/ml G418. Human pcDNA3.1 containing Flag-PXR or luciferase reporter gene under the control of the *CYP3A4* promoter (*CYP3A4*-luc) were constructed as previously described (Luo et al., 2002). Wild-type, Flag-p53, and Flag-p53-RH (R175H) mutants were obtained from Origene (Rockville, MD). p53 mutants containing various mutations at the ^{22}LWKLL^{26} and ^{251}ILTII^{255} motifs were generated by Mutagenex Inc. (Hillsborough, NJ). Standard molecular biology methods were used to generate various deletion mutants of PXR. TK-Renilla luciferase plasmid was purchased from Promega (Madison, WI); p53 reporter plasmids PG13-luc and the reporter containing a mutation at the promoter sequence (MG15-luc) were from Addgene (Cambridge, MA) (Kern et al., 1992). All transfections were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations.

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Immunoprecipitation. 293T cells were transfected with either pcDNA3.1 Flag-PXR or pcDNA3.1-Flag vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. After 48 h of incubation, cells were collected in 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) and incubated for 20 min on ice. Lysates were centrifuged at 10,000 × *g* for 20 min at 4°C to remove cell debris, and the supernatant was incubated with 10 µl of EZview Red anti-Flag M2 affinity gel (Sigma) for 2 h at 4°C. The M2 beads with the bound proteins were then washed twice with lysis buffer, and twice with TBS and then eluted with 100 ng/µl Flag peptide (Sigma) in TBS for 1 h at 4°C. U2OS cells stably expressing GFP-PXR or GFP were collected in lysis buffer and incubated with GFP-Trap agarose beads for 2 h at 4°C. The beads were washed twice each with lysis buffer and TBS then boiled in sample loading buffer (Invitrogen) to release the bound proteins.

Western Blot Analysis. All cell extracts were harvested in lysis buffer, and samples were centrifuged at 10,000 × *g* at 4°C for 20 min. The samples were then boiled in sample loading buffer (Invitrogen) containing SDS, and equal amounts of samples were resolved on 4-12% SDS-PAGE gradient gel and then transferred onto nitrocellulose membrane. The membrane was blocked and incubated with the indicated antibodies overnight at 4°C. All Western blot analyses and quantitation were performed on the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE).

Protein partner identification. After immunoprecipitation using Flag M2 affinity gel, the eluted protein solution was mixed with ice-cold ethanol and incubated overnight at -20°C to precipitate the proteins. The solution was then centrifuged at 6000 × *g* for 30 min and dried using a vacuum evaporator. The protein pellet was then submitted for processing and protein identification by the St. Jude Proteomics & Mass Spectrometry Shared Resource using an LTQ Linear Ion Trap mass spectrometer (Thermo Electron, San Jose, CA) and the data were processed using the Mascot database.

Transactivation Assay. Cells were transfected with PXR or empty vector control along with *CYP3A4*-luc and vector expressing TK-Renilla luciferase as transfection control. To check for p53

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activity, cells were transfected with plasmids expressing either p53 or empty vector control along with luciferase reporter gene under the control of p53 promoter sequence (PG13) and vector expressing TK-Renilla luciferase. After 48 h of transfection, cells were plated in 96 -well plates containing either DMSO or the indicated compound in phenol-red free media containing 10% charcoal-dextran treated FBS. The cells were incubated for 24 h before processing using the Dual-Glo Luciferase Assay System (Promega). Data are reported in relative luciferase units (RLU) by normalizing the firefly luciferase activity to Renilla luciferase activity.

ChIP Assay. HepG2 cells stably expressing Flag-PXR and *CYP3A4*-luc, established using the procedure previously described (Lin et al., 2008), were transiently transfected with wild-type p53, p53-R175H mutant, or empty vector. Cells were incubated with 5 μM rifampicin for 24 h and then treated with 1% formaldehyde to cross-link DNA with the associated proteins. DNA-protein cross-links were sheared and extracted using sonication at 4^{o}C in lysis buffer containing 10% Triton X-100, 4 M NaCl, 0.5 M EDTA, 1 M Tris, 10% SDS, 100 mM PMSF, and protease inhibitor cocktail. Chromatin from 5×10^7 cells was immunoprecipitated using either IgG as negative control or anti-Flag M2 beads overnight at 4^{o}C and eluted using Flag peptide. The eluents were incubated with 200 mM NaCl overnight at 60^{o} C to reverse the cross-linking and then for 2 h at 45^{o}C with 10 mM EDTA, 40 mM Tris (pH 6.5) and 5 μg/ml Proteinase K. DNA was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). PCR was performed using primers designed to amplify the DR3 region (the binding site for PXR) of the *CYP3A4* promoter (Goodwin et al., 2002) using the following primers: forward 5'-ATC CAC TAG TAA CGG CCG-3' and reverse 5'-TTC AGC TTG TGA TTC ACC TG-3'. PCR products were resolved on 1% agarose gel and quantified using the ImageJ image processing and analysis tool.

Statistical Analysis. In all reporter assays, error bars indicate standard errors of measurement (SEM). For ChIP assay, results are expressed as the mean ± standard deviation (SD) of at least 3 independent experiments, and error bars indicate SD. Statistical significant differences between samples (noted with a line in Figure 5B: comparisons between EV and WT, and between EV and RH) are

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indicated by the *p* value, which was determined using the Student's *t*-test and considered significant if $p < 0.05$ (*) or 0.01 (**).

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RESULTS

p53 interacts with PXR. To gain better insight into the regulation of PXR function, we decided to identify protein partners associated with PXR and investigate the effect of such associations on its function. To this end, Flag-tagged PXR (Flag-PXR) was transiently overexpressed in 293T cells and immunoprecipitated using Flag-tag specific antibody covalently attached to agarose beads. The immunoprecipitated samples were then processed, and protein partners associated with Flag-PXR were identified using mass spectrometry. To identify and eliminate false positives, lysates from cells transiently transfected with plasmid expressing only the Flag-tag were used as negative controls for immunoprecipitations. A database search using the Mascot search engine identified 200 proteins associated with PXR. Peptides identified in the negative control samples were eliminated as non-specific binding partners.

Among the specific binding partners, retinoid X receptor protein, a known PXR binding partner, was consistently detected in only the Flag-PXR samples. Tumor suppressor protein p53 was one of the associated proteins identified with PXR but not in the negative control sample (Figure 1A). Using Western blot analysis, we confirmed the association of p53 with Flag-PXR but not with the Flag control by using antibody specific for p53 (Figure 1B). Similar to other NRs, ligand binding to PXR affects its association with co-regulators that can regulate PXR signaling. PXR is activated in 293T cells in response to rifampicin, a compound known to bind and activate PXR (Supplemental Figure S1A); however, the association between PXR and p53 was not altered when cells were treated with rifampicin (Figure 1C).

Wild-type p53 downregulates PXR activity. To assess the functional significance of p53-PXR association, the activity of PXR was tested in the presence or absence of wild-type p53. For this purpose, colon cancer cell line HCT116 was used since PXR is functional in this cellular context and isogenic clones with deleted p53 are available (Bunz et al., 1998; Wang et al., 2011). $p53^{-/-}$ HCT116 cells were used to first show that transiently transfected PXR (Supplemental Figure S1B) and wild-type p53 (Supplemental Figure S1C) were functional, by using the respective reporter gene. Wild-type p53

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significantly downregulated PXR reporter activity, and this downregulation occurred in a p53 dose-dependent manner (Figure 2A). To further test the effect of p53 on PXR activity, a p53 mutant carrying a point mutation at amino acid 175 (R175H) in its DNA binding domain (DBD) was used to determine whether p53 transactivational activity is necessary for its inhibitory effect on PXR. p53 R175H, one of the frequently detected mutants in cancers, is unable to bind to the promoters of p53-regulated genes and is thus considered to be a non-functional protein in regard to transcriptional regulation of p53-dependent genes. As shown in Figure 2B, the p53 R175H had no effect on PXR reporter activity in $p53^{-/-}$ HCT116 cells even with increasing amounts of R175H transfected.

The effect of p53 R175H was further tested by co-transfecting wild-type p53 along with increasing amounts of p53 R175H. As shown in Figure 2C, the inhibitory effect of wild-type p53 on PXR was decreased by the R175H mutant, and completely rescued when larger amounts of p53 R175H were transfected, indicating that the p53 R175H mutant functions in a dominant negative manner. The dominant negative effect of p53 R175H mutant prompted us to determine whether it interacts with PXR. As shown in Figure 3A, p53 R175H mutant interacts with PXR similarly to wild-type p53, suggesting that p53 R175H abolishes the inhibitory effect of wild-type p53 by blocking it from interacting with PXR.

p53 activity is necessary for its inhibitory effect on PXR. Two unique sequences, LWKLL in the DBD and ILTII in the transactivation domain, were identified in the p53 protein that correspond to motifs described in other co-activators and co-repressors of NRs (Figure 3B). The LXXLL motif, which is present in several co-activators of PXR, mediates protein-protein interactions to enhance the activity of the associated receptor (Plevin et al., 2005). The I/LXXI/VI co-repressor nuclear receptor boxes mediate binding of co-repressors such as NCoR and SMRT to NRs, where they attenuate receptor activity (Perissi et al., 1999). To determine whether the LWKLL or ILTII motifs in p53 mediate the protein-protein interaction with PXR, we mutated the LWKLL or ILTII sequences individually (Mt1, Mt2) and in combination (Mt3), as indicated in Figure 3B. Similar to the p53 R175H mutant, Mt1, Mt2, and Mt3 were unable to regulate the activity of the p53 response luciferase reporter gene PG13 (Figure 3C). Mutation at Trp23 within the LWKLL sequence has been shown to result in a loss of p53 transactivation activity and

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is thus critical for p53 function (Lin et al., 1994). The loss of function was observed when either Trp23 or Lys24 within the LWKLL motif was mutated individually or together to an alanine (Mt4, Mt5, and Mt6) (Figure 3B − C).

In immunoprecipitation experiments from U2OS cells stably expressing GFP-PXR or GFP alone, GFP-PXR showed similar association with all the p53 mutants that were used (Figure 3A), suggesting that neither the LWKLL nor the ILTII motif is required for p53 to physically interact with PXR. Consistent with the results observed with the R175H mutant (Figure 2B – C), the inactive p53 mutants did not attenuate PXR activity (Figure 3D) and showed a dominant negative effect on the inhibitory effect of wild-type p53 on PXR (Figure 2C and data not shown).

PXR.2 (PXRΔ174-210) fails to interact with p53. To identify which domains of PXR are responsible for interacting with p53, various truncation mutants of PXR were generated (Figure 4A). PXRΔ1-99, which contains the full LBD (amino acids 142-434) but lacks the DBD, failed to interact with p53 (Figure 4B), suggesting that the N-terminal 99 amino acids are required for the interaction. A more refined study showed that deletion of either amino acids 2-40 (Δ2-40) or amino acids 21-60 (Δ21-60) at the N-terminus did not noticeably affect the interaction of with p53 (Supplemental Figure S2), suggesting that the amino acids 61-99, which are part of the DBD (amino acids 41-107), are critical for p53-PXR interaction. On the other hand, deletion of either the last 14 amino acids (Δ421-434) or the last 134 amino acids (Δ301-434) at the C-terminus, which removes the activation function domain 2 (AF-2), also abolishes the interaction of PXR with p53 (Supplemental Figure S2), suggesting that the AF-2 domain of PXR is critical for its interaction with p53.

Most interestingly, we found that the amino acids from 174-210 on PXR play a significant role in preserving the p53-PXR association (Figure 4B). This sequence is absent in the alternatively spliced isoform of PXR, also referred to as PXR.2 (Lin et al., 2009), indicating that p53 specifically associates with the more abundant isoform of PXR, PXR.1. In agreement with previously reported findings, while PXR.1 was robustly activated by rifampicin, PXR.2 was not (Supplemental Figure S3A − B).

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Interestingly, amino acids 174-210, which are present in PXR.1 but missing from PXR.2, are specific for PXR, and are highly conserved among PXR proteins from different species identified to date (Supplemental Figure S4). The requirement for a highly conserved and PXR-specific sequence for the interaction further indicates the specificity of the p53-PXR association.

Wild-type p53 attenuates PXR binding to CYP3A4 promoter. As transcription factors, both PXR and p53 bind to specific sequences in the promoters of target genes to initiate transcription (Kliewer et al., 2002; Menendez et al., 2009). We used the ChIP assay to determine whether p53 modulates the binding of PXR to its target promoter in *CYP3A4* in hepatocellular carcinoma cells HepG2 stably expressing Flag-PXR, which endogenously express wild-type p53 (Puisieux et al., 1993). Anti-Flag immunoprecipitations from HepG2 cells overexpressing wild-type p53 showed significantly less PXR recruited to the DR3 region of the *CYP3A4* promoter than in immunoprecipitated samples from cells expressing only the control empty vector (Figure 5 A − B). In contrast, overexpression of the p53 R175H mutant did not attenuate, but enhanced the PXR association with *CYP3A4* promoter (Figure 5A − B), consistent with its dominant negative effect (Figure 2C). The levels of endogenous as well as transfected p53 are indicated in Figure 5C.

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DISCUSSION

PXR is a master xenobiotic sensor, whose activity is upregulated by binding to agonists; and the agonist-mediated receptor activity is modulated by the association of PXR with various protein partners. We showed in this study, for the first time, that p53 is one such PXR protein partner whose physical association leads to attenuation of PXR activity. We also show that the decrease in the binding of PXR to its target promoter contributes to such attenuation of receptor activity.

p53 is indisputably one of the most important tumor suppressors, as evidenced by the fact that more than 50% of tumors carry a p53 mutation (Hainaut and Hollstein, 2000). Most of the tumors carry a mutation in the p53 protein that affects either its transactivation function or its ability to bind specific DNA sequences at the promoter of p53 target genes (Soussi and Wiman, 2007). R175 is one of the hotspots for p53 missense mutations. p53 has been shown to interact with various proteins, including NRs, with various functional outcomes. In the case of p53-ER association, p53 enhances the recruitment of ER to its target promoter (Angeloni et al., 2004). In contrast, in our study, the p53-PXR association results in decreased binding of PXR to its target promoter in *CYP3A4* gene, suggesting that the functional outcome of the protein-protein interaction depends on the specific protein partner involved. The effect of p53 on PXR may also vary with different PXR target promoters.

Since p53 interacts with multiple protein partners with different functional outcomes, it is important to determine whether such an interaction is specific, and how this specificity is achieved. Interestingly, we found that the interaction between p53 and PXR is specific to the more abundant PXR isoform PXR.1 (434 amino acids) and does not occur with the alternatively spliced form of the protein PXR.2 (397 amino acids, with amino acids 174-210 deleted). The segment of 37 amino acids (174-210) missing from PXR.2 is unique to PXR and is not present in any other protein. It is also highly conserved among PXR proteins from different species, indicating that the interaction between p53 and PXR.1 is specific and unique. While the physiological function of PXR.2 has not been fully defined, such function is expected to be insensitive to p53 regulation.

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PXR functions as a xenobiotic sensor mainly due to its ability to accommodate structurally diverse xenobiotics. While ligand binding to PXR can alter the interaction of PXR with corepressors such as SMRT and NCoR (Ding and Staudinger, 2005; Johnson et al., 2006), it does not affect the p53-PXR.1 interaction, suggesting that the cellular levels of p53 may affect both the basal and agonist-induced activities of PXR. However, the observation that rifampicin does not alter the p53-PXR.1 association *in vitro* does not exclude the possibility that *in vivo*, rifampicin could affect such an association in a sub-cellular localization-specific manner. Although PXR.2 can retain certain corepressor associations similar to those of PXR.1, PXR.2 fails to activate PXR target genes upon ligand treatment. The deletion of 37 amino acids (174-210) in the LBD is predicted to make PXR.2 unable to retain certain ligands, thereby altering the ligand and protein partner profile (Lin et al., 2009). It is thus not surprising that the integrity of the PXR protein structure is important to retain certain protein-protein interactions such as PXR-p53 binding, as revealed by the loss of interaction with p53 when the AF-2 domain (Δ421-434) or the N-terminal 99 amino acids of PXR (Δ1-99) were deleted. The N-terminal deletion mutant Δ1-99 but not the Δ2-40 or Δ21-60, abolished the interaction, suggesting that the major part of the DBD (41-107), is required for PXR to interact with p53, and is in agreement with the observation that p53 can attenuate the binding of PXR to the *CYP3A4* promoter through its DBD.

However, as we showed in this report, p53 association is not always sufficient to attenuate the activity of the target protein since p53 wild-type and mutant forms can bind to PXR, but only the wild-type form can downregulate the activity of PXR. p53 mutants such as R175H, while inactive, have been shown to attain gain-of-function properties, which can initiate novel protein-protein interactions and transactivation of new target genes, contributing to tumor progression and chemoresistance (Blandino et al., 1999). All the transcriptionally inactive p53 mutants examined in our study maintained their association with PXR, although all of them failed to attenuate PXR-mediated transactivation of target genes such as *CYP3A4*. As expected, these inactive p53 mutants, including p53-R175H, were shown to function in a dominant-negative manner by decreasing the interaction of wild-type p53 and PXR, thereby releasing the inhibitory effect of wild-type p53 on PXR. The dominant-negative effect of the p53-R175H

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mutant was also observed in the ChIP assay; when ectopically expressed from a plasmid, whereas wild-type p53 decreased the binding of PXR to the *CYP3A4* promoter, the R175H mutant actually enhanced the binding, possibly by replacing the endogenous wild-type p53 from the *CYP3A4* promoter. While it is unclear why binding of p53 R175H to PXR did not attenuate the binding of PXR to the *CYP3A4* promoter, it is possible that the gain-of-function of p53 mutants, including the R175H mutant, contributes to the altered functional outcome of protein-protein interactions. Trp23 is necessary for the transactivation of p53 as well as its association with Mdm2 (Lin et al., 1994; Picksley et al., 1994). Interestingly, Trp23 falls within one of the sequences (LXXLL) utilized by coactivators to bind NRs. While p53 also harbors the corepressor association sequence (IXXII), neither motif contributes to PXR binding.

In colon cancer cells, expression of wild-type p53 induces apoptosis (Shaw et al., 1992), while activation of PXR induces colon tumor growth and malignancy (Mani et al., 2013; Pondugula and Mani, 2013; Wang et al., 2011). In this study we showed that in colon cancer cell line HCT116, wild-type p53 downregulates PXR activity. It is conceivable that in colon cancers with wild-type p53, decreased PXR activity due to inhibition by p53, might contribute to p53-mediated growth inhibition. Both the status of p53 and the levels of PXR will be crucial in determining their effect on tumor cell growth and chemosensitivity.

The regulation of a master xenobiotic sensor by a master tumor suppressor raises some interesting questions and exciting possibilities. As the key regulator of the drug metabolism pathway, PXR regulates drug efficacy and contributes to drug resistance in tumors (Chen et al., 2007; Wang et al., 2012). On the other hand, loss of wild-type p53 activity contributes greatly to radioresistance and chemoresistance in tumors containing p53 mutations (El-Deiry, 2003; Fojo, 2002). Our finding that wild-type p53 interacts with and attenuates the activity of PXR suggests that the status of p53 might affect the efficacy of drugs, partially through affecting PXR-regulated pathways. Further investigations, including a comprehensive study on the effect of various naturally occurring p53 mutants on the activity of PXR, and correlation of such effects to clinical observations will help establish the role of p53 in systematic drug metabolism.

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Performed data analysis: Elias, Chen

Wrote or contributed to the writing of the manuscript: Elias, Chen

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. PXR interacts with p53.

A) Proteins and the corresponding number of peptides identified in anti-Flag immunoprecipitated samples from 293T cells transiently overexpressing either Flag-PXR or Flag-tag alone using mass spectrometric analysis. B) Anti-Flag immunoprecipitated samples were prepared from 293T cells transiently overexpressing either Flag-tag (Flag) or Flag-PXR and analyzed using SDS-PAGE. The immunoprecipitates (left panel) and the corresponding lysates (right panel) were immunoprobed using antibodies against p53, Flag, and beta-actin as a loading control. C) Immunoprecipitates prepared from 293T cells overexpressing either Flag-PXR or Flag-tag vector and either left untreated or treated with 2.5, 5, or 10 μM rifampicin for 24 h were probed with antibodies against the Flag-tag and p53.

Figure 2. Wild-type but not the inactive p53 attenuates PXR activity.

HCT116 $p53^{-/-}$ cells were transiently transfected with PXR or an empty vector (Flag), *CYP3A4*-luc, TK-Renilla luciferase as transfection control, and either A) wild-type p53 (p53 WT) or B) p53 R175H (p53 RH) mutant in the amounts indicated for 48 h. Cells were then treated with DMSO as control or rifampicin (2.5, 5, or 10 μM) for 24 h prior to processing using the Dual-Glo luciferase assay system. C) HCT116 $p53^{-/-}$ cells were transiently transfected with Flag-PXR, *CYP3A4*-luc, TK-Renilla luciferase, 0.2 μg of p53WT, and varying amounts of p53 RH. The firefly luciferase signal was normalized to the corresponding Renilla luciferase signal and reported as relative luciferase units (RLU).

Figure 3. Neither LXXLL nor IXXII NR-interacting motif on p53 is required for p53-PXR interaction.

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A) U2OS cells stably expressing GFP-PXR or GFP were transiently transfected with Flag-tagged wild-type p53 (WT) or one of the Flag-tagged p53 mutants (RH, Mt1, Mt2, Mt3, Mt4, Mt5, and Mt6) for 48 h. Cell lysates were immunoprecipitated using GFP-specific beads and resolved using SDS-PAGE and then immunoprobed using anti-GFP (for GFP-PXR) and anti-Flag (for Flag-p53) antibodies. B) Flag-tagged p53 mutants were generated at the LWKLL and ILTII sequences by replacing one or multiple amino acids with alanine. C) Wild-type p53 and the indicated p53 mutants were transiently transfected in HCT116 $p53^{-/-}$ cells along with p53 responsive luciferase reporter (PG13) and TK-Renilla luciferase as a control for 48 h. Luciferase reporter plasmid that does not respond to p53 (MG15) was used as a control to show specificity of the p53 promoter activity. D) HCT116 $p53^{-/-}$ cells were transfected using Flag-PXR, *CYP3A4*-luc, TK-Renilla luciferase plasmid, and either empty vector control (EV), wild-type p53 (WT), or mutant p53 (RH, Mt1, Mt2, Mt3, Mt4, Mt5, and Mt6) for 48 h. Cells were treated with DMSO or with 1.25, 2.5, 5, or 10 μM rifampicin (Rif) for 24 h prior to analysis using the Dual-Glo luciferase assay system. The firefly luciferase signal was normalized to the corresponding Renilla luciferase signal and reported as relative luciferase units (RLU).

Figure 4. **The region of PXR encompassing amino acids 174-210 is important for its association with p53.**

A) Schematic representation of the PXR protein and the truncation mutants that were generated- the LBD Δ1-99 (SEQ1) and Δ174-210 (SEQ2). B) Flag-PXR or Flag-PXR mutants were transfected into 293T cells and immunoprecipitated using the Flag-M2 beads. Left panel shows the Western blot analysis to detect Flag-PXR using anti-Flag (α-Flag) and p53 using anti-p53 (α-p53) antibodies in the immunoprecipitated samples, and the protein levels in corresponding lysates are shown in the right panel. Actin levels are shown to verify equal loading of the lysates.

Figure 5. **Wild-type p53 attenuates PXR's association with CYP3A4 promoter.**

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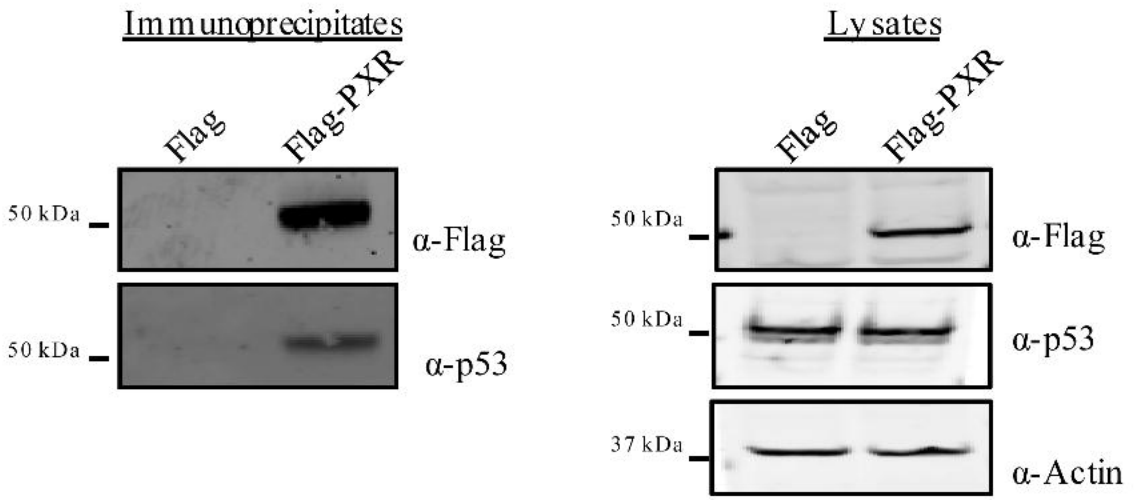
A) Cross-linked Flag-PXR was immunoprecipitated from HepG2 cells stably expressing Flag-PXR and CYP3A4, using anti-Flag M2 agarose beads. Cells were transiently transfected with control vector (EV), wild-type-p53 (WT) or p53 R175H mutant (RH). The levels of *CYP3A4* promoter associated with PXR were amplified using primers against the DR3 region within the promoter. Mouse IgG was used for control immunoprecipitation (right panel). B) Band intensity for ChIP was obtained using ImageJ software and normalized to band intensities generated for the corresponding input sample (lower panel). Error bars indicate ± S.D. and statistical significance was determined by Student's *t*-test where *, $p \leq 0.05$; **, $p \leq 0.01$. C) Cellular lysates used for ChIP assay were immunoprobed with anti-p53 (α-p53) and anti-actin (α-Actin).

Figure 1

A

Protein	Accession number	Flag	Flag-PXR
Nuclear receptor subfamily 1 group I member 2 (NR1I2)	O75469	0	20
Retinoid X receptor (RXR)	P28702	0	5
Cellular tumor antigen p53	P04637	0	4

B



C

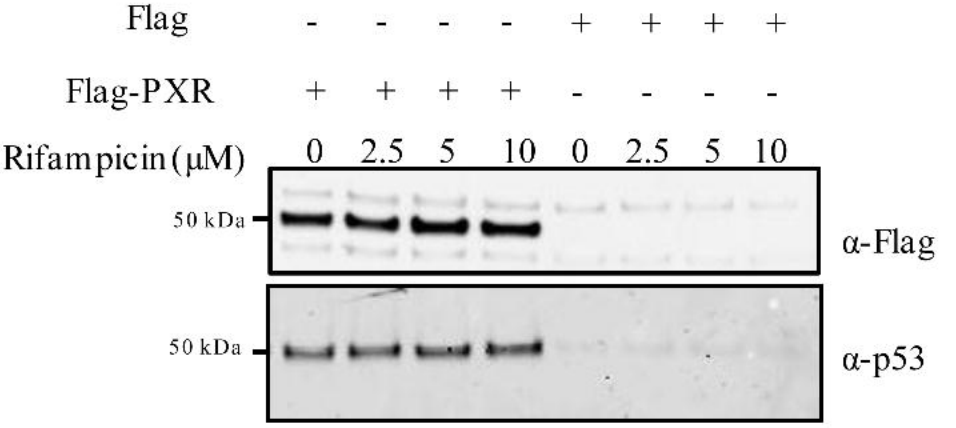


Figure 2

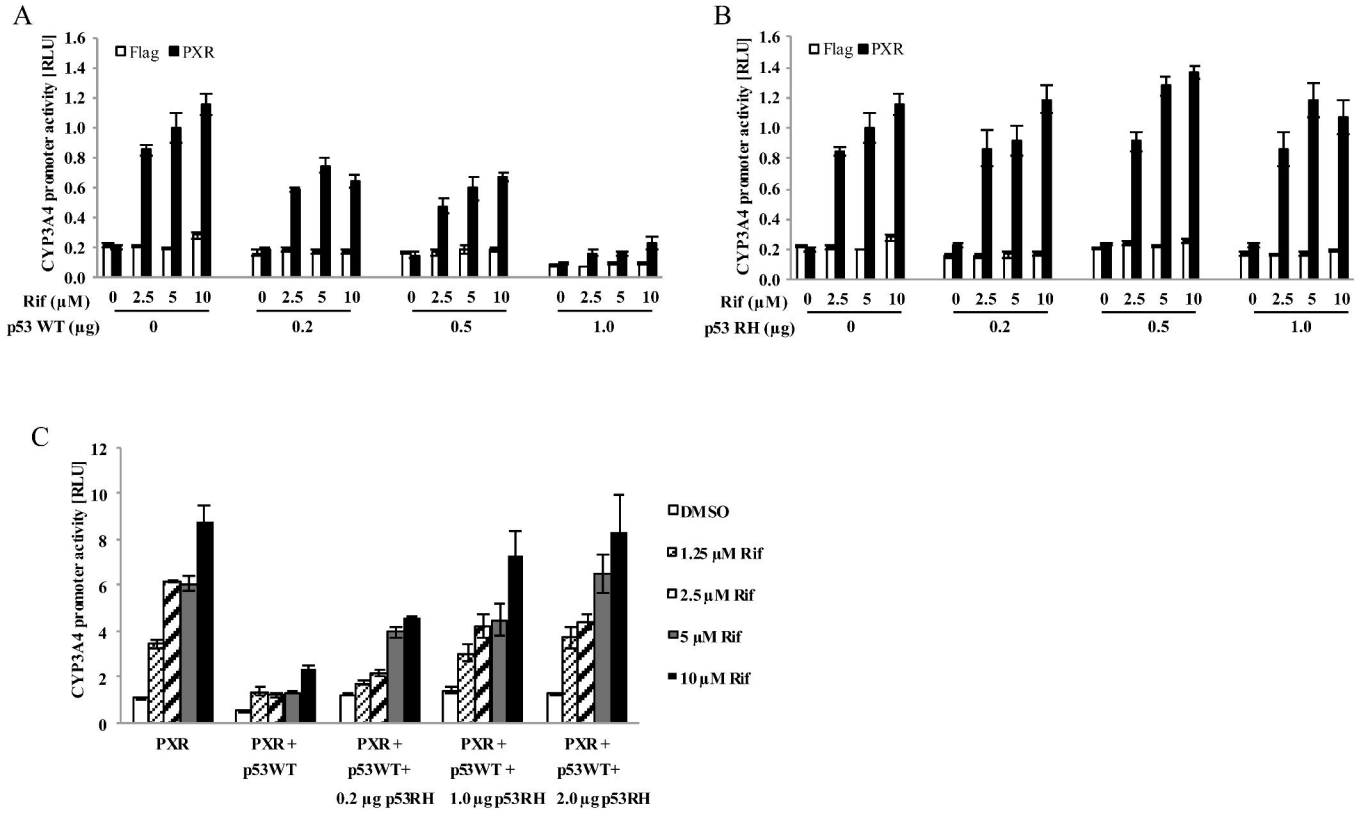
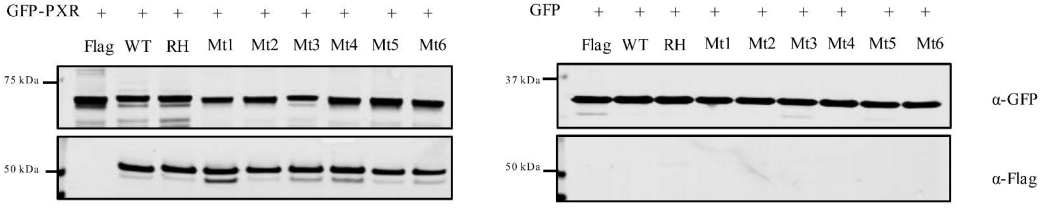

A
Flag
PXR
CYP3A4 promoter activity [RLU]
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Rif (μM)
0 2.5 5 10 0 2.5 5 10 0 2.5 5 10 0 2.5 5 10
p53 WT (μg)
0
0.2
0.5
1.0
B
Flag
PXR
CYP3A4 promoter activity [RLU]
Rif (μM)
0 2.5 5 10 0 2.5 5 10 0 2.5 5 10 0 2.5 5 10
p53 RH (μg)
0
0.2
0.5
1.0
C
CYP3A4 promoter activity [RLU]
12
10
8
6
4
2
0
DMSO
1.25 μM Rif
2.5 μM Rif
5 μM Rif
10 μM Rif
PXR
PXR + p53WT
PXR + p53WT+ 0.2 μg p53RH
PXR + p53WT + 1.0 μg p53RH
PXR + p53WT+ 2.0 μg p53RH

Figure 3

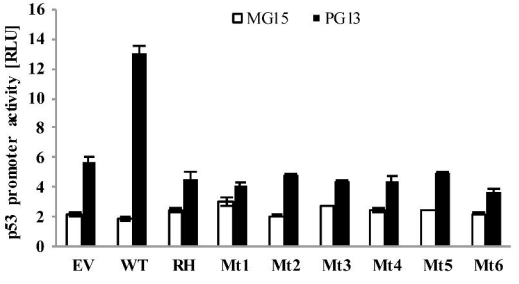
A



B

P53 WILD TYPE (WT)	^{21}DLWKLLPENN^{30} ^{251}ILTIITLEDS^{260}
MUTANT 1 (MT1)	^{21}DAAAAAPENN^{30} ^{251}ILTIITLEDS^{260}
MUTANT 2 (MT2)	^{21}DLWKLLPENN^{30} ^{251}AAAAATLEDS^{260}
MUTANT 3 (MT3)	^{21}DAAAAAPENN^{30} ^{251}AAAAATLEDS^{260}
MUTANT 4 (MT4)	^{21}DLAKLLPENN^{30} ^{251}ILTIITLEDS^{260}
MUTANT 5 (MT5)	^{21}DLWALLPENN^{30} ^{251}ILTIITLEDS^{260}
MUTANT 6 (MT6)	^{21}DLAALLPENN^{30} ^{251}ILTIITLEDS^{260}

C



D

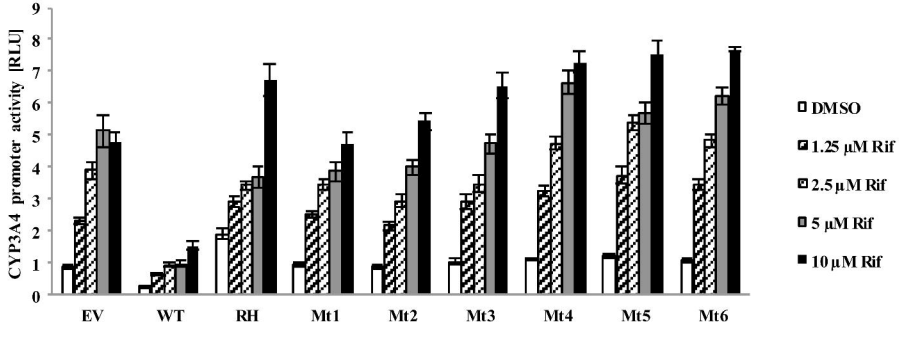
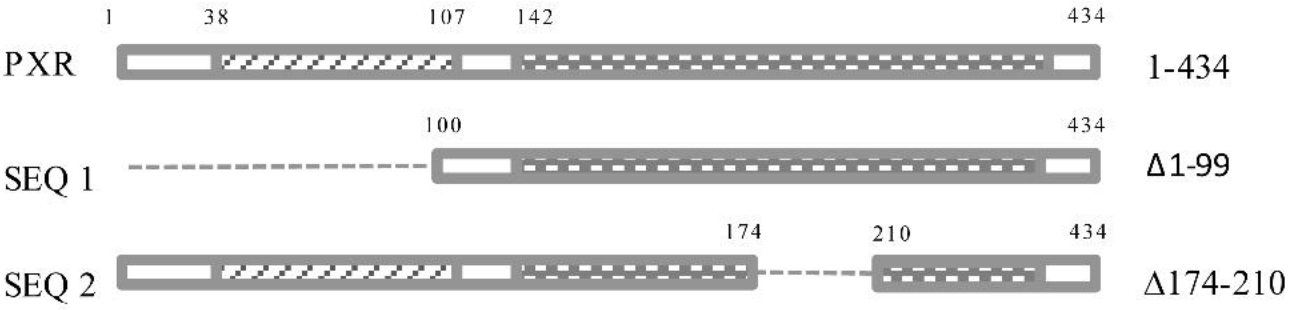


Figure 4

A


1
38
107
142
434
PXR
1-434
100
434
SEQ 1
Δ1-99
174
210
434
SEQ 2
Δ174-210

B

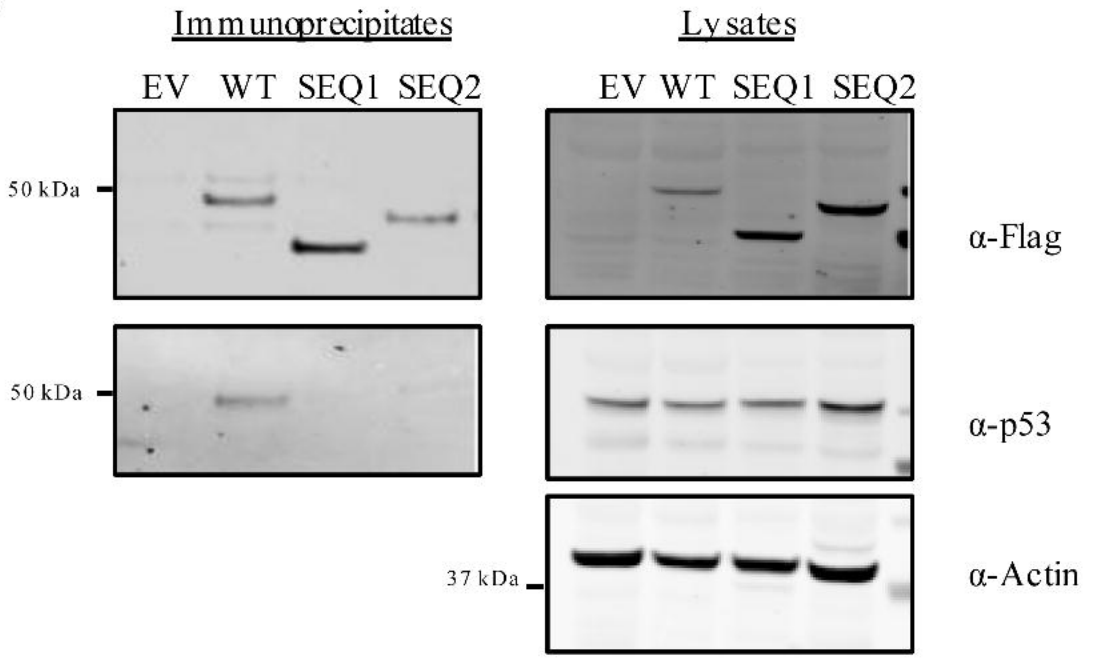
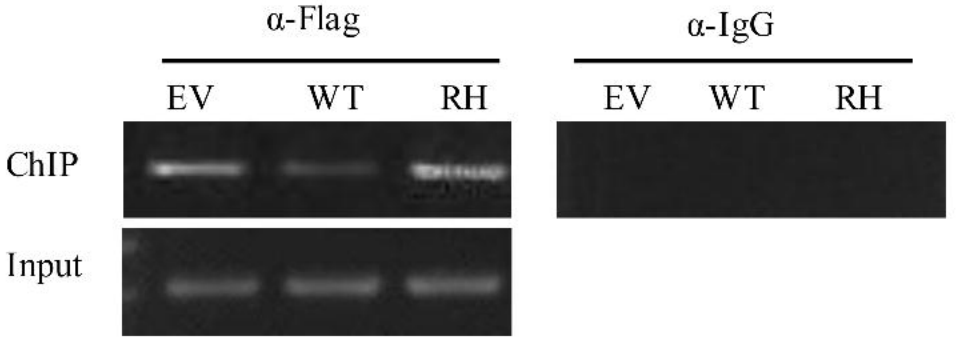
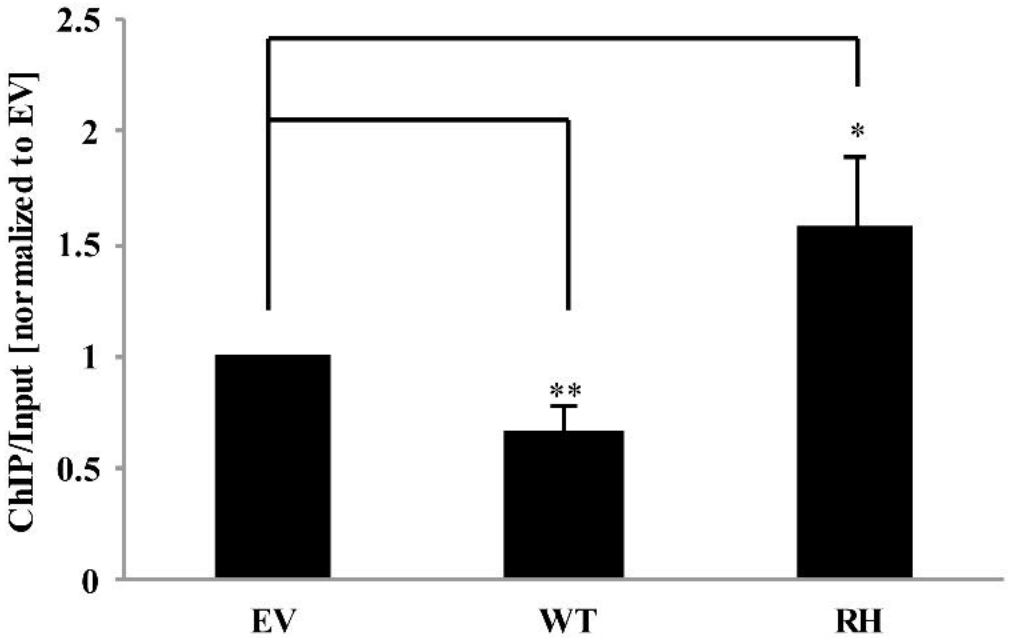

Immunoprecipitates
Lysates
EV WT SEQ1 SEQ2
EV WT SEQ1 SEQ2
50 kDa
50 kDa
37 kDa
α-Flag
α-p53
α-Actin

Figure 5

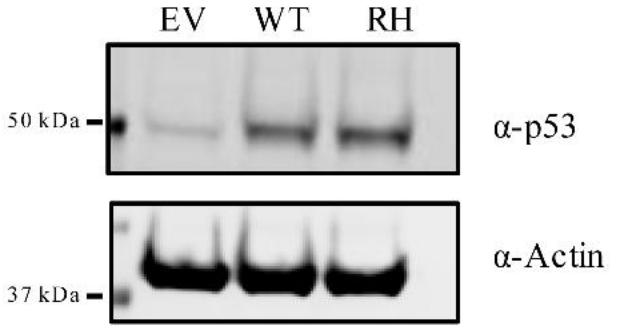
A


α-Flag
α-IgG
EV
WT
RH
EV
WT
RH
ChIP
Input

B


ChIP/Input [normalized to EV]
2.5
2
1.5
1
0.5
0
*
**
EV
WT
RH

C


EV
WT
RH
50 kDa
α-p53
37 kDa
α-Actin