

**A Novel Non-Consensus Xenobiotic Response Element Capable of Mediating Aryl
Hydrocarbon Receptor Dependent Gene Expression**

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Running Title Page

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Abbreviations: **AhR**, Aryl hydrocarbon Receptor; **ChIP**, Chromatin Immunoprecipitation; **EMSA**, Electrophoretic Mobility Shift Assay; **NC-XRE**, Non-Consensus Xenobiotic Response Element; **PAI-1**, Plasminogen Activator Inhibitor-1; **TCDD**, Tetrachlorodibenzo-*p*-dioxin; **XRE**, Xenobiotic Response Element;

Abstract

The Aryl hydrocarbon Receptor (AhR) is a mediator of xenobiotic toxicity, best recognized for conveying the deleterious effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure. Mechanistically, the AhR functions as a ligand-activated transcription factor that binds to a canonical xenobiotic response element (XRE) in association with the heterodimerization partner, the AhR nuclear translocator (Arnt) protein. However, within the repertoire of AhR target genes identified in recent years, many lack a clearly defined XRE highlighting the growing realization that AhR-mediated gene expression appears to involve additional mechanisms distinct from the well-characterized process involving the XRE. The present study characterized a novel non-consensus XRE (NC-XRE) in the promoter of the Plasminogen Activator Inhibitor-1 (PAI-1) gene that recruits a novel protein-DNA complex responsible for TCDD-inducible expression. DNA binding studies and reporter assays identified key residues in the NC-XRE necessary for protein-DNA binding and function, respectively. Functional studies with AhR expression constructs confirm that TCDD-inducibility is AhR dependent and requires direct AhR-DNA binding to the NC-XRE. Chromatin immunoprecipitation and RNA interference studies reveal that the Arnt protein is not a component of the NC-XRE-bound AhR complex, suggesting that in contrast to the XRE, AhR-dependent gene expression mediated through the NC-XRE may involve a new DNA binding partner.

Introduction

The eukaryotic Per-Arnt-Sim (PAS) domain protein family contains several members that function as sensors of extracellular signals and environmental stresses affecting growth and development (Gu et al., 2000). Within this family, the aryl hydrocarbon receptor (AhR) regulates adaptive and toxic responses to a variety of chemical pollutants, including polycyclic aromatic hydrocarbons and polychlorinated dioxins, most notably 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Historically, studies on the AhR focused their efforts toward understand the molecular basis for TCDD toxicity, which manifests as a broad spectrum of biological responses that include tumor promotion, immuno- and hepatotoxicity, teratogenesis, a wasting syndrome, and endocrine disturbances (Poland and Knutson, 1982). Evidence obtained using AhR-defective mouse models confirmed that TCDD toxicity is AhR-dependent, requiring the AhR to function as a soluble cytosolic ligand-activated transcription factor that translocates into the nucleus following agonist activation. Upon nuclear entry, the AhR binds to DNA response elements known as a xenobiotic responsive element (XRE) in conjunction with the heterodimerization partner, the Ah receptor nuclear translocator (Arnt) protein (Lees and Whitelaw, 1999).

To better understand TCDD toxicity, several groups sought to identify AhR target genes through DNA microarray studies using cell culture and whole animal models (Puga et al., 2000a; Frueh et al., 2001; Boverhof et al., 2005; Tijet et al., 2005; Elferink, unpublished). Functional clustering identifies numerous TCDD-regulated genes associated with various physiological responses, which underscores the pleiotropic nature of TCDD toxicity, but fails to provide an unambiguous mechanistic explanation for most of the deleterious outcomes associated with

TCDD exposure. A second general observation made is that despite computational analyzes spanning 3Kb (Boverhof et al., 2005) or 6Kb (Tijet et al., 2005) of the DNA sequence flanking the transcription start sites of responsive genes, many did not contain a readily identifiable XRE based on the consensus sequence. One possible explanation is that expression of genes lacking a XRE reflects indirect AhR-mediated signaling, and indeed, such changes in expression may be attributed to latent secondary effects. However, it is necessary to consider the possibility that the AhR is altering transcription directly through a site(s) distinct from the consensus XRE. For instance, the ligand-activated AhR/Arnt dimer can interact directly with the unliganded estrogen receptor and promote formation of a transcriptionally active complex binding to estrogen response elements (Ohtake et al., 2003). Correspondingly, the AhR can form a quaternary complex with p300, pRb and E2F to suppress S phase gene expression (Puga et al., 2000b; Marlowe et al., 2004), or with an unknown protein(s) upstream of the CYP1A2 gene (Sogawa et al., 2004). Evidence is also accumulating for direct AhR-DNA binding in conjunction with the RelB protein, to a distinct response element located in the interleukin-8 gene regulatory region (Vogel et al., 2007).

The plasminogen activator inhibitor-1 (PAI-1) gene was shown to be a TCDD responsive gene in several DNA microarray studies (Puga et al., 2000a; Frueh et al., 2001; Boverhof et al., 2005; Tijet et al., 2005; Elferink, unpublished). Interestingly, the PAI-1 gene represents an example where TCDD-responsiveness is attributed to a regulatory region devoid of a canonical XRE (Son and Rozman, 2002). Analysis of the PAI-1 promoter using a luciferase reporter system in mouse hepatoma cells demonstrated that a 200 base pair region of the PAI-1 promoter lacking a clearly recognizable XRE (GCGTG) motif conferred AhR-dependent TCDD inducibility. The evidence presented in this report extends upon the previous finding by identifying and characterizing a

novel non-consensus XRE (NC-XRE) located within this PAI-1 promoter that supports direct DNA binding and function by the AhR independently of the Arnt protein. This discovery may help to reconcile some of the confounding results obtained in earlier studies examining the AhR-regulated transcriptome, and serves to illustrate the growing awareness of the complexity associated with AhR biology.

Materials and Methods

Reagents. Anti-AhR (rabbit polyclonal antibody) was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Hypoxia-inducible factor-1 β /Arnt1 antibody was purchased from BD Biosciences (San Jose, CA). Mouse anti-actin monoclonal antibody was purchased from Chemicon (Temecula, CA). [γ - 32 P]GTP was acquired from Amersham Biosciences Inc. (Piscataway, NJ). Anti-rat CYP1A1 was obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). The purified rabbit anti-rat PAI-1 was obtained from Torrey Pines Biolabs, Inc. (Houston, TX). Goat anti-recombinant rat PAI-1 was acquired from American Diagnostica Inc. (Greenwich, CT). Species-specific horseradish peroxidase-conjugated secondary anti-bodies were purchased from Zymed Laboratories (South San Francisco, CA). TCDD was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Kansas City, MO). 1-(1-Propynyl)pyrene was custom synthesized by the UTMB Center in Environmental Toxicology Synthetic Organic Chemistry Core. Micrococcal nuclease was obtained from Worthington Biochemicals (Lakewood, NJ). H3 antibody was obtained from Abcam (Cambridge, MA). Protein-G Agarose, Salmon Sperm DNA and sheep IgG were purchased from upstate (Lake placid, NY). Protein-G coupled Sepharose resin was from Life Technologies Inc. Ribonuclease A, Dulbecco's modified Eagle's medium, glass beads, and Sybr Green were obtained from Sigma-Aldrich (St. Louis, MO). 35 S-methionine was obtained from Amersham Biosciences Inc. (Piscataway, NJ). Oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX).

Production of Adenoviruses. Generation of the virus AdAhRFL (encoding a full-length wild-type rat AhR) and control AdGFP and AdRFP viruses was described previously (Elferink et al.,

2001, Huang and Elferink, 2005). Generation of the DNA-binding defective AhR virus construct (AdAhR-R39/41A) and virus construct used in RNA interference (AdiArnt, encoding a short hairpin RNA) was described previously (Huang, and Elferink, 2005).

Cell Culture, Infections, and Transfections. Mouse primary hepatocytes were isolated by the collagenase type IV perfusion from wild-type C57BL/6 mice as described previously (Park et al., 2005). Cells (5×10^5) were plated in 35 mm plates in Williams' E medium containing penicillin (100 U/ml), streptomycin (100 μ g/ml), insulin (5 μ g/ml), bovine serum albumin (1 mg/ml), and 5% fetal bovine serum. AhR-defective BP8 cells were grown as subconfluent monolayers in medium consisting of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Both cells were incubated in 5% CO₂ atmospheres at 37°C. For viral infection, BP8 cells at 5×10^5 cells per 35 mm dish were infected with a multiplicity of infection of 100 and the infection efficiency monitored using GFP co-expression. Where appropriate, primary hepatocytes and BP8 cells were co-transfected with the indicated plasmids using the LipofectamineTM 2000 (Invitrogen Corporation, Carlsbad, CA) in accordance with the manufacturer's recommendations.

Animal Treatment. All experiments were performed in compliance with the standards established for the care and use of laboratory animals at the University of Texas Medical Branch. TCDD was dissolved in anisole, and diluted in peanut oil. Male adult C57BL/6 mice (10 weeks old, weighing 25 g) were administered via gavage with TCDD at 20 μ g/kg body weight. Control mice received peanut oil spiked with an equivalent amount of anisole. Mice were treated with TCDD or vehicle for the indicated period prior to liver harvest. AhR^{fx/fx} mice (mice containing loxP sites that flank exon 2 of the AhR allele, Walisser et al., 2005), and mice harboring an

albumin promoter-driven *Cre* recombinase on a C57Bl/6 background (Cre^{Alb} mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). $Ahr^{fx/fx}$ mice were crossed with Cre^{Alb} mice to produce conditional knockout (CKO) offspring in which the $Ahr^{fx/fx}$ allele is excised in parenchymal hepatocytes, effectively eliminating AhR protein expression in these cells after birth. Excision of the AhR allele was verified at the genomic level (data not shown). Animals were housed in microisolator cages in a temperature- and light-controlled facility with free access to water and diet. Mice were euthanized at the indicated time by isoflurane overdose followed by cervical dislocation.

Immunoprecipitation and Western Blotting. For assessment of PAI-1 expression, secreted PAI-1 from BP8 cell conditioned media was incubated with Goat anti-recombinant rat PAI-1 antibody for 4 h on ice, followed by precipitation with Protein-G resin. Beads were washed four times in RIPA buffer. Both immunoprecipitated proteins and total cell lysates were fractionated by 10% SDS-PAGE, transferred to the polyvinylidene difluoride membrane, and blocked with 5% bovine lacto transfer optimizer in Tris-buffered saline containing 0.1% Tween 20. Filters were probed with antibodies against the PAI-1, Arnt protein, P4501A1, Actin for 4 h at room temperature, followed by the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature. Immunoreactivity was visualized at room temperature using the enhanced chemiluminescence western blot detection reagent (Amersham Biosciences Inc.). Bands were visualized and quantified by using ChemImages 5500 2.03 software.

Cycloheximide Treatment and Examination of Protein Synthesis. Prior to treatment with TCDD, BP8 cells infected with AdAhRFL or AdGFP at a multiplicity of infection of 100 were exposed to cycloheximide (CHX) (10 μ g/ml) for 1 h. To confirm cessation of nascent protein

synthesis, BP8 cultures \pm CHX where pulsed for 4 h with 0.5 mCi/ml 35 S-methionine in cold-methionine depleted media in the presence of 6 nM TCDD or vehicle (Me₂SO). Cells were harvested and processed for SDS-PAGE and autoradiography to monitor nascent protein synthesis, or total RNA isolation for RT-PCR as described below.

RNA Isolation and Analysis. Total RNA was isolated from liver tissue, mouse primary hepatocytes or BP8 cells using RNAqueous (Ambion, Inc., Austin, TX), and quantitative real-time PCR (qRT-PCR) was performed by the Sealy Center for Cancer Cell Biology and Real-Time PCR Core Facility at the University of Texas Medical Branch. We used Applied Biosystems assays-on-demand 20 assay mix of primers and TaqMan MGB probes (FAMTM dye-labeled) for PAI-1 gene (Assay ID: m00435860_m1) and pre-developed 18S rRNA (VICTM-dye labeled probe) TaqMan assay reagent (P/N 4319413E) for an internal control. The primers were designed to span exon-exon junctions so as not to detect genomic DNA. Validation experiments were performed to test the efficiency of the target and reference amplifications. The absolute value of the slope of log input amount versus CT was <0.1. Separate tubes (singleplex) one-step RT-PCR was performed with 40 ng RNA for both target genes and endogenous control. The reagent we used was TaqMan one-step RT-PCR master mix reagent kit (Applied Biosystems, Foster City, CA). The cycling parameters for one-step RT-PCR was: reverse transcription 48°C for 30 min, AmpliTaq activation 95°C for 10 min, denaturation 95°C for 15s and annealing/extension 60°C for 1 min (repeat 40 times) on ABI 7000. Triplicate C_T values were analyzed in Microsoft Excel (Microsoft, Redmond, WA) using the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta C_T}$) was obtained by normalized to endogenous reference (18S rRNA) and relative to a calibrator (one of the experimental samples).

Genomic DNA Extraction and Constructs and Luciferase Report Assays. Genomic DNA extraction was described previously (Laird, 1991). The mouse PAI-1 promoter (-116/+1) sequence was generated by PCR using Taq polymerase, genomic DNA, and primers 1 and 2 (Table 1). The PCR product was subcloned into pCR 4-Topo vector (Invitrogen Corporation, Carlsbad, CA), and DNA was sequence-verified. The PCR product was subcloned into the KpnI and BglII sites of the firefly luciferase reporter vector pGL3-Basic (Promega Corporation, San Luis Obispo, CA) to generate the resultant plasmid construct pPAI-1/116Luc. The NC-XRE (pGL3-NC-XRE) and mutant constructs (pGL3-M3, pGL3-M4 and pGL3-M5) were generated by PCR using Taq polymerase, pPAI-1/116Luc DNA, and primers 3 and 4 (NC-XRE), 3 and 5 (M4), 3 and 6 (M5), 7 and 8 (M3) (see Table 1). The PCR products were subcloned into pCR 4-Topo vector and the DNA sequence-verified. The PCR products were subsequently cloned into the KpnI and BglII site of the vector pGL3-Promoter.

For transfection experiments the indicated cells were cultured in 6-well plates and transiently co-transfected with the firefly luciferase constructs and the Renilla luciferase-encoding pRL-SV40 vector (Promega Corporation, San Luis Obispo, CA), used as an internal control, according to the LipofectamineTM 2000 method (Invitrogen Corporation, Carlsbad, CA) briefly, 500 μ l of transfection medium containing 200 ng of luciferase reporter plasmid was added per well of 90% of appropriate cells along with 20 ng of the pRL-SV40 plasmid and 10 μ l of LipofectamineTM 2000. After a 24 h period, cells were exposed to TCDD for a further 24 h. Dual luciferase assays (firefly and Renilla) were performed with a Promega kit according to the manufacturer's instructions.

Chromatin Immunoprecipitation (ChIP) Assays. ChIP assays on whole liver tissue were performed as previously described (Nguyen et al., 2005). Briefly, liver tissues from C57BL/6 mice were isolated, finely diced and cross-linked with 1% formaldehyde in phosphate-buffered saline at room temperature for 10 min. Sonication of the cross-linked chromatin was performed with glass beads followed by digestion with micrococcal nuclease to generate fragments with a median length of 400 to 500 bp, as determined empirically by agarose gel electrophoresis of fragmented chromatin samples. The fragmented chromatin lysate was incubated with nonspecific immunoglobulin G (IgG) for 1 h to preclear the lysate. The precleared lysate was divided equally among all samples and incubated overnight at 4 °C with 1µg of the following antibody: Anti- AhR, Hypoxia-inducible factor-1β/Arnt1, H3 antibodies (as a positive control and a measure of chromatin recovery for each lysate) and normal sheep IgG (negative control). Protein G-Sepharose beads with salmon sperm DNA were added and incubated for 1 h, followed by recovery of antibody precipitates.

The recovered DNA was subjected to PCR using primers listed in 9 and 10 or 11 and 12 (Table1) to generate a 234 bp (spanning the PAI-1 promoter NC-XRE) and a 357 bp (Cyp1a1 promoter region spanning several XRE sequences) PCR product, respectively. 28 PCR cycles were performed for each bound DNA fraction and input. PCR products were separated on 6% polyacrylamide gels and stained with SYBR[®] Green. DNA bands were visualized and quantified by using ChemImages 5500 2.03 software. The bound fraction values were calculated as a percentage of the input DNA used in the immunoprecipitation representing 100%.

Electrophoretic Mobility Shift Assays (EMSA). Nuclear extracts were isolated from mouse liver tissue as described previously (Schwartz et al., 1996). Mice were treated with 20 µg/kg

TCDD or vehicle (peanut oil) by gavage 2 hr prior to harvest of liver tissue, and EMSA was performed using a [γ -³²P]GTP end-labeled double-stranded DNA probe containing an AhR-binding site as described by Shen et al. (1991).

Statistical Analyses. Statistical analyses were performed using Prism (version 4.0; GraphPad Software, San Diego, CA). Data were evaluated by two-way ANOVA. *p* values < 0.05 were considered statistically significant.

Results

Several previous DNA microarray reports documented that PAI-1 expression could be induced by TCDD (Puga et al., 2000a; Frueh et al., 2001; Boverhof et al., 2005; Tijet et al., 2005). In microarray studies performed by us, PAI-1 expression was induced 5- to 9-fold in rat 5L hepatoma cells treated with TCDD for 4 h. In primary mouse hepatocytes exposed to TCDD for 12 h PAI-1 expression increased a more modest 3-fold (Fig. 1A), similar to that observed in HepG2 (Puga et al., 2000a; Frueh et al., 2001) and Hepa1 cells (Son and Rozman, 2002). By contrast, *in vivo* liver PAI-1 expression increased over 40-fold following 12 h of TCDD exposure (Fig. 1B). The data clearly identify PAI-1 as a TCDD-responsive gene in liver derived tissues and cells.

In order to establish that PAI-1 induction is AhR dependent, we infected BP8 cells—a variant of the 5L cell line lacking detectable AhR expression (Weiss et al., 1996)—with an adenovirus expressing the AhR (Elferink et al., 2001). A multiplicity of infection of 100, sufficient to infect 100% of the cells was used, and the infection rate monitored through co-expression of a GFP protein (Fig. 2A). A virus (AdGFP) expressing only the GFP served as a control. Activation of the AhR relied on treatment with 1-(1-propynyl)pyrene as previously reported by us (Levine-Fridman et al., 2004). PAI-1 is a secreted protein, therefore its expression was measured following immunoprecipitation of the culture medium at the indicated times. P4501A1 expression was determined concomitantly using the cell lysates. The evidence reveals that increases in secreted PAI-1 are first detectable at 4 h, and peak by 8 h. P4501A1 levels are clearly evident within 4 h following 1-(1-propynyl)pyrene administration. Since expression of both PAI-1 and P4501A1 is restricted to the BP8 cells infected with AdAhRFL, the evidence

clearly illustrates that PAI-1 induction is AhR-dependent. This finding extends upon an earlier observation that TCDD could not induce PAI-1 expression in the AhR-defective Hepa1 variant cell line (Son and Rozman, 2002), by confirming the requirement for the AhR.

Son and Rozman (2002) ascertained that a region of the murine PAI-1 promoter spanning nucleotides -116 to +73 conferred TCDD inducibility upon a luciferase reporter. Our preliminary findings narrowed this region down to the first 116 nucleotides upstream of the transcription start site (Fig. 3). In primary mouse hepatocytes and in BP8 cells infected with AdAhRFL, this promoter sequence mediated TCDD inducible reporter expression. This region also increased basal expression suggesting that other regulatory factors may be interacting with this promoter sequence. Subsequent studies using cyclohexamide (CHX) to inhibit nascent protein synthesis in BP8 cells demonstrated that the AhR-dependent induction persisted in the absence of new protein synthesis (Fig. 4), suggesting that the increased expression is a direct rather than indirect response to TCDD, consistent with a direct role for the AhR reminiscent of a canonical XRE-mediated induction response.

Identification and characterization of the non-consensus XRE.

The evidence indicates that AhR-mediated induction of the PAI-1 gene is a direct response involving a DNA region proximal to the promoter. However, this region lacks an obvious consensus XRE (5'-GCGTG-3'). Electrophoretic mobility shift assays (EMSA) were performed using mouse liver nuclear extracts and overlapping 40 nucleotide-long probes to interrogate the 116 base pair region for a site capable of forming a TCDD-inducible protein-DNA complex (data not shown). These studies identified a single oligonucleotide spanning residues -116 to -76 of the PAI-1 promoter that conferred a TCDD inducible EMSA complex (Fig. 5). The DNA

sequence designated as a non-consensus XRE (NC-XRE), forms two distinct inducible complexes that are refractory to competition by a 10-fold molar excess of unlabeled XRE (Fig. 5, lanes 1-4). In contrast, the unlabeled XRE readily competes for the inducible complex detected using a radiolabeled XRE (Fig. 5, lanes 5-8).

A further characterization of the NC-XRE sought to resolve the precise binding site by identifying important residues for complex formation (Fig. 6). Using the UCSC Genome Browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>), examination of the PAI-1 promoter encompassing this region amongst several mammalian species (human, mouse rat, orangutan, dog) identified two conserved sequences that were independently mutated and analyzed by EMSA (Fig. 6A). The results show that direct binding of the TCDD inducible protein-DNA complex occurred with both the wild-type NC-XRE and M1 mutant oligonucleotide, but not with the M2 sequence. Therefore, the sequence altered in M2 appears to map the NC-XRE binding site. A striking feature associated with this sequence is the repeated 5'-GGGA-3' tetranucleotide motif (Fig. 6B). Subsequent competition EMSA experiments further resolved the key residues involved in protein-DNA binding (Fig. 6C). The evidence suggests that the second tetranucleotide motif is preferentially involved in protein binding because akin to the M2 mutant, the M4 mutant NC-XRE fails to effectively compete for the EMSA complex. In contrast, unlabeled M3 and M5 mutant oligonucleotides proved as effective as the wild-type NC-XRE in competing for binding. Since the unaltered tetranucleotide motifs in the M4 oligonucleotide failed to compete effectively for the complex, we conclude that they do not contribute substantially to protein-DNA binding. In addition, the evidence suggests that the slower migrating complex (Fig. 6C, upper arrow) exhibits higher affinity binding because it persisted

despite challenge with a 100-fold molar excess of the mutant M2 and M4 oligonucleotides. The composition of the protein-DNA complexes bound to the NC-XRE remains undetermined.

To examine the functionality of the mutant NC-XRE sequences, constructs harboring a single copy of the wild-type NC-XRE, M3, M4 or M5 mutant, were introduced into the promoter of a luciferase reporter plasmids and transfected into primary mouse hepatocytes (Fig. 7). The result reveals that the NC-XRE and mutant M3 and M5 binding sites conferred significant TCDD inducibility upon the luciferase reporter, whereas the mutant M4 was unable to confer induced gene expression. The diminished induction seen with the M3 and M5 mutants may reflect a partial disruption in protein-DNA binding, but insufficient to fully abolish function. Therefore, the functional evidence corroborates the *in vitro* protein-DNA binding observations.

AhR binding to the PAI-1 promoter is Arnt-independent

Son and Rozman (2002), using the Hepa1 cell variants surmised that TCDD induction of the PAI-1 gene required both the AhR and Arnt protein, because neither the AhR nor Arnt defective variant cell lines responded to TCDD. While the composition of the protein complex binding to the NC-XRE is unknown, the characteristics of the binding site strongly suggest that the NC-XRE does not recruit the typical AhR/Arnt protein heterodimer known to bind to the XRE. Attempts to interrogate the *in vitro* EMSA complex with antibodies against the AhR or Arnt protein did not produce a supershift complex nor disrupt complex formation (data not shown). However, chromatin immunoprecipitation (ChIP) experiments performed using the whole mouse liver nevertheless revealed a TCDD-inducible association of the AhR with the PAI-1 promoter *in vivo* (Fig. 8). Interestingly, a corresponding association of the Arnt protein was not evident, while concomitant analysis of the Cyp1a1 promoter region harboring XREs confirmed TCDD-

inducible binding by both the AhR and Arnt protein as expected. Therefore, the data suggest that AhR binding to the PAI-1 NC-XRE is Arnt protein independent.

Given the dissimilarity between the NC-XRE and canonical XRE, and the inability to detect Arnt protein binding to the PAI-1 promoter by ChIP, there is some question about the nature of the AhR-NC-XRE interaction. Specifically, whether the receptor binds to the DNA directly, or resides within a quaternary complex through a protein-protein interaction in the absence of direct DNA binding. Indeed, the AhR's association with the E2F/DP complex provides a precedent for the latter scenario (Marlowe et al., 2004). To examine this we tested whether an AhR harboring point mutations in the basic (DNA-binding) domain designed to specifically and exclusively abolish DNA binding (Huang and Elferink, 2005), could still induce PAI-1 expression (Fig. 9). Primary hepatocytes were isolated from $Ahr^{fx/fx}Cre^{Alb}$ CKO mice—lacking the AhR—and infected with a control adenovirus (AdGFP), a virus expressing a wild-type AhR (AdAhRFL), or a virus expressing the DNA binding defective mutant receptor (AdAhR-R39/41A). Following TCDD treatment, RT-PCR was performed on total RNA to monitor AhR expression and PAI-1 and Cyp1a1 inducibility (Fig. 9A). qRT-PCR was performed to quantify the induction of PAI-1 and Cyp1a1 (Fig. 9B). Collectively, the evidence demonstrates that TCDD induction of both target transcripts is abolished in the cell expressing the mutant AhR, suggesting that NC-XRE mediated PAI-1 induction depends on direct DNA binding by the AhR, but seemingly independent of the Arnt protein.

In order to verify that TCDD induction of the PAI-1 gene is indeed independent of the Arnt protein, BP8 cells were co-infected with adenoviruses expressing the AhR and RFP (as a control), or co-infected with viruses expressing the AhR and a short hairpin RNA to knock-down

Arnt protein expression by RNA interference, as previously reported (Huang and Elferink, 2005). The virus-infected cells were subsequently transiently transfected with a control luciferase reporter construct (pGL3) or the construct containing the 116 base pair PAI-1 promoter (pPAI-1/116Luc) and induced with TCDD for 24 h (Fig. 10). As anticipated, partial knock-down of Arnt protein expression caused a marked (>2-fold) attenuation in TCDD induction of the endogenous Cyp1a1 gene (Fig. 10A). However, TCDD inducible luciferase activity driven by the PAI-1 promoter is unaltered despite the marked decrease in Arnt protein expression. This results complements that CHIP data (Fig. 8) and strongly implies that AhR-mediated PAI-1 induction through the NC-XRE is indeed Arnt protein independent.

Discussion

Plasminogen activator inhibitor type 1 (PAI-1) is almost ubiquitously expressed with the highest levels found in endothelial cells, adipocytes and hepatocytes. PAI-1 is a member of the serpin (serine protease inhibitor) family responsible for suppressing the activity of the urokinase (uPA) and tissue-type plasminogen activators that in turn, convert plasminogen to plasmin. Plasmin possesses broad substrate specificity, including extracellular matrix proteins, blood clot fibrin, growth factor precursors and other proteases (Durand et al., 2004), involved in processes important for angiogenesis, wound healing, tumor metastasis, and liver regeneration (Michalopoulos and DeFrances, 1997; Irigoyen et al., 1999). Hence, plasmin formation and action are highly dependent on PAI-1 levels. Specifically, PAI-1 inhibits liver regeneration by forming a complex with uPA thus preventing the activation of hepatocyte growth factor, a key protein in the regenerative process. Since our previous studies showed that TCDD could arrest liver regeneration (Mitchell et al., 2006), it is conceivable that PAI-1 is involved in the TCDD-induced liver growth arrest. As a prelude to this hypothesis we sought to further investigate the finding that PAI-1 is a TCDD-responsive gene (Fig. 1; Puga et al., 2000a; Frueh et al., 2001; Boverhof et al., 2005; Tijet et al., 2005), particularly since the promoter lacks a definable XRE (Son and Rozman, 2002).

Extending upon earlier reported findings (Son and Rozman, 2002), the evidence presented here confirms that the AhR can induce PAI-1 expression through a non-consensus XRE (NC-XRE) devoid of sequence homology with the well-characterized XRE known to regulate CYP1A1 expression (Figs. 5 and 6). The studies further show that AhR activity inducing PAI-1 expression is a direct response not reliant on nascent protein synthesis (Fig. 4), requires direct

DNA binding to the NC-XRE (Fig. 9), but is independent of the Arnt protein (Figs. 8 and 10). It is noteworthy that Son and Rozman (2002) assigned a role for the Arnt protein in TCDD induced PAI-1 induction, based on the observation that induction was not detected in Arnt-defective Hepa 1 mutant cells. Since the cell line was isolated for its resistance to benzo[a]pyrene toxicity following exposure to this potent genotoxin (Miller et al., 1983), it is conceivable that amongst the numerous uncharacterized genetic lesions existing within this cell line, are mutations affecting AhR-mediated PAI-1 induction unrelated to Arnt protein function. In this regard, several features argue against a role for the Arnt protein: 1) the PAI-1 promoter region conferring TCDD responsiveness lacks a canonical XRE capable of binding the AhR/Arnt protein heterodimer, 2) the ChIP assay fails to detect an Arnt protein association with the PAI-1 promoter even though Arnt protein binding to the Cyp1a1 promoter is readily observed (Fig. 8), and 3) the PAI-1 promoter retains TCDD responsiveness when Arnt protein expression—and concomitant P4501A1 protein induction—is suppressed by RNA interference (Fig. 10). The implication for AhR binding to the NC-XRE independently of the Arnt protein imputes a role for a new binding AhR DNA binding partner. Indeed, the AhR is known to interact with other transcription factors including the Retinoblastoma tumor suppressor protein (Ge and Elferink, 1998), E2F1 (Marlowe et al., 2004), and RelB (Vogel et al., 2007). The latter example is noteworthy because the AhR/RelB complex appears to bind DNA directly, although this interaction was not specifically assessed. Moreover, the RelB/AhR response element nucleotide sequence differs markedly from the NC-XRE suggesting that AhR binding to the NC-XRE depends on an as yet unidentified protein partner. Interrogation of the TRANSFAC® database however, did not identify the NC-XRE as a known DNA binding site classified by a defined protein-DNA interaction.

Consistent with the previously observations (Son and Rozman, 2002), TCDD induction of both endogenous PAI-1 gene expression and luciferase reporter expression in the various cultured cell systems is a modest two to three-fold. In contrast, PAI-1 induction *in vivo* revealed a 40-fold increase in gene expression (Fig. 1B) suggesting that under physiological conditions AhR regulation of the PAI-1 gene is indeed robust. This may reflect interplay between multiple transcription factors whose signals are preserved only under *in vivo* conditions. Certainly, gene regulation seldom involves a single transcription factor. Given the involvement of PAI-1 in diverse biological roles, its expression is controlled by several different signals including hormones, cytokines, and both environmental and mechanical stresses. Underpinning the various signaling pathways are several transcription factors that interact with the PAI-1 regulatory region spanning several kilobases upstream of the transcription start site; these include Sp1 (two sites at -73 and -42; Chen et al., 1998), AP-1 (two sites at -60 and -52; Vulin et al., 2004; Al-Nedawi et al., 2004), serum-induced expression element (-165; White et al., 2000), p53 (-136; Parra et al., 2001), and AP-1 like sequence (-62; Guo et al., 2005), HGF responsive element (-158; Imagawa et al., 2006), β -catenin (-425; He et al., 2010), Elk-1 (-42; Wyrzykowska et al., 2010), HIF-1 (two sites at -195 and -3633; Liu, et al., 2004, Ahn, et al., 2010), and the PAS proteins CLOCK and BMAL (at -684 and -565; Schoenhard, et al., 2003; Maemura, et al., 2000). Therefore, the significance of AhR mediated PAI-1 regulation will most likely be context dependent, reflecting the integration of multiple signaling pathways to either enhance or stifle AhR action.

What becomes evident from this and other studies is that AhR-mediated transcriptional regulation is more diverse than the repertoire of target genes responding to the AhR/Arnt protein heterodimer. The effect of AhR activity governing gene expression through multiple DNA *cis*

elements (e.g. XRE, NC-XRE, RelBAhRE) on TCDD toxicity and normal physiological processes still needs to be resolved. Nukaya and coworkers (Nukaya et al., 2010) demonstrated using a conditional knockout Arnt protein mouse line that loss of Arnt protein expression in hepatocytes prevented the occurrence of several well-established toxic endpoints in the liver following TCDD exposure. These findings elegantly show that certain TCDD-induced hepatotoxicities are directly attributable to AhR/Arnt protein activity. Nevertheless, a genomewide analysis of AhR DNA binding target genes (Sartor et al., 2009) revealed that amongst the hundreds of AhR target genes identified by the ChIP-chip analysis, many did not contain canonical XRE sequences in the region flanking the transcription start site. Consequently, there is good cause to speculate that non-XRE mediated Arnt-independent transcriptional responses also contribute to adaptive and toxic AhR-regulated responses. The PAI-1 NC-XRE may constitute an important alternative binding site for receptor action and further characterization of the binding partner(s) is a priority for future study.

Authorship Contributions

Participated in research design: Huang and Elferink.

Conducted experiments: Huang.

Contributed new reagents or analytic tools: Huang and Elferink.

Performed data analysis: Huang and Elferink.

Wrote or contributed to the writing of the manuscript: Huang and Elferink.

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Footnotes

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Figure Legends

Figure 1. TCDD inducible PAI-1 expression in the mouse liver tissue and primary hepatocytes.

A, mouse primary hepatocytes were cultured in the presence of 6 nM TCDD or vehicle (Control) for 4 hr. PAI-1 mRNA expression (normalized to 18s RNA) was then analyzed using quantitative RT-PCR. The data presented are the average of two independent experiments (\pm S.E.M.). B, adult C57BL/6 mice were treated with 20 μ g/kg TCDD or vehicle (peanut oil) by gavage. After 12 h, total RNA was isolated and PAI-1 mRNA levels were analyzed by quantitative RT-PCR. The data shown are representative of three independent experiments. Asterisks indicate a significant difference compared with vehicle group ($p < 0.05$).

Figure 2. PAI-1 induction is AhR dependent. A, AhR-negative BP8 cells were infected with a control adenovirus (AdGFP) or a virus expressing the rat AhR (AdAhRFL) at a MOI of 90 for 24 h as previously described (Elferink et al., 2001). GFP expression was used to monitor infection efficiency. B, Cultures were treated with 1mM 1PP to activate the AhR for the indicated time and western blotting performed on immunoprecipitated PAI-1 from the culture media to measure the secreted PAI-1 protein, and on total cell lysates to assay for P4501A1 expression. Analysis of actin was used as a loading control.

Figure 3. The AhR is required for TCDD-induced PAI-1 expression. A, Mouse primary hepatocytes isolated as described in the materials and methods were transfected with the pGL3 control vector or the pPAI-1/116Luc firefly luciferase reporter construct under the control of the 116 bp PAI-1 promoter region. Cells were co-transfected with the pRL-SV40 vector encoding Renilla luciferase as a transfection control. Cells were treated with vehicle (Control, open bars) or 6 nM TCDD (TCDD, solid bars) for 24 h, and dual luciferase assays (firefly and Renilla)

performed. The data are presented as means \pm S.E.M of four independent experiments for firefly luciferase activity normalized against pRL-SV40 Renilla luciferase activity. The asterisk indicates a significant difference ($p < 0.05$). B, Subconfluent cultures of asynchronous BP8 cells (AhR-negative) were infected with the AdGFP or AdAhRFL at a MOI of 100 for 24 h. The cells were subsequently co-transfected with pGL3 or pPAI-1/116 and pRL-SV40, and treated with vehicle (Control, open bars) or 6 nM TCDD (TCDD, solid bars) for a further 24 h. Dual luciferase activity was determined and the data expressed as means \pm S.E.M of two independent experiments.

Figure 4. TCDD induction of PAI-1 does not require new protein synthesis. A, BP8 (AhR-negative) cells were infected with AdAhRFL or AdGFP at an MOI of 100 for 24 h prior to treatment with PBS (-CHX) or 10 μ g/ml cycloheximide (+CHX) for 1 h. Subsequently, the cells were labeled with 0.5 mCi/ml 35 S-methionine (\pm CHX) as described in the materials and method in the vehicle (-TCDD) or 6 nM TCDD (+TCDD) for an additional 4 h. Cells were harvested to monitor nascent protein synthesis by 10% SDS-PAGE and autoradiography. B, Duplicate cultures of BP8 cells were prepared as described for panel A, except that they were not radioactively labeled with 35 S-methionine. Total RNA was isolated and quantitative RT-PCR performed to measure PAI-1 mRNA levels (normalized to 18S rRNA). The data are presented as the mean (\pm S.E.M.) of two independent experiments.

Figure 5. EMSA on the NC-XRE and XRE reveals distinct protein-DNA complexes. Whole liver nuclear extracts were prepared from vehicle-treated mice (ie., peanut oil administered by gavage, -TCDD) or mice treated with 20 μ g/kg TCDD for 2h (+TCDD). EMSA using the nuclear extracts was performed with 32 P-labeled NC-XRE and XRE oligonucleotide probes as

indicated. Competition experiments used a 10-fold molar excess of the cold XRE probe as described in the materials and methods. or the containing the AhR-binding site. The arrows denote the position of the TCDD-inducible protein-DNA complexes for each oligonucleotide.

Figure 6. Characterization of the NC-XRE binding site. A, Whole liver nuclear extracts were prepared from vehicle-treated mice (ie., peanut oil administered by gavage, -TCDD) or mice treated with 20 μ g/kg TCDD for 2h (+TCDD). EMSA using the nuclear extracts was performed with ³²P-labeled NC-XRE (WT) and two mutant oligonucleotide probes (M1 and M2) depicted in panel B. B, Depicts the sequences of the wild-type and mutant NC-XRE oligonucleotides used in the EMSA analysis. C, Competition EMSA was performed using the ³²P-labeled NC-XRE (WT) with the indicated cold competitor oligonucleotides (at a 10- and 100-fold molar excess) and nuclear extracts from vehicle treated (-TCDD) and TCDD treated (+TCDD) mice. Arrows denote the position of the TCDD inducible complexes.

Figure 7. Functional analysis of the mutant NC-XRE binding sites. A, Mouse primary hepatocytes were co-transfected with pRL-SV40 and reporter constructs lacking a NC-XRE (pGL3), or containing a single copy of the wild-type NC-XRE (pGL3-NC-XRE), or the mutant NC-XREs identified in figure 6B (pGL3-M3, M4 and M5). Cells were cultured in the presence of vehicle (Control) or 6 nM TCDD for 24 h prior to assaying for luciferase expression (firefly and Renilla). The data are expressed as means \pm S.E.M of three independent experiments. Asterisks indicate a significant difference compared with the control ($p < 0.001$).

Figure 8. TCDD-dependent AhR binding to the PAI-1 promoter is Arnt protein independent. ChIP analysis of AhR and Arnt protein binding to the PAI-1 promoter region or Cyp1a1 enhancer in liver tissue from mice treated with vehicle (-) or 20 μ g/kg TCDD (+) for 2 h. IgG

and histone H3 antibodies were used as negative and positive controls, respectively. PCR products were fractionated by 6% PAGE and stained with SYBR® Green, and quantitation presented as a percentage of the input DNA. The data shown are representative of three independent experiments.

Figure 9. PAI-1 induction requires direct DNA binding by the AhR. A, Primary hepatocytes were isolated from $Ahr^{fx/fx}Cre^{Alb}$ CKO mice and infected with a control adenovirus (AdGFP), a virus expressing a wild-type AhR (AdAhRFL), or a virus expressing the DNA binding defective mutant receptor (AdAhR-R39/41A) at a MOI of 100. After 24 h in culture, the hepatocytes were treated with vehicle (-) or 6 nM TCDD (+) for 8 h and AhR, PAI-1, Cyp1a1 and GAPDH mRNA levels measured by RT-PCR. B, Primary hepatocytes were prepared as described above and PAI-1 and Cyp1a1 mRNA measured by qRT-PCR. The data are expressed as means \pm S.E.M of three independent experiments. * $p < 0.05$, ** $p < 0.001$.

Figure 10. The Arnt protein is not required for AhR- mediated PAI-1 expression. A, Asynchronous subconfluent BP8 (AhR-negative) cultures were infected with AdAhRFL at an MOI of 100 for 24 h before subsequent reinfection with AdRFP or AdiArnt (coexpressing RFP, Huang and Elferink, 2005) at MOI of 100 for an additional 24 h. Cells were then transiently transfected with pRL-SV40 and pGL3 or pPAI-1/116Luc for 24h before treatment with Me_2SO or 6 nM TCDD (+) for a further 24 h. Total cell lysates were probed for Arnt protein, P4501A1 and Actin by western blotting and the band densities quantified. The data shown is from a single experiment. B, Cells treated as described above were assayed for dual luciferase activity and the data expressed as means \pm S.E.M of two independent experiments.

Table 1: Oligonucleotides used in PCR and as EMSA probe sets.

Cloning primers

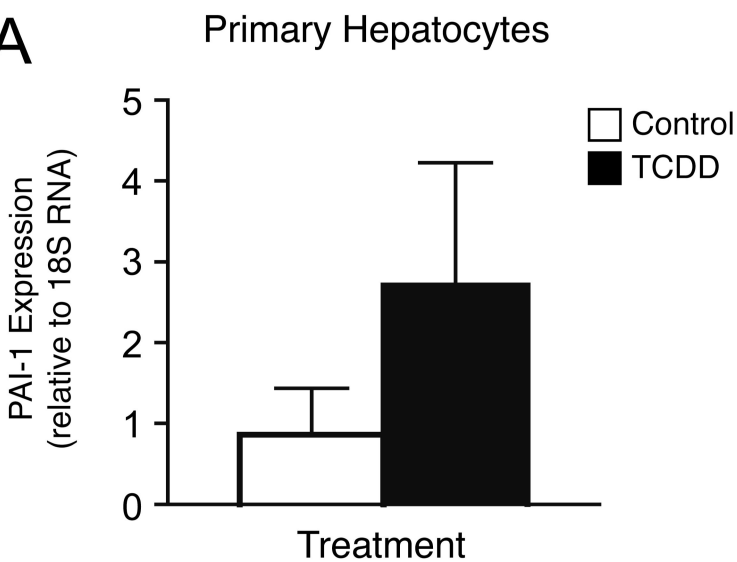
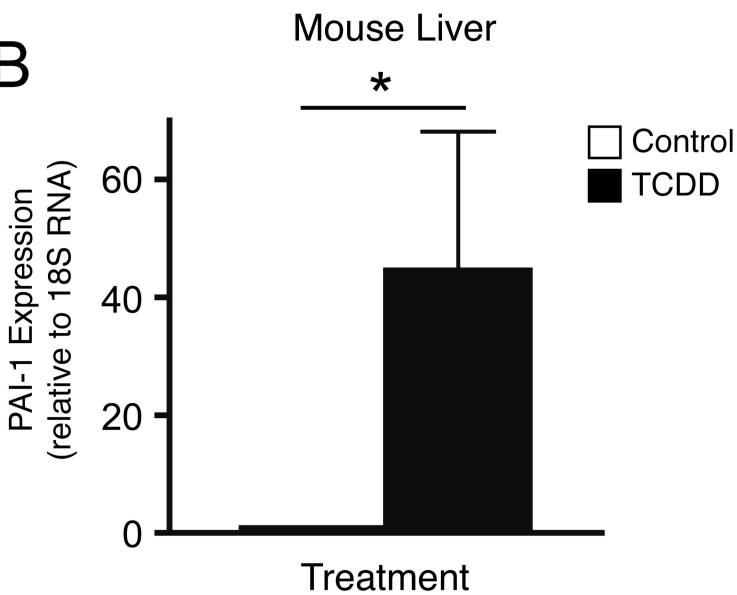
- | | |
|---|---|
| 1 | 5'-GGGGTACCGTCACTGGGAGGGAGGGAGGG-3' |
| 2 | 5'-GGAAGATCTAACTCATGTTCCAGCCCCACCCACTTTC-3' |
| 3 | 5'-GGGTACCGTCCCAGCAAGTCACTGGGAG-3' |
| 4 | 5'-GGAAGATCTCTCCCCCCCCCTCCCTCCCTC-3' |
| 5 | 5'-GGAAGATCTCTCCCCCCCCCTCCCCTTCTC-3' |
| 6 | 5'-GGAAGATCTCTCCCCCCCCCTTTTCCCTC-3' |
| 7 | 5'-GGGTACCGTCCCAGCAAGTCACTGGAGAG-3' |
| 8 | 5'-GGAAGATCTCTCCCCCCCCCTCCCTCCTCT-3' |

ChIP assay PCR primers

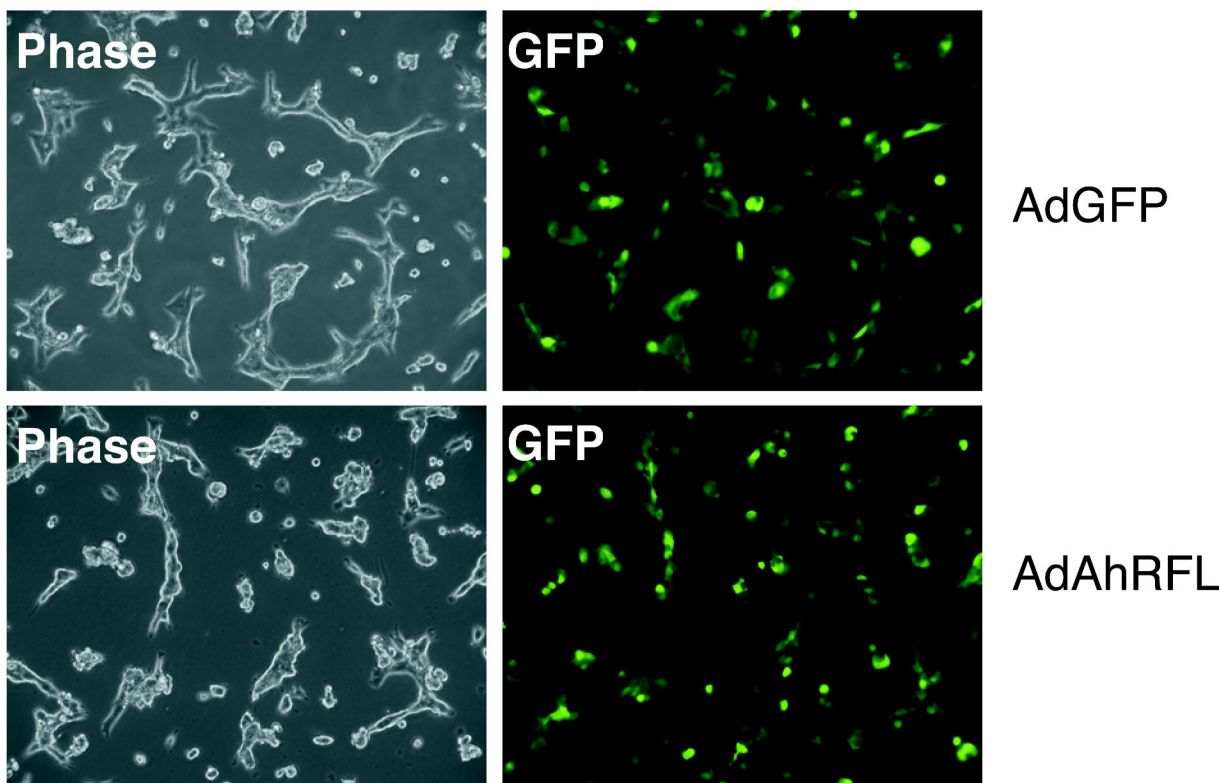
- | | |
|----|--------------------------------|
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| 10 | 5'-CTGGAGGCGGGTGTGCGGCG-3' |
| 11 | 5'-CTATCTCTTAAACCCACCCAA-3' |
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EMSA oligonucleotides

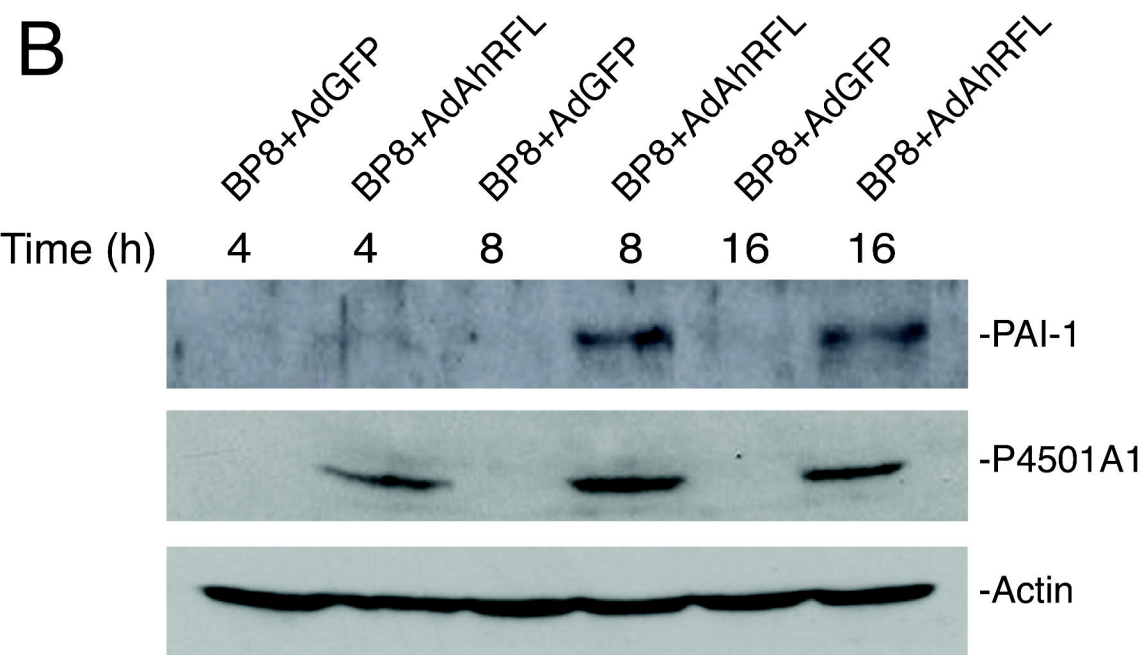
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|----|--|---|
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| 15 | 5'-GTCCCAGCAAGTCACTGGCACCCACCGAGGGGGGGGAG-3' | 5'-CTCCCCCCCCCTCGGTGGGTGCCAGTGACTTGCTGGGAC-3' |
| 16 | 5'-GTCCCAGCAAGTCACTGGAGAGGAGGGAGGGGGGGGAG-3' | 5'-CTCCCCCCCCCTCCCTCCTCTCCAGTGACTTGCTGGGAC-3' |
| 17 | 5'-GTCCCAGCAAGTCACTGGGAGAAGGGGAGGGGGGGGAG-3' | 5'-CTCCCCCCCCCTCCCCGGCTCCCAGTGACTTGCTGGGAC-3' |
| 18 | 5'-GTCCCAGCAAGTCACTGGGAGGGAAAGAGGGGGGGGAG-3' | 5'-CTCCCCCCCCCTTTTCCCTCCCAGTGACTTGCTGGGAC-3' |
| 19 | 5'-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3' | 5'-GATCCGGCTCTTCTCACGCAACTCCGAGCTCA-3' |
-

A**B**

A

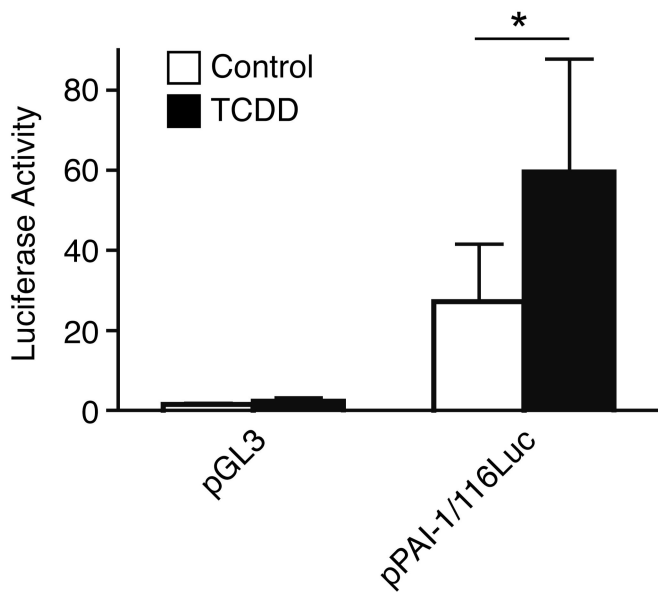


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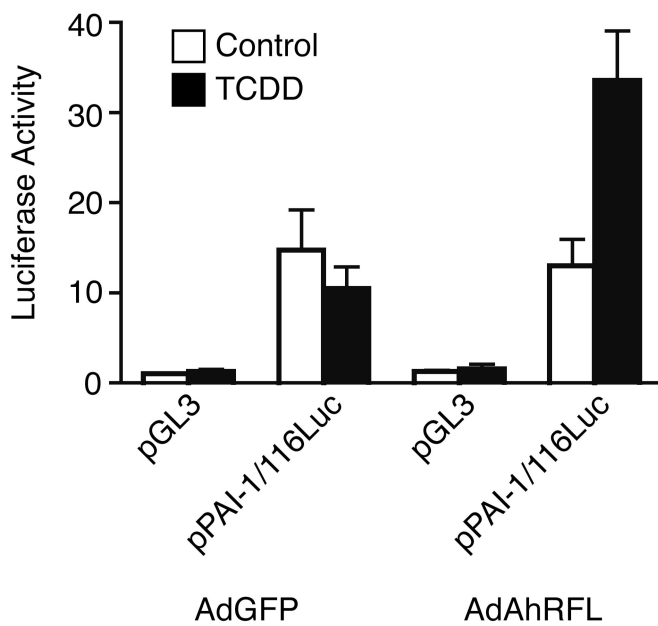
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Primary Hepatocytes



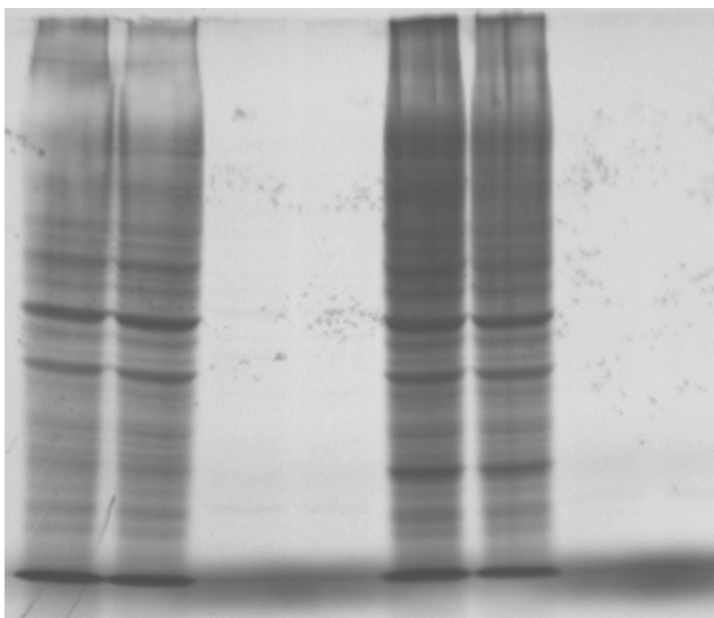
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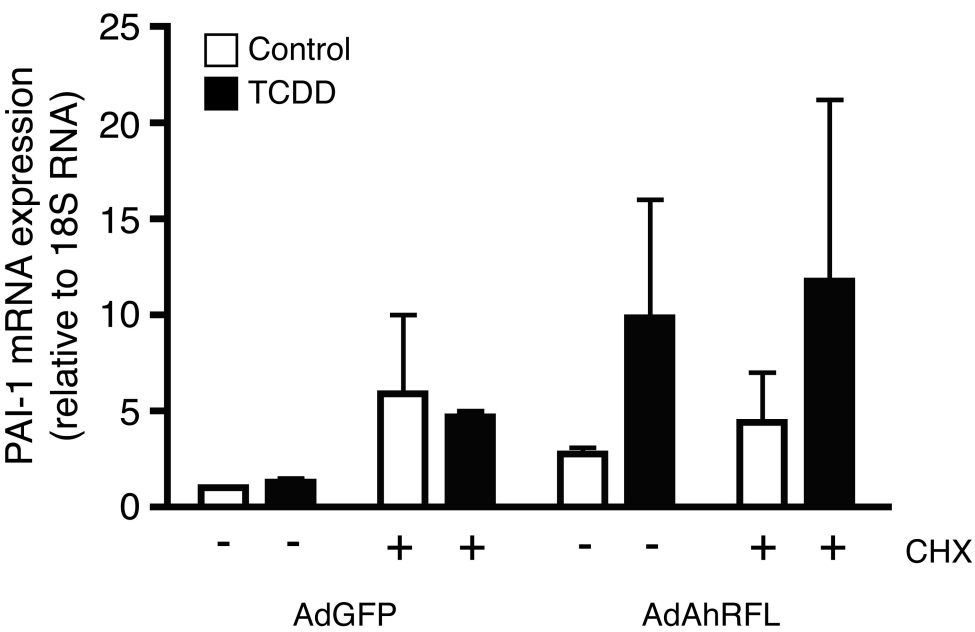


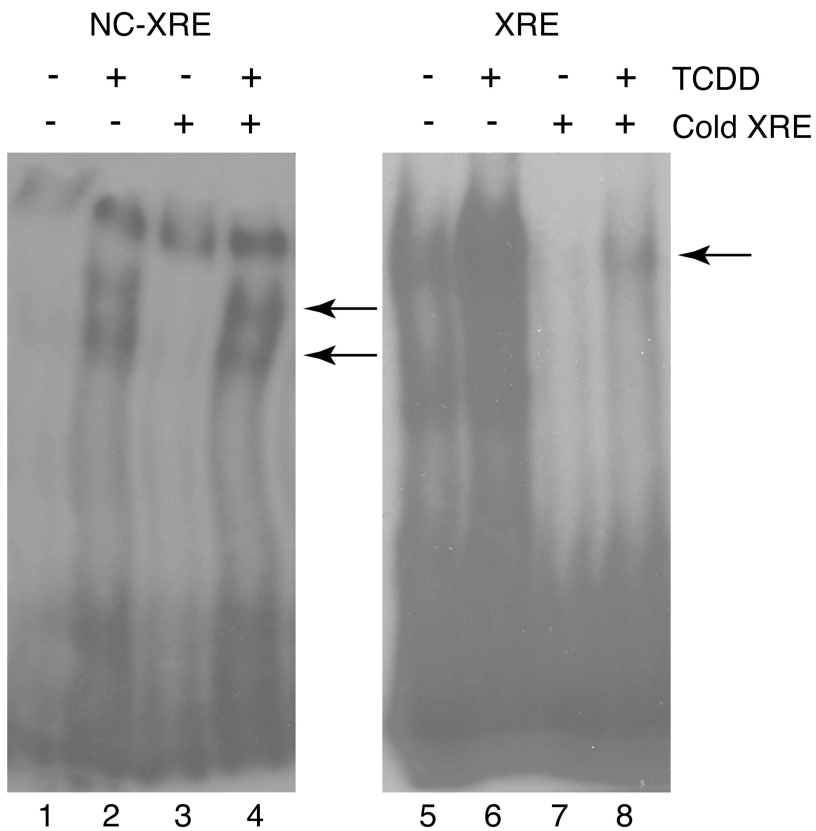
A

	AdGFP				AdAhRFL			
CHX	-	-	+	+	-	-	+	+
TCDD	-	+	-	+	-	+	-	+



B

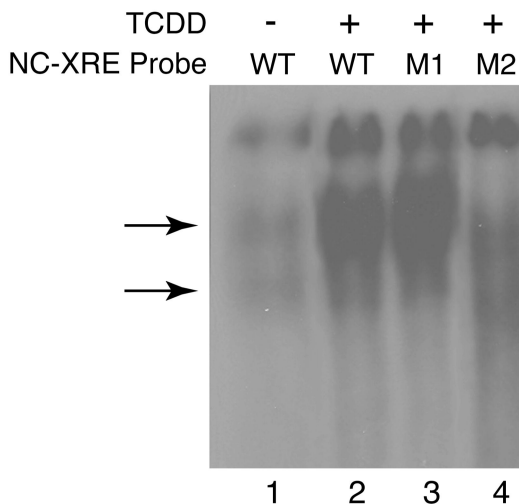




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NC-XRE 5'-GTCCCAGCAAGTCACTGGGAGGGAGGGAGGGAGGGGAG-3'

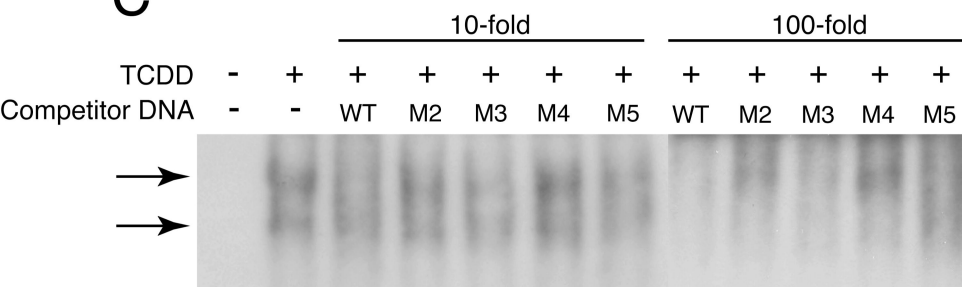
A



B

NC-XRE WT 5'-GTCCCAGCAAGTCACTGGGAGGGAGGGAGGGAGGGGGAG-3'
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 NC-XRE M2 5'-GTCCCAGCAAGTCACTGGCACCCACCGAGGGAGGGGGAG-3'
 NC-XRE M3 5'-GTCCCAGCAAGTCACTGGGAGAGGAGGGAGGGAGGGGGAG-3'
 NC-XRE M4 5'-GTCCCAGCAAGTCACTGGGAGAAGGGAGGGAGGGGGAG-3'
 NC-XRE M5 5'-GTCCCAGCAAGTCACTGGGAGGGAAAAGAGGGAGGGGGAG-3'

C



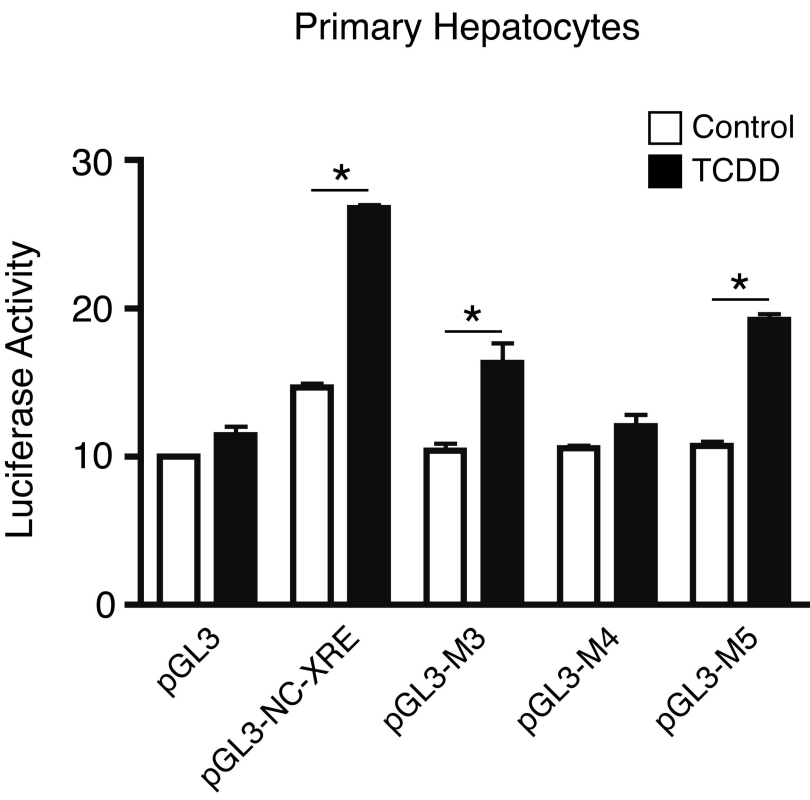
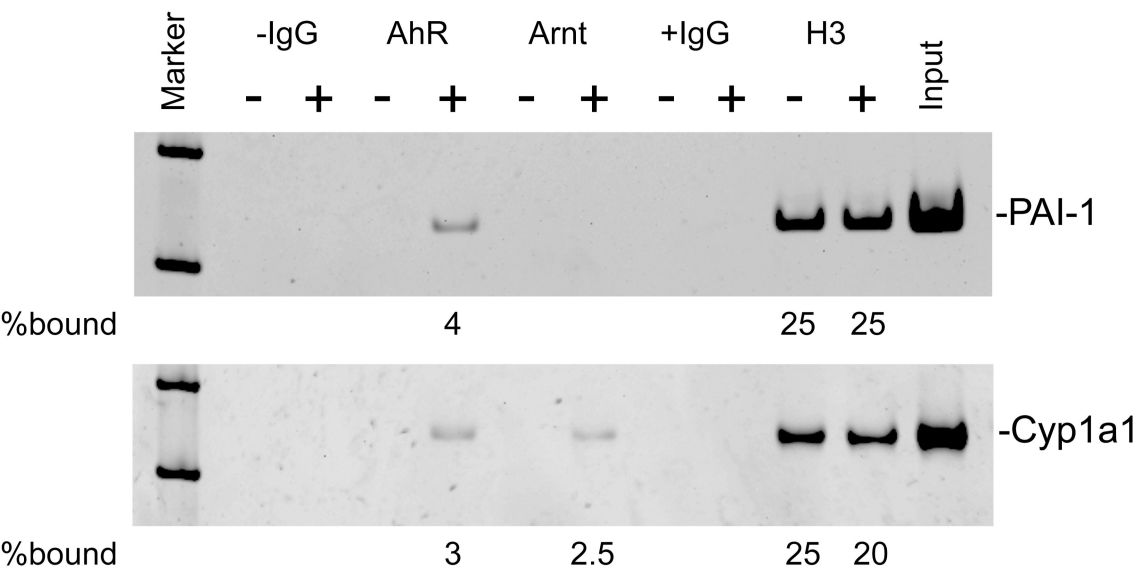
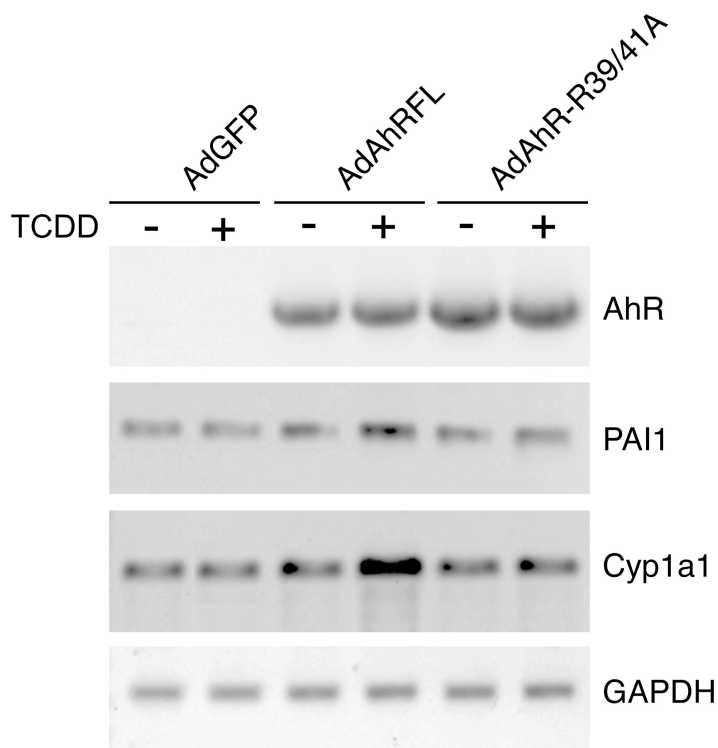


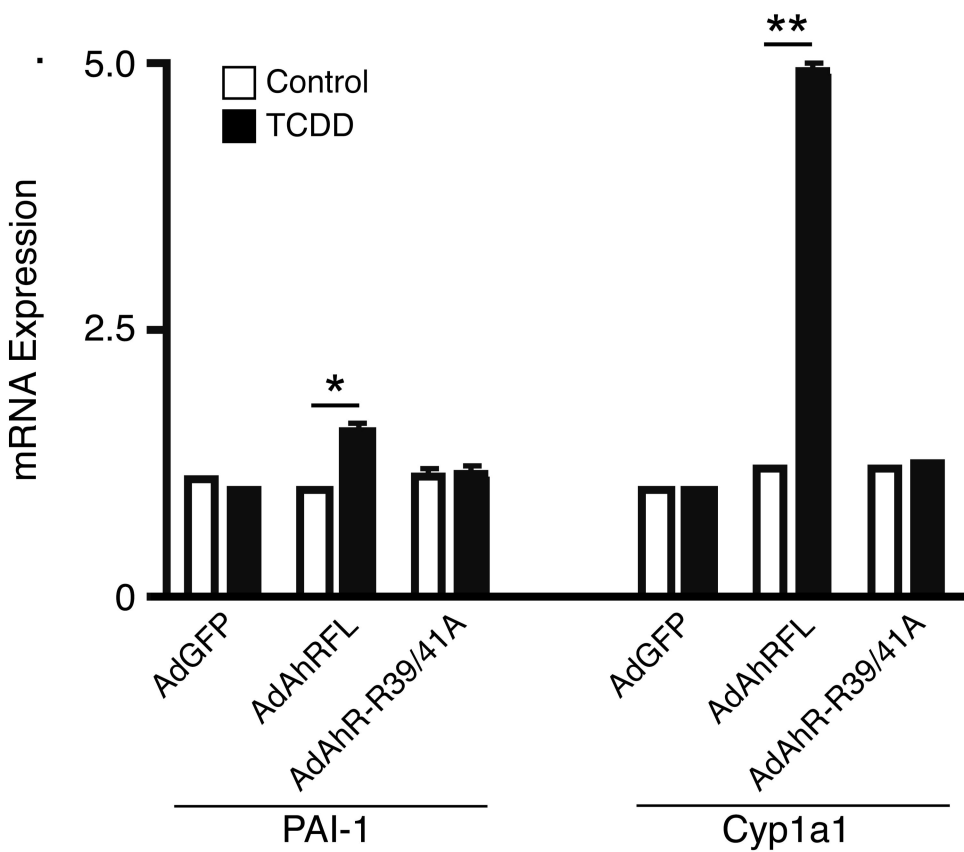
Figure 8



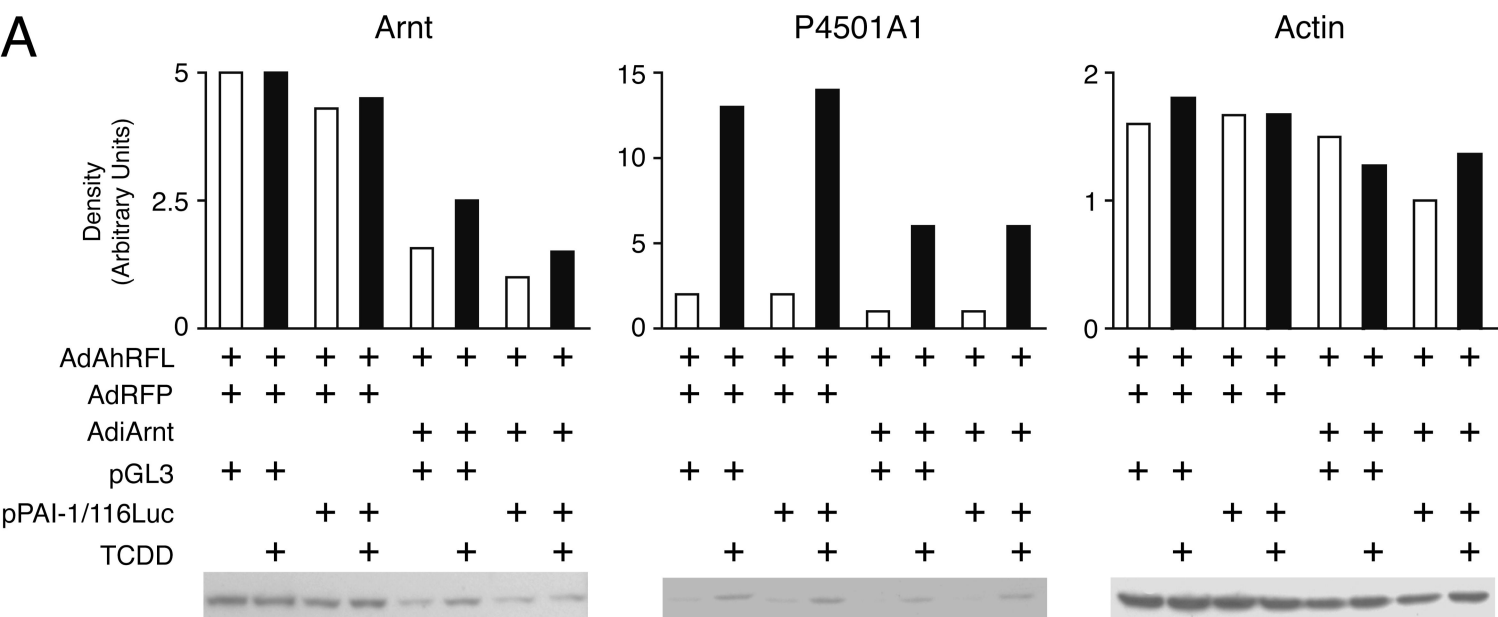
A



B



A



B

