# MOLECULAR MECHANISMS OF ARYL HYDROCARBON RECEPTOR TRANSACTIVATION AND CROSSTALK WITH ESTROGEN RECEPTOR ALPHA

by

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## Abstract

The aryl hydrocarbon receptor (AHR) and estrogen receptor alpha (ERα) are ligand-activated transcription factors. Reciprocal crosstalk between these two receptor systems has been previously established but the exact molecular mechanisms of their interactions remain incompletely understood. Using chromatin immunoprecipitation followed by DNA microarrays (ChIP-chip), I assessed the role of ERα in AHR signalling after dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD) treatment in the T-47D human breast cancer cell line. I determined that ERα is recruited to a subset of AHR target genes suggesting that it is a gene-specific modulator of AHR activity. Transcription factor binding site analysis of our data set also revealed that forkhead motifs were over-represented, implying that they may be important in AHR signalling. To address this, I focused on the regulation of *cyclin G2* (*CCNG2*) to determine the importance of FOXA1 (forkhead box A1) in AHR signalling. CCNG2 is a negative regulator of cell cycle and known to be repressed by ERα. Using ChIP, Co-IP, CCNG2 reporter gene constructs and RNA interference targeting FOXA1, I demonstrated that FOXA1 was important for the AHR-mediated and TCDD-dependent induction of CCNG2. Another finding from the ChIP-chip study was that AHR was recruited to estrogen target genes. To determine the importance of this I used zinc-finger nuclease mediated knockout of AHR and studied ERα signalling as well as the role of AHR in the cell cycle using breast cancer cell lines. Focusing on the regulatory regions of trefoil factor 1 (*TFF-1*) and gene upregulated in breast cancer 1 (*GREB1*) I determined that AHR had an inhibitory effect. Cell cycle analysis indicated that AHR facilitated cell cycle progression with cells accumulating in both the $G_1$ and $G_2$/M phases in the

absence of AHR. My novel findings demonstrated the complexity of AHR-ERα crosstalk, its importance in the cell cycle, and the need for further study.

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# List of Abbreviations

| | |
|---|---|
| 3MC | 3-methylcholanthrene |
| AF1 | Activation function 1 |
| AF2 | Activation function 2 |
| AHH | aryl hydrocarbon hydroxylase |
| AHR | aryl hydrocarbon receptor |
| AHRE | aryl hydrocarbon response element |
| AHRR | aryl hydrocarbon receptor repressor |
| AIP | aryl hydrocarbon receptor interacting protein |
| AP1 | activating protein 1 |
| AR | androgen receptor |
| ARNT | aryl hydrocarbon receptor nuclear translocator |
| B[*a*]P | benzo[*a*]pyrene |
| bHLH-PAS | basic Helix-Loop-Helix-Period-Aryl hydrocarbon receptor nuclear translocator-Single minded |
| BrdU | bromodeoxyuridine |
| CCND1 | cyclin D1 |
| CDK1 | cyclin dependent kinase 1 |
| CDK2 | cyclin dependent kinase 2 |
| CDK4 | cyclin dependent kinase 4 |

| | |
|---|---|
| CCNG2 | cyclin G2 |
| ChIP-chip | chromatin immunoprecipitation coupled with DNA microarray |
| ChIP-seq | chromatin immunprecipitation coupled with DNA sequencing |
| CoCoA | coiled-coil leucine zipper coactivator 1 |
| COX-2 | cyclooxygenase-2 |
| cPLA2 | cytosolic phospholipase A2 |
| CBP | cAMP response element binding protein-binding protein |
| CTCF | CTCC-binding factor |
| CYP1A1 | cytochrome P450 1A1 |
| CYP1A2 | cytochrome P450 1A2 |
| CYP1B1 | cytochrome P450 1B1 |
| DBD | DNA binding domain |
| DCC | dextran coated charcoal |
| DIM | 3,3′-Diindolylmethane |
| DMEM | Dulbecco’s modified Eagle’s medium |
| EGFR | epidermal growth factor receptor |
| ERE | estrogen response element |
| ERα | estrogen receptor alpha |
| ERβ | estrogen receptor beta |
| FACS | fluorescence activated cell sorting |

| | |
|---|---|
| FBS | fetal bovine serum |
| FCS | fetal calf serum |
| FOXA1 | Forkhead box A1 |
| G6Pase | glucose-6-phosphatase |
| GAC63 | GRIP-1-dependent nuclear receptor coactivator |
| GADD34 | Growth arrest and DNA damage-inducible protein 34 |
| HAH | halogenated aromatic hydrocarbon |
| HAT | histone acetyl transferase |
| HDAC | histone deacetylase |
| Hsp27 | 27kDa heat shock protein |
| HSP90 | 90kDa heat shock protein |
| I3C | indole-3-carbinol |
| ICZ | indolo [3,2-b] carbazole |
| Indels | Insertions or deletions |
| LBD | Ligand binding domain |
| MDA | MDA-MB-231 |
| MMTV | mouse mammary tumour virus |
| NCoA1 | nuclear coactivator 1 (SRC1) |
| NCoA2 | nuclear coactivator 2 (SRC2/TIF2) |
| NCoA3 | nuclear coactivator 3 (SRC3/AIB1) |

| | |
|---|---|
| NCoR | Nuclear corepressor |
| NEAA | non-essential amino acids |
| NFκB | nuclear factor kappa B |
| NKX2.1 | NK homeobox 2 protein |
| NLS | nuclear localization sequence |
| p23 | 23kDa co-chaperone protein |
| PAH | polycyclic aromatic hydrocarbon |
| PEPCK | phosphoenolpyruvate carboxykinase |
| PEST | penicillin/streptomycin |
| PI | propidium iodide |
| PKA | protein kinase A |
| PKC | protein kinase C |
| Pol II | RNA polymerase II |
| PP2A | protein phosphatase 2A |
| Rb | Retinoblastoma protein |
| RIP140 | receptor-interacting protein 140 |
| SAhRM | selective aryl hydrocarbon receptor modulator |
| SERM | selective estrogen receptor modulator |
| SHP | small heterodimer partner |
| SMAD3 | SMAD family member 3 |

| | |
|---|---|
| SMARCA1 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 1 |
| SMRT | silencing mediator for retinoid or thyroid-hormone receptor |
| Sp1 | stimulating protein 1 |
| SRB | Sulforhodamine B |
| TAD | transactivation domain |
| TALENs | transcription-activator-like effector nucleases |
| TCDD | 2,3,7,8-tetracholorodibenzo-*p*-dioxin |
| TFBS | transcription factor binding site |
| TFF1 | Trefoil factor 1, also known as pS2 |
| TFIIB | Transcription Factor IIB |
| TFIIF | Transcription Factor IIF |
| TGF-β | Transforming growth factor beta |
| Th17 | T helper 17 cells |
| TiPARP | TCDD-inducible polyADP ribose polymerase |
| TRAP | Thyroid receptor associated protein |
| Tregs | regulatory T cells |
| TSS | transcriptional start site |
| ZFN | zinc finger nucleases |
| αERKO | ERα knockout mice |
| βERKO | ERβ knockout mice |

# Chapter 1: Introduction

## *1* Statement of Research Problem

The aryl hydrocarbon receptor (AHR) and the estrogen receptor α (ERα) are ligand-activated transcription factors. Crosstalk between these two receptor systems has been previously established but the molecular mechanisms of their interactions and its potential impact on breast cancer is currently unknown. It has been proposed that the activation of AHR inhibits ERα signalling through multiple pathways making it a potential therapeutic target for breast cancer. These pathways include: squelching of shared co-activators, synthesis of inhibitory protein(s), increased estrogen metabolism, AHR binding to inhibitory response elements in the promoter regions of ERα target genes, and increased proteasomal degradation of ERα (Reviewed in: Safe and Wormke, 2003). In contrast to these findings, there are reports that show AHR might also enhance ERα signalling (Abdelrahim et al., 2006). The role of ERα in AHR signalling is less studied with its effects ranging from inhibition, to activation, to no effect (Hoivik et al., 1997; Beischlag and Perdew, 2005; Matthews et al., 2005; 2007). Our laboratory has shown recruitment of ERα to the AHR target genes *cytochrome P450 1A1* and *1B1* but it is unclear whether ERα is recruited to other AHR target genes (Matthews et al., 2005). To address this, I will use chromatin immunoprecipitation followed by whole-genome DNA microarrays to determine if ERα is recruited to all AHR target genes after AHR ligand treatment. This will clarify the role of ERα in AHR signalling and help us understand the impact of AHR activation on both signalling pathways.

The aryl hydrocarbon receptor has also been reported to influence the cell cycle (Ge and Elferink, 1998; Elizondo et al., 2000; Marlowe and Puga, 2005; Barhoover et al., 2010). AHR activation inhibits the growth of both ERα positive and negative breast cancer cells suggesting a novel pathway involving AHR with potential to treat breast cancer (Abdelrahim et al., 2003; Zhang et al., 2009; Hall et al., 2010). However, the mechanism of this inhibition is not completely understood. To help elucidate the mechanism, I will study the regulation of a novel AHR target gene, cyclin G2, previously determined to be a negative regulator of the cell cycle and an ERα target gene (Horne et al., 1997; Stossi et al., 2006). I will also assess the impact of cyclin G2 on the AHR-mediated growth-inhibition of breast cancer cells.

Loss-of-function studies to determine the significance of AHR in both ERα signalling and the cell cycle have been previously completed (Abdelrahim et al., 2003). However, these studies have used RNA interference methods, which are associated with multiple limitations including: incomplete knockdown, potential off-target effects, and its transient nature. In order to circumvent the transient nature of RNAi, some researchers have exposed human breast cancer cells to genotoxic agents creating a stable cell line deficient in AHR activity which may inadvertently affect other signalling pathways (Moore et al., 1994; Fong et al., 2005). I propose to use zinc finger-nucleases to knockout AHR in both ERα-positive (MCF-7) and ERα-negative (MDA-MB-231) breast cancer cell lines overcoming the limitations of other methods to study its effects on breast cell proliferation and ERα signalling.

# 2 The aryl hydrocarbon receptor

## 2.1 Discovery

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor. Before the discovery of AHR, scientists were aware that adaptive mechanisms existed to minimize toxicity caused by environmental contaminants (Racker, 1954). This concept was first assessed in early rodent experiments completed in the late 1950s (Conney et al., 1956). In these rodent experiments, treatment with 3-methylcholanthrene (3MC), a polycyclic aromatic hydrocarbon (PAH), led to the induction of a number of liver microsomal enzymes termed the aryl hydrocarbon hydroxylases (AHHs) (Nebert and Bausserman, 1970). The regulation of AHHs was found to vary significantly among inbred mouse strains with the C57BL/6 mice being highly responsive to PAHs, whereas DBA/2 mice were described as non-responsive (Nebert and Bausserman, 1970; 1973). These strain differences led to breeding studies demonstrating that the inheritance of inducibility was an autosomal dominant trait and the genetic locus controlling this inducibility was denoted as the *Ah* locus due to its responsiveness to aryl hydrocarbons (now termed the *Ahr* locus) (Nebert and Bausserman, 1970; Thomas et al., 1972; Nebert, 1989). Further studies were completed to elucidate pathways that regulate AHH expression. This work was made possible with the use of 2,3,7,8-tetracholorodibenzo-*p*-dioxin (TCDD) which was much more potent than 3MC (Poland and Glover, 1974). Using radiolabelled TCDD, Poland and colleagues published the first experimental evidence for a receptor as the cause of AHH induction, now widely referred to as the AHR (Poland et al., 1976).

## 2.2 Structure

The AHR is a member of the bHLH-PAS (basic Helix-Loop-Helix-Period-Aryl hydrocarbon receptor nuclear translocator-Single-minded) family of transcription factors. The AHR is composed of modular domains which include the bHLH, PAS A, PAS B, and transactivation domains (**Figure 1**). The N-terminal bHLH region of AHR is a multi-functional region shown to be important for the nuclear localization and export of AHR (Ikuta et al., 1998; 2000), interaction with both the chaperone protein HSP90 (90kDa heat shock protein (Pongratz et al., 1992)) and its dimerization partner ARNT (aryl hydrocarbon receptor nuclear translocator (Fukunaga et al., 1995; Gu et al., 2000)), and lastly DNA binding (Fukunaga et al., 1995; Ikuta et al., 1998). The PAS region of AHR contains two domains termed PAS A and PAS B. Similar to the bHLH, this region is also a site for HSP90 and ARNT interaction (Antonsson et al., 1995; Fukunaga et al., 1995). The PAS B domain also encompasses the ligand binding domain (LBD) which contains several conserved amino acid residues that are necessary for ligand binding (Goryo et al., 2007).

The C-terminal half of AHR contains the transactivation domain (TAD) that is important for AHR-mediated gene transcription as well as protein-protein interactions (Jain et al., 1994; Rowlands et al., 1996; Watt et al., 2005; Moffat et al., 2007). Within the TAD domain there are three distinct regions: the acidic, glutamine (Q)-rich, and proline-serine-threonine (P/S/T)-rich domains. The acidic region is rich in aspartate (D) and glutamate (E) amino acids which are important in the transactivation of AHR (Jones and Whitlock, 2001). Similar to the acidic region, the Q-rich region is important for the transactivation of AHR but has also been implicated in interactions with coregulatory proteins such as nuclear coactivator 1 (NCoA1) and receptor interacting protein 140 (RIP140) (Kumar and Perdew, 1999; Kumar et al., 1999; 2001). The P/S/T is the least studied region, but has been shown to be important in transactivation albeit less than both the acidic and Q-rich regions (Jain et al., 1994; Rowlands et al., 1996).

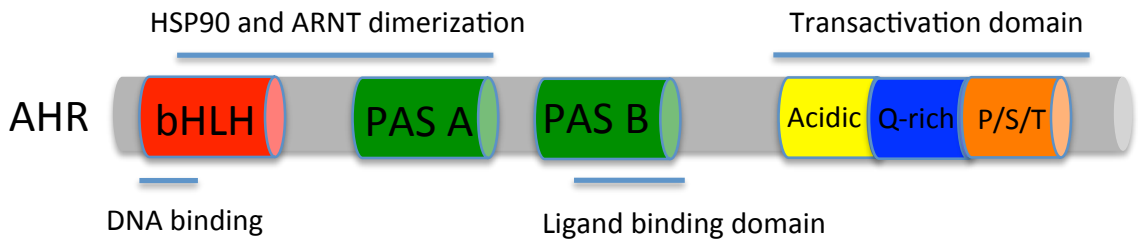



**Figure 1. Schematic of aryl hydrocarbon receptor modular domains.**

**bHLH** (basic-Helix-Loop-Helix), **PAS** (Per (Period) /ARNT (aryl hydrocarbon nuclear translocator) / SIM (Single minded), **P/S/T** (Proline, Serine, Threonine).

## 2.3 AHR ligands

### 2.3.1 Naturally occurring ligands

Ligand binding is considered a critical step for AHR activation (Denison et al., 2002; Denison and Nagy, 2003). Analysis of ligand binding specificity indicates that AHR is a promiscuous receptor, binding a wide variety of structurally diverse chemicals (**Figure 2**) (Hahn, 2002; Denison and Nagy, 2003). Previous work has focused on synthetic ligands to study AHR activation but more recently there has been emphasis on understanding the role of naturally occurring ligands (Stevens et al., 2009; Tilg, 2012). Exposure to naturally occurring ligands comes primarily from our diet (Jeuken et al., 2003). Vegetables or vegetable derived materials are a significant source of AHR ligands. These include indole-3-carbinol (I3C) which can be converted to significantly more potent condensation products ICZ (indolo [3,2-b] carbazole) and the partial agonist DIM (3,3′-diindolylmethane) (Gillner et al., 1993; Jellinck et al., 1993), curcumin (Ciolino et al., 1998), and carotenoids (Gradelet et al., 1996). Flavonoids, including flavones, flavanols, flavanones, and isoflavones are also another group of naturally occurring dietary AHR ligands (Denison and Nagy, 2003); however, the majority of flavonoids act as AHR antagonists. It has been suggested that some flavonoids could be protective by antagonizing dioxin-mediated toxicity (Amakura et al., 2002; Tilg, 2012).

### 2.3.2 Synthetic ligands

The majority of high affinity AHR ligands are synthetic in nature. These include the planar, hydrophobic halogenated aromatic hydrocarbons (HAHs) such as dioxins, dibenzofurans, and biphenyls and the PAHs such as 3MC, benzo[*a*]pyrene (B[*a*]P), and benzoflavones. Of these two

classes of compounds, the HAHs are the most potent and stable AHR ligands with binding affinities in the pico-molar to nano-molar range while the PAHs are more labile with lower binding affinities closer to the nano-molar and micro-molar range (Bandiera et al., 1982; Piskorska-Pliszczynska et al., 1986; Denison and Nagy, 2003). HAHs are persistent in the environment due to their hydrophobicity, chemical stability, and resistance to metabolism by xenobiotic metabolizing enzymes. Detectable levels of these contaminants are present in all humans (Schecter and Olson, 1997). HAHs are released into the environment during combustion processes such as the incineration of industrial and household waste and through the bleaching of paper (Schecter et al., 2006). TCDD represents the prototypical ligand used in the study of AHR signalling and is the most potent activator of AHR (Denison and Heath-Pagliuso, 1998). PAHs are primarily formed by incomplete combustion of carbon-containing material such as coal, diesel fuel, tar, and plant materials (Boström et al., 2002). Due to the high level of exposure to many of these chemicals in our daily life, understanding AHR activation is critical to determine their mechanism of toxicity.

### 2.3.3 Candidate endogenous ligands

There have been many reports suggesting the existence of an endogenous ligand (Carlson and Perdew, 2002; Walisser et al., 2004; McMillan and Bradfield, 2007; Nguyen and Bradfield, 2008; Rannug, 2010). The most striking evidence comes from the fact that AHR has been highly conserved throughout evolution, the manifestation of significant developmental and physiological defects in *Ahr*-null mice, and its role in cell cycle progression. Phylogenetic analysis of the AHR reveals that the primary amino acid sequence of the ligand-binding domain (PAS B) is highly conserved across diverse vertebrate species (Walker et al., 2000). This observation suggests that environmental stressors do not influence ligand specificity since these species are exposed to very different environments but that it is maintained due to a similar endogenous ligand. Additionally, the major defects seen in the *Ahr*-null mice provide evidence of an endogenous activator of AHR. *Ahr*-null mice have a high neonatal lethality rate, inflammation of the bile ducts, depletion of splenic lymphocytes, cardiohypertrophy, skin lesions, portal vascular hypertrophy, and the most consistent phenotype: the patent *ductus Venosus* leading to decreased liver size (Fernandez-Salguero et al., 1995; 1997; Lahvis and Bradfield, 1998; Lahvis et al., 2000; Barouki et al., 2007). There are also reports suggesting that AHR target genes are upregulated during embryonic development. In mice carrying the *Cyp1a1*

promoter driven by a *lacZ* transgene, activity was seen in the hind- and midbrain, heart, kidney, tail, skin, and muscle during various embryonic days (Campbell et al., 2005). Human fetal analysis revealed CYP1A1 expression in the adrenal glands, lung, and liver (Omiecinski et al., 1990). Recent studies have also reported that AHR is important for cell cycle progression in the absence of an exogenous ligand. Progression through the $G_1$ phase of the cell cycle has been shown to require AHR, which physically interacts with cyclin dependent kinase 4 (CDK4) and cyclin D1 (CCND1) serving as a scaffolding protein to promote the CDK-dependent phosphorylation of retinoblastoma protein (Rb) leading to S phase entry (Puga et al., 2000; Barhoover et al., 2010). Candidate endogenous ligands include tryptophan derivatives (Oberg et al., 2005; Rannug, 2010), arachidonic acid metabolites (Schaldach et al., 1999), products of heme degradation (Phelan et al., 1998), cholesterol derivatives (Savouret et al., 2001), and low density lipoproteins (McMillan and Bradfield, 2007). The most potent and highest affinity candidate endogenous ligand is the tryptophan photoproduct FICZ (6-formylindolo [3,2-b] carbazole) (Rannug et al., 1987; Rannug, 2010). FICZ can be formed by photolysis of tryptophan with both visible and UV light. This compound has been found in human urine samples and was shown to transiently induce CYP1A1 and competitively displace TCDD with AHR affinity in nM range (Rannug et al., 1987; Helferich and Denison, 1991). In contrast to TCDD, FICZ is a substrate for the Phase I enzymes CYP1A1, CYP1A2, and CYP1B1 thereby participating in an autoregulatory feedback loop that maintains its own concentrations at low levels (Wincent et al., 2009).

## 2.4 AHR signal transduction

### 2.4.1 Canonical pathway

The aryl hydrocarbon receptor in the absence of ligand is present in the cytoplasm bound to a multi-protein complex. This complex includes HSP90, aryl hydrocarbon receptor interacting protein (AIP, or XAP2/ARA9), and the 23kDa co-chaperone protein (p23) (Kazlauskas et al., 1999; Petrulis and Perdew, 2002; Petrulis et al., 2003). The HSP90 has been shown to be important in stabilizing AHR in the cytoplasm, inhibiting nuclear translocation by masking the nuclear localization sequence (NLS), protecting it against degradation, preventing premature ARNT binding, and stabilizing the high affinity ligand-binding conformation of AHR (Pongratz et al., 1992; Petrulis and Perdew, 2002; Petrulis et al., 2003). The AIP works to further stabilize the chaperone complex, protects AHR against degradation, and influences the nuclear

localization and transactivation of AHR (Meyer and Perdew, 1999; Kazlauskas et al., 2000; Petrulis and Perdew, 2002; Petrulis et al., 2003). The p23 protein has been shown to stabilize the chaperone complex and play a role in the nuclear import of AHR (Kazlauskas et al., 1999). These three proteins also play important roles in the ligand-independent nucleocytoplasmic shuttling of AHR (Ikuta et al., 1998; Pratt et al., 2004; Pollenz et al., 2005).

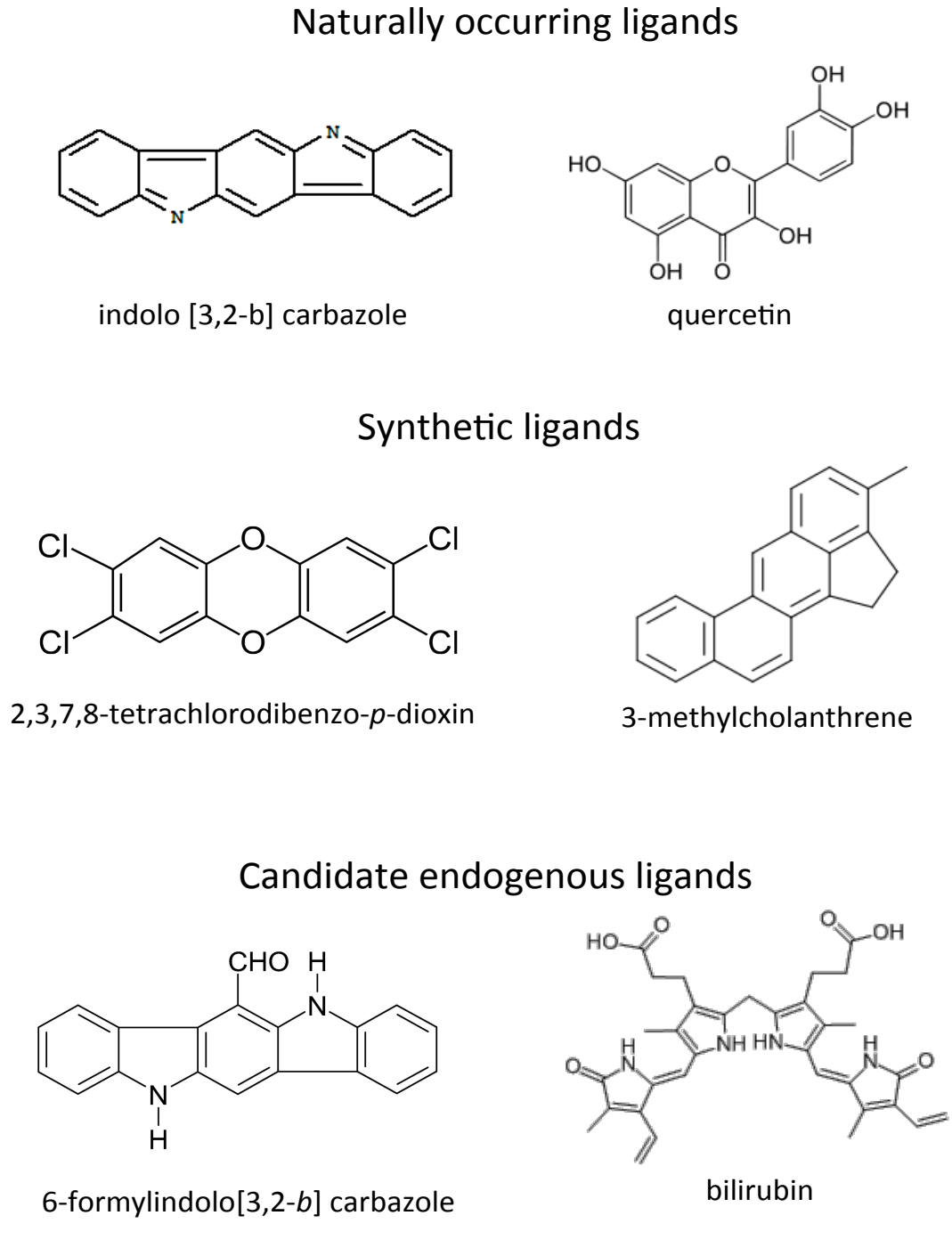



**Figure 2. Structures of select aryl hydrocarbon receptor ligands.**

Most AHR ligands enter the cell through simple diffusion due to their highly lipophilic nature (Denison and Nagy, 2003; Goryo et al., 2007). Ligand binding induces a conformational change in AHR exposing its NLS that was initially masked by HSP90 resulting in nuclear translocation. The mechanism of this translocation is currently unknown but has been suggested to involve importin-β mediated translocation via nuclear pores (Petrulis et al., 2003; Pratt et al., 2004). Once in the nucleus, AHR dissociates from the chaperone complex and heterodimerizes with its dimerization partner aryl hydrocarbon nuclear translocator (ARNT), a related PAS protein. Ligand bound AHR that does not dimerize with ARNT is shuttled back to the cytoplasm and degraded by the ubiquitin-proteasome pathway (Roberts and Whitelaw, 1999). Once complexed with ARNT, AHR is converted into its high affinity DNA binding form, facilitating its binding to specific DNA recognition sites termed aryl hydrocarbon response elements (AHREs) located in the regulatory regions of its target genes (Probst et al., 1993; Bacsi and Hankinson, 1996; Denison and Nagy, 2003). The consensus AHRE has been experimentally determined to be 5`-TnGCGTG-3` while the core GCGTG sequence is required for AHR-ARNT binding (Shen and Whitlock, 1992; Lusska et al., 1993; Swanson et al., 1995). The ligand:AHR:ARNT complex with chromatin then initiates the recruitment of coregulatory proteins which modulate the transcription of target genes. These include coactivators that modify histones (SMARCA1, also known as BRG1, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 1), histone acetyl transferases (Nuclear coactivator 1-3; NCoA1-3), co-integrators such as CBP/p300 (CREB binding protein-binding protein/protein 300), various coactivators whose mechanism of activation is unknown (RIP140, CoCoA, GAC63), basal transcription factors (RNA polymerase II, TFIIB, TFIIF) as well as corepressors (SMRT, SHP) (Hankinson, 2005).

#### 2.4.1.1 Coregulatory proteins

Coactivators encompass a wide variety of proteins that enhance gene transcription either through modifying histones to increase DNA accessibility, or as adaptors to stabilize the pre-initiation complex (Näär et al., 2001). SMARCA1 is a histone modifier that uses ATP to break the hydrogen bonds associating DNA with histones leading to the de-condensation of chromatin. Wang and Hankinson (2002) demonstrated that transient transfection of this protein enhanced reporter gene and endogenous *Cyp1a1* expression and was recruited to the regulatory region of *Cyp1a1*. Another important class of coactivators is the histone acetyl transferases (HATs), which

acetylate lysine residues on histones resulting in the opening of chromatin further facilitating the binding of other proteins to DNA. The most well characterized HATs involved in AHR-mediated gene transcription are the nuclear coactivator family (NCoA1-3), previously known as the p160 class of proteins (Beischlag et al., 2002). NCoA1 enhances nuclear receptor transcription by interacting with other coregulatory proteins that have high HAT activity such as NCoA3, CBP, and TFIIB (Yao et al., 1996; Lemon and Freedman, 1999). Both NCoA2 and NCoA3 have been shown to enhance AHR-mediated gene transcription (Beischlag et al., 2002; Matthews et al., 2005). These studies demonstrated recruitment of the NCoA proteins to the regulatory region of human *CYP1A1*. Furthermore, through Co-IP experiments NCoA proteins were shown to be part of the mouse Ahr/Arnt complex (Beischlag et al., 2002).

The co-integrator protein, CBP has been shown to have intrinsic HAT activity but also facilitates the formation of the pre-initiation complex (Kobayashi et al., 1997). CBP interacts with both AHR and ARNT and is recruited to the mouse *Cyp1a1* regulatory region (Kobayashi et al., 1997; Tohkin et al., 2000; Wang et al., 2004). CBP also interacts with p300, a HAT known to interact with AHR (Tohkin et al., 2000; Hankinson, 2005; Powis et al., 2011).

The coactivator/corepressor RIP140 is involved in both nuclear receptor and AHR-mediated gene transcription. In nuclear receptor mediated transcription, RIP140 mainly represses ligand-dependent gene expression by competing with other coactivators for shared binding sites or recruiting HDACs (histone deacetylases) (Augereau et al., 2006; Carascossa et al., 2006; Gurevich et al., 2007). However, the impact of RIP140 on AHR-mediated transcription is not clearly understood. One group has shown that it enhances AHR-mediated gene transcription (Kumar et al., 1999) unlike nuclear receptor signalling. However, other groups have shown that it is not part of the AHR complex at the *CYP1A1* regulatory region (Matthews et al., 2005; Watt et al., 2005). In a more recent paper (Madak-Erdogan and Katzenellenbogen, 2012), it has been suggested that the coactivator or corepressor function of RIP140 on AHR-mediated transcription is related to the presence or absence of ERα at the regulatory region of target genes. They demonstrate that after TCDD treatment, RIP140 acts as a coactivator at regions occupied by AHR but not ERα, whereas at regions co-occupied by both AHR and ERα, RIP140 acts as a corepressor. This suggests that enzymes in complex with ERα may cause post-translational modifications to RIP140 changing its coregulatory function (Ho et al., 2008; Madak-Erdogan et al., 2011).

CoCoA (coiled-coil leucine zipper coactivator 1) and GAC63 (GRIP-1-dependent nuclear receptor coactivator) are coactivators shown to be involved in AHR-mediated transcription (Kim and Stallcup, 2004; Chen et al., 2006). Both contain C-terminal acidic activation domains that suggest they may recruit other co-regulatory proteins such as the NCoAs to mediate AHR transactivation (Kim and Stallcup, 2004; Chen et al., 2006).

AHR also interacts with basal transcription factors. The assembly of the activated complex requires the association of TFIID complex with the TATA box. Proteins comprising the TFIID complex have been shown to directly interact with AHR, which include TFIIB, TFIIF, and RNA polymerase II (Rowlands et al., 1996; Swanson and Yang, 1998; Hankinson, 2005; Watt et al., 2005).

The mechanism of transcriptional repression occurs through various pathways. Corepressors can interfere with the formation of the activated complex by outcompeting coactivators for binding, condensing chromatin through deacetylation or methylation of histones, or recruiting other proteins that contain HDAC or methyltransferase activity. The role of corepressors in AHR-mediated signalling is not well established and only a limited number of corepressors have been reported to modulate AHR (Marlowe et al., 2008; Pansoy et al., 2010). SMRT (also known as NCoR2) was first characterized as a repressor of nuclear receptor activity (Leo and Chen, 2000; Goodson et al., 2005). SMRT contains several sites for HDAC binding which is thought to be responsible for the repressive activity of SMRT. The inhibition of AHR signalling by SMRT appears to be context specific. One group reported that SMRT directly interacted with the AHR/ARNT complex inhibiting *CYP1A1* reporter gene activity (Nguyen et al., 1999), while another group showed that the repressive actions might be species specific (Rushing and Denison, 2002). They demonstrated that transient transfection of SMRT with mouse *Cyp1a1* reporter gene does not repress luciferase activity but actually enhances it while transient transfection of human *CYP1A1* reporter gene causes a significant reduction in luciferase activity (Rushing and Denison, 2002). SHP or small heterodimer partner is another corepressor and a member of the nuclear receptor superfamily that lacks a DNA binding domain (Kim et al., 2001). It is thought to repress transcription of nuclear receptors through two distinct mechanisms. The first is by associating with other nuclear receptors leading to a non-productive heterodimer unable to bind to DNA as well as competing with coactivators (Båvner et al., 2005). In AHR

signalling, SHP was shown to inhibit AHR transcription by inhibiting AHR:ARNT complexes from binding to DNA (Klinge et al., 2001).

#### 2.4.1.2 Negative regulation of AHR signalling

The attenuation of AHR signalling has been suggested to occur via two distinct mechanisms. The first is that activated AHR is targeted for degradation by the ubiquitin-proteasome pathway after binding to DNA thereby limiting AHR activation (Ma and Baldwin, 2002; Pollenz and Buggy, 2006; Pollenz, 2007). This event has been shown to occur via the 26S proteasome and can be inhibited by treatment with the protein synthesis inhibitor, cycloheximide. The ability of cycloheximide to block the degradation of AHR supports the existence of a labile repressor protein not yet identified (Ma and Baldwin, 2000; Ma et al., 2000). Studies completed in our laboratory and by others demonstrate that after ligand treatment, AHR levels decrease to less than 10 percent by Western blot analysis (Ma and Baldwin, 2002; Pansoy et al., 2010). However, the ligand-dependent degradation of AHR is ligand-structure dependent as well as cell context-dependent (Jin et al., 2012).

The second pathway involves the aryl hydrocarbon receptor repressor (AHRR), another member of the PAS family which is upregulated after AHR ligand treatment (Mimura et al., 1999; Pansoy et al., 2010). Although it is well established that AHRR inhibits AHR signalling, the mechanism by which this is achieved remains unclear. It is proposed that AHRR competes with AHR for dimerization with ARNT and it also competes with AHR:ARNT complexes for DNA binding (Haarmann-Stemmann et al., 2007; Hahn et al., 2009). However, another report has suggested that AHRR-mediated repression is likely to be more complex (Evans et al., 2008). Their studies demonstrate that neither ARNT overexpression nor AHRR mutants defective in AHRE binding abolished the AHRR-mediated repression of AHR (Evans et al., 2008). Their results suggest an additional mechanism may be involved. They hypothesized that the mechanism of inhibition involves AHRR interacting with the AHR complex in a DNA binding-independent manner and required AHRR-dependent protein-protein interactions (Evans et al., 2008; Hahn et al., 2009). However, the specific proteins involved are still unknown.

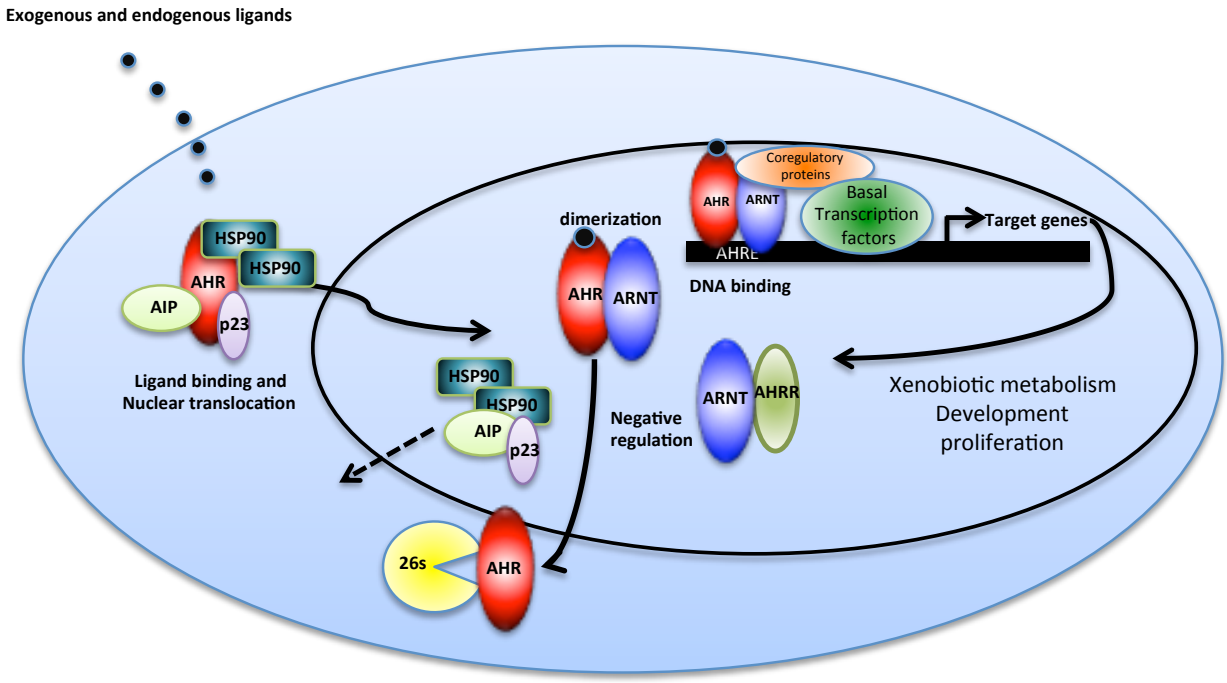



**Figure 3. The canonical pathway of AHR-mediated signal transduction.**

Upon ligand binding, AHR, which is present in the cytoplasm bound to a chaperone complex, translocates to the nucleus where it dissociates from its chaperone complex and dimerizes with ARNT. The ligand:AHR:ARNT complex then binds to AHREs in the regulatory region of its target genes resulting in the recruitment of coregulatory proteins and basal transcription factors. AHR is negatively regulated by the AHRR protein and through proteasomal degradation.

### 2.4.1.3 The Adaptive Response

Activation of the canonical pathway induces the adaptive response. The adaptive response is defined as the induction of xenobiotic metabolizing enzymes (Denison and Nagy, 2003; McMillan and Bradfield, 2007). AHR binding to exogenous ligands leads to the transcriptional activation of a battery of genes that promote the metabolic transformation and excretion of these substances (Nebert et al., 2004; McMillan and Bradfield, 2007; Nguyen and Bradfield, 2008). Some of the genes that are upregulated upon AHR activation are the Phase I and Phase II xenobiotic metabolizing enzymes such as cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1, UDP-glucuronosyl transferase, NAD (P)H: quinone oxidoreductase, aldehyde-3-dehydrogenase, and glutathione S-transferase. The function of the adaptive response is to increase ligand

metabolism and clearance to re-establish homeostatic levels of AHR activators (Beischlag et al., 2008).

A complete understanding of the tight balance cells must maintain to eliminate the potentially harmful chemicals through metabolism while minimizing the generation of more reactive metabolites is not fully understood. The PAH B[*a*]P is an example where a balance must be maintained in order for AHR activation to be beneficial requiring the coordination of Phase I and Phase II enzymes (Boström et al., 2002; Nebert et al., 2004). Metabolism of B[*a*]P by Phase I enzymes first generates a variety of products including benzo[a]pyrene-7,8-epoxide. This product is then further metabolized by epoxide hydrolase to open the epoxide ring to yield benzo[*a*]pyrene-7,8-dihydrodiol which is further metabolized by Phase I enzymes to generate benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide which can form DNA adducts leading to genotoxicity (Jiang et al., 2007). However, conjugation by Phase II enzymes will rescue the cell from these harmful epoxides.

#### 2.4.1.4 The Toxic Response

The toxic response is distinct from the adaptive response but requires the activation of the canonical pathway (Schmidt and Bradfield, 1996; Bunger et al., 2003; 2008). Both HAHs and PAHs have been determined to induce the toxic response; however, HAHs have received more attention due to their environmental persistence and acute toxicity. Exposure to PAHs and HAHs has been shown to have carcinogenic potential. The dioxin-mediated toxic endpoints observed in animals include tumour promotion, teratogenesis, lymphoid involution, and wasting syndrome (Poland and Knutson, 1982). In addition, there are significant species differences in the endpoints observed and the dose of TCDD required to elicit a specific response. For example, the $LD_{50}$ for acute TCDD exposure varies from 1 μg/kg in the guinea pig to 20-40 μg/kg in the rat, 70 μg/kg in the monkey, 114 μg/kg in the mouse and rabbit, and 5000 μg/kg in the hamster (Poland and Knutson, 1982).

Kociba *et al.* (1978) first showed that chronic dietary exposure of TCDD resulted in an increased number of hepatocellular carcinomas and squamous cell carcinomas of the lung, hard palate, and nasal turbinate in female Sprague-Dawley rat. Later studies showed that TCDD can cause these effects through its ability to act as a tumour promoter (Pitot et al., 1980). TCDD is not a tumour initiator since it does not bind directly to DNA to form adducts, a characteristic necessary to be

classified as an initiator (Poland and Glover, 1974). Other proposed mechanisms for the carcinogenic effects of TCDD include Phase I enzyme-mediated activation of other carcinogens or endogenous compounds such as estrogen, DNA single-strand breaks caused by lipid peroxidation, and alteration in cell proliferation through transcriptional regulation of cytokines and growth factors (Huff et al., 1994; Marlowe and Puga, 2005).

TCDD was shown to be a classic teratogen by inducing cleft palate in exposed mice (Courtney and Moore, 1971). TCDD induces cleft palate by altering the proliferation and differentiation of epithelial cells by modulating the expression of TGF-β (transforming growth factor β) proteins shown to be important in both processes. TCDD-treated mice had decreased expression of the TGF-β1 isoform in the palatal epithelium (Abbott and Birnbaum, 1990). During normal embryonic development, mice first form palatal shelves that are then later fused together. However, in mice treated with TCDD, growth of the palatal shelves occurs normally but they do not fuse (Pratt et al., 1984).

Thymic involution and immunosuppression are the most consistent toxic effects of TCDD across species (Poland and Glover, 1980). TCDD-dependent thymic involution is proposed to occur by decreasing the maturation and response to mitogens in the thymic epithelium. TCDD can suppress the immune system by suppressing B lymphocytes responses as well as decreasing antibody production in response to T cell-dependent and –independent antigens (Schmidt and Bradfield, 1996; Esser et al., 2009).

TCDD-activated AHR induces a lethal wasting syndrome in rodents (Schmidt and Bradfield, 1996). TCDD treatment produces a starvation-like state in which gluconeogenesis and food intake are both decreased (Poland and Glover, 1980; Stahl et al., 1993). This is in contrast to normal starvation states which increase gluconeogenesis and stimulate food intake (Yoon et al., 2001). It has been proposed that TCDD decreases gluconeogenesis by upregulating the PARP family member TiPARP (TCDD-inducible poly-ADP ribose polymerase) which then decreases the expression and activity of PEPCK (phosphoenolpyruvate carboxykinase) and G6Pase (glucose-6-phosphatase), two important enzymes involved in gluconeogenesis (Diani-Moore et al., 2010).

Human exposure to dioxin-like compounds occurs through the environment, industrial accidents, occupational contamination, and dietary sources. The most prominent route of human exposure

is through contaminated food primarily from animal fat (van Leeuwen et al., 2000; Huwe et al., 2009). Contamination of food is caused by the deposition of emissions from sources like waste incineration, improper disposal of sewage sludge, disposal of toxic chemicals on farmland and subsequent bioaccumulation in the food chain (van Leeuwen et al., 2000). Accumulation occurs mainly in animal fat due to the lipophilic nature of these compounds. High-level human exposure to dioxin-like compounds has occurred through acute exposures from industrial accidents and pesticide use (Pesatori et al., 2009; Schecter et al., 2009) . The hallmark of high-level TCDD exposure in humans is chloracne (Panteleyev and Bickers, 2006). Other effects of dioxin exposure in humans include: increased mortality from cardiovascular pathologies, altered levels of luteinizing and follicle stimulating hormones, endometriosis, diabetes, immune system disruption, and increased cancer risk (Sweeney and Mocarelli, 2000; Arisawa et al., 2005; Pesatori et al., 2009; Boffetta et al., 2011; Warner et al., 2011).

### 2.4.2 The non-genomic pathway

The canonical pathway of ligand activated AHR leading to direct DNA binding and gene expression does not fully explain some of the toxic and physiological responses seen both *in vitro* and *in vivo* (Matsumura, 2009). Furthermore, some of these responses do not require ARNT, supporting that these downstream effects occur independent of DNA binding (Dong and Matsumura, 2008).

Kinase activity has been implicated as the driving force behind the non-genomic pathway. PKC (protein kinase C), PKA, and Src activation have been documented after TCDD treatment. Hannenman *et al* (1996) have shown that PKCα activation takes place in primary cultures of hippocampal neurons within 10 minutes of TCDD exposure which was accompanied by a rise in intracellular calcium. This group attributed the rise of calcium levels as the trigger for PKC activation. However, it is unclear how calcium levels increase but some have attributed it to direct interaction with calcium channels (Dong and Matsumura, 2008; Dong et al., 2010).

There is growing evidence that TCDD promotes the production of inflammation mediating cytokines and chemokines through the non-genomic pathway (Vogel et al., 2007). These signalling molecules have been shown to activate both cPLA2 (cytosolic phospholipase A2) and COX-2 (cyclooxygenase-2) in order to transduce a signal that activates the inflammatory

pathway, particularly the NFκB (Nuclear factor Kappa B) pathway (Vogel et al., 2007; Sciullo et al., 2008; Matsumura, 2009).

## 2.5 AHR is more than a xenobiotic sensing protein

### 2.5.1 Role in development

Studies in AHR knockout mice revealed that AHR plays an important role in development (Fernandez-Salguero et al., 1997; Lahvis et al., 2005; McMillan and Bradfield, 2007). This is supported by studies in invertebrates, which demonstrate AHR homologs are critical for development. *C. elegans* contain an ortholog of the mammalian AHR (*ahr-1*) which has been shown to be important in neuronal development. *Ahr-1* plays a role in GABAergic ring motor neuron development where the loss of *ahr-1* interrupted the normal distribution and gene expression patterns of the four subtypes of cells (Huang et al., 2004). *D. melanogaster* contains *Spineless*, another AHR ortholog, which belongs to a group of genes involved in homeosis; defined as the process of transforming cells into different body parts (Struhl, 1982; Burgess and Duncan, 1990; McMillan and McGuire, 1992). *Spineless* has been implicated in distal antenna identity (McMillan and McGuire, 1992). Similar to *ahr-1*, *Spineless* may also have an essential role in neuronal development by modulating the morphology of the peripheral nervous system (Kim et al., 2006). Moreover, it is also a master regulator of *D. melanogaster* photoreceptor mosaic required for colour vision (Wernet et al., 2006). The most compelling evidence supporting the notion that AHR is involved in development comes from *Ahr*-null mice (Gonzalez et al., 1995; Schmidt and Bradfield, 1996; Fernandez-Salguero et al., 1997; Lahvis et al., 2000; McMillan and Bradfield, 2007). These mice exhibit many abnormalities including a patent *ductus Venosus*, decreased liver size, resistance to TCDD-induced thymic atrophy and cleft palate, resistance to B[*a*]P induced carcinogenesis, and decreased reproductive success in females (Fernandez-Salguero et al., 1995; Schmidt and Bradfield, 1996; Abbott et al., 1999; Mimura et al., 1999; Lahvis et al., 2000; Shimizu et al., 2000; Lahvis et al., 2005).

The most well characterized phenotype is the reduced liver size due to a persistent fetal vascular structure (Lahvis et al., 2000; 2005). During embryonic development, a shunt termed *ductus Venosus* directs the flow of blood away from the liver portal vein to the inferior vena cava. In normal development, after birth the shunt closes and normal blood flow is returned to the liver. However, in *Ahr*-null mice, the *ductus Venosus* remains open depriving the liver of increased

blood flow. *Ahr*-null female mice also exhibit reproductive abnormalities, including (Abbott et al., 1999; Baba et al., 2005) more difficulties conceiving, reduced litter number, and pup survival. This has been linked to the potential role AHR plays in the ovarian follicle since *Ahr*-null female mice have a reduced number of mature follicles. Disruption of follicle maturation has been previously associated with reduced estradiol synthesis (Baba et al., 2005). Data suggest that AHR has an intrinsic function in the ovaries by adjusting estradiol levels through the regulation of Cyp19 which is not maintained in the *Ahr*-null mice (Baba et al., 2005; Barnett et al., 2007). Resistance to TCDD-induced thymic atrophy in the *Ahr*-null mice has been associated with reduced T cell development and T helper cell differentiation (Fernandez-Salguero et al., 1995). The mechanism of resistance to cleft palate formation in the *Ahr*-null mice is currently unknown but may be linked to the gene *Cmt1 (*chemically-mediated teratogenesis-1)(Mimura et al., 1997; Thomae et al., 2006). Resistance to B[*a*]P-induced carcinogenesis in the *Ahr*-null mice is due to the absence of drug metabolizing enzyme induction thereby reducing the production of metabolically active genotoxic metabolites (Shimizu et al., 2000).

### 2.5.2 Role in cell proliferation

#### 2.5.2.1 Activation of immediate early genes

During the last 15-20 years there has been a large shift in our understanding of AHR signalling. It has become increasingly evident that AHR plays an important role in cell cycle regulation. However, there appears to be two distinct mechanisms at play. In the absence of an exogenous ligand AHR promotes cell cycle progression while in the presence of a high affinity HAH or PAH it causes $G_0/G_1$ arrest and reduces the ability for DNA replication (**Figure 4**) (Puga et al., 2000; Elferink et al., 2001; Puga et al., 2002a; 2002b; Abdelrahim et al., 2003; Ito et al., 2004; Marlowe and Puga, 2005; Marlowe et al., 2008; Chopra and Schrenk, 2011).

The first line of evidence that AHR is involved in the control of cell cycle is that upon AHR agonist treatment there is upregulation of immediate early genes causing proliferation (Enan and Matsumura, 1994; Worner and Schrenk, 1996; Schwarz et al., 2000; Marlowe and Puga, 2005). The activation of immediate early genes has been mainly studied in the liver where AHR has been implicated as a tumour promoter (McGregor et al., 1998). Both dioxins and PCBs (polychlorinated biphenyls) exhibit tumour promoting activity (Anderson et al., 1994; Hemming et al., 1995; Worner and Schrenk, 1996; Moennikes et al., 2004). These compounds cause

promotional activity since they are slowly metabolized leading to sustained activation of AHR (Beebe et al., 1995). The proliferative effects of TCDD in the liver have been tested both *in vivo* and *in vitro* producing some inconsistent results. TCDD has been shown to inhibit hepatocyte proliferation stimulated by partial hepatectomy (Bauman et al., 1995), while others show increases in proliferation of normal hepatocytes (Lucier et al., 1991; Fox et al., 1993). In culture there are also conflicting results where in mouse hepatoma cells (Hepa 1c1c7), the activated AHR upregulates pro-mitogenic signals to induce proliferation while in other cell lines AHR been shown to either decrease or have no effect on proliferation (Gottlicher and Wiebel, 1991; Schrenk et al., 1992; 1994; Ma and Whitlock, 1996).

TCDD has been shown to regulate pro-mitogenic signals (Whitelaw et al., 1991; Köhle et al., 1999; Schwarz et al., 2000; Marlowe and Puga, 2005). One of these signals is the Ras-dependent signalling cascade. Activation of Ras may be a consequence of TCDD-mediated activation of Src kinase (Köhle et al., 1999). It has been shown that Src kinase is in complex with HSP90 and that upon ligand binding both AHR and Src kinases are released from HSP90 (Enan and Matsumura, 1994; Köhle et al., 1999). TCDD-induced nuclear localization of c-Src causes the phosphorylation of EGFR (epidermal growth factor receptor) which can then activate TGF-$\alpha$ and signal Ras as well as other downstream effectors (Gaido et al., 1992; Tullis et al., 1992; Park et al., 1998). A downstream effector of Ras in the nucleus is the AP-1 family of transcription factors in which both c-Fos and c-Jun have been shown to be activated upon AHR stimulation. This family of transcription factors are important for progress out of the $G_1$ phase of the cell cycle (Hoffer et al., 1996)

#### 2.5.2.2 Ligand-independent cell cycle control

Proliferating cells are continuously progressing through the cell cycle with the help of cyclins, cyclin dependent kinases (CDKs), and cyclin dependent kinase inhibitors (CKI). These proteins are responsible for the ordered transition between the phases whose expression is controlled by retinoblastoma (Rb) and the E2F family of proteins (Coqueret, 2002; Trimarchi and Lees, 2002; Marlowe and Puga, 2005). AHR interacts with both CDKs and Rb to promote its ligand-independent effects on the cell cycle (Ge and Elferink, 1998; Barhoover et al., 2010) (**Figure 4**).

Much of the literature supporting the ligand-independent effects of AHR comes from experiments completed using RNAi targeting AHR as well as from *Ahr*-null mice studies. *Ahr*-

null mice display tissue specific effects related to cell cycle control. There is hyperproliferation of hair follicles and liver blood vessels, but increased levels of apoptosis in liver tissue (Gonzalez and Fernandez-Salguero, 1998). Embryonic fibroblasts isolated from *Ahr*-null mice (MEFs) show reduced proliferation rates, increased apoptosis, and an accumulation of cells in the $G_2$/M phase which has been attributed to altered expression of two $G_2$/M kinases: cdc2 (also known as CDK1; cyclin dependent kinase 1) and Plk (polo-like kinase 1) (Elizondo et al., 2000). The mechanism of increased apoptosis has been shown to occur through TGF-β1 signalling (Oberhammer et al., 1992a; 1992b; Schulte-Hermann et al., 1995). TGF-β1 is a cytokine that binds to and activates serine/threonine receptor kinases that send signals into the nucleus via Smad proteins, which then function as transcription factors to induce apoptosis (Schwarz et al., 2000). In order for TGF-β1 to induce apoptosis it must first be activated by protease cleavage mediated by plasmin and transglutaminase II enzymes. *Ahr*-null mice show increased levels of both of these enzymes in the liver (Sutter et al., 1991; Fernandez-Salguero et al., 1995; Andreola et al., 1997; Zaher et al., 1998). There is also evidence indicating that TCDD-activated AHR directly inhibits TGF-β genes which potentially diminish apoptosis suggesting that in the absence of AHR there might be increased TGF-β transcriptional activation (Gaido et al., 1992). Additionally, another group has shown that in *Ahr*-null embryonic fibroblasts, AHR might control proliferation through a CDK-independent pathway (Tohkin et al., 2000). They suggest that AHR interacts with p300 to promote DNA synthesis which is reduced in the *Ahr*-null MEFs leading to the decreased proliferation rates (Tohkin et al., 2000).

Currently, there is growing experimental evidence that the absence of AHR prolongs the cell cycle *in vitro* (Ma and Whitlock, 1996; Abdelrahim et al., 2003). Studies using murine Hepa 1c1c7 variants (c12, c19; cells which lack AHR activity) and the human HepG2 hepatoma cells transfected with siAHR showed reduced proliferation rates due to a delayed $G_1$ to S phase transition (Ma and Whitlock, 1996; Abdelrahim et al., 2003). In contrast, MCF-7 human breast cancer cells transfected with siAHR showed enhanced $G_1$ to S phase transition (Abdelrahim et al., 2003). In addition, the MCF-7 $AH^{R100}$ cells, a variant of the human breast cancer cells with minimal AHR activity, displayed increased proliferation rates when compared to wildtype MCF-7 cells (Spink et al., 2012). MCF-7 $AH^{R100}$ cells were derived from MCF-7 cells exposed to B[a]P for 6-9 months and were characterized to have a 100-fold higher resistance to B[*a*]P than the wildtype cells and contain reduced AHR but normal ARNT levels (Ciolino et al., 2002;

Trapani et al., 2003; Spink et al., 2012). Since these cells were derived by exposure to B[*a*]P it remains possible that other proteins regulating the cell cycle were affected. The increased proliferation rates seen in breast cancer cells were not supported by a recent study (Barhoover et al., 2010) which showed that AHR serves as a scaffolding protein to promote the phosphorylation of Rb by the CDK4 and cyclin D1 (CCND1) complex indicating that AHR is important for $G_1$ to S phase transition. AHR has also been shown to interact directly with the Rb protein promoting its phosphorylation (by cyclin/CDK complexes) leading to increased progression through the cell cycle (Puga et al., 2000; Elferink et al., 2001). Overall, these studies illustrate that AHR has an endogenous function by promoting cell cycle progression in the absence of an exogenous ligand. These findings are further supported by studies of the transgenic mouse line expressing a constitutively active AHR, which show pro-proliferative effects (Andersson et al., 2002). However, when this same constitutively active AHR was transfected into Jurkat cells it causes growth inhibition and apoptosis highlighting the complexity of studying the role of AHR in proliferation (Ito et al., 2004).

#### 2.5.2.3 Ligand-dependent cell cycle control

AHR ligands such as TCDD inhibit cell proliferation but the mechanism of this inhibition has not been clearly defined (Marlowe and Puga, 2005). TCDD-activated AHR has been shown to inhibit DNA synthesis, decrease proliferation rates, block S phase progression, and cause $G_1$ phase arrest (Gierthy and Crane, 1984; Hushka and Greenlee, 1995; Wang et al., 1998; Laiosa et al., 2003; Jin et al., 2004). These effects have been seen in mouse epithelial and thymic progenitor cells, rat primary hepatocytes and 5L hepatoma cells, human estrogen receptor positive MCF-7 cells, SK-N-SH neuronal cells, and pancreatic cancer cells. One potential mechanism by which TCDD has been shown to elicit its effects is through the control of cell cycle genes. TCDD can induce the expression of $p27^{Kip1}$, a CDK inhibitor. This inhibitor will then block the activity of CDK4/6 so they are unable to phosphorylate Rb thereby preventing S phase entry (Kolluri et al., 1999). Another mechanism by which TCDD-activated AHR can cause cell cycle arrest is through its interaction with Rb (Ge and Elferink, 1998; Chan et al., 2001; Elferink et al., 2001). This prevents the phosphorylation of Rb inhibiting the expression of E2F-dependent genes causing $G_1$ phase arrest (Marlowe et al., 2004; Huang and Elferink, 2005). The genes that were affected due to AHR activation were cyclin E, CDK2, and DNA polymerase $\alpha$, all required for S phase entry. These genes were also affected due to AHR interaction with their

promoters. Using chromatin immunoprecipitation assays they showed that TCDD-activated AHR was recruited to these promoters displacing the p300 coregulatory protein leading to transrepression (Marlowe et al., 2004). Furthermore, a more recent study has shown that TCDD-bound AHR can no longer interact with CDK4 leading to reduced CDK-dependent phosphorylation of Rb and $G_1$ arrest (Barhoover et al., 2010). Taken together, these data suggest that ligand-activated AHR works through both protein-protein interactions as well as through direct DNA binding to inhibit cell cycle progression (**Figure 4**). However, more studies are needed to delineate the exact genes and signalling pathways involved in ligand-dependent cell cycle arrest.

#### 2.5.2.4 Ligand-dependent inhibition of apoptosis

Although ligand-activated AHR has been shown to be anti-carcinogenic by preventing cell proliferation, AHR has also been shown to be a tumour promoter by inhibiting apoptosis in both the liver and skin (Dragan and Schrenk, 2000). This has been determined using a two stage initiation-promoter carcinogenesis assay (Dragan and Schrenk, 2000). AHR-mediated inhibition of apoptosis is thought to occur through inhibition of p53, a powerful tumour suppressor protein implicated in promoting DNA repair and apoptosis by regulating the expression of genes involved in these pathways (Worner and Schrenk, 1996; Pääjärvi et al., 2005). Inhibition of p53 accelerates the rate at which DNA-damaged cells convert to a neoplastic phenotype. Another mechanism by which it can inhibit apoptosis is through its direct interaction with E2F-1 (Dick and Dyson, 2003). Under normal circumstances, Rb suppresses apoptosis by repressing E2F-1 target genes (Nahle et al., 2002). However, after DNA damage E2F-1 is stabilized through direct phosphorylation upregulating the expression of pro-apoptotic genes leading to the activation of the apoptotic pathway (Dick and Dyson, 2003). During DNA damage, ligand-activated AHR can directly interact with E2F-1 thereby suppressing the expression of pro-apoptotic genes leading to proliferation and evasion of apoptosis. Overall, it appears that AHR has both a proliferative and inhibitory effect on cell cycle regulation depending on many factors including the tissue studied, ligand treatment, and the induced gene expression patterns. More research is required to fully understand the role of AHR in the cell cycle.

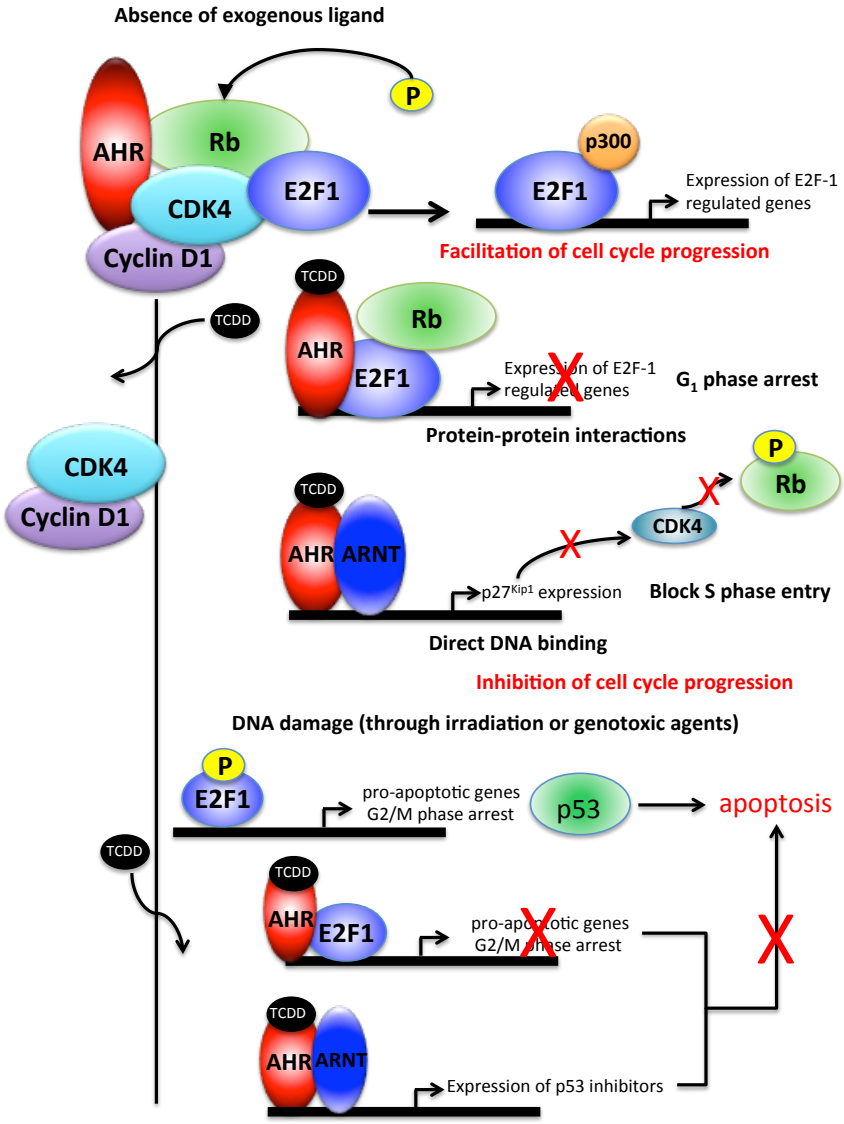



**Figure 4. Proposed pathways by which AHR regulates the cell cycle and apoptosis in the presence and absence of exogenous ligands.**

In the absence of ligand, AHR has been suggested to facilitate cell cycle progression through its interaction with Rb, cyclin D1, and CDK4 to promote the phosphorylation of Rb. However, upon ligand treatment, ligand bound AHR prevents the phosphorylation of Rb and increases the expression of the CDK4 inhibitor $p27^{Kip1}$ so CDK4 is unable to phosphorylate Rb. Taken together, both pathways reduce Rb phosphorylation leading to $G_1$ arrest and inhibition of S phase entry. After DNA damage, normal cells activate proapoptotic pathways through E2F-1 and p53. However, when AHR ligands are added during DNA damage there is evasion of apoptosis through inhibition of p53 and E2F-1 signalling pathways.

### 2.5.3 Role in the immune system

There is considerable evidence to suggest that AHR plays a role in the immune system (Vos et al., 1973; Kerkvliet et al., 1982; 1985; Kerkvliet, 1995; Esser et al., 2009; Stevens et al., 2009). This comes from observations in *Ahr*-null mice and from studies using AHR agonists. Although *Ahr*-null mice do not have an obvious immunological phenotype, they have reduced lymphocyte numbers in the spleen as well as decreased lymphocyte infiltration of the lung, intestine, and urinary tract and were more susceptible to listeria infection and the influenza virus (Fernandez-Salguero et al., 1995; Schmidt et al., 1996; Shi et al., 2007; Teske et al., 2008). TCDD-activated AHR leads to suppression of both humoral and cellular immune responses increasing their susceptibility to infection, as well as thymic involution (Kerkvliet, 1995; Kerkvliet et al., 1996; Laiosa et al., 2003).

Recently, it has been shown that AHR affects regulatory T cells (Tregs). Tregs are important in the immune system because they reduce autoimmune and allergic disease, limit the immune response to infectious disease, and inhibit anti-tumour immune responses (Mottet and Golshayan, 2007). Experimental evidence suggests that ligand-activated AHR promotes Treg differentiation by upregulating the transcription factor FoxP3, enhances TGFβ signalling, or indirectly through dendritic cells (Marshall et al., 2008; Quintana et al., 2008; Stevens et al., 2009). Conversely, AHR has also been reported to play a role in T helper 17 cell (Th17) development which promote an immune response unlike the Treg cells (Kimura et al., 2008). Kimura *et al.* demonstrated that the candidate endogenous ligand FICZ increased the percentage of Th17 cell development induced by TGF-β plus IL-6 addition to naïve T cells. Th17 cells play a major role in autoimmunity and clearance of infectious agents. The mechanism by which AHR affects Th17 cells is through the upregulation of IL-17 and IL-22, two key cytokines secreted by Th17 cells (Veldhoen et al., 2008; 2009). It has been proposed that the balance of Treg/Th17 cells distinguishes an effective immune response from autoimmunity highlighting the potential impact ligand activated AHR can have on the immune system.

# 3 Estrogen Receptor α

## 3.1 Discovery and structure

It was determined fifty years ago that the biological effects of estrogen (17β-estradiol, E2) are mediated by receptor binding (Jensen, 1962). However, the receptor responsible for these effects was not isolated and cloned until 24 years later (Green et al., 1986) termed estrogen receptor (ER). Until 1995, it was assumed that only one ER was responsible for mediating all the biological effects of natural and synthetic estrogens. A second subtype termed ERbeta (ERβ) was discovered and cloned from a rat prostate cDNA library and the original ER was subsequently renamed ERalpha (ERα) (Kuiper et al., 1996).

Estrogens via activation of ERα and ERβ mediate the development and maintenance of normal sexual and reproductive functions, as well as modulate the cardiovascular, musculoskeletal, immune, and central nervous systems (Heldring et al., 2007). Both ER subtypes belong to the nuclear receptor superfamily of ligand-activated transcription factors (Katzenellenbogen and Katzenellenbogen, 1996; Nilsson et al., 2001). ERα and ERβ also have similar affinities for E2 but their distribution within the body determines receptor activation (Nilsson et al., 2001). They display distinct but overlapping expression patterns in a number of tissues with ERα primarily expressed in the uterus, liver, kidney, and heart, while ERβ is primarily expressed in the ovary, prostate, lung, GI tract, bladder, hematopoietic and central nervous systems (Matthews and Gustafsson, 2003). Both receptor subtypes are co-expressed in the mammary gland, epididymis, thyroid, adrenal, bone, and distinct brain regions (Matthews and Gustafsson, 2003). Studies in mice show that ERα mediates most of the estrogen signalling in classic estrogen target tissues such as the uterus, mammary gland, and skeleton, whereas ERβ has a minor role (Harris, 2007). In the uterus, ERα knockout animals (αERKO) have well-documented defects in uterine responses to estrogens (Couse and Korach, 1999). In contrast, ERβ knockout animals (βERKO) still respond to estrogen displaying increased uterine weight after E2 treatment (Harris, 2007). Moreover, microarray studies indicate that both early and late genomic responses to estrogen are indistinguishable between wildtype and βERKO mice (Hewitt et al., 2003). In the mammary gland, αERKO mice display major defects in development (Tekmal et al., 2005). The αERKO mammary glands do not grow beyond the rudimentary duct established at birth. In contrast, the

βERKO mammary gland develops normally and mice are able to lactate (Couse and Korach, 1999). Some studies suggest a cooperation as well as competition between the two subtypes (Reviewed in: Matthews and Gustafsson, 2003). Studies using rat mammary tissue have suggested that one role of ERβ may be to antagonize ERα-mediated actions in epithelial cells (Saji et al., 2000). ERβ mRNA is expressed in breast cancer tissue but there is considerable debate on its role in cancer progression where some have suggested that ERβ mRNA correlates with the development of estrogen-independent tumour growth and a poor prognosis (Dotzlaw et al., 1997; Murphy et al., 1997; Speirs et al., 1999; Iwao et al., 2000). However, there are limited tools available to study ERβ due to its high sequence homology and similar LBD to ERα (Kuiper et al., 1997; Harris, 2007). Despite these limitations, most data suggest the ERα subtype functions independently to modulate estrogen signalling in the mammary gland and will be the focus of my research.

ERα is comprised of seven functional domains (A-F) that work both independently and cooperatively to achieve maximal receptor activity. They are the N-terminal (A/B), DNA binding (C), hinge (D), ligand binding (E), and C-terminal (F) domains **(Figure 5)**. The N-terminal domain contains the ligand-independent activation function 1 (AF1; (A/B)) responsible for both protein-protein interactions and the transactivation of target genes (McInerney and Katzenellenbogen, 1996; McInerney et al., 1996; Nilsson et al., 2001). The AF1 domain of ERα but not ERβ was determined to be active in estrogen response element (ERE)-driven reporter constructs (Cowley and Parker, 1999). Although the AF1 domain can be activated in the absence of ligand, maximal activity is achieved after E2 treatment (Kumar et al., 1987; Tzukerman et al., 1994). This has been attributed to the phosphorylation of serine residues in the AF1 region after E2 treatment leading to the stabilization of ERα at estrogen target genes (Watanabe et al., 2001; Fujita et al., 2003). The DNA binding domain (DBD, (C)) contains two zinc finger motifs with the first zinc finger motif important for receptor dimerization while the second motif assists in the recognition of DNA response elements. (Green et al., 1988; Schwabe et al., 1990; Mader et al., 1993; Schwabe et al., 1995; Klinge, 2001). The next region is the hinge region (D) of ERα, which separates the DBD from the ligand binding domain (LBD; (E)). The hinge region is a stretch of 40-50 amino acids containing sequences important for receptor dimerization, nuclear localization and coregulatory protein interactions (Klinge, 2000). The C-terminal LBD (E) is responsible for ligand binding, receptor dimerization, nuclear translocation, and the

transactivation of target genes. The LBD also harbours the activation function 2 region (AF2; (E)) which mediates the ligand-dependent transcriptional activity of target genes (Nilsson et al., 2001; Heldring et al., 2007). From crystallographic studies of the LBD, it was determined that the amino acids of the AF2 interaction surface are in helix 3, 4, 5 and 12 in the absence of ligand. However, upon ligand binding, the position of helix 12 (H12) is altered (Brzozowski et al., 1997). When ERα is complexed with agonists such as E2, H12 is positioned over the ligand binding pocket and forms a surface for the recruitment and interaction of coactivators (Katzenellenbogen and Katzenellenbogen, 1996; Brzozowski et al., 1997; Nilsson et al., 2001). In contrast, when ERα is complexed with an antagonist, H12 no longer resides over the ligand-binding pocket but instead occupies the hydrophobic groove formed by helix 3, 4, and 5 (Brzozowski et al., 1997). Analysis of all coregulatory proteins shown to interact with ERα will be discussed in a later section (3.2.3 coregulatory proteins).

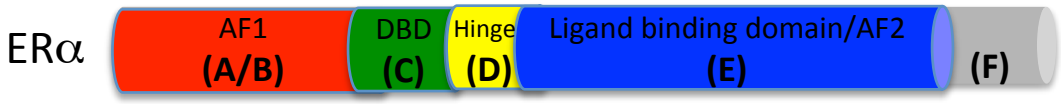



**Figure 5. Functional domain structure of estrogen receptor alpha (ERα).**

AF1 (activation function 1), DBD (DNA binding domain), AF2 (activation function 2). Modular domains A-F are labelled for each section.

## 3.2 ERα signal transduction

### 3.2.1 Direct DNA binding

ERα can modulate transcription by directly interacting with DNA regulatory sequences. In the absence of ligand, ERα is found primarily in the nucleus as a non-activated monomer bound to corepressor proteins to prevent constitutive ER activity (Lavinsky et al., 1998; Nilsson et al., 2001). Since its endogenous ligand E2 is a small lipophilic molecule, it can easily cross the cell membrane and enter the nucleus. The binding of E2 to ERα causes its release from the corepressor complex and induces a conformational change in the receptor exposing the H12 coactivator interaction surface. Activated ERα then homodimerizes and binds to specific DNA response elements termed estrogen response elements (EREs) located in the regulatory regions of its target genes. The ERE was first characterized in the regulatory region of the *Xenopus*

vitellogenin A2 gene and is comprised of a 15 base pair inverted repeat sequence with a 3 base pair spacer: 5`-AGGTCAnnnTGACCT-3` (Klein-Hitpass et al., 1986). This ERE sequence was shown to function in an orientation and distance-independent manner (Klinge, 2001). It has also been documented that ERα can also bind to half-site EREs which contain only one repeat (5`-AGGTCA-3`) (Kato et al., 1992). Once bound to an ERE, ERα interacts with both coregulatory proteins and basal transcription factors to enhance gene expression. ERα signalling is attenuated through ligand-dependent proteasomal degradation of ERα through the 26S proteasome (Lonard et al., 2000). In this pathway, ligand-dependent ubiquitination of ERα and coactivators leads to the subsequent disassembly of the transcriptional complex and cessation of transcription (Reid et al., 2003).

### 3.2.2 Protein-tethering

In the protein-tethering pathway, ERα is activated in the same manner but does not directly bind to DNA. Instead, ERα interacts with other transcription factors already bound to DNA to further stabilize the activated complex and promotes the recruitment of other coactivators (Klinge, 2001). Protein-tethering is not unique to ERα as this pathway is used by many nuclear receptors (Nilsson et al., 2001). ERα has been shown to be in complex with activating protein 1 (AP-1) and stimulating protein 1 (Sp1) family of transcription factors (Paech et al., 1997; Porter et al., 1997). AP-1 is a heterodimeric transcription factor composed of proteins belonging to the c-Fos, c-Jun, ATF, and JDP families. ERα has been shown to interact with the c-Jun and c-Fos components of AP-1 in an E2-dependent manner (Paech et al., 1997; Kushner et al., 2000). This interaction requires the AF1 and AF2 domains of ERα to not only stabilize the activated complex but to also recruit coregulatory proteins leading to the enhancement of AP-1 activity (Webb et al., 1999). In the Sp1 protein-tethering pathway, ERα can interact with Sp1 in a ligand-independent manner. The tethering of ERα enhances Sp1 binding to its GC-rich response elements and the transactivation of its target genes. It is unclear if protein tethering is utilized in other signalling pathways.

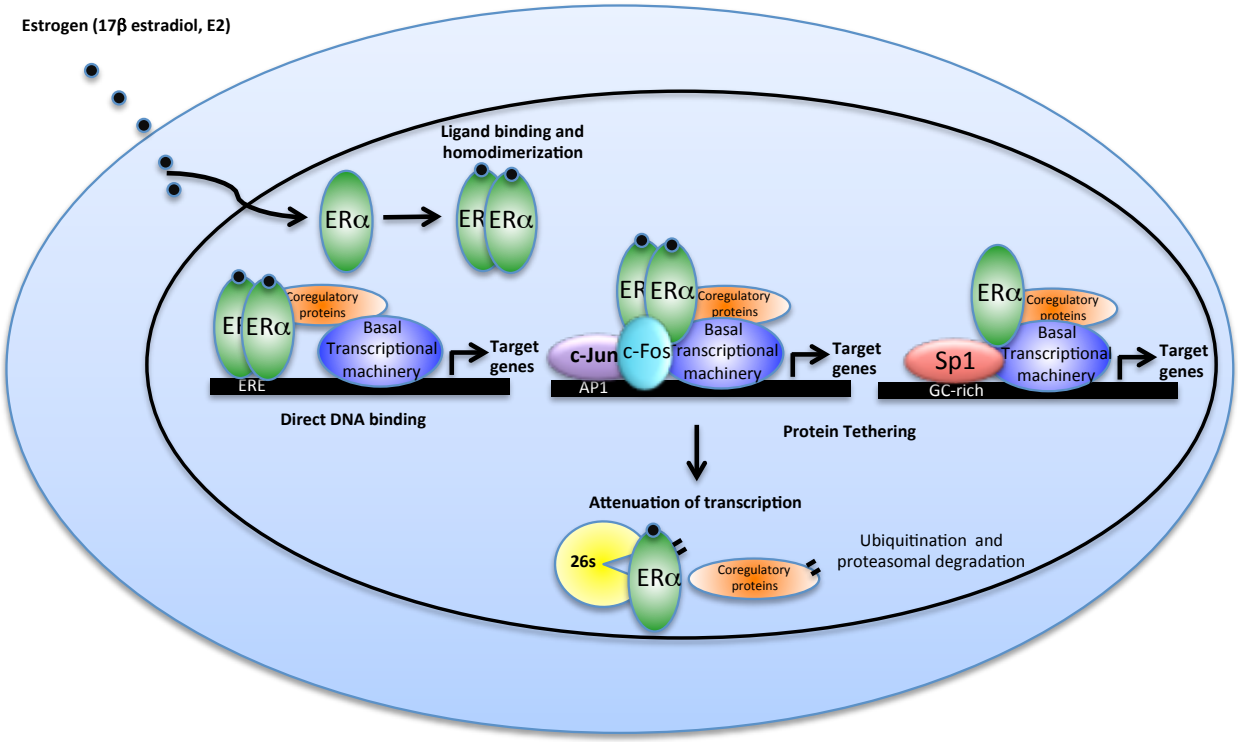



**Figure 6. Estrogen receptor signalling via direct DNA binding and protein tethering**

Estradiol diffuses into the nucleus where it binds and activates ERα. In the direct DNA binding pathway, activated ERα homodimerizes and binds to estrogen response elements (EREs) in the regulatory regions of its target genes. In the protein-tethering pathway, ligand-activated ERα can interact with the transcription factors c-Fos and c-Jun, which are part of the AP-1 transcriptional complex to enhance the transcription of AP-1 specific genes. Unliganded ERα can also interact with Sp1 bound to GC-rich DNA regions to enhance transactivation of Sp1 specific genes.

### 3.2.3 Coregulatory proteins

Similar to AHR, ERα requires coactivators, corepressors, and cointegrators to stabilize the activated complex, modify histones, and de-condense chromatin to achieve maximal gene expression. Coactivators involved in ERα-mediated gene expression include the NCoA family of proteins (NCoA1-3). These proteins and many others interact with the AF2 region of ERα via short leucine-rich motifs (LXXLL) termed 'NR boxes' (Nilsson et al., 2001). These motifs represent the primary docking sites to the AF2 domain and each coregulator 'NR box' may differ in affinity for ligand-bound ERα (Suen et al., 1998). NCoA proteins also contain activation domains that are involved in the recruitment of CBP/p300 proteins that relax chromatin structure

through their intrinsic HAT activity. CBP/p300 can also directly interact with ERα via their N-terminal NR-boxes (Hanstein et al., 1996; Nilsson et al., 2001). ERα has also been shown to interact with the TRAP (Thyroid Hormone Associated-Protein) complex implicated in connecting ERα with basal transcription factors (Ito et al., 1999) through its interaction with the C-terminal domain of RNA polymerase II (Björklund and Gustafsson, 2005).

Studies also indicate that ERα interacts with corepressor proteins. The corepressors RIP140 and SHP have been shown to inhibit ERα signalling by competing with the nuclear coactivator proteins for AF2 binding sites (Treuter et al., 1998; Johansson et al., 2000). The co-repressors NCoR and SMRT also associate with antagonist-bound ERα and may be required for mediating their repressive actions (McKenna et al., 1999; Nilsson et al., 2001). These proteins contain CoRNR (corepressor nuclear receptor) boxes, similar to the 'NR' boxes of coactivators to interact with ERα (Hu and Lazar, 1999). It has been suggested that NCoR and SMRT repress target gene expression by recruiting HDACs by the realignment of the H12 region to expose a corepressor binding epitope (Nichols et al., 1998; Nilsson et al., 2001). However, this corepressor epitope on ERα has not been fully elucidated.

### 3.2.4 FOXA1 as a pioneer factor in ERα signalling

Our knowledge of ERα activity has evolved in recent years through the use of chromatin immunoprecipitation coupled with genome-wide microarrays (ChIP-chip), which allows researchers to map ER binding sites in an unbiased manner (Carroll et al., 2005; Carroll and Brown, 2006; Carroll et al., 2006; Hurtado et al., 2011). Using this approach, they have identified several new features of ERα transcription including the binding of ERα to distal *cis*-regulatory enhancer regions mediated by the pioneer protein Forkhead box A1 (FOXA1) (Lupien et al., 2008; Hurtado et al., 2011). Pioneer proteins are defined by their ability to interact with condensed chromatin facilitating the binding of other transcription factors (Cirillo et al., 2002; Lupien et al., 2008). FOXA1 is a member of the forkhead family of winged-helix transcription factors (FOXA1-3) and was initially identified as a transcription factor required for the development of various tissues including the liver, lung, and prostate (Friedman and Kaestner, 2006). FOXA1 binds to target DNA as a monomer using a helix-turn-helix motif, which creates a winged structure when bound to DNA. FOXA1 binds to the consensus sequence 5`-A(A/T)TRTT(G/T)RTUTU-3`. Currently, FOXA1 has been shown to interact with other

transcription factors through its winged helix DBD. These include SMAD3 (SMAD family member 3), NKX2.1 (NK1 homeobox transcription factor) and the androgen receptor (AR). However, its ability to bind to condensed chromatin is likely responsible for its effects on ERα signalling, where the winged helix structure displaces linker histones H1 and disrupts the H3/H4 tetramer nucleosome structure creating an open chromatin state (Cirillo et al., 1998; 2002; Yu et al., 2005). Current studies suggest that the binding of FOXA1 to chromatin is governed by epigenetic modifications of histones (Carroll et al., 2005; Yu et al., 2005; Carroll et al., 2006; Carroll and Brown, 2006; Lupien et al., 2008). Recent genome-wide binding site analysis of FOXA1 in MCF-7 human breast cancer cells report that FOXA1 binding correlates with regions poor in histone 3 lysine 9 dimethyl marks (H3K9me2) but rich in histone 3 lysine 4 mono/dimethyl marks (H3K4me1/2) (Lupien et al., 2008). The authors indicate that these modifications could guide FOXA1 binding by directly interacting with FOXA1 leading to its subsequent interaction with other transcription factors (Nakshatri and Badve, 2009). FOXA1 can also facilitate chromatin looping through its interaction with insulator proteins such as CTCF (CTCC-binding factor) to connect distal enhancer regions to basal transcription factors (Splinter et al., 2006; Hurtado et al., 2011). FOXA1 has been shown to interact with both ERα and AR to regulate transactivation. In androgen signalling, FOXA1 directly interacts with AR to promote the expression of prostate specific genes (Yu et al., 2005; Lupien et al., 2008; Wang et al., 2009). In ERα signalling its direct interaction has not been confirmed but using siFOXA1 coupled with ChIP and high-throughput sequencing (ChIP-seq) it was determined that FOXA1 was required for almost all ER binding events irrespective of the proximity of their binding sites (Hurtado et al., 2011). The authors also demonstrate that FOXA1 was required for tamoxifen:ERα (a selective estrogen receptor modulator (SERM) used for the treatment of breast cancer) binding to chromatin and not just E2:ERα binding. This might explain why high FOXA1 levels predict a positive prognosis in patients by facilitating tamoxifen-meditated transrepression (Badve et al., 2007; Wolf et al., 2007). Another mechanism by which FOXA1 may correlate with positive prognosis is that it controls ERα expression. Tumours with low FOXA1 expression have low ERα transcriptional activity and protein levels and therefore their growth is less dependent on ER (Badve et al., 2007; Wolf et al., 2007; Bernardo et al., 2010). However, the ability of FOXA1 to influence chromatin binding of other transcription factors has not been determined.

### 3.2.5 Role of ERα in breast cancer development and treatment

Estrogen is considered a stimulant for the initiation and promotion of mammary gland tumours. Epidemiological studies demonstrate an increased risk for developing breast cancer in women with prolonged exposure to estrogen through early menarche, late menopause, and estrogen replacement therapy (Clemons and Goss, 2001). Although the exact mechanisms have not been elucidated; the alkylation of cellular molecules, generation of active radicals that can cause damage to DNA, as well as the potential genotoxicity of estrogen metabolites have all been implicated (Cavalieri et al., 2000; Liehr, 2000; Ikeda and Inoue, 2004). Estrogens can also promote tumourigenesis through their role in proliferation. In this pathway, tumour formation may result from excessive stimulation of the mammary gland via ERα activation which when over-stimulated will cause the progression from normal growth to hyperplasia to neoplasia (Clemons and Goss, 2001). This effect can be due to higher estrogen levels but also higher ERα levels. Two-thirds of human breast tumours have higher ER levels than normal breast tissue (Ikeda and Inoue, 2004), leading to therapies targeting the receptor and its endogenous ligand. The main goal of such therapies is to block the interaction between estrogen and ER. The first way this can be achieved is by blocking estrogen synthesis using aromatase inhibitors, thereby reducing the formation of estrogen from steroidal precursors (Pearce and Jordan, 2004). ERα can also be targeted using selective estrogen receptor modulators (SERMs), functioning as anti-estrogens in some tissue while estrogens in others. These compounds are believed to be anti-estrogenic by interrupting the H12 coactivator interaction surface unlike agonist bound ERα (Nilsson et al., 2001; Pearce and Jordan, 2004). Others SERMs have been implicated in antagonizing ERα signalling through direct protein degradation.

### 3.2.6 Breast cancer classification

Breast cancer is a heterogeneous disease in terms of histology, therapeutic response, and patient outcomes (Reis-Filho et al., 2005; Simpson et al., 2005). Global gene expression analysis has helped explain this heterogeneity and provided classifications to account for its diversity (Cleator and Ashworth, 2004; Andre and Pusztai, 2006; Geyer et al., 2009). Receptor status has been traditionally considered when classifying breast tumours by determining estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) status (Geyer et al., 2009). Expression profiling analyses demonstrated that breast cancers can be classified

into at least five groups: luminal A, luminal B, normal breast-like, HER2, and basal-like. Luminal tumours are described as those that show expression patterns similar to normal luminal epithelial cells of the breast by having low molecular weight cytokeratins 8/18, ER, PR, and genes associated with ER activation (Perou et al., 2000; Sorlie et al., 2003). Within this group, luminal A cancers usually have a low histological grade, good prognosis and show high levels of expression of ER-activated genes while luminal B have a much higher histological grade and higher proliferation rates with a poorer prognosis (Sorlie et al., 2003; Geyer et al., 2009). The luminal A molecular subtype has been intensely studied *in vitro* with many immortalized cell lines including the ER+ PR+ MCF-7, T-47D, BT483 and the ER+ but PR- ZR-75 (Neve et al., 2006). Normal breast-like cancers are poorly characterized tumours, but one significant feature is that they cluster together with samples of fibroadenomas and normal breast samples (Geyer et al., 2009). HER2 positive tumours are usually ER-negative and characterized by the over-expression of HER2 and genes associated with the HER2 pathway (Sorlie et al., 2003; Piccart-Gebhart et al., 2005; Guarneri et al., 2010). The most widely used immortalized cell line is the ER-PR-HER+ AU565 (Neve et al., 2006). This cancer subtype is very aggressive, but therapies are available that target HER2 using humanized monoclonal antibodies (Guarneri et al., 2010). The basal-like cancers are another group of ER, PR, and HER2 negative whose cells consistently express genes normally expressed in basal cells of the breast, including high molecular weight cytokeratins, P-cadherin, and epidermal growth factor receptor (Nielsen et al., 2004; Gusterson et al., 2005; Rakha et al., 2006; Geyer et al., 2009). They are usually of high histological grade and have poor prognosis due to the heterogeneity of their molecular profile. Various basal-like subtype immortalized cell lines have been created including the MDA-MB-231, -435, -436, and -468 (Neve et al., 2006).

# *4* Reciprocal AHR-ERα crosstalk

Crosstalk has been observed between AHR and a number of signalling pathways, but its actions on ERα signalling are perhaps the most well-documented (Kuil et al., 1998; Kim et al., 2000; Safe and Wormke, 2003; Marlowe and Puga, 2005; Esser et al., 2009). Despite many studies, the mechanisms of reciprocal AHR-ERα crosstalk remain poorly understood. The inhibitory effects of AHR on ERα have been well documented but some groups have demonstrated that AHR can have estrogenic effects on ERα signalling (Abdelrahim et al., 2003; Safe and Wormke, 2003). The role of ERα in AHR signalling ranges from inhibition, to activation, to no effect (Hoivik et

al., 1997; Beischlag and Perdew, 2005; Matthews et al., 2005; 2007). Below is a summary of the current literature on the reciprocal AHR-ERα crosstalk.

## 4.1 Inhibitory effects of AHR on ERα signalling

Inhibitory AHR-ERα crosstalk was first suggested in early rodent experiments completed by Kociba *et al* (1978) which examined the long-term effects of dietary exposure to TCDD. It was observed that after a two-year treatment with dioxin, exposed rodents exhibited a lower incidence of spontaneous mammary and uterine tumours compared to control animals (Kociba et al., 1978). This observation led to the suggestion that ligand-activated AHR may inhibit the formation and growth of E2-dependent tumours (Safe and Wormke, 2003). Additional studies have shown that ovariectomized rodents and mice when treated with E2 alone had increased uterine wet weight, DNA synthesis, and induction of multiple uterine genes (Romkes et al., 1987; Romkes and Safe, 1988; Umbreit and Gallo, 1988; Astroff and Safe, 1990; Astroff et al., 1990). However, in animals co-treated with TCDD, the E2-dependent responses were inhibited. This included reduced uterine wet weight, DNA synthesis, inhibition of progesterone receptor binding, peroxidase activity, EGFR binding and mRNA, and c-fos mRNA (Romkes et al., 1987; Romkes and Safe, 1988; Umbreit and Gallo, 1988; Umbreit et al., 1988; Astroff et al., 1990; 1991). The role of AHR in modulating the TCDD-dependent inhibition of E2-induced uterine changes was confirmed by studies completed using *Ahr*-null mice (Buchanan et al., 2000; 2002). In these studies TCDD failed to inhibit E2-induced uterine changes in *Ahr*-null animals implicating ligand-bound AHR in modulating the effects. Administration of TCDD has also been shown to inhibit mammary tumour growth in carcinogen-induced rodent mammary tumours and in athymic nude mice bearing human breast cancer cell xenografts (Gierthy et al., 1993; Holcomb and Safe, 1994; Tritscher et al., 1995).

AHR agonists inhibit the expression of several E2-regulated genes and proteins and/or their related activities including cathepsin D, c-fos, TFF-1 (trefoil factor 1, also known as pS2), Hsp27 (27kDa heat shock protein), prolactin receptor, progesterone receptor, and CCND1 (Harper et al., 1994; Krishnan et al., 1994; Zacharewski et al., 1994; Krishnan et al., 1995; Lu et al., 1996; Wang et al., 1998; Duan et al., 1999; Porter et al., 2001) using ERα positive breast, ovarian, and endometrial cancer cell lines. TCDD and other AHR ligands also decreased ERα levels in T-47D, MCF-7, and ZR-75 human breast cancer cells and this down-regulation correlated with

ligand-activated AHR (Harris et al., 1990; Merchant et al., 1993; Zacharewski et al., 1994; Wormke et al., 2000; Ohtake et al., 2007). Despite the numerous *in vivo* and *in vitro* studies, the precise molecular mechanisms of inhibition remains unclear. Several mechanisms have been proposed **(Figure 7)** including squelching of shared co-activators, synthesis of inhibitory protein(s), increased estrogen metabolism, AHR binding to inhibitory response elements in the promoter regions of ERα-target genes and increased proteasomal degradation of ERα (Reviewed in: Safe and Wormke, 2003). However, many of these mechanisms imply global inhibition because they indiscriminately disrupt ERα signalling (e.g. reduced estrogen levels and reduced protein levels) which would lead to inhibition of all ERα signalling. Further examination of the inhibitory role of AHR on ER-mediated gene expression in the uterus has indicated that this is not the case and that inhibition is restricted to a subset of estrogen-regulated genes (Boverhof et al., 2008). This suggests that previous mechanisms do not fully describe how AHR elicits its selective antiestrogenic effects. Some reports have indicated that ERα is recruited by AHR agonists, but not antagonists to AHR target genes (Beischlag and Perdew, 2005; Matthews et al., 2005; Abdelrahim et al., 2006). The presence of ERα in these complexes may have important regulatory effects on ERα signalling and provides a new mechanism for AHR-mediated inhibition of estrogen activity. Collectively, current data support the notion that AHR exerts a gene-specific modulation of ERα activity; although additional studies are needed to confirm its selectivity.

## 4.2 Estrogenic effects of AHR ligands and AHR activation

AHR agonists have also been reported to enhance estrogen-dependent responses (Ohtake et al., 2003; Abdelrahim et al., 2006; Boverhof et al., 2006; Shipley and Waxman, 2006a). Ohtake and colleagues showed that 3MC-activated AHR associates with unliganded ERα to form a functional complex with p300 that can activate E2-responsive genes (Ohtake et al., 2003). 3MC was able to induce estrogenic responses, including increased c-fos and VEGF expression as well as increased uterine wet weight *in vivo* (Ohtake et al., 2003). They concluded that AHR functions as a coregulator of ERα signalling promoting the expression of ERα target genes (Ohtake et al., 2003). However, this novel mechanism of crosstalk has been questioned by others (Abdelrahim et al., 2003; 2006; Shipley and Waxman, 2006b). RNAi-mediated knockdown of AHR in MCF-7 breast cells and rodent hepatoma cell line 5L and its AhR-deficient variant BP8 (Weiss et al., 1996) demonstrated that the estrogenic activity of 3MC was dependent on ERα expression and

not AHR (Shipley and Waxman, 2006a). 3MC was also shown to induce estrogenic responses in *Ahr*-null mice illustrating that 3MC estrogenic activity is independent of AHR (Abdelrahim et al., 2006). The estrogenic effects of 3MC are most likely due to its hydroxylated metabolites which have been shown to be estrogenic and not the parent compound (Swedenborg et al., 2008).

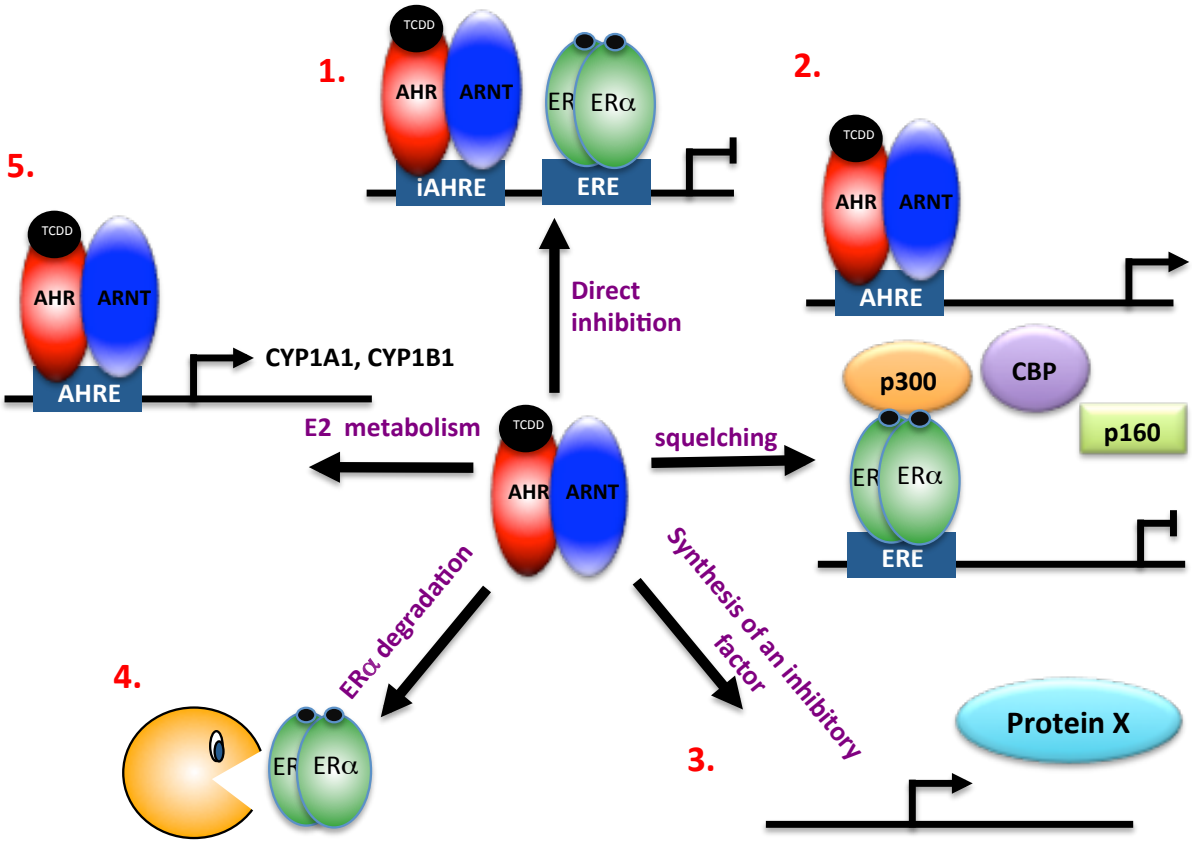



**Figure 7. Proposed mechanisms for the AHR-mediated inhibition of ERα signalling.**

AHR has been shown to inhibit ERα signalling through a combination of several mechanisms: 1. Direct inhibition through inhibitory AHREs (iAHREs) at estrogen target genes 2. Squelching of shared activators leading to reduced ERα signalling 3. Synthesis of an unknown inhibitory protein 4. Increased proteasomal degradation of ERα 5. Altered estrogen metabolism through increased CYP1A1 and CYP1B1 expression. Adapted from (Matthews and Gustafsson, 2006) .

This is consistent with another PAH, B[*a*]P which does not bind to ERα but was able to induce ERα-mediated reporter gene activity in MCF-7 breast cancer cells (Fertuck et al., 2001). Fertuck et al., demonstrated that the ability of B[*a*]P to induce ERα-mediated reporter gene activity was due to its monohydroxylated metabolites which were able to bind to ERα (Fertuck et al., 2001).

It was reported that TCDD, a compound that does not undergo rapid metabolism *in vivo* or *in vitro*, exhibits estrogenic activity through the induction of a small subset of estrogen responsive genes in mouse uteri (Watanabe et al., 2004; Boverhof et al., 2006). Furthermore, using competitive binding assays, TCDD was shown to not directly bind to ERα highlighting that these effects are mediated by AHR activation (Klinge et al., 1999). These findings raise the possibility that AHR could mimic ERα activity in a gene specific manner.

### 4.2.1 ARNT is a coactivator of ERα signalling

The dimerization partner of AHR, ARNT, has been reported to be an ERα coactivator (Brunnberg et al., 2003; Labrecque et al., 2012) through direct interactions with ER. It was shown that the C-terminal transactivation domain of ARNT was required for its coactivator function whereas its bHLH or PAS domains had little effect on the ability of ARNT to enhance ER-dependent transcription. This demonstrates that the interaction of ER-ARNT is distinct from the AHR-ARNT interaction sites which have been documented to occur at both the bHLH and PAS domains (Whitelaw et al., 1993a; 1993b; Brunnberg et al., 2003) RNAi-mediated knockdown of ARNT resulted in reduced ERα transactivation, which was proposed to be due to reduced ARNT recruitment to estrogen target genes (Brunnberg et al., 2003). Interestingly, Ruegg *et al.* also showed that activation of AHR signalling (via TCDD treatment) caused reduced ERα transcriptional activity. They ascribed this effect to TCDD-activated AHR outcompeting ERα for ARNT binding (Rüegg et al., 2008). However, a recent study suggests that TCDD-dependent transrepression is independent of ARNT where the loss of ARNT failed to abrogate the repressive effects of TCDD on E2-inducible transcription and protein expression (Labrecque et al., 2012). Using RNAi-mediated knockdown of ARNT it was demonstrated that ARNT had coactivator activity in MCF-7 breast cancer cells but displayed corepressor activity in endometrial cancer cells illustrating a complex mechanism for the role of ARNT in ERα signalling (Labrecque et al., 2012).

## 4.3 Inhibitory effects of ERα on AHR signalling

A recurring controversy in the field of AHR-ERα crosstalk is the effects of estrogen on AHR-mediated transcription, as studies have reported activation, inhibition, or no effect (Thomsen et al., 1994; Kharat and Saatcioglu, 1996; Hoivik et al., 1997; Beischlag and Perdew, 2005;

Matthews et al., 2005). Ricci and colleagues demonstrated that the induction of CYP1A1 by TCDD in both MCF-7 human breast cancer cells and ECC-1 endometrial cancer cells was significantly reduced after cotreatment of E2 with ER antagonists restoring TCDD-dependent CYP1A1 induction (Ricci et al., 1999). It was suggested that the mechanism of this inhibition was due to the squelching of a limiting factor involved in CYP1A1 transactivation, namely nuclear factor 1 (Ricci et al., 1999). In addition, in mouse Hepa-1 cells E2-activated ERα inhibited TCDD-induced CYP1A1 expression by interfering with the binding of AHR to AHREs (Kharat and Saatcioglu, 1996). In a more recent paper, it was reported that E2-activated ERα functions as a corepressor by directly interacting with the AHR-ARNT complex at the regulatory region of *CYP1A1* and *CYP1B1* (Beischlag and Perdew, 2005). However, there are studies that show minimal inhibition is achieved after E2 addition (Hoivik et al., 1997; Wormke et al., 2000; Matthews et al., 2005). The addition of estrogen did not affect TCDD-mediated CYP1A1 mRNA expression in both MCF-7 and Hepa-1 cells unlike what was previously reported (Hoivik et al., 1997). In Ishikawa endometrial cells, estrogen did not inhibit TCDD induced CYP1A1-dependent EROD (ethoxyresorufin O-deethylase) activity (Wormke et al., 2000). Further studies are needed to confirm the inhibitory role of ERα in AHR signalling since it appears to be context and cell line dependent.

## 4.4 ERα positively impacts AHR activation

Some studies have demonstrated that ERα positively modulates AHR signalling (Thomsen et al., 1994; Spink et al., 2003; Matthews et al., 2007). It has also been shown that ERα in the absence of ligand affects AHR activation. The introduction of exogenous ERα into the ERα-negative breast cancer cells MDA-MB-231 cells restores TCDD-dependent gene expression indicating a positive role for ERα in AHR transactivation (Thomsen et al., 1994). Further analysis of different human cancer cell lines have shown a positive correlation between ERα expression and AHR activity (Vickers et al., 1989; Angus et al., 1999; Sladek, 2003; Spink et al., 2003). Chronic E2 treatment in the ERα positive MCF-7 breast cancer cells was required to maintain high levels of AHR expression as well as CYP1A1 and CYP1B1 inducibility (Spink et al., 2003). Some *in vivo* studies have observed that female rats that received a chronic dose of TCDD had increased incidence of liver hyperplasia compared to their male counterparts, highlighting the involvement of estrogen in exacerbating AHR-mediated effects (Kociba et al., 1978; Lucier et al., 1991).

Moreover, E2 co-treatment potentiated TCDD-induced CYP1A1 expression in ovariectomized rats providing further evidence that ERα may be involved in AHR signalling (Kociba et al., 1978; Lucier et al., 1991; Sarkar et al., 2000; Wyde et al., 2001). Taken together, these studies suggest ERα positively modulates AHR signalling.

### 4.4.1 ERα as a modulator of AHR activity

The discrepancies in the literature on the role of ERα in AHR signalling may be due to its potential role as a general or gene specific coregulator of AHR signalling. Our laboratory has recently shown that TCDD treatment induces the recruitment of ERα to the regulatory region of *CYP1A1* and *CYP1B1*; two AHR target genes. The recruitment of ERα was further enhanced after cotreatment with E2 which suggests that E2 may positively influence AHR activity (Matthews et al., 2005). Furthermore, it was also shown that RNAi-mediated knockdown of ERα reduced the TCDD-induced CYP1A1 expression (Matthews et al., 2005). These findings highlight the potential influence ERα recruitment can have on AHR transactivation. However, it is unclear whether ERα is a general component of the active AHR complex or is only present at select promoters.

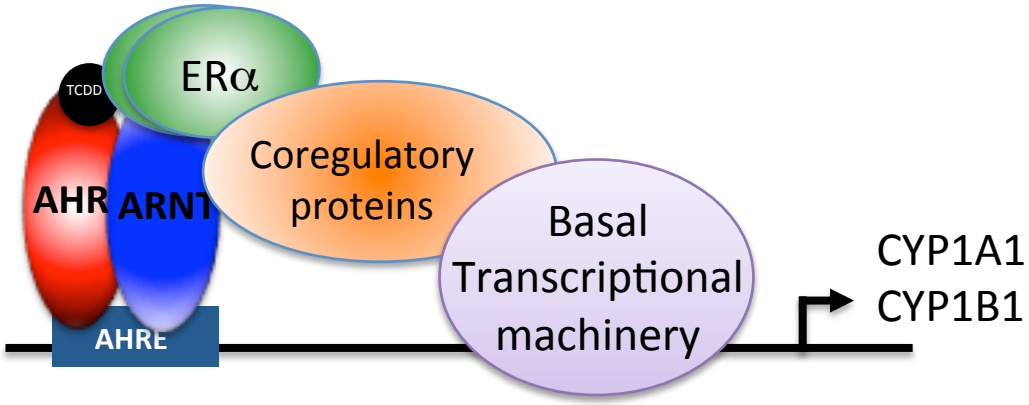



**Figure 8. Potential coregulator function of ERα in AHR signalling.**

TCDD treatment induces the recruitment of ERα to the AHR target genes *CYP1A1* and *CYP1B1*. This may be a novel mechanism by which ERα elicits its effects on AHR signalling.

# 5 Role of AHR in breast cancer

The antiestrogenic effects of AHR in the mammary gland have led to studies investigating its potential as a therapeutic target for breast cancer. AHR agonists are potent inhibitors of mammary tumour growth in rodent animal models, in athymic nude mice bearing human breast cancer cell xenografts, and in DMBA-induced carcinogenesis (Gierthy et al., 1993; Holcomb and Safe, 1994). The inhibitory effects on ERα positive breast cancer cells (MCF-7, T-47D, ZR-75) have provided further support for its usefulness in breast cancer since AHR activation can inhibit E2-responsive genes, cause ERα protein degradation, and inhibit E2-dependent cell proliferation, invasiveness, and anchorage-independent growth (Safe and Wormke, 2003; Matthews and Gustafsson, 2006; Ohtake et al., 2007; Hall et al., 2010). The natural AHR ligands such as I3C and DIM have exhibited inhibition of mammary tumour development and growth in rat models (Safe et al., 1999; 2000). Their effectiveness in reducing tumour formation has led to the development of selective aryl hydrocarbon receptor modulators (SAhRMs). Two classes of compounds are currently under investigation for their therapeutic value. One class is composed of compounds structurally related to I3C and the second group of compounds is composed of alternate-substituted alkyl polychlorinated dibenzofuran (PDCFs) structures (Safe et al., 1999; Safe and McDougal, 2002; Murray et al., 2010). These compounds inhibit E2-induced responses *in vivo* and *in vitro* and decrease mammary tumour development and growth using mouse and rat animal models (Harris et al., 1990; Astroff and Safe, 1991; Dickerson et al., 1995; McDougal et al., 1997).

Recent studies suggest that AHR may be a potential therapeutic target for ERα-negative breast cancer (Zhang et al., 2009) which currently does not have a good prognosis and there are limited treatment options. AHR is expressed in ERα-negative breast cancer cells but its role in cell proliferation and growth has not been intensely studied (Wang et al., 1997). Using the triple negative cell line MDA-MB-468, TCDD was shown to inhibit cell proliferation through the induction of TGFα, determined to have a growth inhibitory function in this cell line (Wang et al., 1997). AHR ligands also induced differentiation responses in ERα-negative breast cancer cells and inhibited invasion (Hall et al., 2010). The SAhRM MCDF inhibited tumour growth in athymic nude mice in which MDA-MB-468 cell were injected directly into the mammary fat pad (Zhang et al., 2009). Interestingly, AHR activation after tranilast treatment (an AHR ligand) has been documented to inhibit mammosphere formation and cancer stem cell marker expression in

the ERα negative MDA-MB-231 cell line (Prud'homme et al., 2010). These preliminary studies highlight the potential value AHR may play in treating ERα negative breast cancer.

# 6 Rationale and Research Objectives

Despite many studies, the molecular mechanisms of reciprocal AHR-ERα crosstalk are not completely understood. Many studies have focused on a small subset of genes to describe their interactions. These include the AHR target genes *CYP1A1* and *CYP1B1* as well as the ERα target genes *TFF-1* and *Cathepsin D*. Studies investigating AHR-ERα crosstalk at other AHR and ERα-regulated genes are needed. Moreover, the molecular mechanisms governing the gene selective inhibition of ERα activity remain unclear. Understanding AHR signal transduction and how AHR modulates ERα signalling beyond this small subset of genes will improve our understanding of their crosstalk and the potential significance of AHR in breast cancer. The role of ERα in AHR signalling is less studied with its effects ranging from inhibition, to activation, to no effect (Kharat and Saatcioglu, 1996; Hoivik et al., 1997; Matthews et al., 2005). Our laboratory has shown recruitment of ERα to the AHR target genes *CYP1A1* and *CYP1B1* but it is unclear whether ERα is recruited to other AHR target genes, suggesting a modulatory role in AHR signalling (Matthews et al., 2005).

In addition, the role of AHR in cell cycle progression and in ERα-negative breast cancer remains unknown. Experiments completed in this thesis outline a novel gene involved in the TCDD-dependent cell cycle arrest named cyclin G2 which was previously characterized to be inhibited by E2-activated ERα (Stossi et al., 2006). This gene highlights the complex interplay between AHR and ERα activity related to cell cycle regulation since they have opposing actions on this gene. The role of AHR in both ER-positive and -negative breast cancer cells has not been fully elucidated due to the lack of good *in vitro* models to study breast cancer in the absence of AHR. To address this, we have created an ERα positive cell line using MCF-7 cells and an ERα negative cell line using MDA-MB-231 cells that have AHR knocked out to characterize the role of AHR in breast cancer cells.

**To address these questions I have three Research Aims:**

1) Determine if ERα is an important modulator of AHR activity

2) Determine the mechanism by which AHR specifically regulates cyclin G2

3) Investigate the role of AHR in ERα positive and negative breast cancer cell lines

## 6.1 Determine if ERα is an important modulator of AHR activity

Previous studies in our laboratory indicated that ERα was an important mediator of AHR activity and part of the activated AHR complex at the regulatory regions of well characterized AHR target genes, namely *CYP1A1* and *CYP1B1* (Matthews et al., 2005). Whether ERα is a general component of the activated AHR complex or is only present at select promoters is still not known. To determine if ERα is a general or gene specific modulator of AHR activity in an AHR ligand dependent manner we used chromatin immunoprecipitation (ChIP) followed by whole genome but promoter focused microarrays (chip) known as ChIP-chip on the Affymetrix human promoter 1.0R array.

## 6.2 Determine the mechanism by which AHR specifically regulates cyclin G2

The ChIP-chip studies completed in Aim 1 identified that AHR was bound to the regulatory regions of numerous genes involved in diverse cellular pathways, including metabolism, differentiation and cell cycle regulation. One gene of interest we decided to focus on was cyclin G2. The expression of cyclin G2 (CCNG2) has been shown to inhibit cell cycle progression by preventing $G_1$ to S phase transition (Martinez-Gac et al., 2004; Xu et al., 2008; Stossi et al., 2009). In our previous ChIP-chip study, we identified an AHR-bound AHRE containing site in the upstream regulatory region of *CCNG2*. CCNG2 is repressed in response to estrogen by the ERα-dependent recruitment of a complex containing nuclear co-repressor (NCoR) and histone deacetylases to the *CCNG2* promoter resulting in the repression of CCNG2 mRNA expression (Stossi et al., 2006). In contrast, TCDD and 3MC induced AHR recruitment to *CCNG2* causing an increase in CCNG2 mRNA levels. However, the molecular mechanisms and characterization of the AHR-dependent regulation of CCNG2 as well as the interplay of these two receptor systems remains elusive. Using, chromatin immunoprecipitation, reporter gene constructs, RNAi, and Co-IP experiments we delineated its regulation and its role in AHR-mediated cell cycle arrest.

## 6.3 Investigate the role of AHR in ERα positive and negative breast cancer cell lines

Current literature suggests that both TCDD and selective AHR modulators (SAhRMs) inhibit mammary tumour cell growth through a complex inhibitory AHR-ERα crosstalk pathway involving multiple general and gene-specific mechanisms. More recently it has been shown that AHR might even have a role in ERα negative breast cancer by inhibiting cancer cell growth and invasion (Zhang et al., 2009; Hall et al., 2010). However, most of these studies investigating the role of AHR in breast cancer development and progression have used RNAi-mediated knockdown of AHR to study its function *in vitro*. These methodologies do not completely eliminate AHR mRNA, leaving some protein to induce a functional response. In order to circumvent the transient nature of RNAi based approaches, laboratories have exposed both human MCF-7 breast cancer cells and the murine Hepa1c1c7 hepatoma cells to low levels of B[*a*]P for 6-9 months in order to generate a stable cell line that is deficient in AHR activity (MCF-7 $AH^{R100}$ and Hepa1 c12, c19,). B[*a*]P is a highly toxic chemical in which its genotoxicity requires metabolic activation through AHR-dependent upregulation of cytochrome P450 enzymes. Culturing cells in the presence of B[*a*]P functions as a selective pressure promoting the survival of cells resistant to B[*a*]P genotoxicity due to a non-functional AHR. However, it is unknown what prolonged exposure to B[*a*]P will do to other cellular functions. A recent study has investigated the basal gene expression pattern in the Hepa1 variants indicating there were differences beyond reduced AHR activity including genes involved in cellular morphology, mitochondrial activity, and proliferation (Fong et al., 2005). I used zinc finger nucleases to knockout AHR in both ERα-positive (MCF-7) and ERα-negative (MDA-MB-231) breast cancer cells to study the effects of AHR loss on breast proliferation and ERα signalling. A description of zinc-finger nuclease technology is described below.

### 6.3.1 Genomic editing using Zinc Finger Nucleases

Genomic editing with engineered nucleases refers to a method of using highly specific molecular scissors to cut and create double strand breaks at desired locations in the genome which will then be repaired incorrectly using the cell's endogenous error prone repair mechanism of nonhomologous end joining (DeFrancesco, 2011; Isalan, 2012). This will eventually lead to frameshift mutations and the abolishment of target gene protein expression. The dependence of nucleases on the cell's error prone double strand break (DSB) repair pathways is an efficient

system since it has been shown that 50% of the double strand breaks induced in mycobacteria caused mutations at the repair site (Gong et al., 2005). There are currently two families of engineered nucleases: Zinc Finger Nucleases (ZFNs) and the Transcription Activator-Like Effector Nucleases (TALENs) (DeFrancesco, 2011; Isalan, 2012). These nucleases are different than RNAi methodologies in that they are able to modify DNA directly inducing complete and reproducible removal of the target gene. Both nucleases contain a DNA binding domain engineered to recognize the gene of interest fused with a non-specific DNA cutting enzyme (DeFrancesco, 2011; Isalan, 2012). The restriction enzyme FokI is used in both systems. However, they differ in their DNA recognition peptides. ZFNs rely on Cys2-His2 zinc fingers while TALENs rely on TALEs. Zinc fingers are more challenging to build in that it is not possible to target just any desired DNA sequence since they preferentially bind to G-rich sequences and require a 5-7 base pair gap between the two zinc finger pairs for proper FokI cleavage domain function (Isalan, 2012). The DNA binding domain of individual ZFNs contain between three and six individual zinc finger repeats with each repeat recognizing 3 base pairs (Isalan, 2012). TALENs are composed 17-18 repeats of 34 amino acids with its DNA specificity determined by amino acids at positions 12 and 13 within each repeat (DeFrancesco, 2011). This allows for the design of TALENs where each repeat recognizes one base pair unlike the ZFN triplet reducing potential off-target effects and increasing the amount of genes that can be targeted. I used ZFNs targeting AHR in exon 1 to generate MCF-7 and MDA-MB-231 *AHR*-null cell lines.

# Chapter 2: Materials and Methods

## 7 Materials

### 7.1 Chemicals and biological agents

A 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 nutrient mixture, (DMEM/F-12, Wisent Bio Products; 319-075-CL), DMEM with 1g/L of glucose (Wisent Bio Products; 319-010-CL), MEM non-essential amino acids (Invitrogen; 11140050), fetal bovine serum (FBS, Wisent Bio Products; 090-150), penicillin/streptomycin antibiotic mixture (PEST, Wisent Bio Products; 450-201-EL), 0.25% Trypsin-EDTA (Wisent Bio Products; 325-042-CL), Phosphate Buffered Saline (PBS, Wisent Bio Products; 311-013-CL), DNase/RNase-Free distilled sterile water (Wisent Bio Products; 609-115-CL) were all used to maintain cells. Dextran coated charcoal stripped FBS (DCC-FBS) was made in-house using DCC purchased from Sigma (St. Louis MO; C6241). The chemicals dimethyl sulfoxide (DMSO, Sigma Aldrich; D8418), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Accustandard, New Haven CT) and 17β-estradiol (E2, Sigma Aldrich; E2758) were used for all experiments. Protein A-Agarose Fast Flow 50% (v/v) was used for both ChIP and Co-IP experiments (Sigma Aldrich; P3476).

The antibodies used for ChIP, Co-IP, and Western Blot analysis include: AHR (H-211), ARNT (H-172), ERα (HC-20), NCoA3 (M-397), CYP1B1 (H-105) and IgG (rabbit immunoglobulin, sc-2027); were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies FOXA1 (ab23738), ARNT (ab2), CCNG2 (ab54901) were all purchased from Abcam (Cambridge, MA). β-actin was used as loading control for Western blots (Sigma Aldrich, A2228). Secondary antibodies used for both Western blots and Co-IP experiments include: Horseradish Peroxidase (HRP)-conjugated donkey anti-rabbit IgG whole antibody (NA934, GE Healthcare; Buckinghamshire, UK), HRP-conjugated chicken anti-mouse IgG (sc2954, Santa Cruz Biotechnology), and HRP-conjugated Clean Blot IP Reagent (Thermo Scientific, 21230). ECL Advance Western blotting detection system (GE Healthcare) was used to visualize proteins for both Western blots and Co-IP experiments. For the purification of PCR products generated from ChIP experiments Buffer PB (Qiagen, 19066) and EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc. Markam, ON; BS664) were used. For RNA extraction and cDNA synthesis, Aurum ™ total RNA mini kit (BioRad, Hercules, CA; 732-6820) and Superscript III reverse

transcriptase (Invitrogen, 18080044) were purchased. For the isolation of reporter gene constructs and mutagenesis products, DNA miniprep (Sigma, PLN350) and maxiprep (Qiagen, 12663) kits were purchased. The *pfu* DNA polymerase used for site-directed mutagenesis was purchased from Stratagene (La Jolla, CA). SsoFast EvaGreen Supermix was used for all quantitative real-time PCR reactions (Q-PCR) (BioRad, 1725200). The ONE-glo™ luciferase assay system was used to detect firefly luciferase reporter gene expression (Promega, 6110). All primers used for real-time PCR and the design of reporter gene constructs were synthesized by Integrated DNA Technologies (IDT, Coralville, IA).

## 7.2 Plasticware

Plasticware used for cell culture was purchased from Sarstedt (Newton, CT) which include: T-25, T-75, and T-175 tissue culture flasks, 10 cm tissue culture dishes, 6-well tissue culture plates, and 2 mL cryovials. The 12-well and 96-well clear flat bottom plates were purchased from BD Biosciences-Falcon (San Jose, CA). The 96-well black untreated flat bottom plates used for luciferase assays were purchased from Corning (Lowell, MA; 07-200-590). The 1.5 ml (Axygen) and 1.7 mL low-binding (Progene) microcentrifuge tubes were purchased from Ultident Scientific (St. Laurent, QC). 96-well non-skirted PCR plates were purchased from D-Mark Biosciences (Toronto, ON; 130104)

## 7.3 Instruments

Cells were maintained in HERAcell® 150 incubators (Kendro, Langenselbold, Germany). The water bath used to warm media was purchased from VWR International (Plainfield, NJ; 1228). Cells were centrifuged with Centrifuge 5702 (Eppendorf, Hamburg, Germany). All protocols that required the spinning of microcentrifuge tubes were preformed using Centrifuge 5424 (Eppendorf). A Locator JR Cryo Biological Storage System (Thermolyne, VWR International) was used to store all human immortalized cell lines. To visualize and count cells for experiments the Bright-Line Hemocytometer (Hausser Scientific, Horsham, PA) and Vistavision Light Microscope (VWR International) were used. The concentration and purity of both DNA and RNA samples were measured with the Ultrospec 2100 *pro* Spectrophotometer (Biochrom, Cambridge, England). The Bradford protein quantification assay as well as β-gal levels were determined using the Thermo Scientific 96 well Multiskan EX Photometer (Waltham, MA). Luciferase levels from reporter gene constructs were measured using GLO-max 96-well

microplate luminometer (Promega, Madison, WI). Fluorescence-activated cell sorting (FACS) to determine cell cycle progression of various cell lines was measured using the BD Biosciences FACS Calibur Analyzer (San Jose, CA). For the sonication of ChIP samples as well as for the lysing of cells for the preparation of protein extracts the Branson Digital Sonifier 450 using a 1/8" inch tapered microtip (VWR, 40000-686) was used. Amplification of ChIP DNA fragments and cDNA generated from the reverse transcription of RNA samples was performed on the Chromo4 RT-PCR detector (BioRad). The DNA Engine Peltier Thermal Cycler (BioRad) was used for cDNA synthesis and site directed mutagenesis protocols.

# *8* Methodology

## 8.1 Maintenance of T-47D, MCF-7 and MDA-MB-231 human breast cancer cells

T-47D is an immortalized human ductal breast epithelial carcinoma cell line. These cells were purchased from American Type Culture Collection (ATCC, HTB-133) and were cryopreserved and stored in liquid nitrogen. In order to preserve cells, approximately 1 million cells were suspended in 1mL of 90% FBS/10% DMSO mixture and placed in liquid nitrogen for long-term storage. To prepare cells for usage, they were rapidly thawed at 37$^{o}$C and transferred into a T-25 coated tissue culture flask containing a 1:1 mixture of DMEM (low glucose)/F-12 medium supplemented with 10% (v/v) FBS and 1% PEST. Once cells reached 90% confluency, they were trypsinized using 1mL of 0.25% trypsin and transferred into a T-75 flask containing the same media. Again, once the T-75 became confluent its contents were trypsinized and transferred into a T-175 tissue culture flask. To maintain cells, they were subcultured ever 2-3 days or when they reached 90% confluence by: aspirating media, rinsing with PBS to remove any residual medium, adding 4 mL of trypsin and once cells detached the trypsin was neutralized with equal volumes of complete media. Cells were then transferred into a new flask and 25 mL of complete media was added. Cells were then placed in an incubator set at 37$^{o}$C and 5% $CO_2$.

MCF-7 is an immortalized human breast adenocarcinoma cell line that was purchased from ATCC (HTB-22). Similar to T-47D cells, 1 million cells were cryopreserved and placed in liquid nitrogen for long-term storage and were propagated using the same methods. Cells were cultured in DMEM low glucose (1g/L) supplemented with 10% (v/v) FBS and 1% PEST. Cells were subcultured every 2-3 days or when they reached 90% confluence by: aspirating media, rinsing

with PBS to remove any residual medium, adding 4mL of trypsin and once cells detached the trypsin was neutralized with equal volumes of complete media. MCF-7 cells are sensitive to trypsinization and were exposed to trypsin at room temperature with careful watch. Cells were then transferred into a new flask and 25 mL of complete media was added. Cells were placed in an incubator set at 37$^{o}$C and 5% $CO_2$.

MDA-MB-231 is an immortalized human basal epithelial breast adenocarcinoma cell line that was purchased from ATCC (HTB-26). It was also stored in liquid nitrogen and thawed similar to T-47D cells. These cells were cultured in DMEM low glucose supplemented with 10% FBS (v/v) and 1% PEST and 1% of NEAA. Cells were subcultured every 2-3 days or when they reached 90% confluence by: aspirating media, rinsing with PBS to remove any residual medium, adding 4 mL of trypsin and once cells detached the trypsin was neutralized with equal volumes of complete media. Trysinization was completed at room temperature. Cells were then transferred into a new flask and 25 mL of complete media was added. Cells were then placed in an incubator set at 37$^{o}$C and 5% $CO_2$.

## 8.2 ChIP-chip experiments

For the ChIP-chip studies T-47D cells were seeded at density of 3 million cells per 10 cm dish containing 10 mL of a 1:1 mixture of DMEM/F-12 supplemented with 10% FBS (v/v) and 1% PEST and placed in the incubator set at 37$^{o}$C and 5% $CO_2$. After 48 h, cells were treated with either 10 μL of DMSO (vehicle control, 0.1% final concentration) or 10 nM of TCDD for 1 h. After treatment, protein-chromatin complexes were cross-linked with 1% of formaldehyde. Cells were left for 10 minutes with constant rocking. Cross-links were quenched using 125 mM of glycine and left for an additional 5 mins with constant rocking. The medium was then aspirated and the cells were washed twice with PBS. After the final wash, 750 μL of PBS/0.1% Tween 20 was added to each dish and cells were scraped and pelleted by centrifugation for 3 mins at 10,000 rpm at 4$^{o}$C. The supernatant was aspirated and the cell pellet was resuspended in 400 μL of TSE I buffer (50 mM Tris-base [pH 8.0], 150 mM of NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with 1X protease inhibitor cocktail (PIC, Sigma). Cells were then sonicated to achieve chromatin fragments approximately 500 bp in length. To achieve this fragment size, cells were sonicated eighty times (four cycles of 2 x 10 second sonications). Solubilized chromatin was then separated from cellular debris by centrifugation at 13,000 rpm for 10 mins at

4°C. The supernatant was then collected in a new microcentrifuge tube and 30 μL of a 50% Protein-A Agarose (Sigma) slurry was added to the chromatin and incubated at 4°C for 2 h to allow for binding to any non-specific sites. After this step the agarose beads were pelleted by centrifugation and 100 μL of chromatin was aliquoted into microcentrifuge tubes containing 0.5 μg/μL BSA and 0.05 μg/μL of salmon sperm DNA diluted in TSE I buffer. One microgram of IgG, AHR (H-211), or ERα (HC-20) was added to each sample and chromatin was immunoprecipitated overnight at 4°C with constant rotation. The following day, 25 μL of Protein A Agarose beads (50% slurry) were added to each sample and left to rotate for another 2 hours at 4°C to allow for binding to any antibody:chromatin complexes. Subsequently, Protein A beads were pelleted by centrifugation at 5,000 rpm for 1 min at room temp and washed with buffers for 5 mins each. Specifically, beads were washed 3 times with 1 mL of TSE I, once with 1 mL of TSE II (20 mM Tris-base [pH=8.0], 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), once with 1mL LiCl buffer (Tris-Base [pH=8.0], 250 mM LiCl, 1mM EDTA, 1% NP-40, 1% Na-deoxycholate), and twice in 1 mL of TE (10 mM Tris-Base [pH=8.0], 1 mM EDTA). After the final wash, protein-DNA complexes were eluted in 110 μL of TE +1% SDS for 1 h, and the cross-links were reversed by overnight incubation at 65°C. Following the incubation period, chromatin was purified using EZ-10 Spin Column PCR Purification Kit (Bio Basic) according to the manufacturer's protocol with some modifications. One hundred micro-liters of eluted chromatin was resuspended in 500 μL of Buffer PB (Qiagen), transferred to the spin tubes provided and centrifuged at 13,000 rpm for 1 min. Columns were washed twice with wash buffer (containing 95% ethanol) and dried by centrifugation. In the final step, DNA was eluted using 50 μL of elution buffer. Immunoprecipitated DNA from 10-cm dish per antibody was linearly amplified using a random hexamer linear amplification protocol with primer A: GTTTCCCAGTCACGGTC(N)9 and primer B: GTTTCCCAGTCACGGTC according the manufacturer's instructions (Affymetrix, Santa Clara, CA). Linearly amplified DNA (7.5 μg) was fragmented by limited DNAseI digestion and hybridized to Affymetrix human promoter tiling arrays 1.0 R (Affymetrix, Santa Clara, CA). Hybridization and washing steps were performed according to the manufacturer's protocol by The Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada).

### 8.2.1 Data analysis

Data analysis was completed by our collaborators Albin Sandelin and Eivind Valen using

CisGenome (Ji et al., 2008). Enriched peaks at a false detection rate of 0.2 were determined by comparing triplicate samples of $AHR_{TCDD}$ and $ER\alpha_{TCDD}$ to triplicate $IgG_{TCDD}$ using TileMAP v2 by a moving average approach using default settings (Ji and Wong, 2005). Regions were merged if the gap between them was <300 bp and the number of probes failing to reach the cut-off was <5. Regions were discarded if they were <120 bp or did not contain at least 5 continuous probes above the cut-off. Enriched AHR and ERα regions were labeled as AHR_TCDD region # and ERα_TCDD region # with the number reflecting their relative ranking in the analysis.

To determine the overlap between $AHR_{TCDD}$ and $ER\alpha_{TCDD}$ data sets, the ChIP regions were first clustered so that regions between the sets that overlapped with >50% of the width of the smallest regions were merged. Each region was then labeled with the gene symbol of the closest transcript regardless of strand using the all_mRNA and unigene annotation from the UCSC Genome Browser (Karolchik et al., 2008). This analysis can potentially give multiple regions per gene but in most cases this assignment was trivial since the tiling array was focused on promoter regions, but there are cases of misannotation. Therefore, gene-region pairs that were further than 10kb away were excluded. This analysis was performed using CisGenome software and human gene build 36 version hg18.

### 8.2.2 Transcription factor binding site analysis

Transcription factor binding site prediction was done by searching potential TFBSs using motif models from the JASPAR database (Bryne et al., 2008) and the ASAP framework (Marstrand et al., 2008) as previously described by our collaborators (Liu et al., 2008). Briefly, all the hits were counted that scored above a threshold t, set to 85% of the total scoring range for a given model. We then performed two types of over-representation tests, one using a background and one comparing differences between the three data sets ($AHR_{TCDD}$, $ER\alpha_{TCDD}$ and the intersect). To address the different lengths of the ChIP regions within each set, we used two different strategies. When comparing to background, we constructed a specific background similar in length and position to the set it was compared to, which was constructed using the transcription start sites (TSSs) of 10,000 transcripts samples randomly from Ensembl (Flicek et al., 2008). In order to avoid bias caused by proximity to genes, we sampled the background sequence relative to the TSS of this transcript based on empirically observed distributions of distances (to the closest TSS) and region lengths for each set. We then counted the number of sequences that had

at least one subsequence that scored above the threshold (t). For the inter-set comparison, we used simulations to achieve Z scores as described in (Liu et al., 2008). By using this matrix of Z values, we clustered the transcription factor models (rows) and the data sets (columns) with a hierarchical clustering method. The Z value was transformed into a colour and presented in a heat map. A trace was added to make it easier to distinguish similar colours. The heatmap.2 function in the R gplots() package was used to draw the heat map (Ihaka, 1996). To focus only on the most strongly overrepresented and underrepresented patterns, we filtered out any pattern that did not have score of at least 3 SD.

## 8.3 ChIP and re-chip experiments

To confirm the recruitment of AHR and ERα to the regions isolated from the ChIP-chip studies we completed ChIP and re-chip experiments. ChIP experiments were done as described above. For re-chip experiments, the first antibody-bound protein-chromatin complexes were released by incubation with 50 mM of dithiothreitol/1% SDS for 1 h at 37$^{o}$C. The supernatant was then collected and diluted 40 times to a final volume of 2 mL. Two micrograms of the second antibody was added to 900 μL of the supernatant and rotated for 2 h at room temperature before ChIP washes were complete.

The ChIP DNA was quantified by Q-PCR using SsoFast EvaGreen SYBR supermix (BioRad) with specific primers targeting the region of interest. Primers were designed by entering the isolated regions from the ChIP-chip analysis into Primer Express 3.0 software using the default settings (Applied Biosystems, Carlsbad, CA) and were verified using the NCBI Blast program. Samples were run in triplicate on the Chromo4 Real-Time PCR detector (Bio-Rad). Cycling conditions consisted of an initial denaturation step of 3 mins at 95$^{o}$C. This step is also required to activate the SYBR Green enzyme. This was then followed by 45 cycles of 95$^{o}$C for 5 s (denaturation of double strands) and then 60$^{o}$C for 20 s (annealing of primers and elongation). After each cycle the plate was measured. Data were analyzed using Opticon Monitor 3 software (Bio Rad) and the obtained results were normalized to 5% total input and reported as percent recruitment relative to 5% total chromatin input (in some cases the results were normalized to 100% input). Fold change was calculated using the comparative $C_T$ method of analysis. Percent recruitment was calculated using the difference between the cycle threshold ($C_T$) value of each treatment condition compared to $C_T$ value of each total (average $C_T$ for total input-$C_T$

sample=$\Delta C_T$). This was then transformed using the following equation: $2^{\wedge CT}$ x 100 to obtain a percentage value that was compared to a 5% total input. The primers used for the ChIP confirmation studies as well as re-chip studies are found in **Table 1**.

**Table 1. Q-PCR primers used for ChIP-chip confirmation, ChIP analysis, and re-chip experiments.**

| AHR region | ERα region | Closest gene | Sequence 5'-3' |
|---|---|---|---|
| 18 | 22 | RERG | TCCTTCCCTTATTGCCTTTGG |
| | | | TCTAGGCTTGAGGCTGACCATT |
| 1057 | 547 | HES1 | GCGTGCAGTCCCAGATATATATAGAG |
| | | | CCAGCTCCGGATCCTGTGT |
| 1 | 28 | TiPARP | GGGAGGGCAGTCACGCTAT |
| | | | GTCCTCCCCCGGTGAACT |
| 6 | 111 | SYCP2 | AAGCGTGGATTTACCTGACAAGTAA |
| | | | CCAGTCCCCCAGTTCAGTAGAC |
| 60 | 328 | MSMB | CAGTCTCTGGGACAATCCATTG |
| | | | AACTATGCACAGAACCCAGGAACT |
| 102 | 452 | JAK1 | TGTGAGTTTATGGACAGCAGGAA |
| | | | CAGAATGCCTATGGCTGCAA |
| 156 | N/A | HDAC7A | GCACGCACCCTCGCATA |
| | | | TGATTCTGCCCCTTGTAAACATG |
| 3 | 85 | TMEM30a | TGAAGGGAGTCGGAGCATTT |
| | | | CCTCCCCCGACTCAATCC |
| 4 | 4 | SYT12 | CGACAGCCAGAGGCCAGAT |
| | | | ACAGCACAACAGGCGTGGTA |
| 9 | 12 | TiPARP | CACAGGCCGGTTTGGTATG |
| | | | GGCACAATTGGCACAGATTG |
| 37 | 100 | CCNG2 | GTGTGAAAGTGTCTCTGGCCTAAG |
| | | | TCCCAGGACGGGACAAAA |
| 54 | 335 | ITPR1 | AATCTCAGAGCCGGCAAGTC |
| | | | GACCTCTCCGGTGCACAAA |
| 1184 | N/A | PROX1 | CCGGTGAAAGGGACGTTCTA |
| | | | CTCCTTGGGCTTTGCAAGTT |
| 326 | 601 | PROX1 | CGTGAGCATACACCGAGCAA |
| | | | CATGCATCTGGTTCTGGGTTAA |
| 204 | 118 | ESR1 | GGCTGGCTGCGTATGCA |
| | | | CACTGTAACCGAGATAGGGCAGAT |
| 210 | 183 | RERG | GGGCAAGCAAGCGTCTTTAT |
| | | | GATGAGCGCATGCAAACAGA |
| 56 | 322 | PSG2 | AATGCACAACATTCTGCAAACAT |
| | | | GGAATCCACGCCAACTCTGA |
| 78 | 369 | PSG5 | TGTCTTCCGTGGCAATCATTAT |
| | | | AAAGCCATTTGACTCTAGGACACA |

| | | | |
|---|---|---|---|
| 51 | 253 | PSG9 | GTGGCTTCCGTGACAATCATT |
| | | | AAAGCCATTTGACTCTAGGACACA |
| 14 | 78 | SORL1 | TTGTCAGATGTACCCTTGTTTTTACTG |
| | | | CCAGCCTGAAGCACAGCAA |
| 43 | 10 | ITPR1 | ACCCATGTTAGCCACTCCAAA |
| | | | TCAAGCATTGGGCCAGAAG |
| 252 | 40 | GREB1 | CCAGGCTGCCAGCTGACT |
| | | | CAAAGGGTCAGGAGAAGAACACA |
| 111 | N/A | CYP1A1 | GGAGGGAGAGGAACTGTCGAA |
| | | | TGCACCGCATCCCCTTATT |
| 35 | N/A | FOXN4 | TGTGGCTTTGGCTTTCCTTT |
| | | | AACCAAGACGCAGATTAGAGCAT |
| 48 | N/A | TBL1XRL1 | CGCGGCATCATATGGACAT |
| | | | TTTACGCACACAAAGGTGTTCAC |
| 351 | 17 | HDAC11 | CAAGACCTTTGCAGGCAGTACTG |
| | | | GAGTGAGCGGGCTTGAAAAA |
| 257 | 539 | GPRC5 | CCAGCAACTCCGGGTGAT |
| | | | GCCGCGGTCTACGACAAC |
| 10 | 107 | CYP1B1 | ATATGACTGGAGCCGACTTTCC |
| | | | GGCGAACTTTATCGGGTTGA |
| 63 | 286 | HOXC10 | CCCCAGGGTTGGATAATGCT |
| | | | TTCTGGCGGGCTCTCAGT |
| CCNG2 AHRE2 | | distal | TGGGTTACCAAGGACCAAGAA |
| | | | CCAGAGGTTGTAGTGCTGTTGTTT |
| CCNG2 AHRE1 | | distal | AACTCTCCCGTGGCTGAAAA |
| | | | CGCGGCGCTTCTCCTAA |
| CCNG2 TATA | | proximal | GGGAGGCCGCGAGAGA |
| | | | GGGAGGCCGCGAGAGA |
| CYP1B1 | | distal | ATATGACTGGAGCCGACTTTCC |
| | | | GGCGAACTTTATCGGGTTGA |
| CYP1B1 | | proximal | GTTACCGCACAATGGAAACGT |
| | | | GGAGCTCTACCAGCAGGCTTT |
| TFF-1 | | proximal | CCGGCCATCTCTCACTATGAA |
| | | | CCTCCCGCCAGGGTAAATAC |
| GREB1 | | distal | GAAGGGCAGAGCTGATAACG |
| | | | GACCCAGTTGCCACACTTTT |

### 8.3.1 ChIP experiments to determine recruitment to *CCNG2*

Three million T-47D cells were seeded in 10 cm dishes in 1:1 mixture of DMEM: F-12 phenol red free media supplemented with 5% (v/v) dextran-coated charcoal (DCC) stripped serum and 1% penicillin/streptomycin. Serum was prepared in-house by first suspending 3.12 g of dextran-coated charcoal (Sigma) in 50 mL of 10 mM Tris-base (pH 7.4) then centrifuged at 4000 rpm for

10 mins. The supernatant was aspirated and the washing with Tris-base (pH 7.4) was repeated 2 more times. After the final wash, DCC suspended in 10 mM Tris (pH7.4) was stored overnight at 4$^{o}$C. Fifteen milliliters of DCC solution was added to 500 mL of heat-inactivated fetal calf serum (FCS, Invitrogen) and incubated overnight at 4$^{o}$C with constant stirring. FCS was then centrifuged for 10 mins at 4,000 rpm and the supernatant transferred to a new bottle and then incubated with 15 mL of DCC solution with constant stirring at 56$^{o}$C for 45 mins. The overnight followed by 45 mins at 56$^{o}$C incubation with fresh DCC solution was repeated again to ensure serum was stripped. After the final incubation, the stripped serum was syringe filtered using a 0.2 μm filter and transferred to conical tubes for storage at -20$^{o}$C. Seventy-two hours after plating using stripped-serum, cells were treated with DMSO (final concentration 0.1%), 10 nM TCDD, 10 nM E2 or E2 + TCDD (10 nM) for 0.75 h. ChIP and re-ChIP assays were performed as described above and recruitment was determined by Q-PCR using primers targeting *CCNG2 AHRE2 (distal)*, *CCNG2 AHRE1 (proximal)*, *CCNG2 TATA (proximal) (***Table 1***)*.

### 8.3.2 MCF-7 and MDA-MB-231 ChIP experiments

Four million MCF-7 AHR$^{+/+}$/AHR$^{-/-}$, MDA AHR$^{+/+}$/AHR$^{-/-}$, and MDA cells stably expressing ERα (generously provided by Dr. Craig Jordan; (Jiang et al., 1992)) were seeded in 10 cm dishes in DMEM phenol red free media supplemented with 5% (v/v) DCC-stripped serum and 1% PEST (MDA cells were also supplemented with 1% NEAA). After 72 h, cells were treated with DMSO (final concentration 0.1%), 10 nM TCDD, 10 nM E2 or E2 + TCDD (10 nM) for 0.75 h. ChIPs were performed as described above. Enrichment levels (relative to 100% total input) were determined by Q-PCR using primers targeting *CYP1B1* proximal and distal regulatory regions, as well as *TFF-1* and *GREB1* regulatory regions (**Table 1**).

## 8.4 Gene expression analysis: mRNA time course

Approximately 300,000 T-47D cells were seeded in six-well plates and grown in a 1:1 mixture of DMEM and F-12 Ham's nutrient mixture. Cells were treated with either 10 nM of TCDD or DMSO (0.1% final concentration) for 1.5, 3, 6, or 24 h. After treatment, cells were washed once with PBS and lysed with 350 μL of cell lysis buffer (Aurum Total RNA mini kit, BioRad) containing 1% β-mercaptoethanol. After lysis, cells were scraped and placed in microcentrifuge tubes. RNA binding was completed by adding 350 μL of 70% ethanol and mixed by gentle pipetting. RNA was then isolated using the Aurum Total RNA mini kit following the

manufacturers protocol. Briefly, RNA was bound to the spin columns by centrifugation at 13,000 rpm for 30 sec. The column was then washed once with 700 μL of low stringency wash buffer and centrifuged for 30 s at 13,000 rpm. The column membrane was then dried and 5 μL of reconstituted DNase1 diluted in 75 μL of DNAse1 dilution buffer was added to each column and left for 15 mins at room temperature. This reaction was stopped with the addition of 700 μL of high stringency wash buffer and then centrifuged for 30 s at 13,000 rpm. The column membranes were washed again with 700 μL of low stringency wash buffer and centrifuged for 1 min at 13,000 rpm and the flow-through discarded. To dry the membrane, columns were centrifuged again for 1 min at 13,000 rpm. To elute the RNA, columns were placed in new microcentrifuge tubes and 40 μL of elution buffer was added and samples were centrifuged for 2 mins at 13,000 rpm.

The concentrations of RNA samples were measured using a spectrophotometer (Biochrom) and all samples were diluted to a concentration of 50 ng/μL in DNase/RNase-free distilled water. To synthesize cDNA, 500 ng of extracted RNA (10 μL) was reverse transcribed using Superscript III (Invitrogen) in a total volume of 20 μL containing 1 μL of 50 mM random hexamers, 1 μL of 10 μM dNTP mixture, and 2 μL of 0.1 mM DTT, and 4 μL 5X concentrated first strand buffer. Complementary DNA was synthesized for 1 h at 50$^{o}$C and then increased to 70$^{o}$C for 15 mins after which samples were diluted with 60 μL of DNase/RNase free water. Q-PCR was preformed in triplicate using 1 μL of cDNA samples using the SsoFast EvaGreen SYBR supermix (BioRad) and primers were generated using Primer Express 3.0 (Applied Biosystems) **(Table 2).** Data were analyzed using Opticon Monitor 3 Software (BioRad). All target gene transcripts were normalized to ribosomal 18s levels. Fold change was calculated using the comparative $C_T$ ($\Delta\Delta C_T$) method of analysis and the expression level of each gene was compared to time-matched DMSO samples.

**Table 2. Q-PCR primers used for mRNA expression analysis**

| mRNA primers | Sequence 5'-3' |
|---|---|
| CYP1A1 | TGGTCTCCCTTCTCTACACTCTTGT |
| | ATTTTCCCTATTACATTAAATCAATGGTTCT |
| CYP1B1 | CCAGATCCCGCTGCTCTACA |
| | TGGACTGTCTGCACTAAGGCTG |
| PROX1 | CCTTATTCGGGAAGTGCAATG |

| | |
|---|---|
| | TGAGCTTTGCTTTTTTCAAGTGAT |
| CYP2A6 | TCAAGTCCTCCCAGTCACCTAAG |
| | GGCAAAGCCCACGTGTTT |
| CYP2A7 | CTGAACACAGAGCACATATGTG |
| | GTGTTCACCATTCACTTGGGG |
| LGI1 | ATGGAACGGAAACGGATTCTAC |
| | CCACATCAGTGTCCCTGTACCA |
| MSMB | TGGGCAGCGTTGTGATCTT |
| | TGAAATAGCATGATGCATTGCA |
| CLPS | CCTGACCTGTGAGGGAGACAA |
| | GCCAAAGTTGGTGTTGGTGAT |
| JAK1 | TGGCCAGATGACAGTCACAAG |
| | CACGGCAGGCGTTTTCC |
| TOP2A | GCAGTCACAAGCAAGAAATCCA |
| | GGAGCCACAGCTGAGTCAAAG |
| CYP2B6 | CGTGCGGAATTGTTCCTCTT |
| | GGGCTGGCCATGGAGAA |
| HDAC7A | CCCATCGCCCGAGAGTT |
| | CAGCATCAAATCCAGCAGACA |
| RERG | ACTAGACACTGCTGGTCAGGAAGA |
| | CCCCCCATCGCATGTG |
| HES1 | GCTGAGCACAGACCCAAGTG |
| | GGGTCACCTCGTTCATGCA |
| TiPARP | GGCAGATTTGAATGCCATGA |
| | TGGACAGCCTTCGTAGTTGGT |
| SYCP2 | GGGACAGCCAAGCTCTAAAATG |
| | GGAATTGTCTTGTCCATCTTTTTTG |
| CCNG2 | TGCAACTGCCGACTCATCTT |
| | AAATTGAGAAGGCACAAGGCTAA |
| IL6ST | TGGGATGGTGGAAGGGAAA |
| | GTGTTGCCCATTCAGATTTTAAAGT |
| SORL1 | GAAGTGCGACGGGATGGAT |
| | GGTTTTCGCAGTTGGCTTCA |
| ITPR1 | GCAGAGGTGGACCCTGATCA |
| | CCATCTTTTCTTGGGCATTCC |
| COL9A2 | GTCGCCCGGGAACCAT |
| | TTGGTTGGACACAGGAAATCC |
| TMEM30a | GCCGATACTCTTTGAATGTCACAT |
| | TCATCCGTTTTCGTCCATCA |
| TBLXR1 | TGGTTCCTATGATGGGTTTGC |
| | GCTGCCCTAAGGTGCTAGCA |
| HDAC11 | TGTGCCTATGCGGACATCAC |
| | GCCCTCCACACGCTCAAA |
| GPRC5 | CGGCGCTTCCTCTTTGG |
| | TGAGCCGCCAGACAAGAGA |
| HOXC10 | CCTGCTCCTACCCACCTAGTGT |

| | |
|---|---|
| | CTTCTCTGCGCTGTACATGCA |
| SMAPL1 | CGGAAAAGCTCCCCGAAA |
| | TCAAGGCCCAACAAATCCA |
| ESR1 | CTTGATACACTGCAGATTCAGATGTG |
| | GGAACCCATGACCGGAAAG |
| GREB1 | CAAAGAATAACCTGTTGGCCCTGC |
| | GACATGCCTGCGCTCTCATACTTA |
| FOXN4 | TGCCATCCACCGGAGTATG |
| | CGGTCGGAGATCAGCCTTGTC |
| IL6ST | TGGGATGGTGGAAGGGAAA |
| | GTGTTGCCCATTCAGATTTTAAAGT |
| FOXA1 | GAAGATGGAAGGGCATGAAA |
| | GCCTGAGTTCATGTTGCTGA |
| TFF-1 | CATCGACGTCCCTCCAGAAGAG |
| | CTCTGGGACTAATCACCGTGCTG |
| VEGF | TCCTCACACCATTGAAACCA |
| | GATCCTGCCCTGTCTCTCTG |
| CA9 | ACTTCAGCCGCTACTTCCAA |
| | AGAGGGTGTGGAGCTGCTTA |
| FOXA2 | CACCACTACGCCTTCAACCA |
| | GCTGCTCCGAGGACATGAG |
| FOXA3 | TCCCTTACTACCGGGAGAATCA |
| | CAGCGAGTGGCGAATGG |

### 8.4.1 CCNG2 mRNA expression time course

T-47D cells were seeded in six-well plates and grown in a 1:1 mixture of DMEM: F12 supplemented with 5% DCC and 1% PEST. Cells were treated with either 10 nM TCDD, or pre-treated for 1 h with 1 μM CH223191(Sigma) for 0.75, 1.5, 3, 6, or 24 h. RNA was isolated as described above and all target gene transcripts were normalized to ribosomal 18s RNA levels and fold inductions where calculated using time-matched DMSO treated samples and the $\Delta\Delta$ $C_T$ method was used for data analysis. Q-PCR primers used to measure CCNG2 levels are found in **(Table 2).**

### 8.4.2 mRNA expression analysis in MCF-7 and MDA $AHR^{+/+}$ & $AHR^{-/-}$ cells

MCF-7 and MDA $AHR^{+/+}$ and $AHR^{-/-}$ were seeded in 6-well plates and grown in DMEM phenol red free media supplemented with 5% DCC-stripped serum and 1% PEST. After 72 h, cells were treated with either DMSO (final concentration 0.1%), 10 nM TCDD, 10 nM E2 or E2 + TCDD (10 nM) for 6 and 24 h or 100 μM of $CoCl_2$ for 24h. RNA was isolated using illustra RNAspin

mini columns (GE Healthcare) and reverse-transcribed as described above. All target gene transcripts were normalized to ribosomal 18s RNA and fold inductions were calculated using DMSO control samples and the $\Delta\Delta C_T$ method. Q-PCR primers used in these experiments are available in **Table 2**.

## 8.5 Western blot analysis

Approximately 3 million T-47D cells were plated in 10 cm dishes using complete media and treated with 10 nM TCDD or DMSO for 1 h after which cells were washed twice with PBS and scraped using 750 μL of PBS/0.1% Tween 20 for AHR and ERα protein extraction. For analysis of CCNG2 and FOXA1 protein levels, 3.5 million T-47D cells were plated in stripped-serum and treated DMSO or 10 nM TCDD for 6 h. For analysis of AHR, ARNT, ERα, and CYP1B1 in MCF-7 and MDA-MB-231 cells, 3.5 million cells were plated in 10 cm dishes using DCC-stripped serum. After plating, cells were pelleted by centrifugation at 10,000 rpm for 3 mins at 4$^{o}$C. The supernatant was aspirated and cell pellets were first frozen then resuspended in 200 μL of cell lysis buffer (50 mM HEPES, 10% glycerol, 100 mM KCl, 2 mM EDTA, 0.1% NP-40) containing 1X PIC. Cell lysates were sonicated on ice for 10 s at 20% amplitude and rotated for 20 mins at 4$^{o}$C. Solubilized protein was then quantified by the Bradford reaction. For immunoblots 50 μg of protein extract was resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane in 25 mM Tris base (pH 8.3) containing 19.2 nM glycine and 20% (v/v) methanol. The membrane was blocked in 2% (w/v) ECL-Advanced blocking agent for 1 h at room temperature with constant rocking and then incubated with 1:5,000 anti-ERα (HC-20), 1:5,000 anti-AHR (SA-210/H-211), 1:5000 anti-ARNT (ab2), 1:5000 anti-FOXA1 (ab23738), 1:5000 anti-CCNG2 (ab54901), 1:5000 anti-CYP1B1(H-105) overnight at 4$^{o}$C with constant rocking. The membrane was then washed three times in PBS/0.1% Tween 20 and incubated with 1:200,000 horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (NA934, GE Healthcare) for 1h at room temperature with constant rocking diluted in the 2% blocking solution. For detection of β-actin, membranes were stripped using Restore Plus Western Blot Stripping solution (Thermo Scientific). Briefly, membranes were incubated with 10 mL of stripping solution for 20 mins and washed 5 times with PBS/0.1% Tween 20. After washing, membranes were blocked again and 1:500,000 dilution of primary mouse anti-β-actin antibody (Sigma) was incubated for 2 h at room temperature followed by 30 mins wash using PBS/0.1% Tween 20. After washing, membranes

were incubated with 1:200,000 HRP-conjugated anti-mouse secondary antibody (Santa Cruz) for 1h at room temperature and then washed again. Proteins were visualized using the ECL-Advance chemiluminescent substrate (GE Healthcare) according to the manufacturer's instructions. The membranes were exposed to autoradiography film for 30 sec-5 min.

## 8.6 RNAi-mediated knockdown studies

### 8.6.1 SMARTpool siRNA against AHR, ERα, and CCNG2

Three hundred thousand T-47D cells plated in 6 well dishes using complete media were transiently transfected with siRNA targeting AHR (L-00490-00-0020) and ERα (L-003401-00-0020) using the ON-TARGETplus SMART pool system and DharmaFECT1. For CCNG2 (L-003217-00-0005) studies, cells were plated in DCC-stripped serum. Twenty-four hours after plating, 2 μM of siRNA against AHR and ERα, CCNG2 or non-targeting pool (NTP, D-0011810-10-20, Dharmacon) were transfected using 4 μL of DharmaFECT 1 and 400 μL of Opti-MEM. Briefly, 2 μM of siRNA was diluted in 100 μL of DNase/RNase free water and then 100 μL of Opti-MEM was added which was then left for 5 mins. In a separate tube, 4 μL of DharmaFECT 1 was added to 200 μL of Opti-MEM and left for 5 mins. The DharmaFECT 1 solution was added to the diluted siRNA and left for an additional 20 mins. After this incubation period, 400 μL of mixture was added to each well in a drop-wise fashion to make the final siRNA concentration 100 nM. ChIP assays, mRNA isolation, and whole cell extracts were prepared 48h after transfection as described above.

### 8.6.2 Single siRNAs targeting FOXA1, ERα, and CYP1B1

Three hundred thousand T-47D cells plated in 6 well dishes using media supplemented with DCC-stripped serum were transiently transfected with single siRNAs targeting FOXA1 denoted as siFOXA1 seq2 (SASI_Hs01_00168404), siFOXA1 seq3 (SASI_Hs01_00168403) and a universal negative control (Sigma). Single siRNAs targeting ERα were purchased from Dharmacon and were labeled siERα11 (J-003401-11-0050) and siERα14 (J-003401-14-0050). Single siRNA were transfected using DharmaFECT 1. Transfection was completed similar to SMARTpool siRNA and ChIP and mRNA experiments were completed 48 h after transfection.

siCYP1B1 seq1 (SASI_Hs01_00020014), seq2 (SASI_Hs01_00301876), and seq3 (SASI_Hs01_00020012) were purchased from Sigma-Aldrich (St. Louis, MO). Briefly, MCF-7 or MDA cells were seeded at 350,000 cells in a 6-well plate in medium containing 5% DCC-stripped serum. After 24 h, 50 nM of pooled siCYP1B1 or universal negative control were transfected using 2 μL of DharmaFECT (Dharmacon) and 400 μL Opti-MEM.

### 8.6.3 shRNA-mediated AHR knockdown

The T-47D shAHR cell line was generated using the pSuperior Tet-ON inducible vector system (OligoEngine, Seattle, WA). Briefly, T-47D cells already stably expressing Tet-R (T-47D Tet-ON cell line; Clontech, 631144) were transfected with the pSUPER.shAHR plasmid (sense 5`-GATCCCCGAACTCAAGCTGTATGGTATTCAAGAGATACCATACAGCTTGAGTTCTTT TTC-3`; antisense 5`- TCGAGAAAAAGAACTCAAGCTGTATGGTATCT CTTGAATACCATACAGCTTGAGTTCGGG-3`). Cells were selected after addition of 4μg/ml puromycin. T-47D shAHR cells were treated with 1μg doxycycline (Clontech, 631311) for 1 week to achieve maximal AHR knockdown prior to RNA extraction.

## 8.7 CCNG2 reporter gene construct and mutagenesis

The plasmid pGL4-*CCNG2* containing -1.402 kb to +179 of the upstream regulatory region of *CCNG2* as well as plasmids pGL4-*CCNG2* ΔAHRE1 and ΔAHRE2 were generously provided by Dr. Chun Peng (York University, Ontario, Canada; (Fu and Peng, 2011)). The numbering of the cloned *CCNG2* regulatory region was relative to CCNG2 mRNA (NM_004354). Site-directed mutagenesis targeting the AHRE2 and upstream FKH sites using the *CCNG2* full-length plasmid were completed using 50 ng of the plasmid, 1 μL *pfu* Turbo, 5 μL 10X *pfu* Buffer, 1 μL 10 mM dNTPs, 40 μL of water, and 1 μL 10μM of the forward and reverse PCR primers (**Table 3**, mutated residues underlined). PCR was amplified by an initial heating to 95$^{o}$C for 5 min, followed by 25 cycles of 95$^{o}$C for 1 min, 50$^{o}$C for 1 min, and 70$^{o}$C for 10 mins, then a cooling at 72$^{o}$C for 7 mins and final cool down at 4$^{o}$C for 10 mins. After the reaction was complete 1 μL of DpnI (NEB) restriction enzyme was added to each PCR reaction and incubated for 1 h at 37$^{o}$C. Ten microliters of the PCR reaction was transformed using DH5-α bacterial cells and incubated overnight at 37$^{o}$C. Colonies were selected and checked through DNA sequencing.

**Table 3. PCR Primers used for site-directed mutagenesis experiments.**

| | |
|---|---|
| AHRE2 | CTCATCAGCGCGCTAAGTTTT |
| | AAAACTTAGCGCGCTGATGAG |
| FKH3 | TAGGAGGGAG AGAGTCCCAAAATAAATGTTCCAG |
| | CTGGAACATTTATTTTGGGACTCTCTCCCTCCTA |
| FKH4 | CCGTAATTATT AGATTGGGACGTACCCTCATCAG |
| | CTGATGAGGGTACGTCCCAATCTA ATAATTACGG |

## 8.8 Luciferase reporter gene activity

Approximately 125,000 T-47D cells were plated in 12-well dishes using a 1:1 DMEM: F-12 mixture containing 5% DCC-stripped serum. Twenty-four hours after plating, cells were transfected with luciferase reporter vectors using Lipofectamine LTX (Invitrogen, Burlington, Canada). Briefly, 200 ng of luciferase reporter gene constructs, 100 ng of CH100-βgal (GE Healthcare, used to normalize for transfection efficiency), and 700 ng of empty vector (pGL3basic, to achieve a total of 1 μg of DNA) was added to 50 μL of OptiMEM supplemented with 1 μL of Plus Reagent (Invitrogen) and was left for 5 mins. In a separate tube, 2 μL Lipofectamine LTX was added to 50 μL of OptiMEM and left for 5 mins. Following the incubation period, the lipofectamine mixture was added to the DNA mixture and incubated for another 20 mins and then the 100 μL mixture was added to each well in a drop-wise manner. After 24 h, the media was changed and cells were dosed with either DMSO (0.1% final concentration) or 10 nM TCDD and left for another 24 h. The following day, cells were lysed and luciferase activity was determined using the ONE-Glo system according to manufacturer's recommendations (Promega, Madison, WI). Briefly, 250 μL of the 1X passive lysis buffer was added to each well with constant shaking for 10 mins. After lysis was complete, 25 μL of sample in duplicate and 25 μL of ONE-Glo were added to black flat bottom 96-well plates (CoStar). Luciferase activity was measured using the GLO-max luminometer using the manufacturer's recommended settings (Promega). Data were first normalized to β-galactosidase levels by adding 20 μL of lysed sample, 100 μL of β-gal buffer (0.6 M $Na_2HPO_4$, 0.04 M $NaH_2PO_4$, 0.01 M KCl, 1 mM $MgSO_4$, 0.3% β-mercaptoethanol), 25 μL of 4 mg/ml of ONPG (*ortho*-Nitrophenyl-β-galactoside) into a clear flat bottom 96-well plate. The plate was then incubated at 37$^{o}$C for 30 min to 4 h, until it turned a light yellow after which 25 μL of 1M $Na_2HCO_3$ was added to each

well to stop the reaction. The plate was then read using the Multiskan EX Photometer (ThermoScientific) at 420 nm. Data were also normalized to DMSO control samples.

## 8.9 Co-immunoprecipitation studies

For Co-IP studies, 3 million T-47D cells were seeded in 10-cm dishes in 1:1 mixture of DMEM:F-12 supplemented with 5% DCC-stripped serum. After 72 h, cells were treated with either 10 nM TCDD or DMSO (final concentration 0.1%) for 1 h and cross-linked using 1% formaldehyde for 10 min and quenched using 125 mM glycine for 5 mins. Cell lysates were pre-cleared using 30 μL of a 50% slurry of Protein A and protein complexes were immunoprecipitated using 2 μg of rabbit IgG (Sigma), AHR (H-211), FOXA1 (Abcam 23738), or NCoA3 (M-397) for 2 h. Beads were washed four times for 5 mins each with 1 mL of wash buffer (10 mM Tris HCl pH 8.0, 150 mM NaCl, 10% glycerol, and 1% NP-40, and 2 mM EDTA). Eighty micro-liters of 1X sample buffer with 100 mM of DTT were added to the beads and samples were heated to 70$^{o}$C for 10 mins. Samples were loaded on an 8% SDS-PAGE gel, transferred to nitrocellulose and visualized using the Clean Blot anti-rabbit HRP (Thermo Scientific) and ECL-Advanced.

## 8.10 Generation of zinc-finger mediated AHR$^{-/-}$ cell lines

MCF-7 and MDA-MB-231 AHR$^{-/-}$ cells were generated using a CompoZr knockout ZFN targeting AHR plasmid (catalogue no. CKOZFN26436) purchased from Sigma-Aldrich (St. Louis, MO). Briefly, 2 x $10^6$ MCF-7 or 1 x $10^6$ MDA-MB-231 cells were transfected with 2μg of each vector encoding the ZFN targeting AHR using nucleofector kit V and Amaxa nucleofector (Lonza, Mapleton, IL) according to the manufacturer's recommendations. Three days post transfection, cells were serial diluted into 2 x 96-well plates from an initial seeding density of 100,000 cell/well. The CEL-1 assay was then performed to determine zinc-finger nuclease activity. Briefly, genomic DNA was isolated from transfected MCF-7 and MDA-MB-231 cells using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, G1N70) following the manufacturer's recommendations. Primers designed around the ZFN binding site were then used to amplify the genomic DNA. DNA was amplified using 28 μL of dd$H_20$, 1 μL of GC rich PCR buffer, 1 μL 10 mM dNTPs, 1 μL of GC rich DNA polymerase (Invitrogen), 1 μL of the 25 μM forward (5`-CACTGTCCCGAGAGGACG-3`) and reverse (5`-GGGAATGGACCTAATCCCAG-3`) primers, and 200 ng of genomic DNA diluted in 8 μL

volume with the following protocol: initial denaturation for 5 mins at 95°C, followed by 33 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s followed by a final extention of 72°C for 5 mins then a cooling to 4°C. After cooling, 10 μL of the amplified DNA was then tested for the presence of aberrant repair induced by the ZFN-mediated double strand breaks. The amplified DNA is denatured and re-annealed (95°C for 10 mins, 95°C to 85°C at a rate of -2°C/s, 85°C to 25°C –0.1°C/s) creating heteroduplex formations between wildtype and modified amplicons (due to NHEJ). The CEL-1 mismatch endonuclease was then added which will cleave the heteroduplex molecules (Transgenomic SURVEYOR kit, 706025). CEL-1 enzyme digests were then resolved by running samples on a 10% PAGE-TBE gel. At least 24 clones were screened for the presence of indels (insertions or deletions) at the zinc finger recognition site in exon 1 of *AHR*. The ability of TCDD to induce CYP1A1 mRNA expression levels by Q-PCR was assessed in clones with genetic alterations that result in frame shift errors. Two clones from each cell line that displayed significantly reduced AHR transactivation were transfected a second time with the ZFN plasmids and the screening procedure repeated as described above. Clones that displayed no TCDD-dependent increases in CYP1A1 mRNA levels and that did not have any AHR expression in Western blots were used in subsequent assays.

## 8.11 Cell proliferation using the Sulforhodamine B assay

The Sulforhodamine B (SRB) colourimetric assay was used for the determination of cell proliferation (Vichai and Kirtikara, 2006). Four thousand MCF-7/MDA $AHR^{+/+}$ or $AHR^{-/-}$ cells were plated in DCC-stripped serum in 96 well plates. The next day, cells were treated with DMSO, 10 nM TCDD, 10 nM E2 (MCF-7 only), or 10 nM E2+TCDD (MCF-7 only) for 4, 6, or 8 days with media being replaced every 2 days. At the end of the growth period, cells were fixed using 100 μL of 3% formaldehyde for 10 minutes then washed twice with 100 μL of 1% acetic acid. After washing, 100μL of 0.057% SRB was added for 30 minutes with mild shaking. After the incubation period, cells were rinsed 3 times with 1% acetic acid and left to dry. When dry, 100 μL of 10 mM Tris-base (pH 8.0) was added and the plates were read at 560 nm. All data were normalized after 8 h of plating, which was considered to be 100%.

## 8.12 Cell cycle analysis

Cell cycle analysis by bromodeoxyuridine (BrdU) and propidium iodide (PI) double staining was completed on T-47D, MCF-7 $AHR^{+/+}$/$AHR^{-/-}$, and MDA $AHR^{+/+}$/$AHR^{-/-}$. 1 million cells

were seeded in DCC-stripped serum and dosed for 48h with DMSO (final concentration 0.1%), TCDD (10 nM), E2 (10 nM; MCF-7 only), and E2+TCDD (MCF-7 only) and then pulsed for 1 h with 10 μg/mL of BrdU (Sigma). Cells were then collected and fixed in 70% ethanol for 20 mins at -20$^{o}$C. Cells were then rinsed in wash buffer (PBS +0.5% BSA) and resuspended in 2 N HCl for 20 mins, washed, incubated with 0.1 M sodium borate (pH 8.5) for 2 mins, washed again, then incubated with FITC-conjugated anti-BrdU (10 μL of antibody per sample) (BD Biosciences) in PBS+0.5% BSA +0.5% Tween20 and left in the dark for 30 mins. Cells were then washed with wash buffer and stained with 50 μg/ml PI for another 30 mins. FACS analyses and data acquisition were completed by Nishani Rajakulendran using a FACS Calibur flow cytometer (BD Biosciences) and FLOJO software (Treestar).

## 8.13 Statistical Analysis

All data were expressed as the mean ± the standard error of the means (SEM) of three independent replicates unless otherwise stated. Statistical analysis was performed using GraphPad Prism 5 or Microsoft Excel. A one-way Analysis of Variance (ANOVA) or Student's t-test were used where appropriate. Statistical significance was assessed at $P<0.05$.

# Chapter 3: Results

## 9 TCDD induces ERα recruitment to a subset of genomic regions bound by AHR

### 9.1 Defining AHR and ERα-bound regions isolated by ChIP-chip

In order to determine if AHR-induced recruitment of ERα occurs at all or just a subset of AHR target genes we performed ChIP-chip assays on T-47D human breast cancer cells grown for 48 h in 10% FBS containing medium prior to 1 h treatment with 10 nM TCDD or solvent control DMSO (0.1% final concentration). Chromatin was isolated using specific antibodies against AHR (H-211) and ERα (HC-20) and the isolated DNA was linearly amplified and hybridized to Affymetrix Human tiling 1.0R microarrays, which contain 25,500 human promoter regions tiled at 35-bp resolution with probes spanning 7.5 kb upstream and 2.5 kb downstream from the TSS (cancer target genes were probed an extra 2.5 kb upstream). We performed three biological replicates from which enriched peaks were identified by comparing the triplicate $AHR_{TCDD}$ or $ER\alpha_{TCDD}$ samples to $IgG_{TCDD}$ using a false detection rate of 0.2. This analysis resulted in the identification of 412 regions bound by $AHR_{TCDD}$ (Appendix; **Table A1**) and 364 regions bound by $ER\alpha_{TCDD}$ (Appendix; **Table A2**) which were performed by our collaborators (Eivind Valen & Albin Sandelin) and Dr. Matthews. AHR and ERα regions are referred to as AHR_number and ERα_number where the number indicates the relative rank of the region within each of the respective analyses. Since the enriched regions were determined by comparing AHR or ERα bound regions to IgG, these regions may or may not be dependent on TCDD treatment.

### 9.2 Overlap between AHR- and ERα-bound genomic regions and putative target genes

In order to determine regions that were bound by both AHR and ERα, we merged regions that overlapped (>50% sequence identity) between the data sets. We found that of the 364 ERα-bound regions 110 overlapped with the 412 AHR-bound regions, representing a 30% overlap. I will refer to these subsets of regions as the intersect set (110 regions), AHR-only set (302) and ERα-only set (254). A Venn diagram illustrating the relation among these sets is found in **Figure 9A**.

We then investigated putative target genes for the identified regions by determining the gene with the closest TSS, regardless of strand. In this process we noted that several known AHR target genes, *CYP1A1* and *CYP1B1*, as well as known ERα target genes cyclin G2 (*CCNG2*), estrogen receptor α (*ESR1*), gene regulated in breast cancer 1 (*GREB1*), and carbonic anhydrase XII (*CA12*) were identified in both the $AHR_{TCDD}$ and $ER\alpha_{TCDD}$ data sets. Since each of the isolated regions could be labeled with a target gene, we also assessed the overlap between the experiments in terms of target genes. This type of analysis is not the same as simply considering enriched regions, since many ChIP regions may be located in the upstream regulatory region of a single gene (**Figure 9B**). Interestingly, in ~97% of cases (96/99) where a gene was targeted by both ERα and AHR, the two regions in question also overlapped.

The annotated function of the target genes (GO terms) in the respective sets was not significantly different when comparing the sets to one another, although compared to a general background (all genes) some differences were observed (Appendix, **Table A3 A-C**).

## 9.3 Validation of ChIP-chip regions using conventional ChIP

To validate the enriched regions identified by ChIP-chip, conventional ChIPs were performed on a subset of 26 identified regions using Q-PCR. The regions were chosen to cover a range of enrichment values but also included regions near a select number of known AHR and ERα regulated genes. All 26 regions verified the recruitment of AHR and ERα (or lack thereof) from the ChIP-chip study with the level of enrichment varying among the regions. The results shown in **Figure 10A** reveal a strong ligand-dependent recruitment of AHR to a subset of the total identified AHR-bound regions. For the most part there was weak binding of AHR to the tested regions in the absence of TCDD. However, in agreement with other studies AHR occupied the *CYP1B1* (AHR_10) upstream regulatory region in the absence of TCDD when compared to IgG (Yang et al., 2008). Significant ligand independent AHR occupancy was also observed at the upstream regulatory regions of synaptotagmin XII (*SYT12*, AHR_4), transmembrane protein 30A (*TMEM30a*, AHR_3), pregnancy specific beta-1-glycoprotein 9 (*PSG9*, AHR_51) and gene amplified in breast cancer 1 (*GREB1*, AHR_252). AHR exists simultaneously in the nucleus and

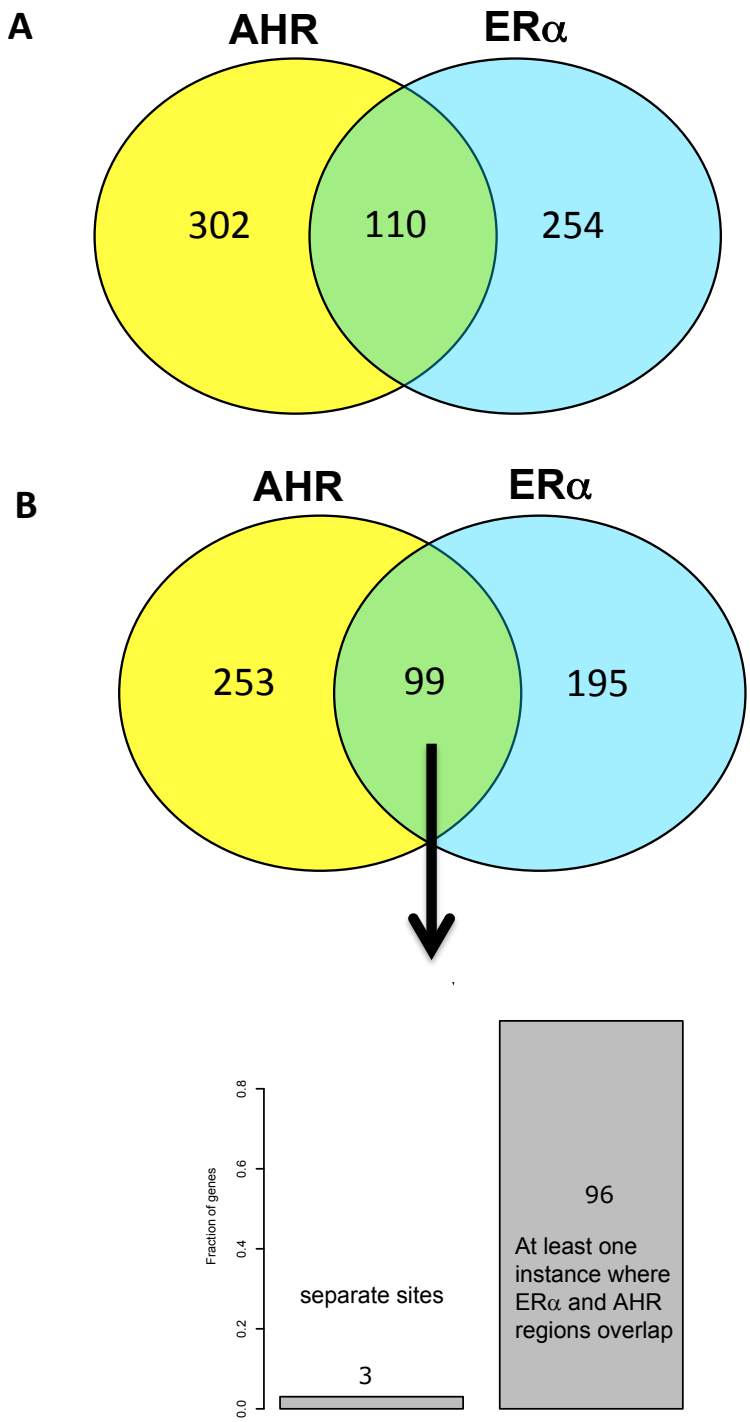



**Figure 9. Overlap between ChIP sets and target genes.**

(**A**) Venn diagram showing the number of ChIP regions from respective experiments that overlap with more than 50% of the length of the smallest region. (**B**) Overlap of the experiments in terms of the identity of the closest gene. In 96 of the 99 genes where the gene has both AHR and ERα chip regions, the ChIP regions overlap physically.

cytoplasm in human breast cancer cells (Wang et al., 1998), which might explain the occupancy of AHR at these regions in the absence of TCDD. The highest ranked region bound by AHR was also bound by ERα and mapped to a sequence approximately 100 kb downstream of the *TiPARP* (AHR_1) transcriptional start site, suggesting that this gene might be regulated by a distal 3` enhancer. A 3` enhancer has also been reported to regulate the *CYP1A2* (Okino et al., 2007). We also identified a number of novel AHR bound sites upstream of prospero homeobox 1 (*PROX1*, AHR_326), Forkhead box N4 (*FOXN4*, AHR_35), homeobox 10 (*HOXC10*, AHR_63), sortilin-related receptor (*SORL1*, AHR_14), inositol 1,4,5-triphosphate receptor, type 1 (*ITPR1*, AHR_43) genes.

There was good agreement between the ChIP-chip regions and the confirmation of TCDD-induced ERα recruitment to shared regions in the intersect group. In contrast to the AHR confirmation data (**Figure 10A**), promoter occupancy of ERα in DMSO samples was observed at a number of analyzed regions. This was due to the fact that cells were cultured in 10% fetal bovine serum and not DCC-stripped serum, which is required to observe robust estrogen-dependent responses in breast cancer cell lines. However, steroid deprivation is not necessary to observe robust TCDD-dependent activation of AHR transcription (Hankinson, 1995). The occupancy of ERα at the promoter regions of the well-characterized ER target genes *GREB1* and *ESR1* was consistent with previously published ChIP-chip studies (Carroll et al., 2005; 2006; Kwon et al., 2007; Krum et al., 2008; Lupien et al., 2008).

TCDD-dependent recruitment of ERα was observed to a number of regions including those upstream of RAS-like estrogen-regulated growth inhibitor (*RERG*, ERα_22), *CCNG2* (ERα_100), *CYP1B1* (ERα_107) and synaptotagmin XII (*SYT12*, ERα_4). TCDD also induced recruitment of AHR to known estrogen responsive genes including *GREB1* (AHR_252), *RERG* (AHR_18), *CCNG2* (AHR_37) and *ESR1* (AHR_204). These findings indicate that AHR influenced the recruitment of ERα to AHR regulated genes but also that AHR is recruited to genomic regions occupied by ERα where the binding of ERα is independent of AHR activation. We observed three false negatives in that our ChIP-chip experiment failed to detect recruitment of ERα to *CYP1A1* (AHR_111), transducin (beta)-like 1 X-linked receptor 1 (TBL1XR1; AHR_48) and Janus kinase 1 (JAK1; AHR_102), but ERα binding to these regions was detected by conventional ChIP. This may have been due to the thresholds applied in the ChIP-chip

experiments. Sequential ChIPs were done on a subset of six regions in the intersect set (bound by both AHR and ERα) confirming the simultaneous binding of both AHR and ERα to the regions examined (**Figure 10B**).

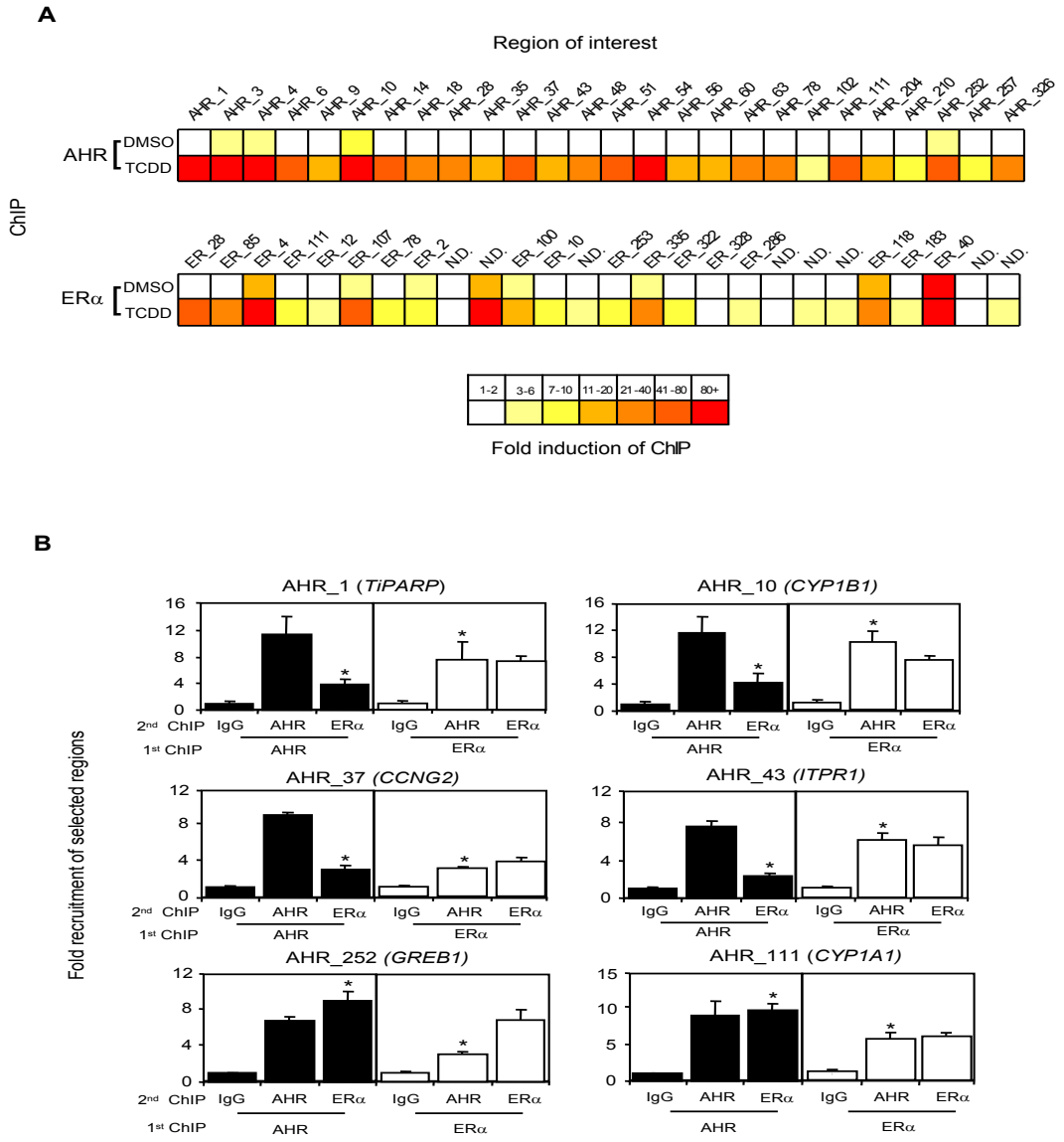
## 9.4 Chromatin binding and correlation with gene expression of TCDD-responsive genes

We were then interested to determine if the binding of AHR and/or ERα to genomic regions resulted in changes in mRNA levels of the closest genes that map to the isolated genomic fragments. We treated T-47D cells with 10 nM TCDD for 1.5, 3, 6, and 24 h, isolated RNA and determined changes in mRNA levels using Q-PCR. A subset of the examined genes is shown in **Figure 11**. We observed that mRNA expression of the predicted target genes displayed TCDD-dependent increases, decreases or no change at the time points examined. A table summarizing the mRNA changes for the closest genes corresponding to the confirmed ChIP-chip regions is provided in **Table 4**. As expected, TCDD increased the mRNA expression levels of CYP1A1 and CYP1B1. We also observed TCDD-dependent increases in CCNG2, PROX-1 and ITPR1 mRNA expression levels **(Figure 11)**. In support of the anti-estrogenic action of TCDD, the estrogen responsive genes GREB1 and ESR1 were both inhibited by TCDD treatment but quickly rebounded at the later time points **(Figure 11).**

## 9.5 Transcription factor binding site analysis of the ChIP regions

We then investigated the density of putative transcription factor binding sites in the AHR-only, intersect and ERα-only regions, and calculated over- or under-representation of transcription factor binding sites compared to either a sampled promoter background (**Figure 12A**), or among sets (**Figure 12B**). In the first type of analysis we obtained an "absolute" measure of over-representation, where one factor can be over-represented in all sites, while in the second type of analysis we determined the different binding sites among the different data sets.

Our collaborators measured the over-representation as a *Z*-score statistic, and visualized which transcription factor binding sites were significantly over- and under-represented by hierarchically clustered heat maps as in (Liu et al., 2008), where the rows are the JASPAR database (Bryne et al., 2008) transcription factor binding sites and the columns are the ChIP sets. As expected, when compared to a generic promoter sequence background, the ERα-only set had a strong over-




## Figure 10. TCDD-induced recruitment of both AHR and ERα to ChIP-chip identified regions.

**(A)** Quantification of AHR and ERα binding was determined as fold induction above IgG DMSO and is expressed as the mean of three independent replicates. Regions were chosen to cover a range of enrichment values and included a select number of sites near AHR and ERα target genes. N.D. refers to regions that were not detected in the ERα ChIP-chip experiment. T-47D cells were treated with 10 nM TCDD for 1 h. ChIP assays were performed with the indicated antibodies and the immunoprecipitated DNA was measured by Q-PCR using primers targeting regions isolated in the ChIP-chip study. **(B)** T-47D cells were treated with 10 nM TCDD for 1 h. Sequential ChIPs were performed with the indicated antibodies. Immunoprecipitated DNA was measured by Q-PCR using primers targeting regions isolated in the ChIP-chip study. Quantification of binding was determined as fold induction above IgG DMSO. Each error bar represents the standard error of the mean of three independent replicates. Asterisks indicate statistically significant differences compared to IgG DMSO control samples (Student's t-test, $P<0.05$).

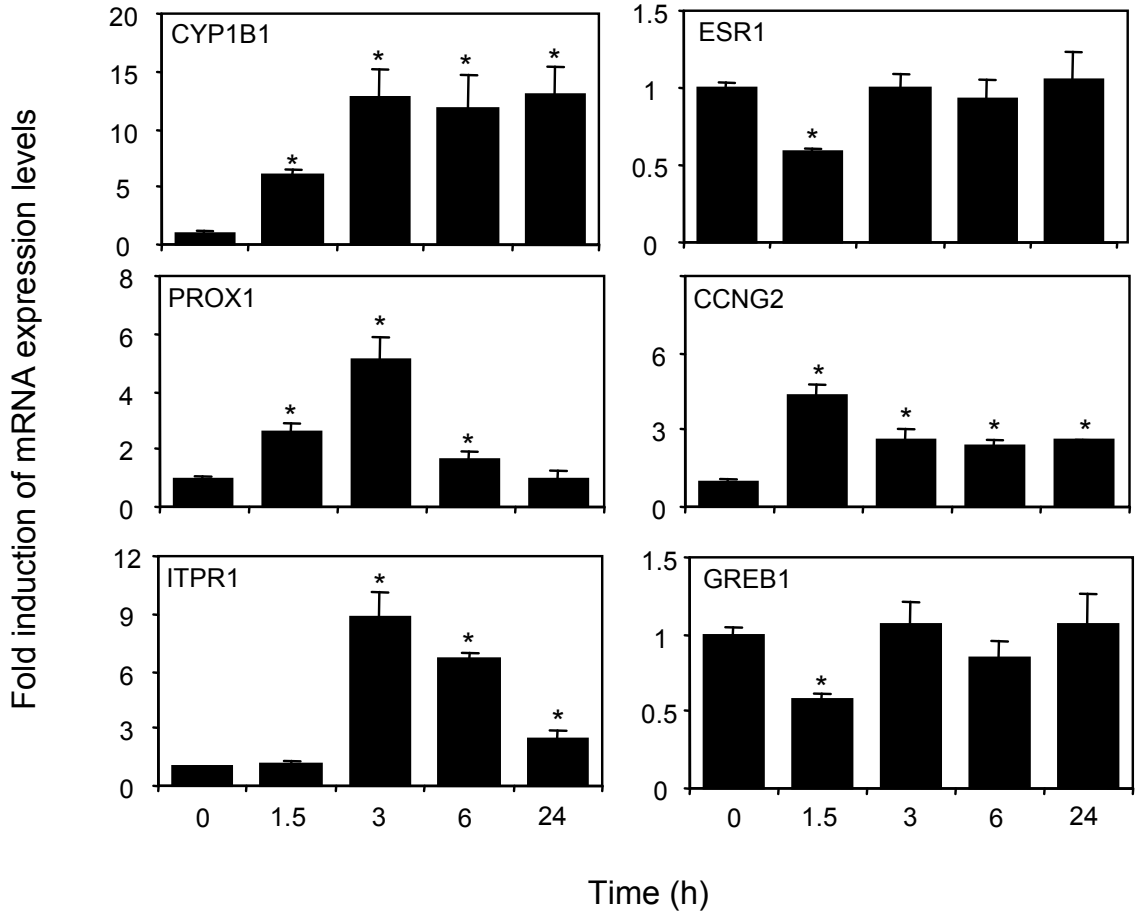



**Figure 11. Chromatin profiles correlate with expression status in TCDD-responsive genes.**

After TCDD treatment for the indicated time periods, RNA was isolated and reverse transcribed. mRNA expression was then determined using Q-PCR. Data were normalized against time matched DMSO and to ribosomal 18s levels. Each error bar represents the standard error of the mean of three independent replicates. Asterisks indicate statistically significant differences compared to time-matched DMSO control samples (Student's t-test, $P<0.05$).

representation of the ERE pattern represented by the ESR1 JASPAR model, whereas the AHRE pattern, represented by the Arnt-Ahr JASPAR model, was over-represented in all sets but most evident in the intersect set (**Figure 12A**). This was also consistent when assessing over-representation between sets (**Figure 12B**). In that analysis the intersect set was strongly enriched in AHREs (Arnt-Ahr JASPAR model) when compared to the $AHR_{TCDD}$ or the $ER\alpha_{TCDD}$ sets, while as expected the ERE (ESR1 JASPAR model) was strongest in the $ER\alpha_{TCDD}$ set. Of the 110 regions in the intersect group 57 contained an AHRE, 24 contained an ERE, but surprisingly only 10 regions contained both response elements. Since the AHRE was particularly over-represented in the intersect set, we hypothesized that it was likely that AHR was contributing at least in part to the recruitment of ERα to regions in the intersect set.

## 9.6 AHR modulates recruitment of ERα to the shared regions.

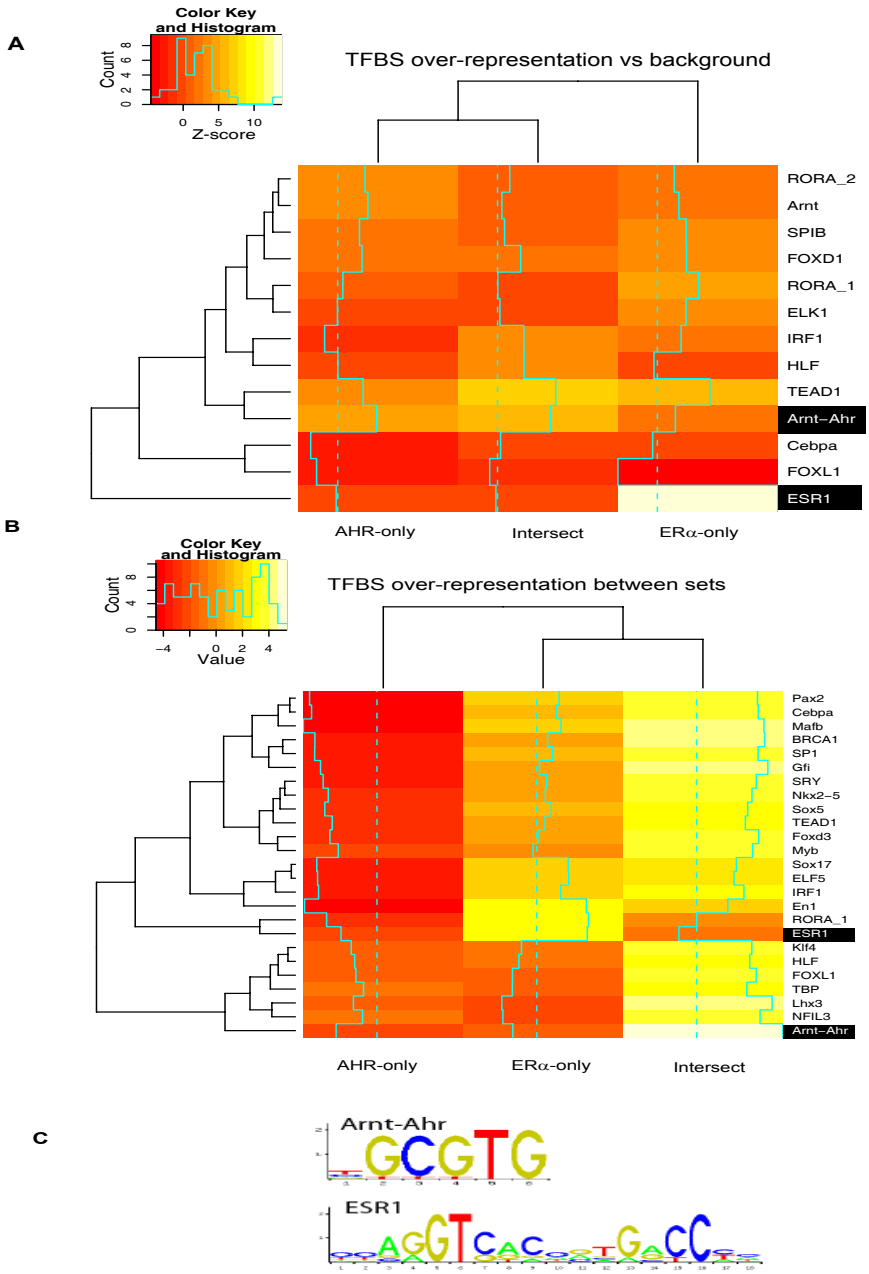
To test the hypothesis that AHR was influencing the recruitment of ERα to the regions in the intersect set, we used RNAi-mediated knockdown of AHR or ERα and determined the recruitment of each of these factors to a subset of regions in the intersect group as well as changes in mRNA expression levels. Following transfection of siRNA oligos into T-47D we determined that 48 h post-transfection both AHR and ERα protein levels were undetectable and mRNA expression levels were reduced to 20% compared to controls (**Figure 13A, 13B**). Western blots of ERα levels after 1 h TCDD treatment showed that any reduction in recruitment levels of ERα were not due to TCDD-dependent proteolysis of ERα (**Figure 13B**). ChIP assays and RNA isolation were then done on siRNA transfected T-47D cells exposed to 10 nM TCDD for 1 h and 6 h, respectively. As expected, knockdown of AHR or ERα reduced their respective recruitment to the genomic regions examined. AHR knockdown reduced the TCDD-dependent recruitment of ERα to AHR_10 (*CYP1B1*), AHR_54 (*ITPR1*) and AHR_37 (*CCNG2*) compared to non-targeting pool controls (**Figure 14A**). All three of these genes have been reported to also be responsive to estrogen treatment (Kirkwood et al., 1997; Tsuchiya et al., 2004b; Stossi et al., 2006). Further studies completed in DCC-stripped serum confirmed the TCDD-dependent recruitment of ERα to these regions (**Figure 15**). Knockdown of ERα had no effect on TCDD-dependent induction of ITPR1 and CCNG2 (**Figure 14B**), but caused a significant reduction of TCDD-induced CYP1B1 (**Figure 14B**) mRNA levels. Interestingly, knockdown of ERα resulted in increased basal mRNA levels of CCNG2 (**Figure 14B**). *CCNG2* is negatively regulated by

ERα, which may explain the increase in basal expression following ERα knockdown (Stossi et al., 2006). As expected, the occupancy of ERα at all regions examined was significantly reduced in cells transfected with siERα compared to controls. AHR knockdown, however, had no effect on the recruitment of ERα to upstream regulatory regions for AHR_252 (*GREB1*), AHR_204 (*ESR1*) and AHR_18 (*RERG*), demonstrating that AHR exhibits region-specific modulation of ERα genomic binding profiles (**Figure 16A**). These three genes have been reported to be estrogen target genes (Castles et al., 1997; Finlin et al., 2001; Lin et al., 2004; DeNardo et al., 2005) and ERα occupied these regions in the absence of TCDD, which may explain why AHR had no effect on the recruitment of ERα to these genes. TCDD increased the overlap of ERα and AHR to these genes through the recruitment of AHR to genomic sequences bound by ERα in the presence of DMSO. The recruitment of AHR was unaffected by knockdown of ERα for all regions examined with the following exceptions; TCDD-dependent recruitment of AHR to *GREB1* (**Figure 16A**) was decreased, while recruitment of AHR was increased at *CYP1B1* (**Figure 14A**). These results indicate that ERα influences the AHR transcription in a promoter and context specific manner. These data also show that TCDD-mediated activation of AHR modulates the recruitment of ERα to a number of genomic regions in a gene specific manner, but also that AHR is recruited to many genomic regions regulated by ERα.

## Table 4. Chromatin profiles correlate with expression status in TCDD-responsive genes

After TCDD treatment for the indicated time periods, RNA was isolated and reverse transcribed. mRNA expression was then determined using Q-PCR. Data were normalized against time matched DMSO and to ribosomal 18s levels. Each error bar represents the standard error of the mean of three independent replicates (Student's t-test, b=$P$<0.01 a=$P$<0.05).

| Gene Name | 1.5 hr | 3 hr | 6 hr | 24 hr |
|---|---|---|---|---|
| ***TCDD treated activated genes*** | | | | |
| *CYP1A1* | 505 ±157[b] | 900 ± 125[b] | 528 ± 54[b] | 1685.8 ± 173.5[b] |
| *CYP1B1* | 6.00 ± 0.45[b] | 12.74 ± 2.46[b] | 11.87 ± 2.77[b] | 12.91 ± 2.58[b] |
| *TiPARP* | 6.35 ± 0.48[b] | 2.77 ± 0.28[b] | 3.93 ± 0.72[b] | 2.9 ± 0.22[b] |
| *PROX1* | 2.60 ± 0.31[b] | 5.12 ± 0.79[b] | 1.67 ± 0.24[a] | 0.94 ± 0.27 |
| *ITPR1* | 1.09 ± 0.25 | 8.87 ± 1.20[b] | 6.59 ± 0.31[b] | 2.52 ± 0.35[b] |
| *TMEM30a* | 1.29 ± 0.13 | 1.18 ± 0.17 | 1.54 ± 0.09[b] | 1.38 ± 0.11[b] |
| *CCNG2* | 4.37 ± 0.42[b] | 2.65 ± 0.35[b] | 2.40 ± 0.19[b] | 2.55 ± 0.05[b] |
| *SYCP2* | 0.76 ± 0.07[a] | 1.45 ± 0.24 | 1.16 ± 0.11 | 1.94 ± 0.17[b] |
| *GPRC5C* | 1.51 ± 0.34 | 1.31 ± 0.17 | 1.72 ± 0.10[b] | 2.28 ± 0.26[b] |
| ***TCDD treated unchanged genes*** | | | | |
| *FOXN4* | 1.06 ± 0.38 | 1.06 ± 0.14 | 0.64 ± 0.12 | 0.62 ± 0.24 |
| *HOXC10* | 0.97 ± 0.14 | 0.88 ± 0.04 | 0.82 ± 0.16 | 0.95 ± 0.11 |
| ***TCDD treated repressed genes*** | | | | |
| *ESR1* | 0.60 ± 0.01[b] | 1.01 ± 0.08 | 0.92 ± 0.12 | 1.06 ± 0.17 |
| *SORL1* | 0.60 ± 0.05[b] | 1.06 ± 0.12 | 1.62 ± 0.19[a] | 0.97 ± 0.08 |
| *JAK1* | 0.65 ± 0.02[b] | 1.03 ± 0.06 | 0.71 ± 0.05[b] | 0.64 ± 0.07[b] |
| *MSMB* | 0.44 ± 0.03[b] | 0.82 ± 0.20 | 1.06 ± 0.25 | 0.75 ± 0.11 |
| *GREB1* | 0.58 ± 0.03[b] | 1.06 ± 0.14 | 0.85 ± 0.12 | 1.07 ± 0.18 |
| *RERG* | 0.70 ± 0.12[a] | 1.10 ± 0.12 | 1.04 ± 0.07 | 1.75 ± 0.55 |
| *TBL1XR1* | 0.81 ± 0.15 | 0.65 ± 0.08[b] | 0.80 ± 0.09 | 0.63 ± 0.08[b] |




## Figure 12. Transcription factor binding site analysis.

Heat maps showing the most over-and under-represented transcription factor binding site patterns in each set, either compared to large promoter background **(A)** or compared between sets **(B).** Heat map A can be viewed as an "absolute" measure of over-representation, while heat map B shows what patterns that are significantly different between at least two sets in terms of occurrence. Over/under-representation is expressed as a *Z*-score, where a negative value means under-representation (coded red) and high values indicate over-representation (coded white). *Z* scores were translated into a color range from red to white. Rows (transcription factor binding patterns from JASPAR) and columns (ChIP regions as in Fig. 1A) are ordered by similarity to each other. The Arnt-Ahr pattern (corresponding to an AHRE) and the ESR1 pattern (corresponding to an ERE) are highlighted. **(C)** Sequence logo for the Arnt-Ahr and ESR1 matrices from JASPAR.

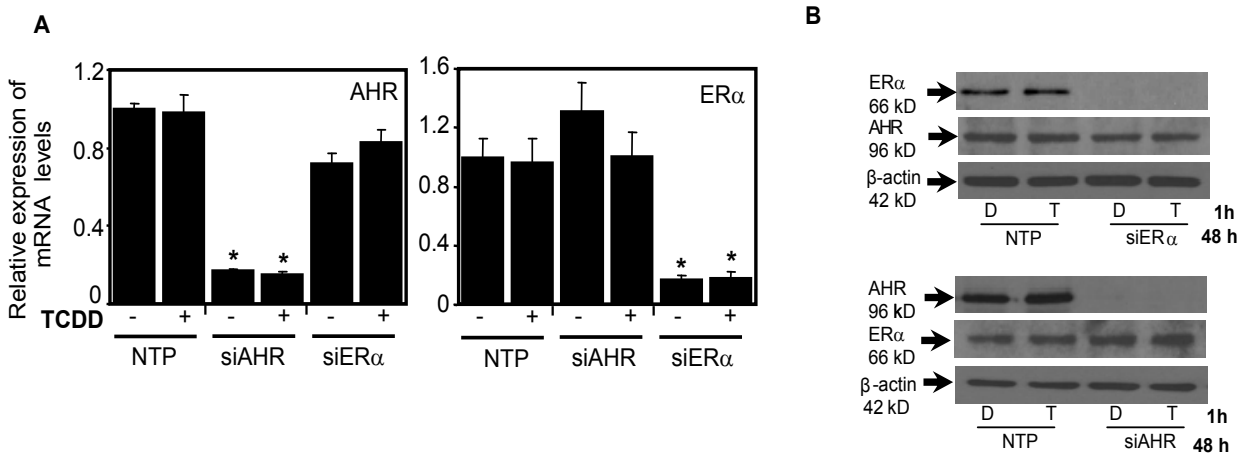



**Figure 13. Analysis of AHR and ERα knockdown in T-47D cells: Protein and Transcript levels.**

**(A)** T-47D cells were transfected with specific siRNA against AHR and ERα for 48 h. RNA was isolated and reverse transcribed. mRNA expression was then determined using quantitative PCR. Data were normalized against time matched DMSO and to ribosomal 18s levels. Each error bar represents the standard error of the mean of three independent replicates. Significance was determined by comparison to NTP (non-targeting pool) TCDD treatment $P<0.05$. **(B)** Western blot analysis of AHR and ERα knockdown in T-47D cells following 48 h transfection then 1 h treatment with either DMSO or 10 nM TCDD. Cell extracts were probed with rabbit antibody against AHR and ERα. β-actin was used as loading control.

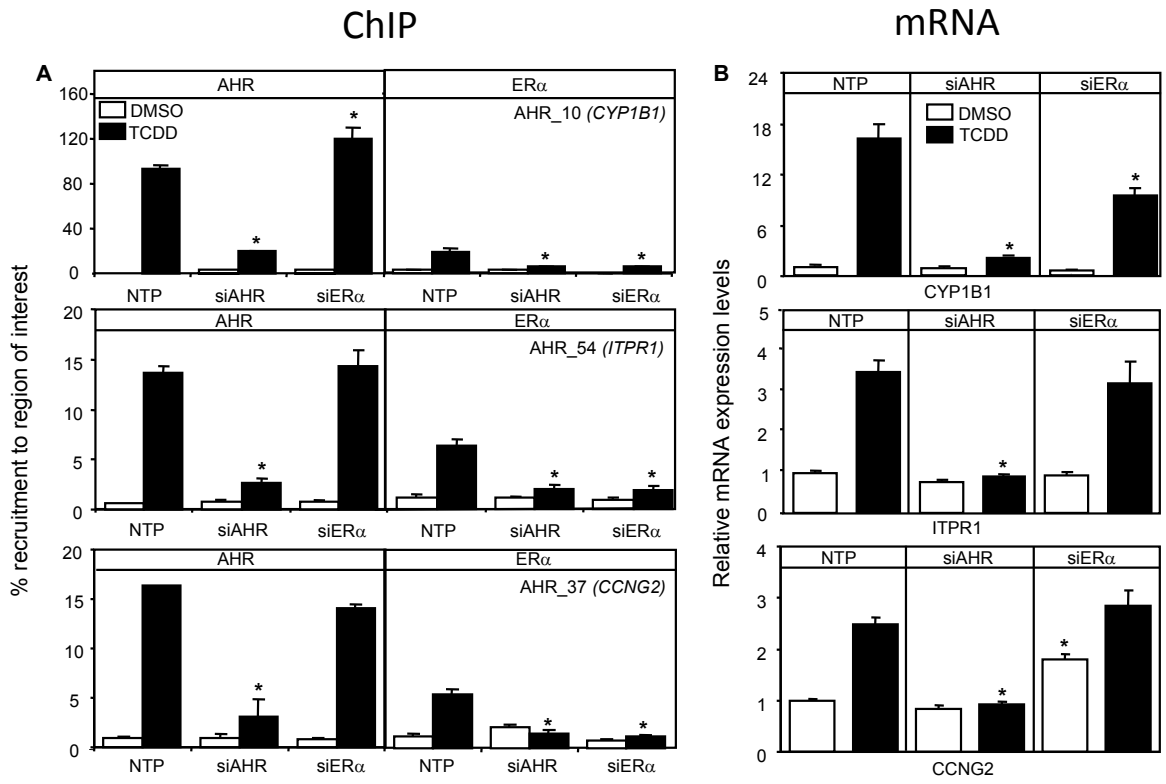



**Figure 14. AHR is required for TCDD-dependent recruitment of ERα to a subset of co-occupied AHR and ERα target genes.**

(**A**) T-47D cells were transfected for 48 h with siRNA and then treated for 1 h with TCDD. ChIP assays were performed with the indicated antibodies, and the immunoprecipitated DNA was measured by Q-PCR using primers targeting regions isolated in the ChIP-chip study. Quantification of binding was determined as a percent of input DNA and is expressed as the mean of three independent replicates. (**B**) Gene expression profiles were completed on T-47D cells transfected for 48 h with siRNA and then treated for 6 h with TCDD. RNA was isolated and reverse transcribed. mRNA expression was then determined using Q-PCR. Data were normalized against time matched DMSO and to ribosomal 18s levels. Each error bar represents the standard error of the mean of three independent replicates. Asterisks indicate statistically significant differences ($P$<0.05) compared to NTP treatment matched samples.

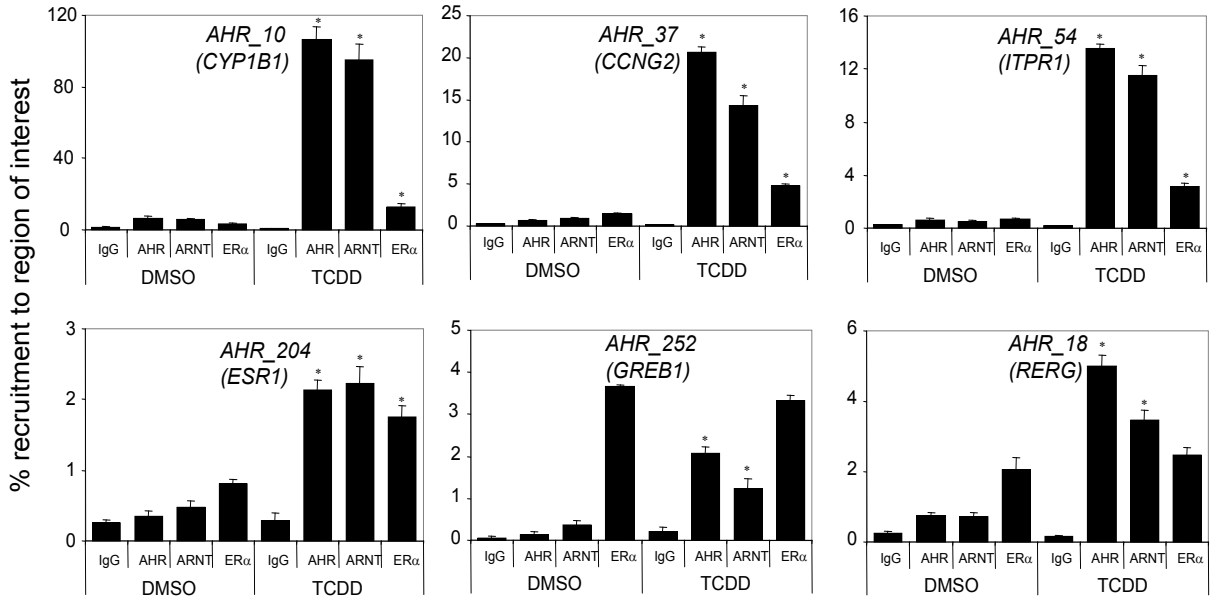



**Figure 15. Effects of DCC-stripped serum on the TCDD-dependent recruitment of ERα.**

T-47D cells were plated in 5% DCC-stripped serum for 3 days prior to treatment. Cells were subsequently treated with either DMSO or TCDD for 1 h and ChIP assays were performed with the indicated antibodies, and the immunoprecipitated DNA was measured by Q-PCR using primers targeting regions isolated in the ChIP-chip study. Quantification of binding was determined as a percent of input DNA and is expressed as the mean of two independent replicates. Each error bar represents the standard error of the mean of the two independent replicates. Asterisks indicate statistically significant differences ($P$<0.05) compared to DMSO treatment sample.

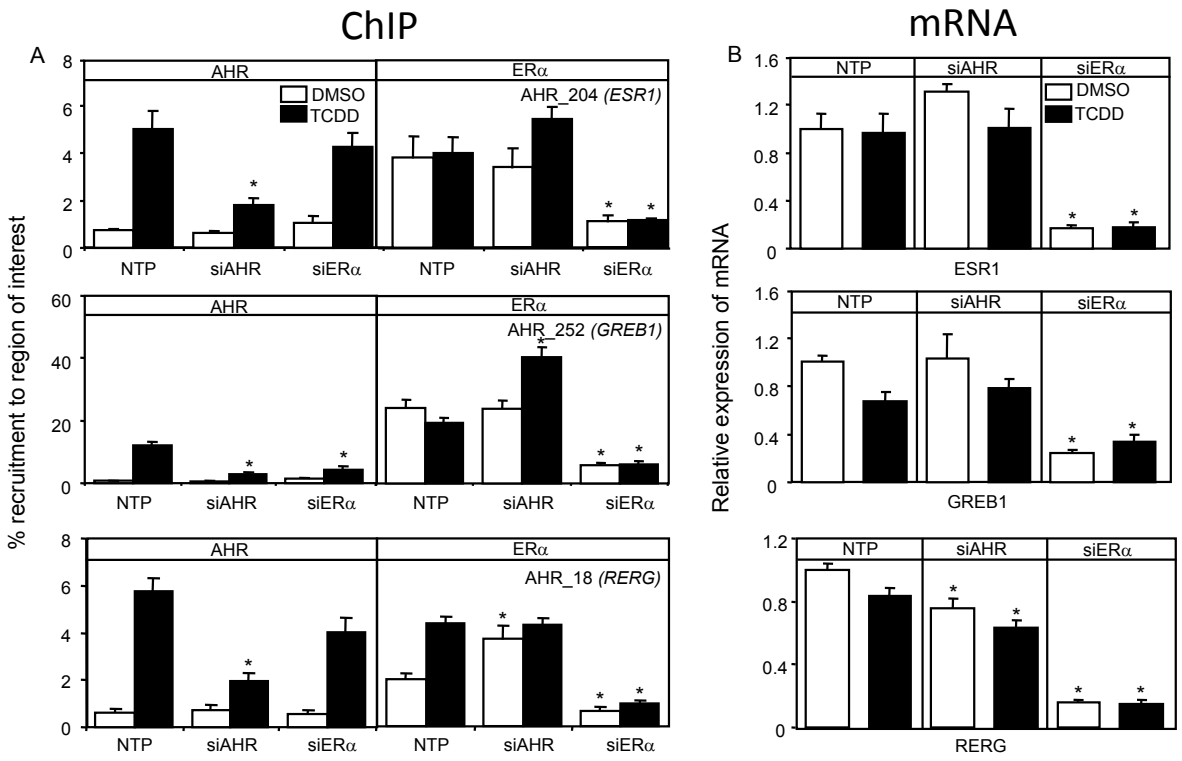



**Figure 16. AHR is not necessary for ERα binding to a subset of co-occupied AHR and ERα target genes.**

(**A**) T-47D cells were transfected for 48 h with siRNA and then treated for 1 h with TCDD. ChIP assays were performed with the indicated antibodies, and the immunoprecipitated DNA was measured by Q-PCR using primers targeting regions isolated in the ChIP-chip study. Quantification of binding was determined as a percent of input DNA and is expressed as the mean of three independent replicates. (**B**) Gene expression profiles were completed on T-47D cells transfected for 48 h with siRNA and then treated for 6 h with TCDD. RNA was isolated and reverse transcribed. mRNA expression was then determined using quantitative PCR. Data were normalized against time matched DMSO and to ribosomal 18s levels. Each error bar represents the standard error of the mean of three independent replicates. Asterisks indicate statistically significant differences ($P$<0.05) compared to NTP treatment matched samples.

# *10* AHR-dependent regulation of cyclin G2 requires FOXA1

## 10.1 TCDD- and AHR-dependent regulation of CCNG2

The ChIP-chip studies (Aim 1) identified that AHR bound to the regulatory regions of numerous genes involved in diverse cellular pathways, including metabolism, differentiation and cell cycle regulation (Ahmed et al., 2009). One gene of interest we decided to focus on was *cyclin G2* (*CCNG2*). The expression of *CCNG2* has been shown to inhibit cell cycle progression by preventing $G_1$ to S phase transition (Martinez-Gac et al., 2004; Xu et al., 2008; Stossi et al., 2009). From our previous ChIP-chip study we identified an AHR-bound AHRE containing site located in the upstream regulatory region of *CCNG2*. To investigate the mechanism of the AHR-dependent regulation of *CCNG2*, we first performed time course mRNA expression analysis of TCDD-treated T-47D cells cultured for 72 h in medium containing 5% DCC-stripped serum to increase the percentage of cells in $G_0/G_1$ (Mason et al., 2004) and to reduce the concentration of potential AHR activators in the FBS. As shown in **Figure 17A**, CCNG2 mRNA levels were increased following 1.5 h treatment and remained elevated until 24 h. TCDD-dependent increases in CCNG2 mRNA levels were reduced after co-treatment with the selective AHR antagonist CH223191 (Zhao et al., 2010) at all time points examined. Western blots confirmed TCDD-dependent increases in CCNG2 protein levels after 6 h treatment (**Figure 17B**).

To assess the impact of CCNG2 upregulation and its role in the TCDD-dependent cell cycle arrest, we performed cell cycle analysis. T-47D cells transiently transfected with RNAi targeting CCNG2 (approximately 70% knockdown was achieved at the mRNA level; **Figure 18**) were double stained with BrdU and PI after 48h treatment with DMSO or 10 nM TCDD and analyzed using FACS. We observed that in cells transfected with universal negative control (neg. control) and treated with TCDD resulted in an increase in the number of cells in $G_1$ when compared to DMSO (**Figure 19A**, **B**). However, in cells transfected with RNAi-targeting CCNG2 there was an increased amount in the S phase (**Figure 19C**) but TCDD treatment did not alter the distribution of cells (**Figure 19D**). The ability of TCDD to increase the number of cells in $G_1$ and reduce the number of cells in S phase was lost following CCNG2 knockdown (**Figure 19E, F**), suggesting that CCNG2 is an important contributor to the TCDD-dependent cell cycle inhibition in T-47D cells

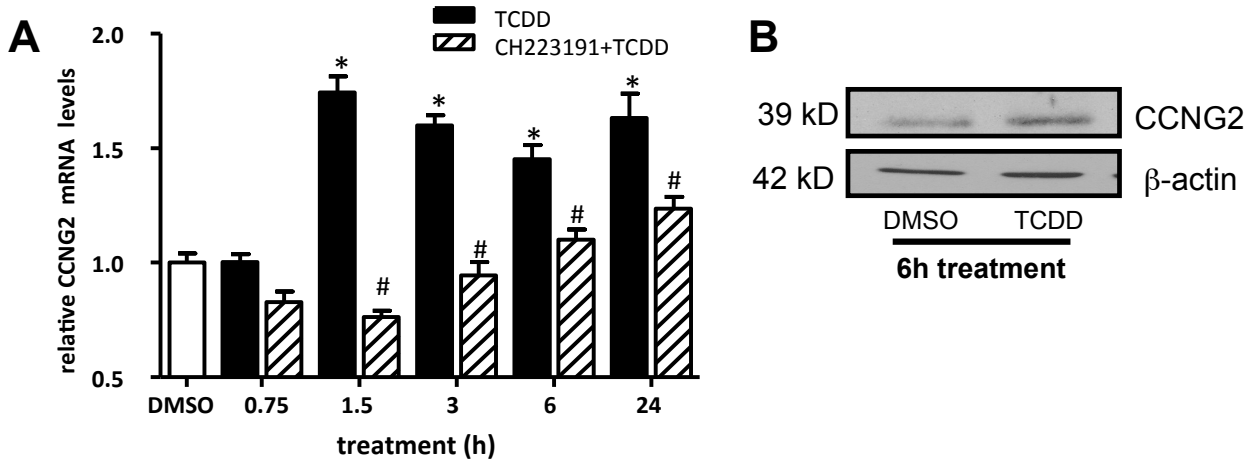



**Figure 17. TCDD-dependent regulation of *CCNG2*.**

(**A**) Time course analysis of the TCDD-dependent gene regulation of CCNG2. T-47D breast cancer cells were treated (10 nM TCDD or pre-treatment for 1 h with 1 μM CH223191) for the indicated time period and RNA was isolated and reverse transcribed. Changes in mRNA expression levels were then determined using Q-PCR. Data were normalized against time-matched DMSO and to ribosomal 18s levels. Each error bar represents the SEM of three independent replicates. Asterisks represent statistical significance compared to time-matched DMSO and pound sign represent statistical significance compared to time-matched TCDD treatment ($P<0.05$, one-way ANOVA) (**B**) Western blot analysis of CCNG2 protein levels. T-47D cells were treated with DMSO or 10 nM TCDD for 6 h. Cell extracts were probed with rabbit antibody against CCNG2. β-actin was used as loading control.

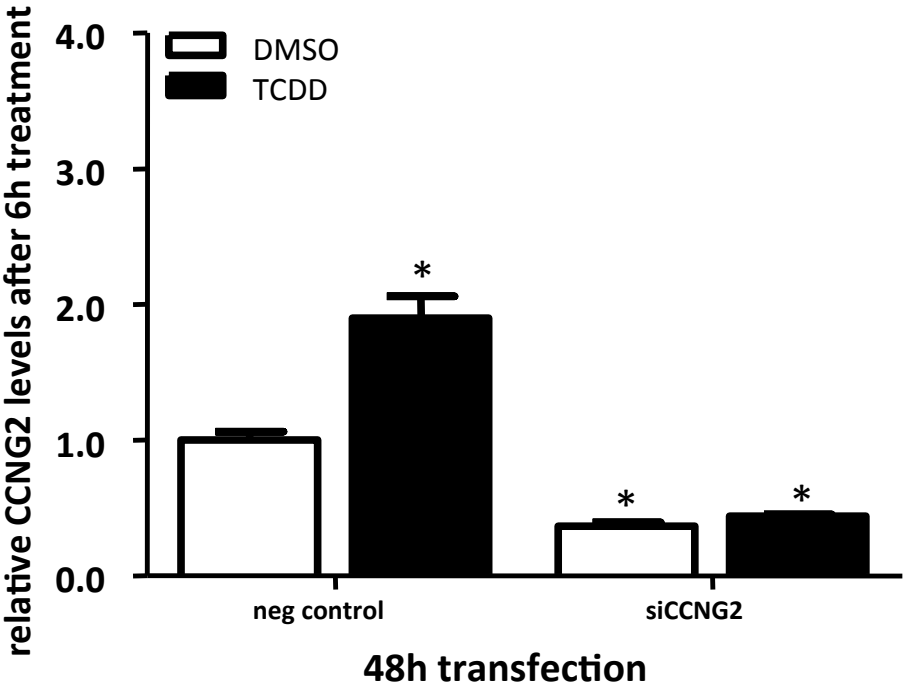



**Figure 18. RNAi-mediated knockdown of CCNG2.**

T-47D cells were plated in DCC-stripped serum and transfected with either negative control or siRNA targeting CCNG2. 48 h post-transfection cells were treated with DMSO or 10 nM TCDD for 6 h. RNA was isolated and reverse transcribed Changes in mRNA expression levels were determined using Q-PCR. Data were normalized against negative control DMSO and to ribosomal 18s levels. Each error bar represents the SEM of three independent replicates. Asterisks represent statistical significance compared to negative control ($P$<0.05, Student's t-test)

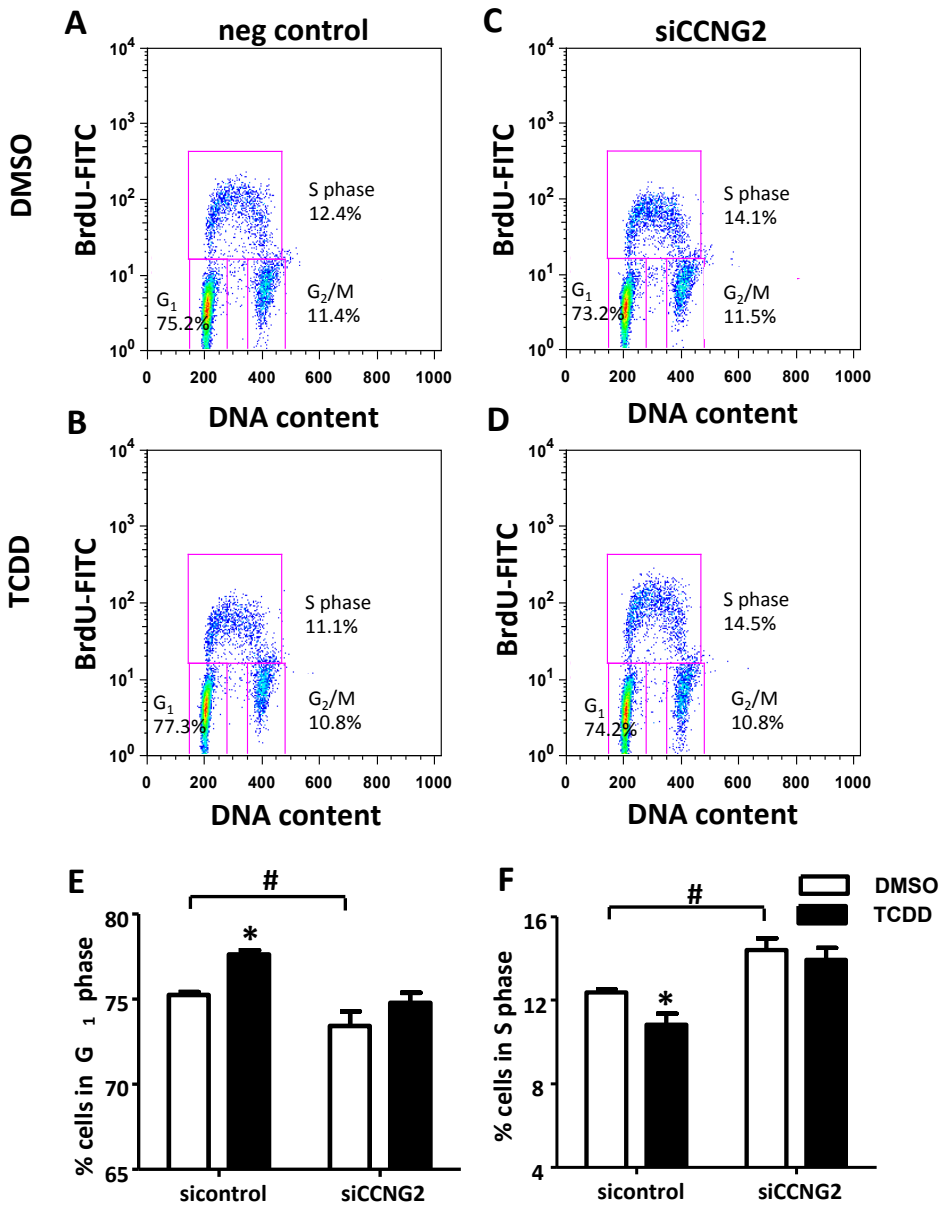



**Figure 19. CCNG2 is important for the TCDD-dependent $G_1$ phase arrest.**

(**A-D**) Cell cycle analysis of T-47D cells transiently transfected with universal negative control or siCCNG2 were exposed to DMSO, or 10 nM TCDD for 48 h and harvested for FACS analysis. Cells were pulsed with 10 µg/ml of BrdU before being collected. For each treatment BrdU-PI bivariate plot with numbers corresponding to the percentage of cells in $G_1$, S, and $G_2$/M phases of the cell cycle were generated. Data shown (**A-D**) are representative graphs of three experiments. Each box represents a different phase of the cell cycle. The data presented in (**E, F**) are compiled data from three independent experiments and indicate the percentage of cells in each phase of the cell cycle. Asterisks represent statistical significance compared to RNAi-matched DMSO treatment cells, whereas the pound signs represent statistical significance compared to negative control DMSO treatment ($P$<0.05, one-way ANOVA).

## 10.2 TCDD induces the recruitment of AHR and FOXA1 to CCNG2

Since CCNG2 was shown to be important in mediating the TCDD-dependent $G_1$ phase arrest, we wanted to characterize its regulation. To identify AHREs and other transcription factor binding sites that might be important in the TCDD-dependent regulation of *CCNG2*, we performed transcription factor binding site (TFBS) analysis on the -1.4 kb *CCNG2* regulatory region using MatInspector (Genomatix). This analysis identified two AHRE sequences that were positioned in close proximity to multiple forkhead (FKH) sites. We designated the AHRE sites, AHRE1 and AHRE2, and the FKH sites, FKH1-4 (**Figure 20**). Since FOXA1 is an important transcription factor in ERα positive breast cancer cells, we then determined the role of FOXA1 as well as each of the individual AHREs in the AHR-dependent regulation of *CCNG2* (Lupien et al., 2008). To examine AHR and FOXA1 recruitment to the *CCNG2* promoter in T-47D cells, we performed ChIP experiments. Cells were treated with DMSO or 10 nM TCDD for 1 h, cross-linked and protein-DNA complexes were immunoprecipitated with antibodies directed against AHR, FOXA1, H3K4Me2 or H3K9Ac. H3K9Ac was examined since this modification correlates to actively transcribed genes (Wang et al., 2008). H3K4Me2 was tested to identify functional FOXA1 binding sites, since H3K4Me2 has been reported to correlate with FOXA1 binding to its FKH site (Lupien et al., 2008). As shown in **Figure 21A**, TCDD treatment significantly and preferentially induced the recruitment of AHR and FOXA1 to AHRE2, whereas only a modest, albeit significant increase in AHR recruitment to AHRE1, was observed (**Figure 21A**). ChIP studies revealed constitutive binding of FOXA1 to the distal region, which was increased after ligand treatment. Treatment with TCDD resulted in increased levels of H3K4Me2 or H3K9Ac at AHRE2 but not at AHRE1 (**Figure 21B**). These findings suggest that AHRE2, but not AHRE1, is the dominant AHRE driving the TCDD-dependent regulation of *CCNG2*. In agreement with TCDD-dependent increases in CCNG2 mRNA levels, ChIP assays confirmed increases in the recruitment of RNA polymerase II to the proximal promoter region of *CCNG2* after TCDD treatment (**Figure 21C**). Although the expression of FOXA2 was comparable to FOXA1 levels, FOXA2 was not recruited to *CCNG2*, whereas FOXA3 was not detected in T-47D cells (**Figure 22A, B**).

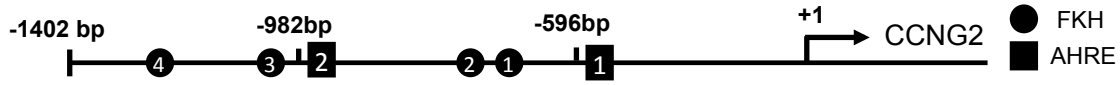
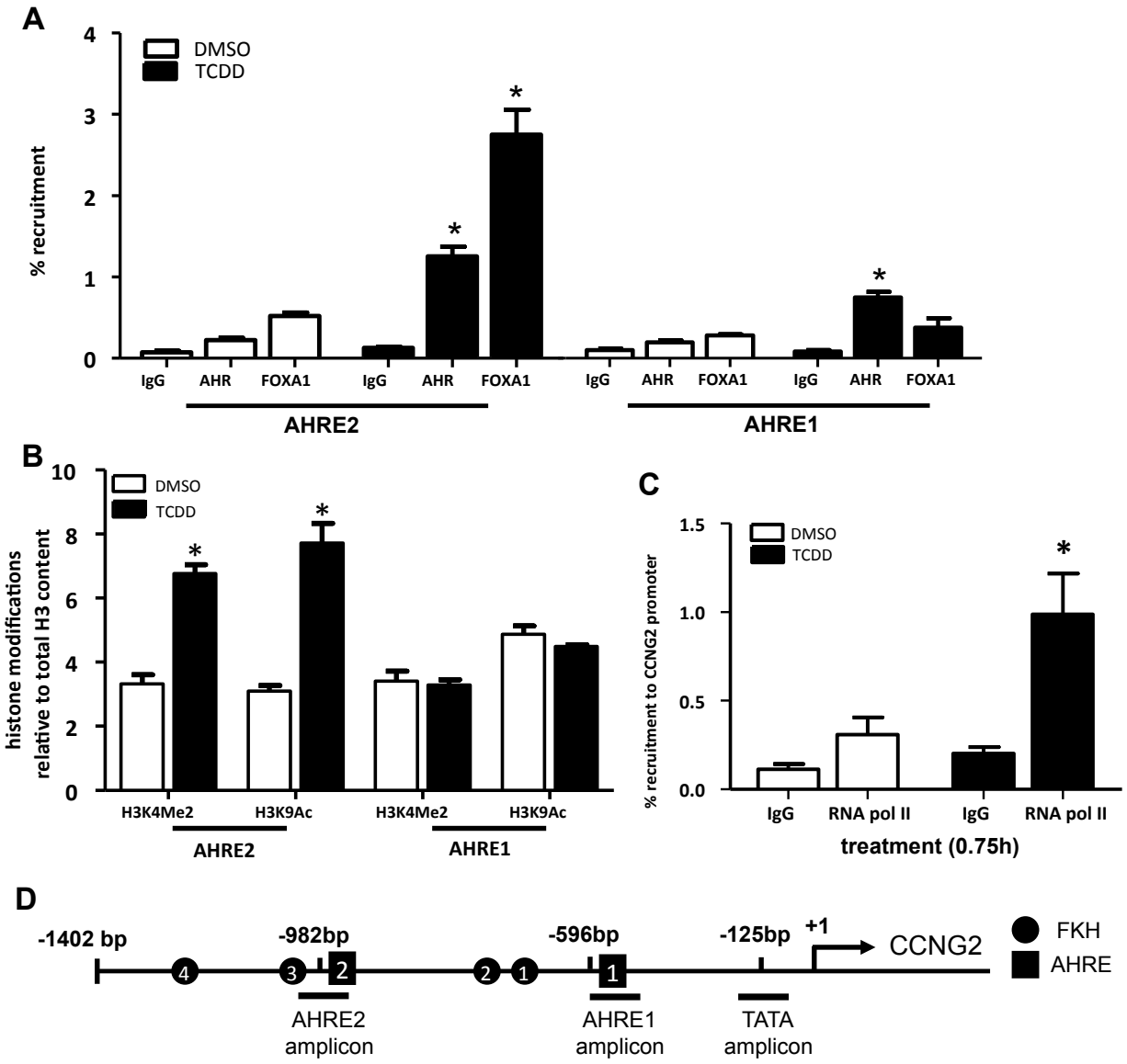



**Figure 20. Diagram of the *CCNG2* regulatory region.**

We performed transcription factor binding site (TFBS) analysis on the -1.4 kb *CCNG2* regulatory region using MatInspector (Genomatix). This analysis identified two AHRE sequences that were positioned in close proximity to multiple forkhead (FKH) sites. We designated the AHRE sites, AHRE1 and AHRE2, and the FKH sites, FKH1-4. AHRE2 was isolated from the ChIP-chip study.




## Figure 21. TCDD induces the recruitment of AHR and FOXA1 to *CCNG2.*

**(A)** Quantification of AHR and FOXA1 recruitment to AHRE1 and AHRE2 using the ChIP assay. Briefly, T-47D cells were treated with 10 nM TCDD for 45 mins and immunoprecipitated using the antibodies indicated. The immunoprecipitated DNA was measured by Q-PCR with primers designed around each response element. **(B)** Determining the relative levels of the histone modifications H3K4Me2 and H3K9Ac following TCDD treatment at AHRE1 and AHRE2 using the ChIP assay **(C)** Recruitment of RNA polymerase II to the TATA box in the proximal promoter region of *CCNG2* after 45 mins treatment. **(D)** Diagram of *CCNG2* regulatory region with location of amplicon designated. Each error bar represents the standard error of the mean of three independent replicates. All data are relative to 100% total input. Asterisks indicate statistically significant differences compared to DMSO control samples using a one-way ANOVA ($P$<0.05).

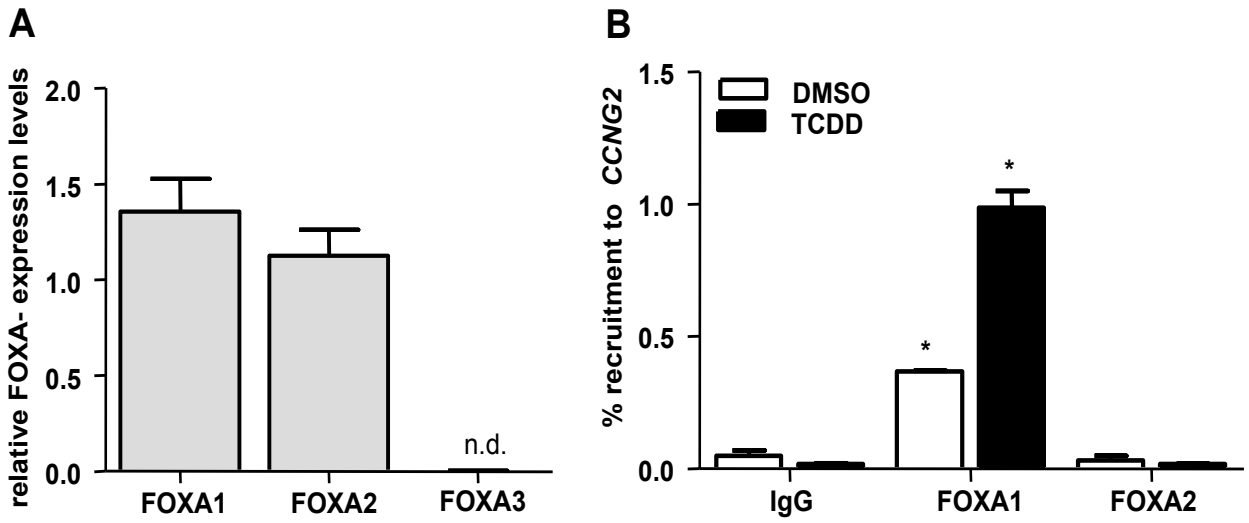



**Figure 22. FOXA- expression and recruitment in T-47D cells.**

(**A**) Expression of FOXA1, 2, 3 in T-47D cells. RNA was isolated and reverse transcribed. All data are relative to ribosomal 18s levels. (**B**) Recruitment of FOXA1 and FOXA2 using the ChIP assay. Briefly, T-47D cells were treated with 10 nM TCDD for 45 mins and immunoprecipitated using the antibodies indicated. The immunoprecipitated DNA was measured by Q-PCR with primers targeting AHRE2. Data are relative to 100% total input. Asterisks indicate statistically significant differences compared to DMSO IgG samples using a one-way ANOVA ($P$<0.05).

## 10.3 TCDD-dependent interactions between FOXA1 and AHR

To determine if FOXA1 and AHR were present in the same protein complex, we performed sequential ChIP and co-immunoprecipitation (Co-IP) experiments. Sequential ChIPs revealed that AHR and FOXA1 were recruited simultaneously to *CCNG2* (**Figure 23A**). Furthermore, AHR and FOXA1 were shown to be part of the same protein complex in Co-IP assays completed in the presence or absence of TCDD (**Figure 23B**). Time-course analysis of AHR and FOXA1 also revealed that both proteins follow the same recruitment pattern over time (**Figure 23C).**

## 10.4 AHR mediates the TCDD-dependent regulation of CCNG2 utilizing FOXA1

To determine how AHR regulates *CCNG2* transcription and to identify the key response element(s) involved in this regulation, we performed promoter deletion and site-directed mutagenesis analyses. Treatment with 10 nM TCDD resulted in an approximate 1.5-fold increase in activity of the full-length promoter, pGL4-*CCNG2* (**Figure 24A**). Deletion of AHRE2 abolished the TCDD-dependent regulation of *CCNG2* (**Figure 24A**). Site-directed mutagenesis of AHRE2 inhibited the TCDD-mediated luciferase activity (**Figure 24B**). These findings suggest that AHRE1 is not required for AHR-dependent regulation of *CCNG2*. We then mutated the FKH3 and FKH4 sites to evaluate the role of FOXA1 in modulating the AHR-dependent regulation of *CCNG2*. Mutation of either FKH site significantly decreased, but did not abolish the TCDD-dependent increase in luciferase activity (**Figure 24B**). However, mutation of both FKH sites prevented the TCDD-dependent increase in luciferase activity. Taken together, these data show that AHRE2 and FOXA1 play key roles in the AHR-dependent regulation of *CCNG2*. The AHR binding observed at AHRE1 in the ChIP analysis (**Figure 21A**) may just represent larger immunoprecipitated DNA fragments containing AHRE2, since the resolution of our ChIP assay is 500-800 bp.

## 10.5 FOXA1 but not ERα is required for the AHR-dependent regulation of CCNG2

FOXA1 is an important modulator of ERα and AR transactivation in breast and prostate cancer cells respectively (Gao et al., 2003; Carroll et al., 2005; Yu et al., 2005; Carroll et al., 2006; Lupien et al., 2008; Belikov et al., 2009). Since FKH sites were found to be important in the TCDD-mediated regulation of the *CCNG2* luciferase reporter plasmid, we hypothesized that

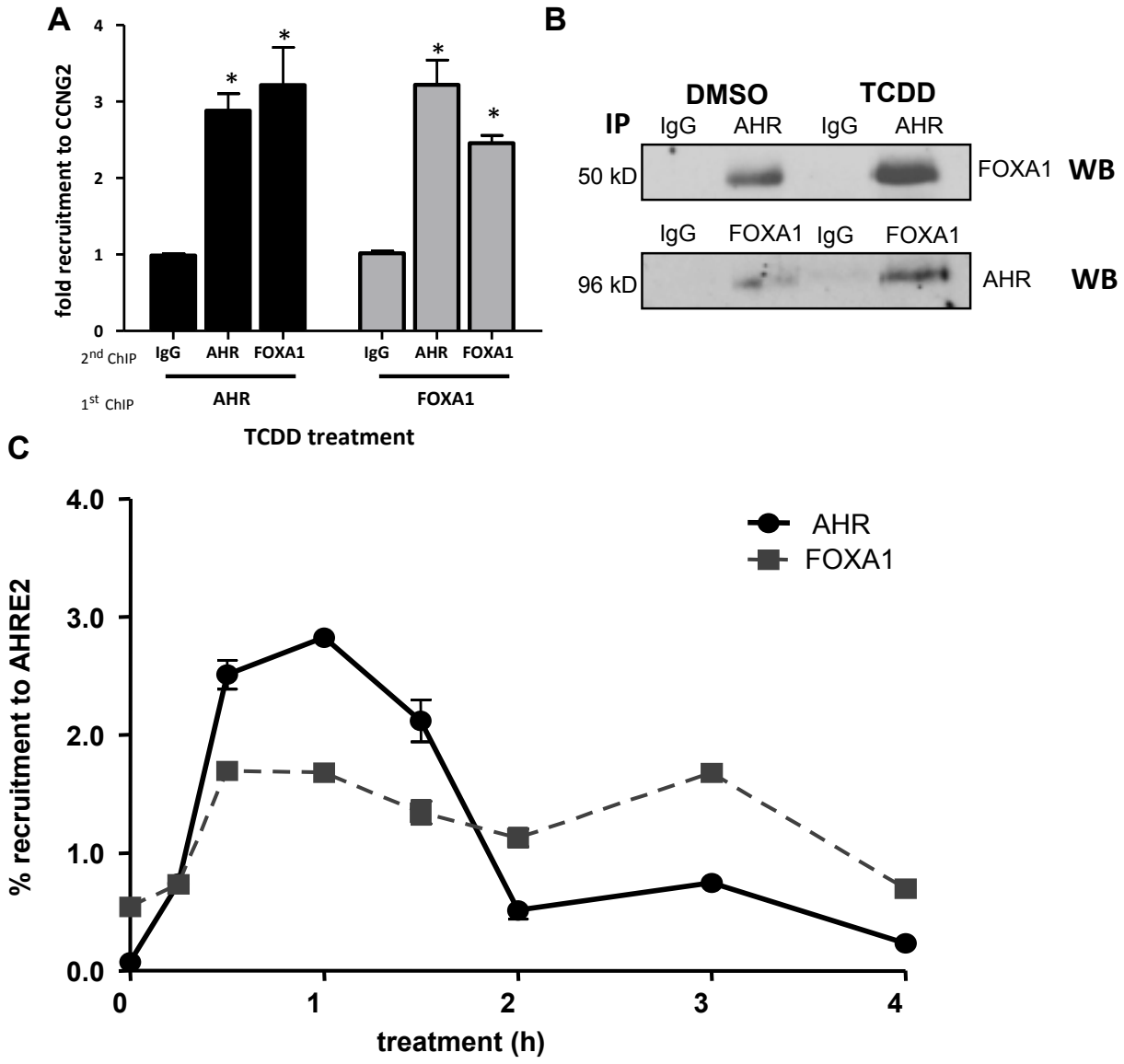



**Figure 23. FOXA1 and AHR are part of the same protein complex.**

(**A**) Sequential ChIPs were preformed with the indicated antibodies. Immunoprecipitated DNA was measured by Q-PCR using the CCNG2 enhancer primers (AHRE2). Quantification of binding was determined as fold induction above IgG DMSO. Each error bar represents the SEM of three independent replicates. Asterisks indicate statistically significant differences compared to IgG DMSO control samples ($P<0.05$, one-way ANOVA). (**B**) Co-immunoprecipitation studies were completed in T-47D cells. Cells were treated for 1 h with either DMSO or TCDD then cross-linked using formaldehyde. Cell lysate was immunoprecipitated using antibodies against AHR and FOXA1. IgG was used as the negative control. Western blot was then completed using the reciprocal antibody. (**C**) Time course analysis of FOXA1 and AHR recruitment to AHRE2 following 10nM TCDD treatment. Immunoprecipitated DNA was measured by Q-PCR using the CCNG2 enhancer primers (AHRE2).

FOXA1 may have a similar role with AHR-chromatin interactions at *CCNG2*. To test this hypothesis we used RNAi-mediated knockdown of FOXA1 and measured mRNA expression as well as the recruitment patterns of AHR, FOXA1, and ERα; ERα was investigated since it is known to negatively regulate *CCNG2* (Stossi et al., 2006). Following transient transfection of two distinct siRNA oligos into T-47D cells, we determined that 48 h post-transfection FOXA1 protein levels were greatly reduced (**Figure 25B**) and mRNA expression was reduced to 20% compared to control cells (**Figure 25A**). Interestingly, the loss of FOXA1 caused a marked decrease in ERα protein levels, which has been previously reported (Bernardo et al., 2010), but did not cause any changes in AHR protein levels. Similar findings were observed in MCF-7 ERα positive breast carcinoma cells (**Figure 25B**). RNAi-mediated knockdown of FOXA1 inhibited the TCDD-dependent gene expression supporting my promoter deletion and mutagenesis results described above (**Figure 26A**). As indicated by our ChIP studies, treatment with 10 nM of TCDD treatment resulted in increased recruitment of FOXA1, AHR, and ERα (**Figure 26B, C, D**). Interestingly, we observed constitutive binding of both FOXA1 and ERα when compared to IgG controls. RNA-mediated knockdown of FOXA1 abolished the TCDD-dependent recruitment of both AHR and ERα (**Figure 26C, D**). The reduced recruitment of ERα to *CCNG2* was most likely due to reduced protein expression levels rather than the loss of FOXA1. FOXA1, however, was necessary for the AHR-mediated regulation of *CCNG2*, although we cannot exclude the possibility that the reduced ERα protein levels influence AHR transactivation, since our lab and others have shown that ERα modulates AHR activity (Safe and Wormke, 2003; Matthews et al., 2005; Ahmed et al., 2009).

We then performed RNAi-mediated ERα knockdown studies to determine the role of ERα in AHR-mediated regulation of CCNG2 expression. The loss of ERα had no effect on either FOXA1 or AHR protein levels (**Figure 26E**). In agreement with our previous findings, the knockdown of ERα significantly increased the constitutive levels of CCNG2 mRNA levels but did not affect the TCDD-mediated increase in CCNG2 mRNA levels (Ahmed et al., 2009) (**Figure 26E**). ChIP analysis showed that ERα was recruited to *CCNG2* in the absence of ligand, suggesting that under these conditions ERα modulates CCNG2 gene expression (**Figure 26E, F**). Knockdown of ERα did not affect the TCDD-dependent recruitment of AHR or FOXA1 to *CCNG2* (**Figure 26G, H**). Together, these data provide evidence that FOXA1 is driving the AHR-mediated regulation of *CCNG2* irrespective of ERα levels.

## 10.6 TCDD-dependent recruitment of NCoA3 to CCNG2

Previous studies showed that CCNG2 was negatively regulated by ERα through the recruitment of an NCoR complex leading to the hypoacetylation of histones and the release of RNA polymerase II (Stossi et al., 2006). Based on these results, we hypothesized that the TCDD-dependent positive regulation of CCNG2 must overcome this inhibition through the recruitment of nuclear coactivators to promote gene expression. Since NCoA3 is over-expressed in breast cancer, we determined the ability of TCDD to induce recruitment of NCoA3 to *CCNG2* in T-47D in the presence or absence of RNAi-mediated knockdown of FOXA1 or ERα. TCDD treatment resulted in increased NCoA3 recruitment to *CCNG2*, which was significantly reduced only after knockdown of FOXA1 but not ERα (**Figure 27A, B**). Co-IP studies provided further evidence that NCoA3 is part of the activated multi-protein AHR containing complex, since it was found to interact with both AHR and FOXA1 (**Figure 27C**).

## 10.7 AHR prevents the ERα–dependent negative regulation of CCNG2

In support of a previous report, estrogen-bound ERα inhibited CCNG2 mRNA expression levels (**Figure 28A, B**) (Stossi et al., 2006). However, this repression was overcome by co-treatment with TCDD, and required FOXA1 but not ERα (**Figure 28A, B**). Co-treatment of TCDD+E2 prevented the E2-dependent removal of NCoA3 from the *CCNG2* resulting in increased recruitment of both AHR and FOXA1 (**Figure 28C-E**). The TCDD-induced recruitment of NCoA3 was dependent on FOXA1 (**Figure 28C-E**). RNAi-mediated knockdown of ERα had no effect on the ability of AHR to block the repression caused by E2 treatment (**Figure 28G, H, I**). Overall, our findings show that FOXA1 facilitated the binding of TCDD-induced AHR and NCoA3 to *CCNG2*, leading to increased CCNG2 gene expression and preventing the previous described repressive actions of ERα on *CCNG2* expression (Stossi et al., 2006) (**Figure 29).**

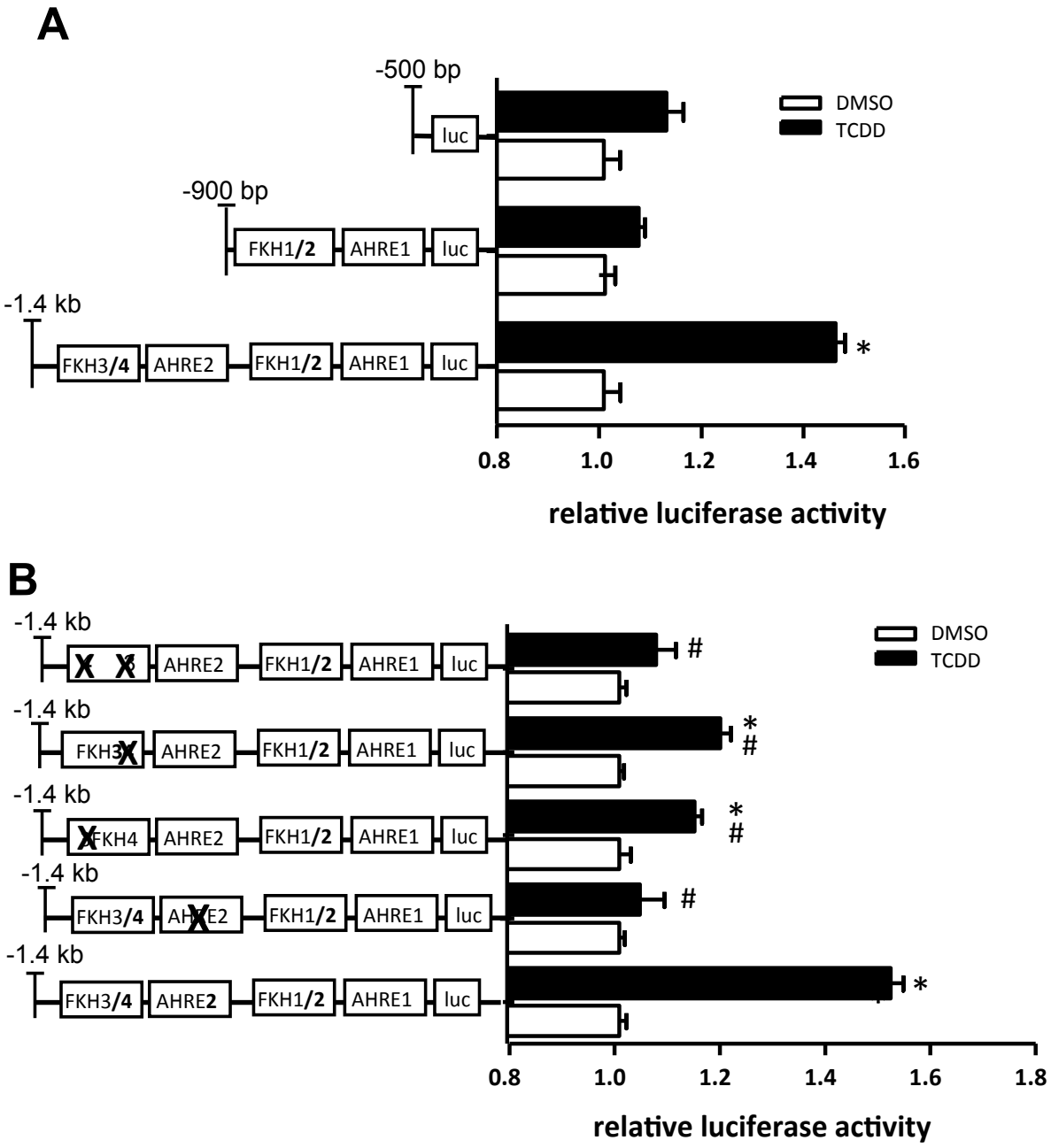
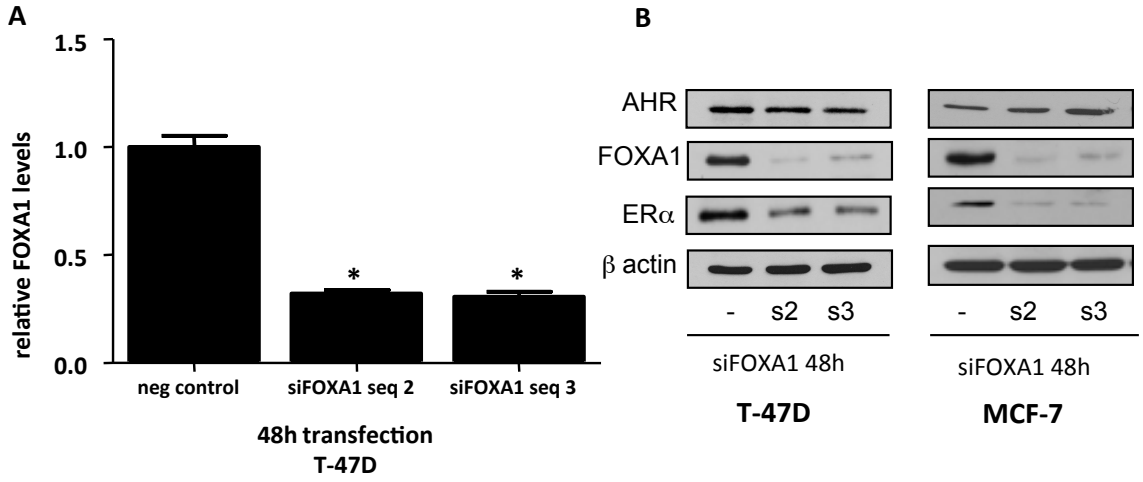



**Figure 24. AHR mediates the TCDD-dependent regulation of CCNG2.**

(**A**) Deletion fragments of pGL4-*CCNG2* were tested to deduce the functional significance of AHRE1 and AHRE2. Two hundred nanograms of each vector were transfected in T-47D cells and luminescence was measured following a 24 h 10 nM TCDD treatment. (**B**) Site-directed mutagenesis of AHRE2 as well as to the FKH recognition sites was generated in pGL4-*CCNG2*. T-47D cells were transfected with 200 ng of the single and double response element mutants and treated with either DMSO or 10 nM TCDD for 24 h. Results represent the mean of three independent replicates with the asterisks indicating luciferase activity that was statistically different compared to DMSO pGL4-*CCNG2* control and the pound signs indicate luciferase activity statistically different compared to TCDD pGL4-*CCNG2* ($P<0.05$, one-way ANOVA).




## Figure 25. Knockdown of FOXA1 affects ERα protein levels.

**(A)** RNAi-mediated knockdown of FOXA1 was measured in T-47D cells 48 h post transfection. Cells were transfected with 50 nM of siRNA and RNA was isolated and reverse transcribed. All data are relative to ribosomal 18s levels. Asterisks indicate statistically significant differences compared to negative control samples using the Student's t-test ($P$<0.05). (**B**) Western blot analysis of AHR and ERα protein levels after FOXA1 knockdown using two distinct sequences in T-47D and MCF-7 cells. Cell extracts were probed with rabbit antibody against all three proteins. β-actin was used as loading control

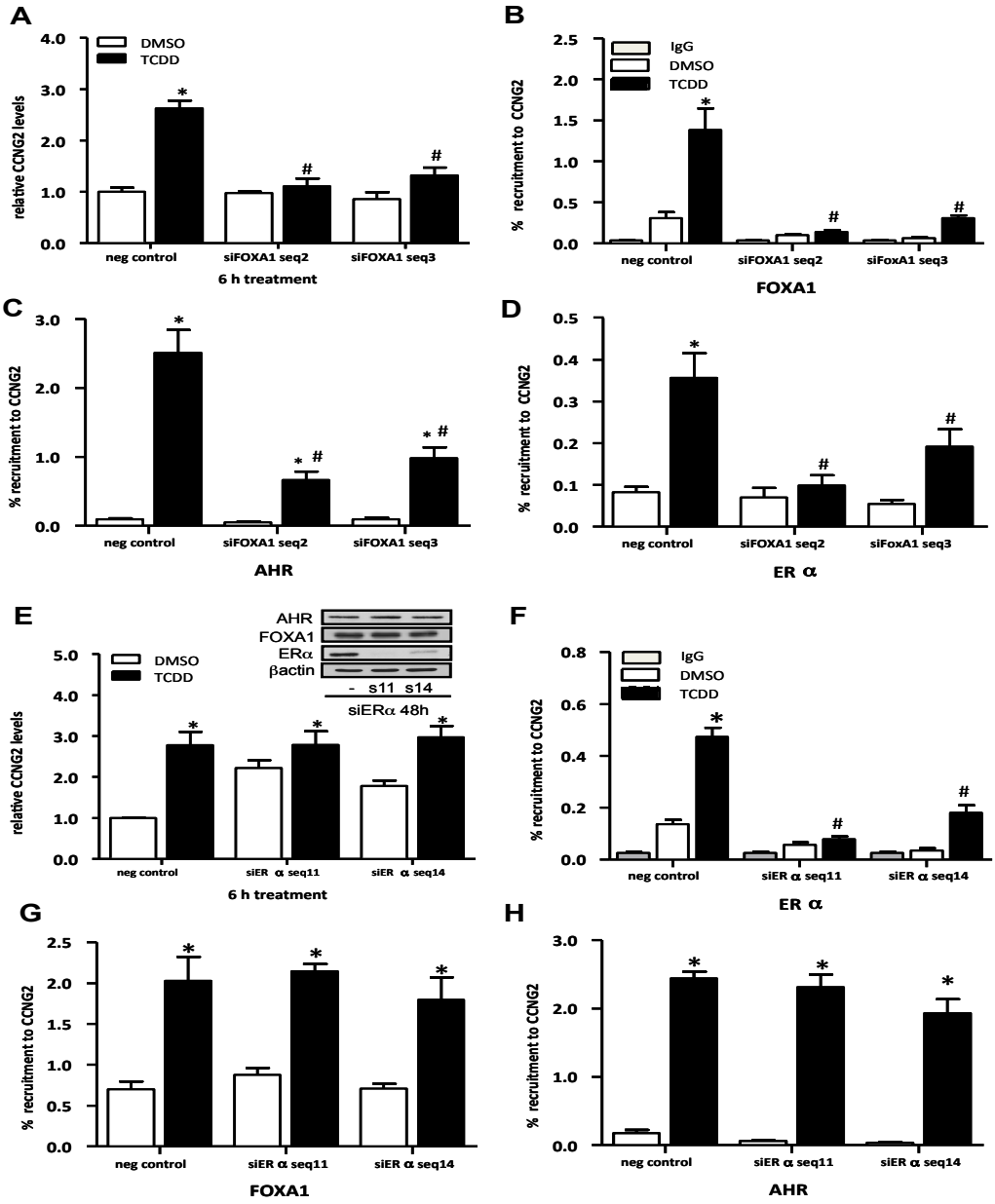



**Figure 26. FOXA1 but not ERα is essential for the TCDD-dependent recruitment of AHR to *CCNG2.***

Gene expression profiles were completed on T-47D cells transfected for 48 h with siRNA then treated for 6 h with TCDD. RNA was isolated and reverse transcribed. mRNA expression was then determined using Q-PCR. Data were normalized against time-matched DMSO and to ribosomal 18s levels. Data represent the mean of three independent replicates and the pound sign is compared to TCDD negative control ($P$<0.05, one-way ANOVA). Recruitment of FOXA1 (**B**), AHR (**C**) and ERα (**D**) following siRNA mediated knockdown of FOXA1 using the ChIP assay. Briefly, T-47D cells were transfected for 48 h with siRNA and then treated for 45 mins with TCDD and immunoprecipitated using the antibodies indicated. The immunoprecipitated DNA was measured by Q-PCR with primers targeting the enhancer region (relative to 100% total input). Each graph represents the mean of three independent replicates with asterisks indicating statistically significant differences compared to DMSO negative control while the pound sign indicates statistically significant differences compared to TCDD negative control ($P$<0.05, one-way ANOVA). (**E**) CCNG2 mRNA expression levels were completed on T-47D cells transfected for 48 h with siERα then treated for 6 h with TCDD. Data represent the mean of three independent replicates and the asterisks are compared to DMSO negative control ($P$<0.05, one-way ANOVA). (**Inset**) Analysis of ERα knockdown in T-47D cells after 48 h. Cell extracts were probed with rabbit antibody against AHR, ERα and FOXA1. β-actin was used as loading control. Recruitment of ERα (**F**), AHR (**G**) and FOXA1 (**H**) was determined following RNAi-mediated knockdown of ERα using ChIP assays. Each graph represents the mean of three independent replicates with the asterisks indicating statistically significant differences compared to DMSO negative control ($P$<0.05, one-way ANOVA)

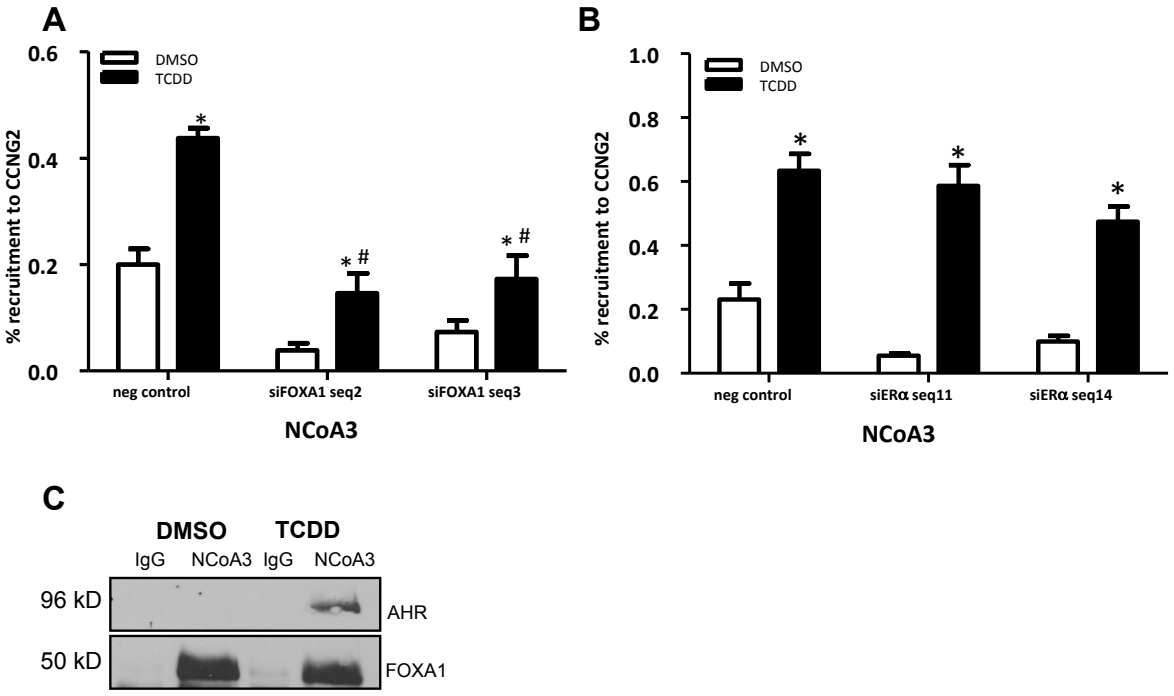
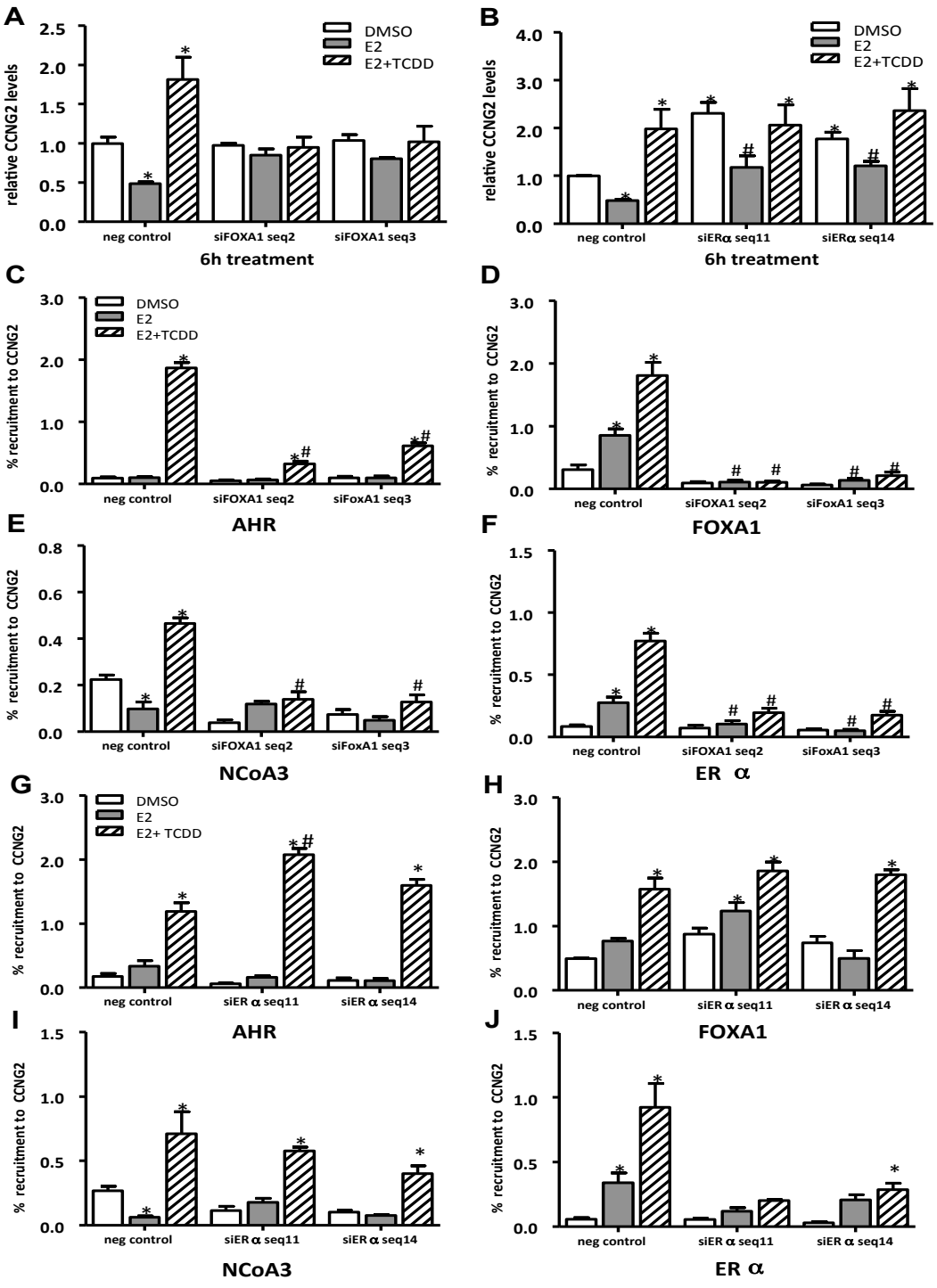



**Figure 27. NCoA3 is part of the complex formed at *CCNG2*.**

Recruitment of NCoA3 was determined after knockdown of FOXA1 (**A**) and ERα (**B**). Briefly, cells were transfected with siRNA targeting both factors followed by treatment with 10 nM TCDD, after which chromatin was immunoprecipitated using an antibody against NCoA3. The immunoprecipitated DNA was measured by Q-PCR with primers targeting the enhancer region. Each graph represents the mean of three independent replicates with the asterisks indicating statistically significant differences compared to DMSO negative control while the pound sign indicates statistically significant differences compared to TCDD negative control ($P<0.05$, one-way ANOVA). (**C**) Co-immunoprecipitation studies were completed in T-47D cells. Cells were treated for 45 min with either DMSO or TCDD then cross-linked using formaldehyde. Cell lysate was immunoprecipitated using a selective NCoA3 antibody. IgG was used as the negative control. Western blot was done using antibodies against AHR and FOXA1.




## Figure 28. AHR can overcome the ERα−dependent negative regulation of *CCNG2*.

CCNG2 mRNA expression levels were determined from T-47D cells transfected for 48 h with siFOXA1 (**A**) or siERα (**B**) then treated for 6 h with 10 nM E2 or 10 nM E2 + 10 nM TCDD. RNA was isolated and reverse transcribed and mRNA expression levels were determined using Q-PCR. Data were normalized against time-matched DMSO and to ribosomal 18s levels. Data represent the mean of three independent replicates with the asterisks representing statistically significant differences compared to DMSO and the pound sign represent statistically significant differences compared to treatment-matched samples ($P$<0.05, one-way ANOVA). Cells were treated with 10 nM E2 or 10 nM E2 + 10 nM TCDD for 45 mins and the recruitment of AHR (**C**), FOXA1 (**D**), NCoA3 (**E**) and ERα (**F**) was determined 48 h post siFOXA1 transfection using ChIP assays and Q-PCR. Similar experiments were also performed 48 h after siERα transfection using antibodies against AHR (**G**), FOXA1 (**H**), NCoA3 (**I**), ERα (**J**).

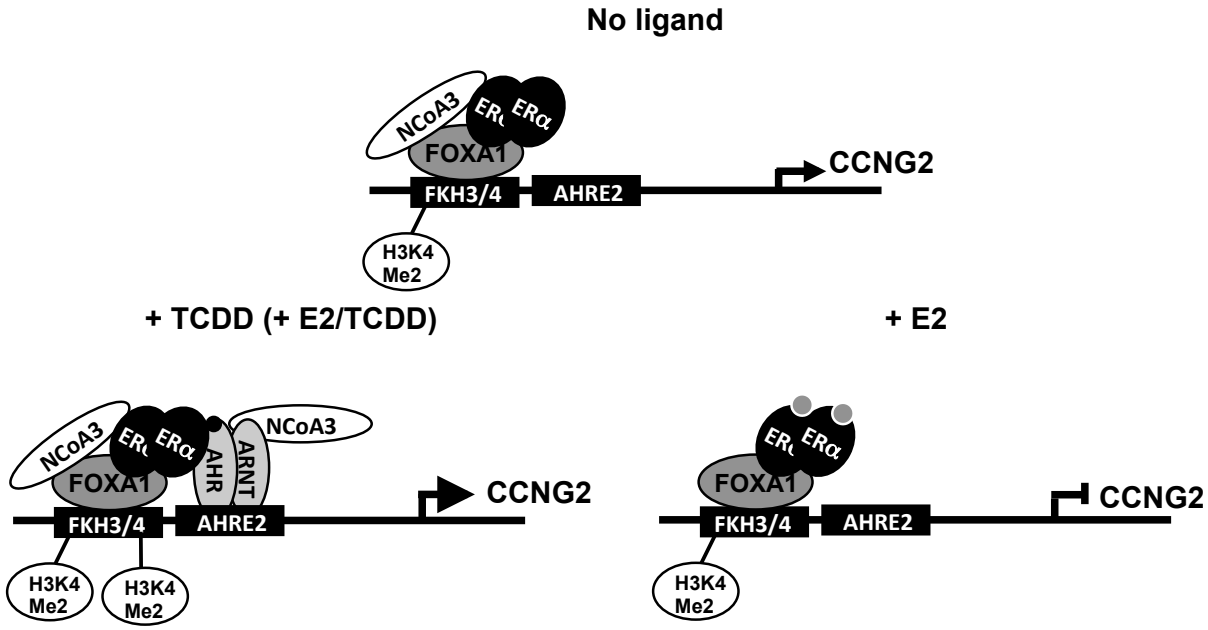



**Figure 29. Proposed mechanism for the FOXA1- and AHR-dependent regulation of CCNG2.**

In the absence of ligand, FOXA1 is bound to the FKH recognition sites in the enhancer region of CCNG2. ERα and NCoA3 occupy the upstream regulatory region of *CCNG2*. Treatment with E2 reduces the constitutive NCoA3 but increases E2-bound ERα occupancy at *CCNG2* resulting in transcriptional repression as previously described (Stossi et al., 2006). However upon TCDD or E2+TCDD treatment, FOXA1 binding is increased facilitating the recruitment of AHR and increased recruitment of NCoA3 leading to transcriptional activation of CCNG2. FOXA1 mediates these effects through direct interactions with AHR. The relative transcriptional activity of *CCNG2* is represented by the magnitude of the arrow.

# *11* AHR knockout in MCF-7 and MDA-MB-231 alters ERα signalling, proliferation and depletes constitutive CYP1B1 levels.

## 11.1 Targeted disruption of AHR in MCF-7 and MDA-MB-231 human breast cancer cell lines

Loss-of-function studies using RNA interference have been used previously to study AHR signalling (Abdelrahim et al., 2003; Yang et al., 2008; Ahmed et al., 2009; Zhang et al., 2009); however AHR expression is reduced but not eliminated with this approach. Our laboratory, like many others have shown a marked reduction in protein levels following RNAi-mediated knockdown of AHR, but we still observed a TCDD-dependent induction of CYP1A1 and CYP1B1 mRNA levels, albeit less than control cells (**Figure 30**). This low level of expression might mask important cellular and regulatory roles of AHR. Gene knockout, rather than knockdown, is well recognized as a powerful approach to determine gene function. With this in mind, we used a zinc finger nuclease approach to knockout AHR in ERα positive (MCF-7) and negative (MDA-MB-231 abbreviated MDA herein) human breast cancer cells. We chose these cells to further investigate the role of AHR in the regulation of ERα transactivation and protein levels, as well as to determine the importance of AHR in MDA cells, an *in vitro* model for triple negative breast cancer.

The ZFN proteins targeted exon 1 of AHR causing either deletions or insertions, resulting in genetic changes causing shifts in reading frame to generate premature stop codons. Unlike RNAi-mediated knockdown that reduces mRNA levels, no measureable changes in mRNA expression levels were observed but the frameshift led to the abolishment of protein expression (**Figure 31**). Deletions were observed more frequently than insertions. However, due to the randomness associated with the change, the MCF-7 $AHR^{-/-}$ clones contained 22-bp or 4-bp deletions while the MDA $AHR^{-/-}$ had 2-bp or 4-bp deletions. Sequence alignment of the 22-bp deletion in MCF-7 $AHR^{-/-}$ is shown in **Figure 32**. To determine if the ZFN targeting AHR might bind to other sequences in genome, we used the ZFN-Site web-based interface (Cradick et al., 2011). Despite allowing for the up to 2 mismatched bases and flexible spacing, no additional or potential off-target binding sites were identified across the human genome.

AHR has been reported to act as a ligand-activated regulator of ERα protein levels as an integrated component of the Cul4B ubiquitin ligase complex (Ohtake et al., 2007). Despite this, AHR knockout did not affect ERα (MCF-7) or ARNT (MCF-7 and MDA cells) protein levels, which is in agreement with previous RNAi-mediated knockdown of AHR in T-47D cells and low AHR expressing MCF-7 $AH^{R100}$ human breast cancer cells (Ciolino et al., 2002; Ahmed et al., 2009) (**Figure 31**). AHR loss in the MCF-7 breast cancer cells caused a marked decrease in constitutive CYP1A1 and CYP1B1 levels (**Figure 33A, B**). Similar effects were seen in MDA cells (**Figure 33C, D**). CYP1B1 was of particular interest since it is implicated in breast cancer because of its ability to metabolize endogenous estrogen into the mutagenic 4-hydroxl catechol OH-E2 metabolite (Hayes et al., 1996; Belous et al., 2007). The reduced constitutive levels observed for CYP1A1 and CYP1B1 was selective, since other known AHR responsive genes, such as TiPARP and NFE2L2 (Nuclear factor (erythroid-derived 2)-like 2 or NRF2) showed significant loss in TCDD-dependent increase in mRNA expression but no changes in constitutive levels (**Figure 33E, F**).

## 11.2 Constitutive and ligand-induced CYP1B1 levels are dependent on AHR expression.

To determine if the depletion of constitutive CYP1B1 levels was directly related to reduced AHR expression levels we compared the CYP1B1 expression levels following RNAi-mediated AHR knockdown using transient siRNA and a stable inducible shRNA with that of MCF-7 and MDA $AHR^{-/-}$ cells. Reduced CYP1B1 mRNA levels were detected in siAHR and shAHR experiments with levels comparable to those achieved after using RNAi against CYP1B1 (**Figure 34A**). However, knockout of AHR in both MCF-7 and MDA cells caused a far greater decrease in constitutive CYP1B1 levels than that achieved by siRNA or shRNA, illustrating the key role of AHR in regulating constitutive CYP1B1 expression levels in breast cancer cells (**Figure 34A-C**). We next examined if the loss of constitutive CYP1B1 expression could be rescued with ectopic AHR expression in MCF-7 $AHR^{-/-}$ or MDA $AHR^{-/-}$. For these experiments cells were transfected with AHR or $AHR_{DBDmut}$ (an AHR insertion mutant that does not bind to AHREs), and CYP1B1 expression levels determined. Transfection with increasing amounts of AHR or $AHR_{DBDmut}$ resulted in concentration dependent increases in AHR mRNA using Q-PCR primers that amplified both forms of AHR (**Figure 34D, F**). Transient overexpression of AHR but not $AHR_{DBDmut}$ restored the constitutive and TCDD inducible mRNA levels of CYP1B1 (**Figure**

**34E, G**) demonstrating that AHR regulates constitutive CYP1B1 mRNA levels through DNA binding and requires an intact DNA-binding domain.

CYP1B1 is regulated by AHR, but also by E2 through ERα (Shehin et al., 2000; Tsuchiya et al., 2004; Yang et al., 2008); however, it is unclear if loss of AHR would influence the ability of ERα to modulate CYP1B1. Moreover, we have recently reported that activated AHR induces the recruitment of ERα to *CYP1B1*, suggesting that AHR modulates the genomic binding profiles of ERα (Matthews et al., 2005; Ahmed et al., 2009). To investigate the recruitment profile of AHR, ARNT, and ERα to modulate CYP1B1 in the presence and absence of AHR, we performed ChIP assays in MCF-7 and MDA cells. The ligand-dependent recruitment of all three factors was determined to two different functional regions of *CYP1B1*, a distal region (-900 bp) and a proximal region (-250 bp). The distal region contains AHREs that have been previously used to study AHR-dependent regulation of *CYP1B1* (Shehin et al., 2000; Tsuchiya et al., 2003; Matthews et al., 2005), whereas the proximal region (-250 bp) contains an AHRE and a half-site ERE shown to be important for the E2-dependent regulation of CYP1B1 (Tsuchiya et al., 2004).

TCDD induced recruitment of AHR, ARNT and ERα to the *CYP1B1* distal region. E2 alone did not significantly induce recruitment of AHR or ARNT to *CYP1B1;* however, a small but significant increase in ERα occupancy at *CYP1B1* distal region was observed (**Figure 35A-C**). Co-treatment of TCDD with E2 did not affect the recruitment levels of AHR or ARNT, but enhanced the recruitment of ERα to the *CYP1B1* distal region. TCDD and E2 alone induced the recruitment of AHR and ARNT to the *CYP1B1* proximal region, which was significantly increased after TCDD+E2 co-treatment. ERα exhibited constitutive binding to the CYP1B1 proximal region that was increased with exposure to E2 and further increased with TCDD+E2 co-treatment (**Figure 35E, F**). No ligand-dependent recruitment of AHR, ARNT, or ERα to the distal or proximal regions was observed in $AHR^{-/-}$ cells (**Figure 35A-F**). Despite the increased recruitment of AHR and ARNT at the CYP1B1 proximal region, co-treatment with TCDD+E2 did not result in a further increase in CYP1B1 mRNA expression levels compared to TCDD alone (**Figure 35G**). These results demonstrated that AHR expression regulates the recruitment of ARNT and ERα at both the distal and proximal regions, including the E2-dependent recruitment of ERα to *CYP1B1*.

To establish the importance of ERα in mediating the increased recruitment of AHR and ARNT at the *CYP1B1* proximal region, we performed ChIP and mRNA expression analyses in the MDA, MDA AHR$^{-/-}$ and MDA cells stably expressing ERα (Pearce and Jordan, 2004). TCDD and E2+TCDD as well as ERα status had no effect on AHR and ARNT recruitment to the *CYP1B1* distal region (**Figure 36A, C**). E2 alone did not alter the recruitment patterns of AHR or ARNT compared to DMSO. TCDD, E2 and TCDD+E2 induced recruitment of ERα to *CYP1B1* distal region was only observed in MDA cells stably expressing ERα (**Figure 36E**). In MDA cells, we observed constitutive, but not ligand induced, occupancy of AHR and ARNT at *CYP1B1* proximal region. No recruitment of AHR or ARNT was observed in the AHR$^{-/-}$ cells (**Figure 36A-F)**. Similar to that observed at the distal region, E2 and TCDD+E2 treatment induced the recruitment of ERα to the CYP1B1 proximal region. Interestingly, stable expression of ERα resulted in TCDD+E2-dependent increase in recruitment of AHR and ARNT to the *CYP1B1* proximal region (**Figure 36B, D, F)**. Although E2 did not affect TCDD-dependent increases in CYP1B1 mRNA levels, higher CYP1B1 expression levels were observed in MDA cells stably expressing ERα (**Figure 36G**). Moreover, the expression of ERα resulted in a weak but significant increase in CYP1B1 mRNA levels. No ligand induced CYP1B1 mRNA expression levels was observed in the MDA AHR$^{-/-}$ cells. These data demonstrated that ERα was important in mediating the interactions of AHR and ARNT at the *CYP1B1* proximal region, but AHR rather than ERα was absolutely required for CYP1B1 expression in these two breast cancer cell lines.

## 11.3 Loss of AHR alters ERα signalling in a gene-dependent manner

Both AHR and ARNT have been previously reported to impact ERα signalling but it is unknown if they function independently or together to affect ERα (Brunnberg et al., 2003; Safe and Wormke, 2003). Since MCF-7 cells endogenously express AHR, ARNT, and ERα, the *AHR*$^{-/-}$ MCF-7 cells allow us to study the role of ARNT independently of AHR in ERα signalling. Previous reports have implicated ARNT as a potent coactivator of ERα transactivation (Brunnberg et al., 2003; Labrecque et al., 2012) while activated-AHR has been shown to inhibit ERα signalling (Harper et al., 1994; Krishnan et al., 1994; Zacharewski et al., 1994).

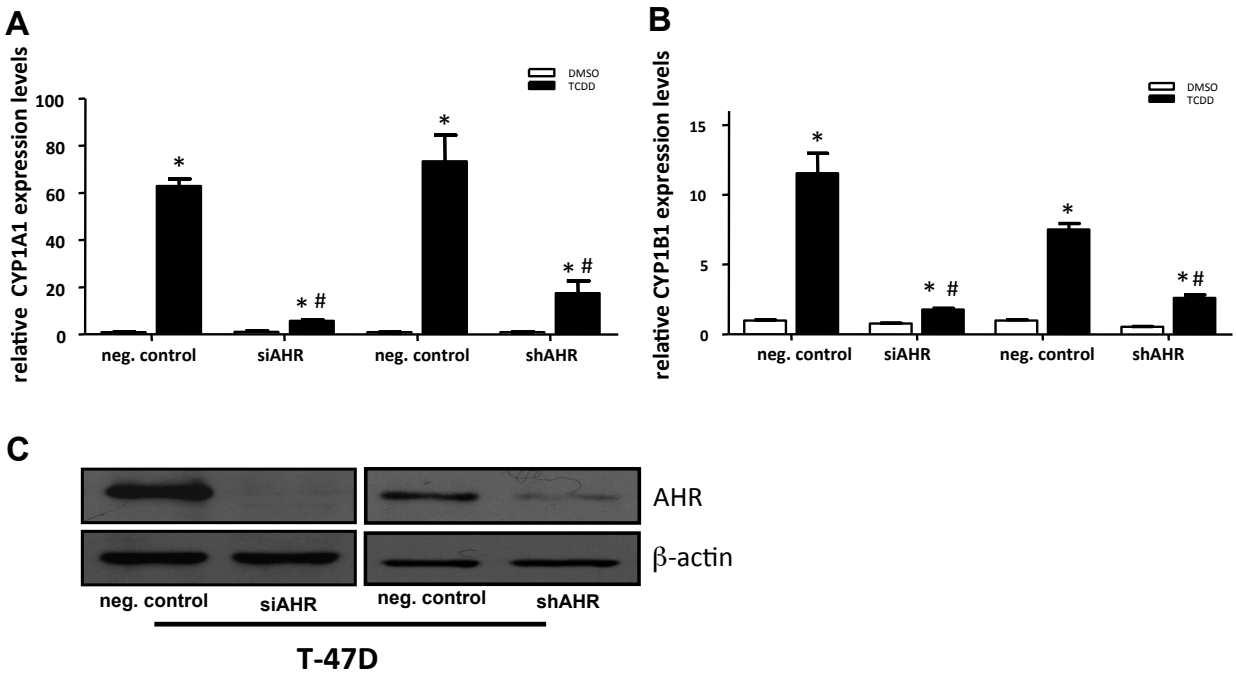



**Figure 30. RNAi-mediated knockdown of AHR still induces a functional response.**

Comparison of the functional response after RNAi-mediated knockdown of AHR by measuring **(A)** CYP1A1 and **(B)** CYP1B1 levels. T-47D cells were transiently transfected with siRNA against AHR for 48 h followed by a 6 h treatment with 10 nM TCDD. T-47D stably expressing an inducible shRNA against AHR was shown to have reduced levels of AHR after 1 week treatment with 1 μM of doxycycline. After knockdown was achieved, cells were treated with 10 nM TCDD. After 6 h treatment, all cells had their RNA isolated and reverse transcribed. Data were normalized against DMSO and to ribosomal 18s levels. Each error bar represents the SEM of three independent replicates. Asterisk is compared to negative control DMSO and pound sign is compared to negative control TCDD treatment ($P$<0.05, one-way ANOVA). **(C)** Western blot analysis of AHR knockdown in each cell line. Cell extracts were probed with rabbit antibody against AHR. β-actin was used as loading control.

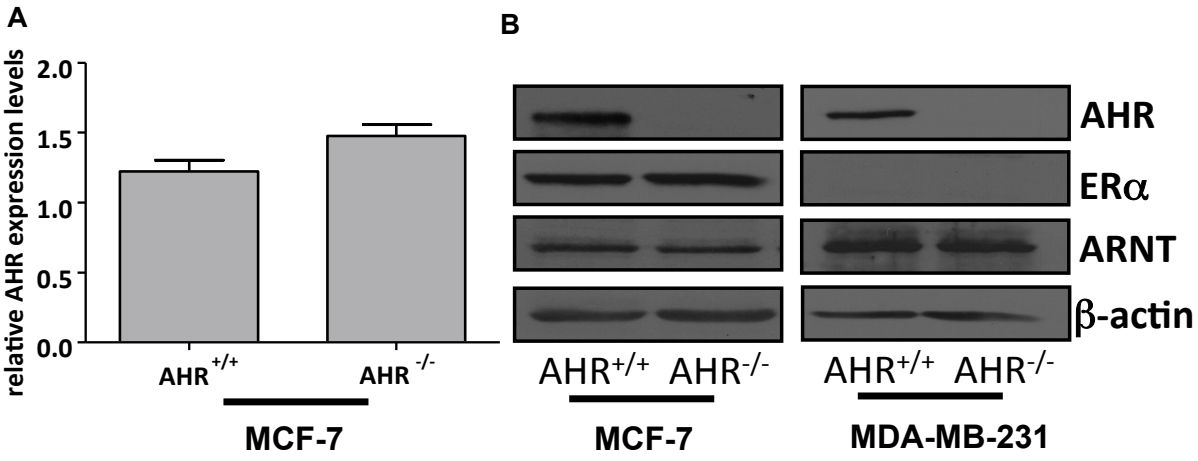



**Figure 31. Zinc finger nuclease-mediated AHR knockout in MCF-7 and MDA cell lines.**

**(A)** AHR expression levels in MCF-7 wildtype and AHR knockout cells using Q-PCR. Similar results were seen in the MDA cells. Data represent the mean of three independent replicates. **(B)** Western blot analysis of AHR, ERα, and ARNT protein levels in both MCF-7 and MDA-MB-231 wildtype and AHR knockout cells. β-actin was used as loading control.

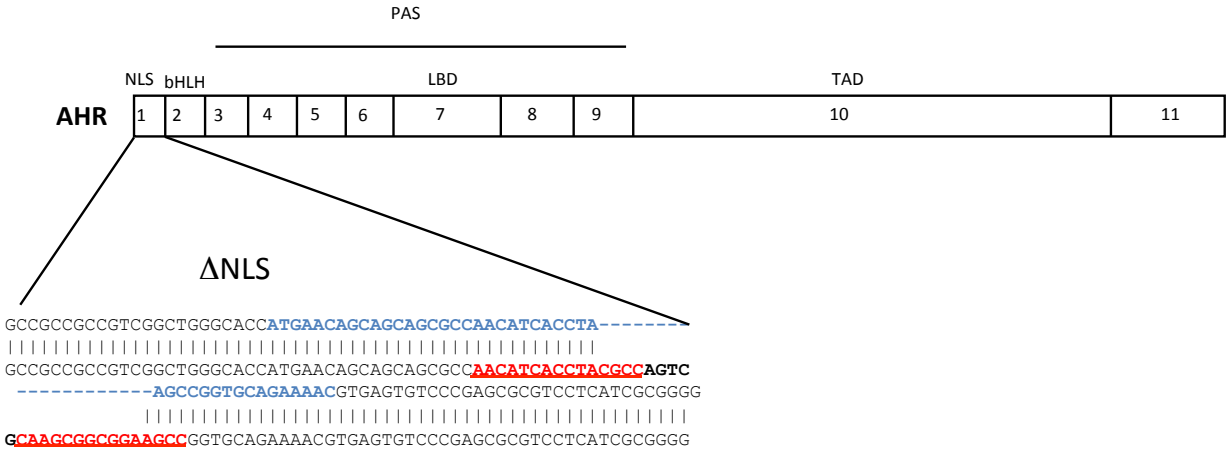



**Figure 32. Zinc finger nuclease pair targets exon 1 of AHR.**

A representation of the AHR locus as approximated from NCBI. Each exon is denoted by a number. The domains are the basic-helix-loop-helix (bHLH), ligand binding domain (LBD), transcriptionally active domain (TAD), and the Per-ARNT-Sim domain (PAS). Our ZFNs targeted the nuclear localization sequence in exon 1. Sample sequence showing a deletion of 22 bp. Red denotes ZFN pair binding site.

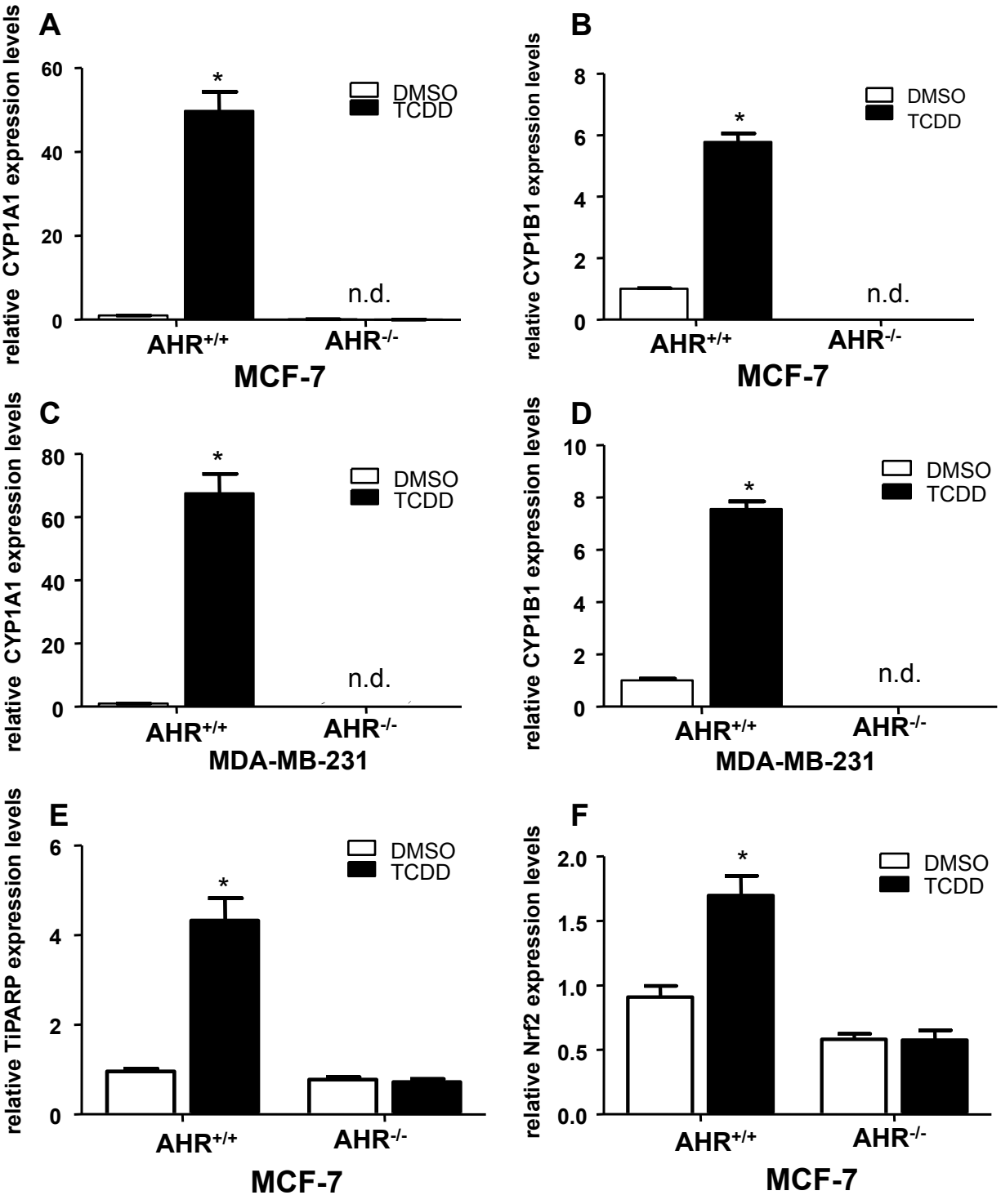



**Figure 33. Gene expression profiles of AHR target genes in wildtype and *AHR*-null cells.**

MCF-7 and MDA $AHR^{+/+}$/$AHR^{-/-}$ were treated with DMSO or 10 nM TCDD for 6 h and their RNA were subsequently isolated and reverse transcribed. Data were normalized against DMSO and to ribosomal 18s levels. Each error bar represents the SEM of three independent replicates. CYP1A1 (**A, C**), CYP1B1 (**B, D**), TiPARP (**E**), and Nrf2 (**F**) mRNA levels were measured using Q-PCR. Asterisk is compared to DMSO in the wildtype cells ($P$<0.05 one way ANOVA)

We used the MCF-7 $AHR^{+/+}$/$AHR^{-/-}$ to study ERα recruitment to and gene expression of *TFF-1* and *GREB1,* two estrogen target genes.

The MCF-7 cells were first assessed for perturbations in ARNT signalling. ARNT is a general heterodimerization partner for other bHLH-PAS proteins including hypoxia inducible factor alpha (HIF1-α). HIF1-α together with ARNT mediates the cellular response to hypoxia. Our results suggest that ARNT interaction with HIF1-α, to activate hypoxia responsive genes (VEGF, CA9, (Vengellur et al., 2005)) was unchanged with the loss of AHR after 24h $CoCl_2$ treatment (**Figure 37A, B**). Treatment of cells with cobalt promotes a response similar to hypoxia (Ho and Bunn, 1996). This indicated that both ARNT-dependent signalling and protein were unaffected by AHR loss.

As expected, in the MCF-7 $AHR^{+/+}$ cell line, AHR and ARNT were recruited to the regulatory region of *TFF-1* after TCDD, E2, and E2+TCDD treatment (**Figure 38 A, B**). Loss of AHR prevented the TCDD-dependent increases in the recruitment of ARNT, but there was an increase in the basal promoter occupancy of ARNT at *TFF-1* (**Figure 38B**). ERα binding was increased in an E2 and E2+TCDD dependent manner in the MCF-7 $AHR^{+/+}$ cell line (**Figure 38C**). After AHR removal, there was a significant increase in the basal binding of ERα as well as an enhancement of the E2 and E2+TCDD-dependent recruitment (**Figure 38C**). Investigation of TFF-1 mRNA expression levels mimicked the changes in recruitment of ERα, where the loss of AHR caused increased basal levels and E2-dependent induction (**Figure 38G**) with no documented changes in ERα protein levels (**Figure 38I**). Characterization of the *GREB1* regulatory region revealed similar recruitment patterns of AHR, ARNT, and ERα in the wildtype MCF-7 cells (**Figure 38D-F**). However, in the MCF-7 $AHR^{-/-}$ cells we did not observe increased constitutive binding of ARNT or ERα but did see enhanced E2-dependent binding (**Figure 38E, F**). Gene expression analysis confirmed these findings, where there was no increase in the constitutive levels of GREB1 but there was higher E2 induction in the AHR-null cells (**Figure 38 H**). Taken together, these results suggested that AHR inhibits ERα signalling at the *TFF-1* and *GREB1* regulatory region with ARNT appearing to play no discernable role.

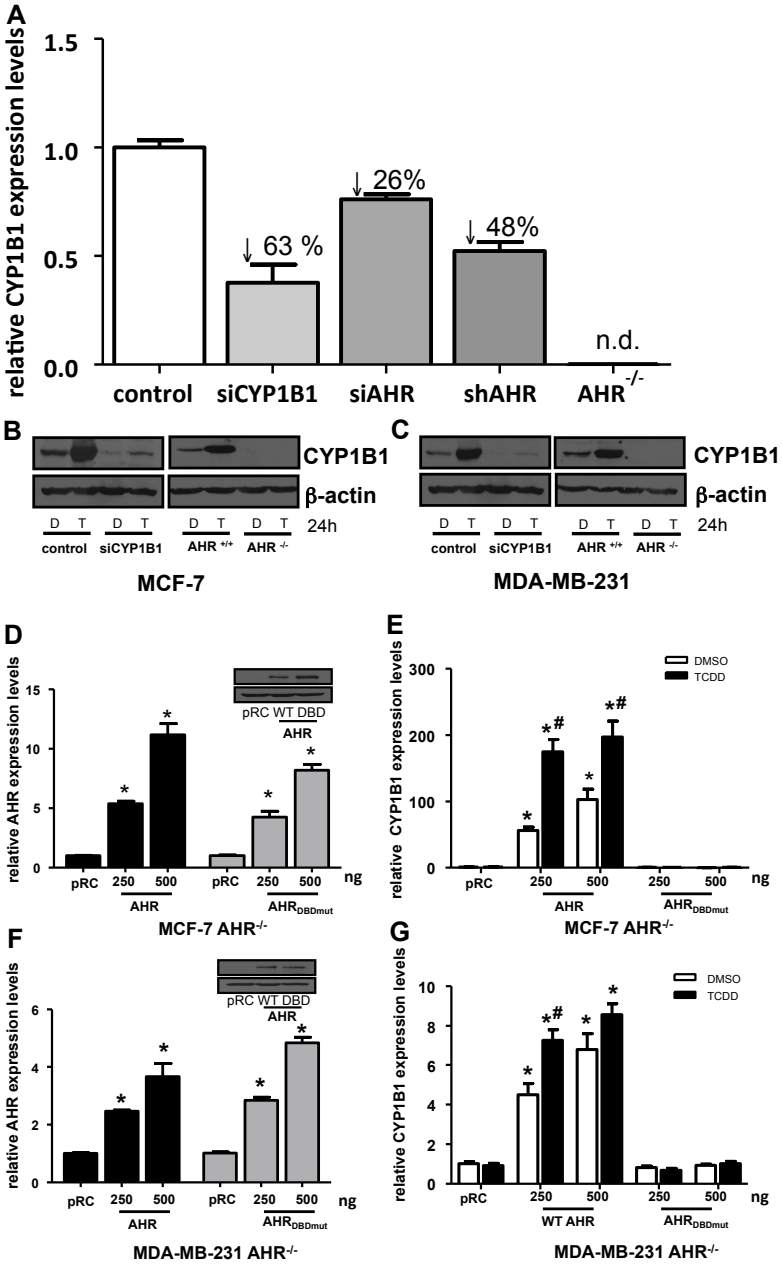
## 11.4 Loss of AHR reduces proliferation rates of MCF-7 and MDA cells and causes $G_1$ and $G_2$/M phase accumulation

AHR has been previously shown to affect the cell cycle (Elizondo et al., 2000; Abdelrahim et al., 2006; Zhang et al., 2009; Barhoover et al., 2010). To investigate this further, we determined the consequence of AHR knockout on the proliferation of MCF-7 and MDA cells. The loss of AHR significantly reduced the proliferation of both cell lines (**Figure 39A and 40A**). In MCF-7 cells, E2 enhanced the proliferation of both wildtype and $AHR^{-/-}$ cells but was less effective at inducing growth in the $AHR^{-/-}$ cells (**Figure 39A**). TCDD alone had no effect on proliferation but as expected co-treatment of TCDD+E2 reduced cell proliferation was significantly reduced compared to E2 treatment (**Figure 39A**).

In the MDA cells, cell proliferation was not affected by TCDD treatment. Since MDA cells are not responsive to E2 and do not express ERα, E2 and TCDD+E2 co-treatment experiments were not performed. (**Figure 40A**).

Cell cycle analysis using BrdU and PI double stain confirmed the reduced proliferation rates in both cell lines. Compared to wildtype MCF-7 cells the MCF-7 $AHR^{-/-}$ showed an accumulation of cells in both the $G_1$ and $G_2$/M phase suggesting a decrease in growth (**Figure 39B,C**). Upon E2 treatment in the MCF-7 wildtype cells there was an increase in the percentage of cells in the S phase, which decreased after TCDD co-treatment (**Figure 39C**). However, with the loss of AHR, more than 90% of the cells were captured in the S phase indicating either a decrease in cycling or an S phase arrest (**Figure 39D**).

Similar to the proliferation results in the MDA cells, cell cycle was unaffected by treatment but was altered after AHR removal (**Figure 40B-D**). Unlike the MCF-7 cells, AHR knockout decreased the amount of cells in the $G_1$ phase but increased the percentage of cells in the $G_2$/M phase (**Figure 40B,C**). As stated above, E2 and TCDD+E2 co-treatment experiments were not performed due to the lack of ERα expression in these cells. Our results confirm the important role AHR plays in the cell cycle but further studies are required to fully elucidate the mechanism.




## Figure 34. The constitutive levels of CYP1B1 are dependent on AHR.

**(A)** CYP1B1 expression after different cell treatments. Data represent the mean of three independent replicates. CYP1B1 protein levels after siRNA targeting CYP1B1 in **(B)** MCF-7 and **(C)** MDA cells compared to AHR knockout cells. β-actin was used as loading control. Transient transfection of vector control (pRC) wildtype AHR(AHR) and the DNA binding domain mutant ($AHR_{DBDmut}$; contains an insertion of glycine and serine in the DNA binding domain of AHR) in both MCF-7 $AHR^{-/-}$ **(D, E)** and MDA $AHR^{-/-}$ **(F, G)** cells were completed to assess constitutive CYP1B1 levels. Each error bar represents the standard error of the mean of three independent replicates. Asterisks denotes statistically different than DMSO treated vector control while the pound sign denotes statistically different than DMSO treated wildtype AHR transfected cells (one-way ANOVA; $P<0.05$).

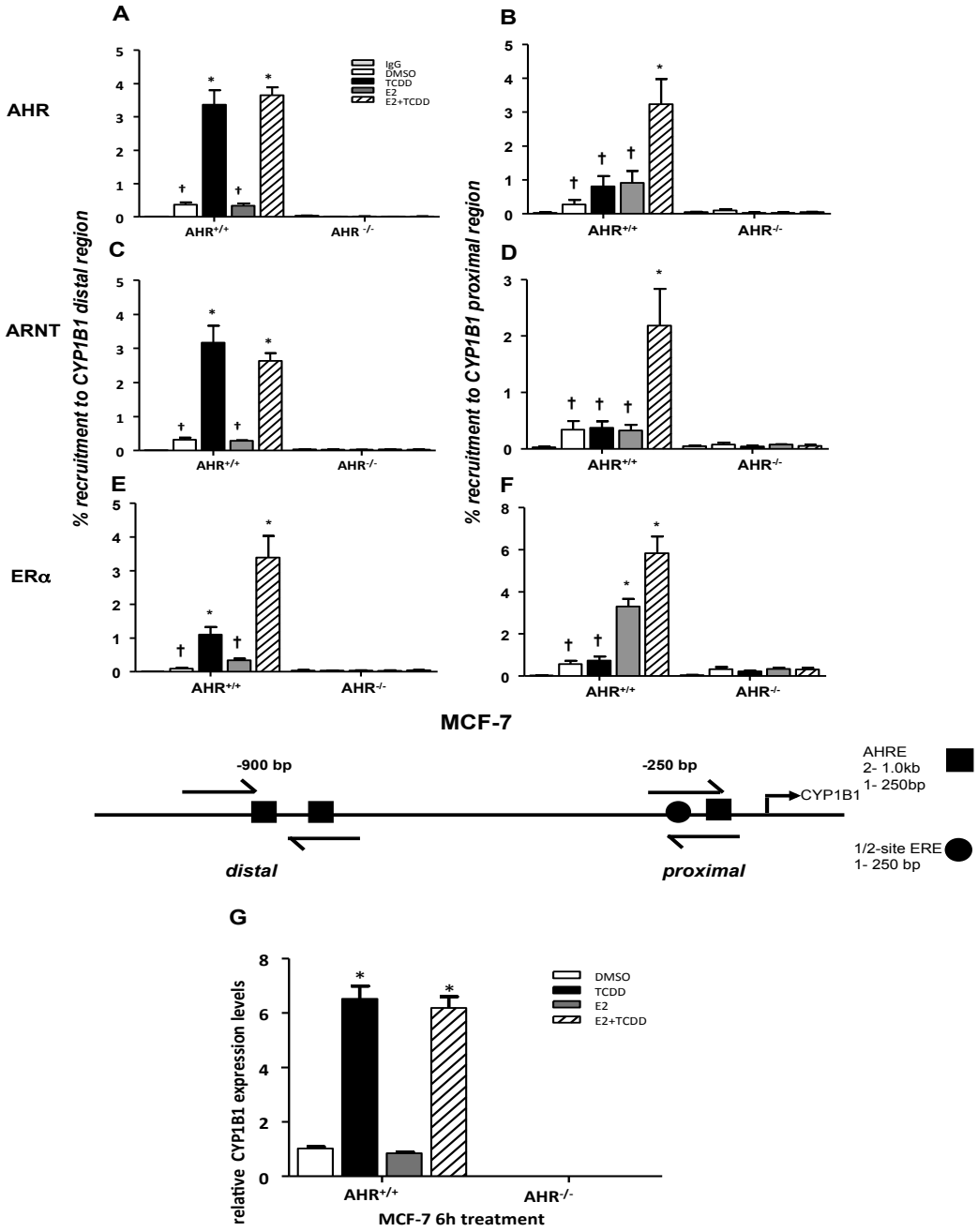



**Figure 35 Recruitment to the regulatory regions of *CYP1B1* is dependent on AHR in MCF-7 ERα positive breast cancer cell lines.**

Quantification of AHR **(A, B),** ARNT **(C, D),** and ERα **(E, F)** recruitment to the distal and proximal regions of *CYP1B1* using the ChIP assay. Briefly, MCF-7 AHR$^{+/+}$ and MCF-7 AHR$^{-/-}$ were treated with DMSO, 10 nM TCDD, 10 nM E2, or E2+TCDD for 45 mins and immunoprecipitated using antibodies against the proteins indicated. Each error bar represents the standard error of the mean of three independent replicates. All data are relative to 100% total input. Asterisks indicate statistically significant differences compared to DMSO control samples while † is compared to IgG control samples using a one-way ANOVA ($P$<0.05). (**G**) Expression analysis of CYP1B1 levels in MCF-7 wildtype and *AHR*-null cells. Briefly, cells were treated with ligand for 6 h and RNA was isolated and reversed transcribed. All data were normalized to 18s levels and DMSO. Asterisks indicate significantly different than DMSO using a one-way ANOVA ($P$<0.05).

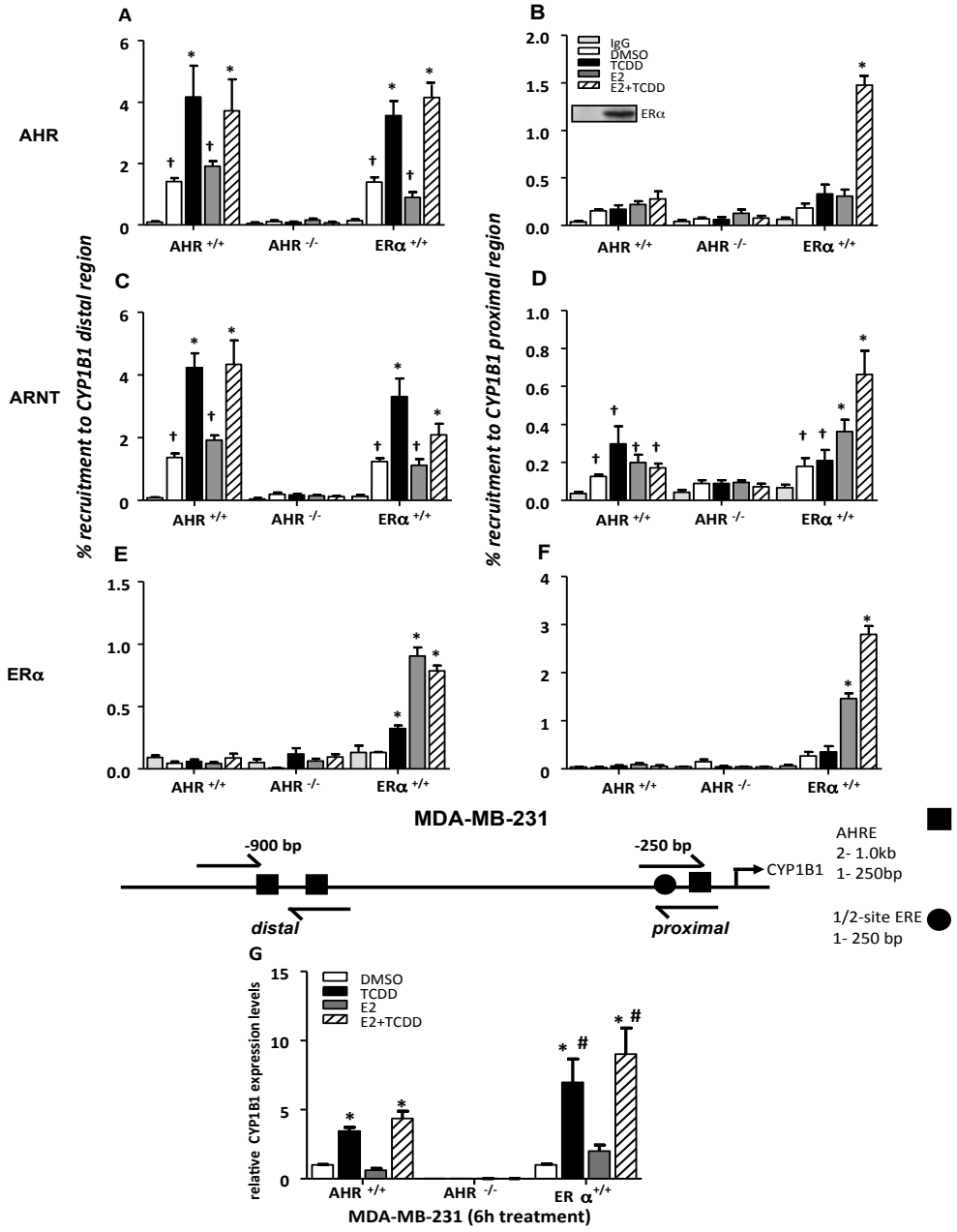



**Figure 36. Recruitment to the regulatory regions of *CYP1B1* in the MDA-MB-231 ERα negative breast cancer cell line.**

Quantification of AHR **(A, B),** ARNT **(C, D),** and ERα **(E, F)** recruitment to the distal and proximal regions of *CYP1B1* using the ChIP assay. Briefly, MDA AHR$^{+/+}$, MDA AHR$^{-/-}$, and MDA cells stably expressing ERα were treated with DMSO, 10 nM TCDD, 10 nM E2, or E2+TCDD for 45 mins and immunoprecipitated using antibodies against the proteins indicated. Each error bar represents the standard error of the mean of three independent replicates. All data is relative to 100% total input. Asterisks indicate statistically significant differences compared to DMSO control samples while † is compared to IgG control samples using a one-way ANOVA ($P$<0.05). (**G**) Expression analysis of CYP1B1 levels in MDA wildtype, AHR-null, and MDA cells stably expressing ERα. Briefly, cells were treated with ligand for 6 h and RNA was isolated and reversed transcribed. All data are normalized to 18s levels and DMSO. Asterisks indicate significantly different than DMSO while the pound sign is significantly different than wildtype using a one-way ANOVA ($P$<0.05)

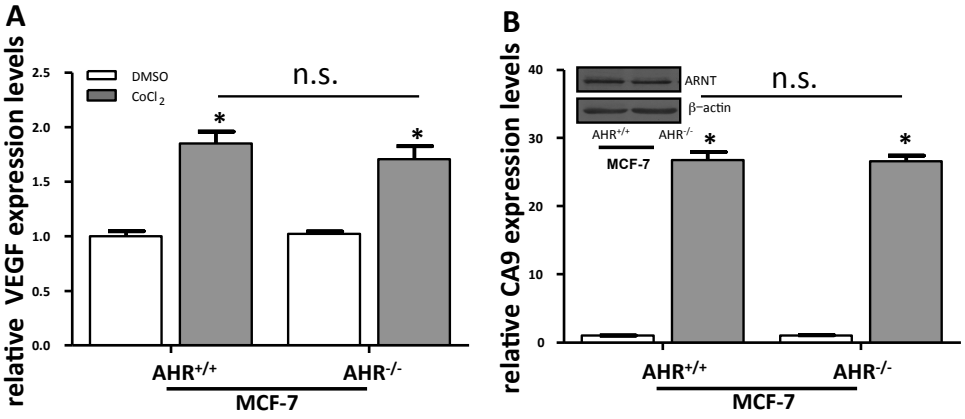



**Figure 37 Knockout of AHR does not affect ARNT signalling.**

Gene expression profiles were completed on MCF-7 $AHR^{+/+}$ and MCF-7 $AHR^{-/-}$ exposed to 100 μM $CoCl_2$ for 24 h. After treatment, RNA was isolated and reverse transcribed. Gene expression was then determined using Q-PCR. Data were normalized against DMSO and to ribosomal 18s levels. Each error bar represents the SEM of three independent replicates. Asterisk is compared to DMSO MCF-7 $AHR^{+/+}$ cells ($P<0.05$, one-way ANOVA). The target genes VEGF (**A**) and CA9 (**B**) were analyzed for ARNT function.

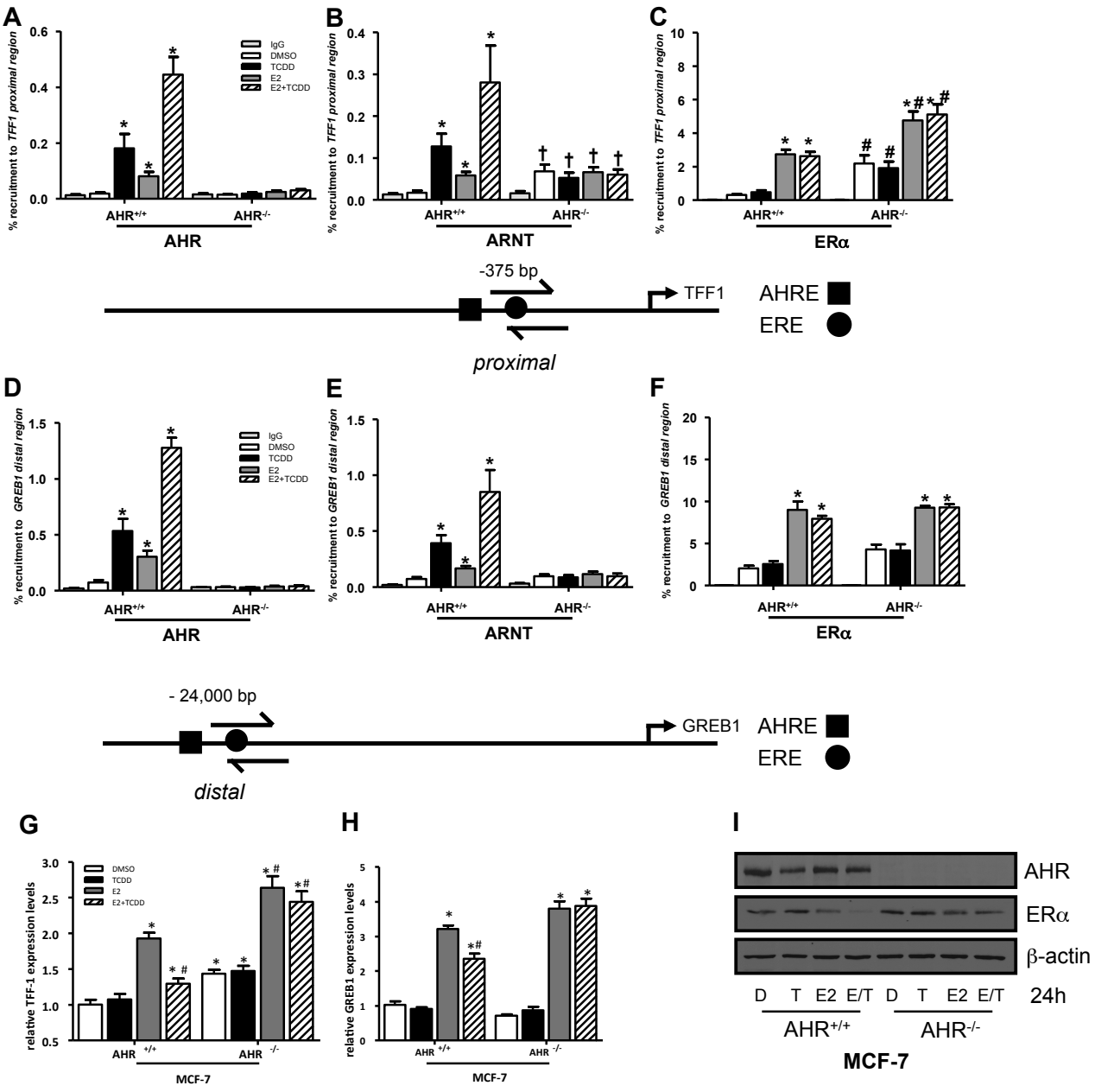



**Figure 38. Regulation of *TFF-1* and *GREB1* was affected by AHR knockout in MCF-7 cells.**

Quantification of AHR **(A, D),** ARNT **(B, E),** and ERα **(C, F)** recruitment to the regulatory region of *TFF1* and *GREB1* using the ChIP assay. Briefly, MCF-7 AHR$^{+/+}$ and MCF-7 AHR$^{-/-}$ were treated with DMSO, 10 nM TCDD, 10 nM E2, or E2+TCDD for 45 mins and immunoprecipitated using antibodies against the proteins indicated. The immunoprecipitated DNA was measured by Q-PCR with primers targeting *TFF1* and *GREB1* regulatory region. Each error bar represents the standard error of the mean of three independent replicates. All data is relative to 100% total input. Asterisks indicate statistically significant differences compared to DMSO control samples while † is compared to IgG control samples using a one-way ANOVA ($P$<0.05). **(G, H)** mRNA expression of TFF1 and GREB1 levels after 24 h treatment **(I)** Western blot analysis of AHR and ERα levels after 24 h treatment with DMSO, TCDD, E2, and E2+TCDD to determine ligand-dependent protein degradation in both wildtype and AHR knockout cells. β-actin was used as loading control.

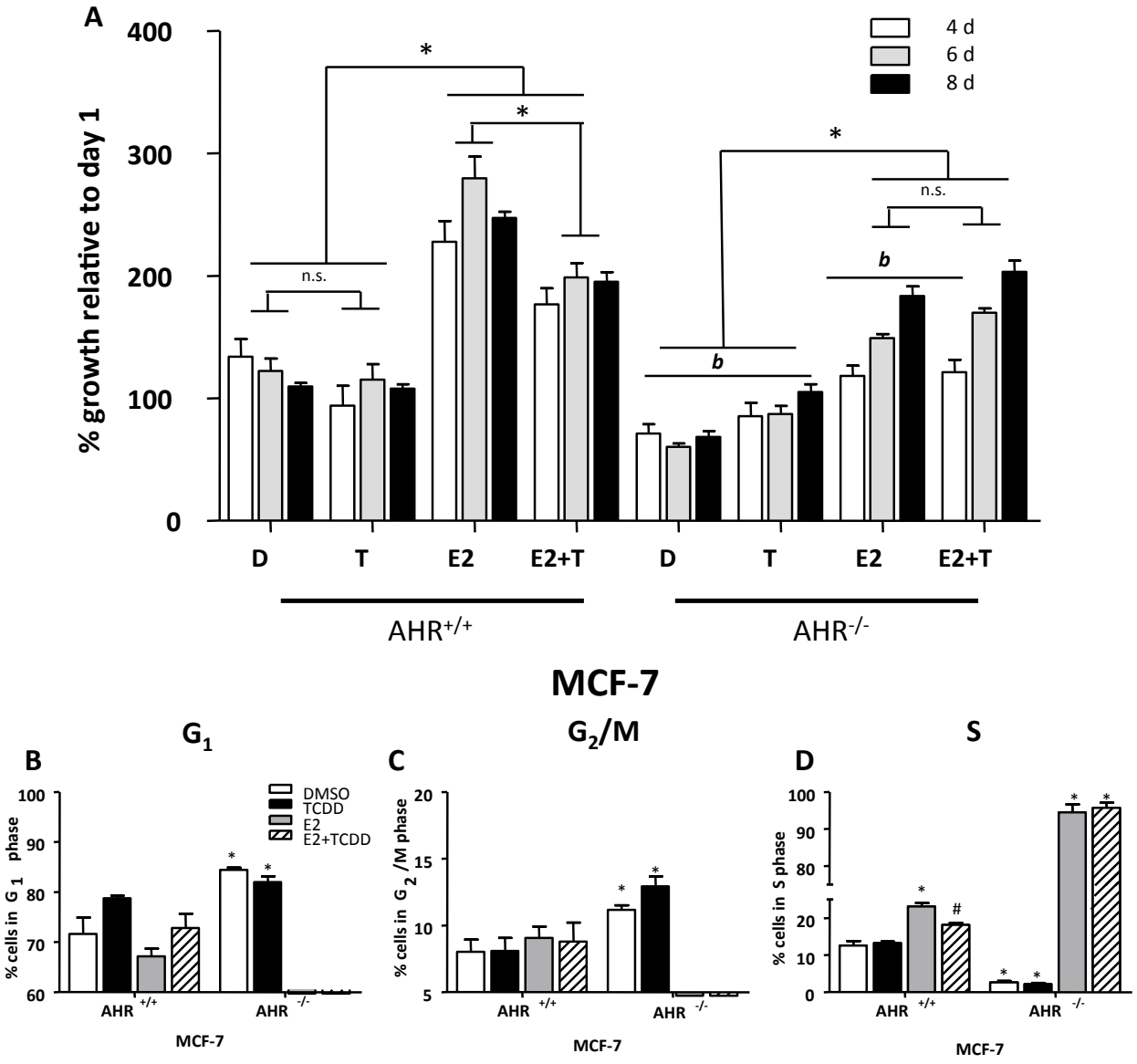



**Figure 39. Proliferation and cell cycle analysis of MCF-7 AHR$^{+/+}$/AHR$^{-/-}$ cells.**

(**A**) Cells were seeded in 96-well plates at 4,000 cells per well, and the media were replenished every 3 days with DCC-stripped serum. Cells were treated with DMSO, 10 nM TCDD, 10 nM E2, 10 nM E2+ TCDD for 4, 6 or 8 days. Proliferation was measured at the indicated times using the Sulforhodamine B assay. Data represent the mean of three independent replicates. Significance was determined using a one-way ANOVA ($P$<0.05). (**B**) Cell cycle analysis of cells exposed to DMSO, 10 nM TCDD, 10 nM E2, or 10 nM E2+TCDD for 48 h and harvested for FACS analysis. Cells were pulsed with 10 µg/ml of BrdU before being collected. For each treatment BrdU-PI bivariate plot with numbers corresponding to the percentage of cells in $G_1$, S, and $G_2$/M phases of the cell cycle were generated. Asterisks represent statistical significance compared to wildtype DMSO treatment cells, whereas the pound signs represent statistical significance compared to wildtype E2 treatment ($P$<0.05, one-way ANOVA).

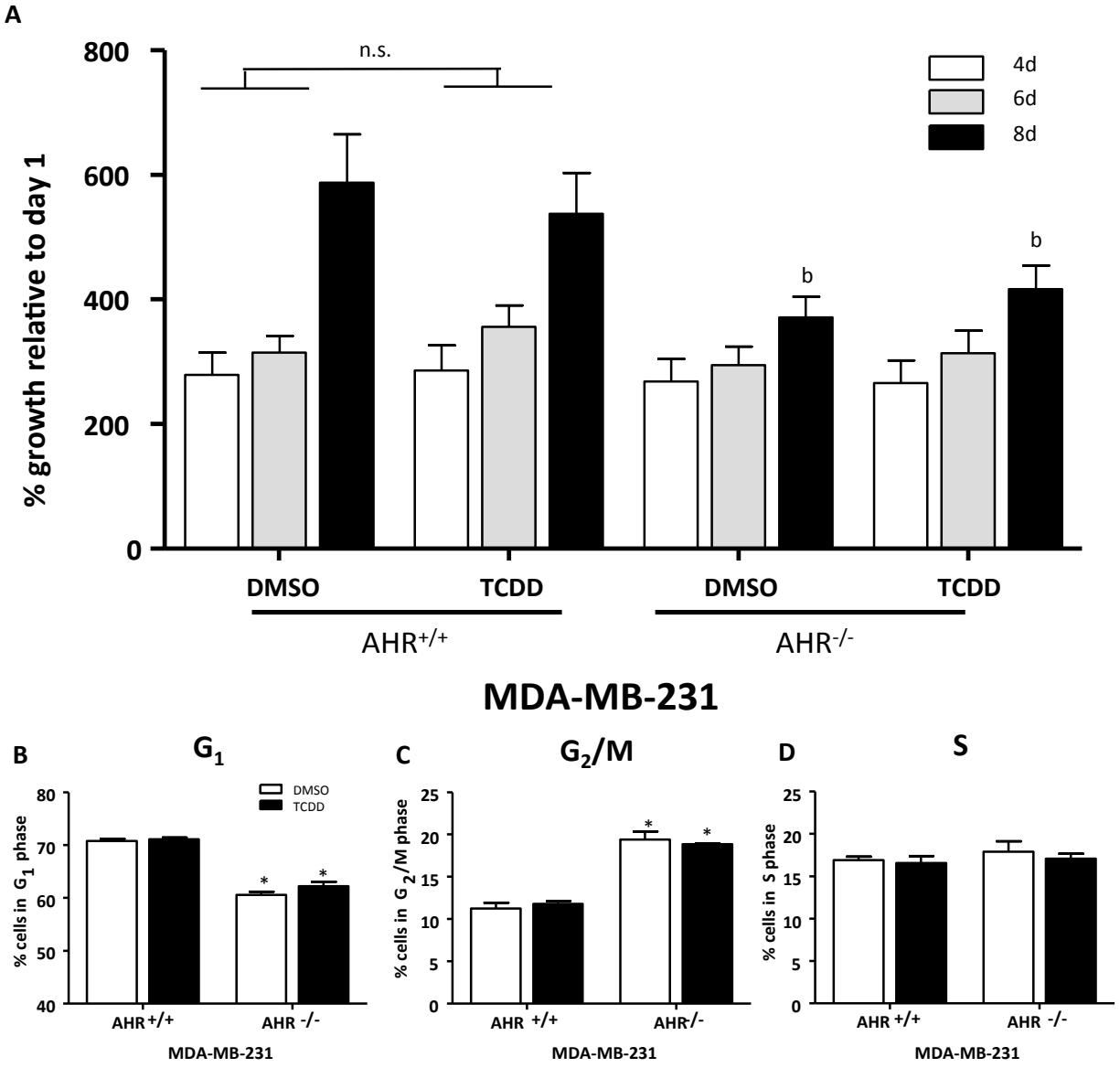



**Figure 40. Proliferation and cell cycle analysis of MDA AHR$^{+/+}$/AHR$^{-/-}$ cells**

(**A**) Cells were seeded in 96-well plates at 4,000 cells per well, and the media were replenished every 3 days with DCC-stripped serum. Cells were treated with DMSO, 10 nM TCDD for 4, 6 or 8 days. Proliferation was measured at the indicated times using the Sulforhodamine B assay. Data represent the mean of three independent replicates. Significance was determined using a one-way ANOVA. (**B**) Cell cycle analysis of cells exposed to DMSO, 10 nM TCDD for 48 h and harvested for FACS analysis. Cells were pulsed with 10 μg/ml of BrdU before being collected. For each treatment BrdU-PI bivariate plot with numbers corresponding to the percentage of cells in $G_1$, S, and $G_2$/M phases of the cell cycle were generated. Asterisks represent statistical significance compared to wildtype DMSO treatment cells ($P$<0.05, one-way ANOVA).

# Chapter 4: Discussion

## *12* TCDD-activated AHR recruits ERα to a subset of genomic regions

### 12.1 Recruitment pattern of AHR following TCDD treatment

The coupling of chromatin immunoprecipitation with DNA microarrays has allowed researchers to create high-resolution genome-wide maps of transcription factor and DNA-associated protein interactions with chromatin. The binding of several of these proteins in both human and mouse cell lines has been investigated (Ren et al., 2000; Bourdeau et al., 2004; Carroll et al., 2006; Lupien et al., 2008; Cao et al., 2011; Lanz et al.). However, there have been few studies that have used this methodology to characterize the binding profile of AHR (Sartor et al., 2009). Instead most of the research has focused on gene expression arrays to assess AHR signalling after ligand treatment (Frueh et al., 2001; Adachi et al., 2004; Guo et al., 2004; Ito et al., 2004; Karyala et al., 2004; Fong et al., 2005; Boverhof et al., 2006; Coe et al., 2006; Tijet et al., 2006; Boverhof et al., 2008). A limitation of expression array studies is that they cannot distinguish between genes that are directly regulated by AHR and those caused by downstream effects (Wu et al., 2006). To address this problem and to increase our understanding of the genomic binding profile of AHR we used genome-wide but promoter focused human tiling arrays to identify AHR binding sites in T-47D human breast cancer cells treated with TCDD. We were also interested in determining the impact of TCDD-activated AHR on ERα binding on a genome-wide level. Our analysis identified a number of novel TCDD-responsive genes that were directly regulated by AHR (**Figure 10, 11**).

AHR was recruited to genes whose expression was increased or decreased in response to TCDD (**Figure 11, Table 4**), which is consistent with AHR serving either as an activator or repressor of transcription in a context-specific manner (Okey, 2007). This may be attributed to the contents of the activated AHR complex and whether it is in complex with coactivators and corepressors (Hankinson, 1995; 2005; Beischlag et al., 2008). TCDD-bound AHR has been shown to interact with both types of coregulatory proteins in a gene-dependent manner (Nguyen et al., 1999; Rushing and Denison, 2002; Matthews et al., 2005; Beischlag et al., 2008; Powis et al., 2011). In a few cases, we observed AHR occupancy at genes that were not TCDD responsive (**Table 4**).

This implies that AHR binding was not the limiting factor for the regulation of these genes and suggests potential cell type–specific regulation. Similar findings have been reported for genome-wide glucocorticoid receptor binding in response to dexamethasone (So et al., 2007) and from a recent study examining TCDD-induced AHR binding in mouse Hepa1c1c7 cells (Kinehara et al., 2008). This could also be due to the time points studied (1.5, 3, 6, 24 h treatment; **Figure 11** and **Table 4**) in which some of these genes could have been up or down regulated but were not captured in our experiment. In addition, the determination of gene expression is also dependent on mRNA stability which could have also influenced our results (Cheadle et al., 2005).

Our laboratory has also performed ChIP-chip experiments in T-47D cells in response to 3MC treatment (Pansoy et al., 2010). A comparison of $AHR_{3MC}$-bound regions to $AHR_{TCDD}$-bound regions in my study revealed a 53% overlap between the data sets. This percentage was increased when the top 50 (100% overlap) and top 100 (87% overlap) $AHR_{3MC}$-bound regions were analyzed (Pansoy et al., 2010). We expected an overall higher degree of overlap between both data sets. The lower than anticipated percent overlap may be due to the methods utilized during the amplification of ChIP fragments as well as variations in hybridization. In my study we used a random hexamer linear DNA amplification technique while in the 3MC study whole genome DNA amplification was used (Ahmed et al., 2009; Pansoy et al., 2010). The use of two different amplification techniques could have introduced a bias in the number of regions detected by ChIP-chip as some genomic sequences may have been more easily amplified by the random hexamer method as opposed to the whole genome method. Moreover, the ChIP-chip experiments were completed at different times and therefore their hybridizations to the tiled probes may have been different.

Comparisons were also made to mouse tissue and cell lines. In a study completed by Sartor and colleagues (Sartor et al., 2009), they used Hepa1 mouse cells treated with B[*a*]P and TCDD to determine AHR-bound regions. There was a low degree of overlap between $AHR_{3MC}$ and $AHR_{TCDD}$ -bound regions completed in our laboratory using T-47D human breast cancer cells (Ahmed et al., 2009; Pansoy et al., 2010). This result may be attributed to cell type differences (liver hepatoma vs. breast carcinoma), species (mouse vs. human), and ligand differences (TCDD and 3MC vs. B[a]P). However, when we compared the identified $AHR_{B[a]P}$ and $AHR_{TCDD}$ regions (Sartor et al., 2009) to those determined in the same cell line (hepa1) but using high-throughput southwestern chemistry-based ELISA there was also very low overlap in the

identified AHR target genes (4 for B[*a*]P and 5 for TCDD) . The variations seen within the same cell line highlight that ChIP methodologies influence genomic binding sites and that more standardized methodologies are required to determine the AHR gene battery. Comparison of our results to the data set generated in mice treated with 30μg/kg of TCDD for 2h with ChIPs using liver tissue also showed a low degree of overlap (Lo et al., 2011). Of the 412 $AHR_{TCDD}$-bound sites in T-47D cells only 40 overlapped with the 1642 $AHR_{2h}$-bound sites in the mouse liver. Among the 40 were the classical AHR target genes such as the Phase I enzymes CYP1A1 and CYP1B1. This may be attributed to the differences in AHR signalling *in vivo* and *in vitro* as well as differential human and mouse regulation of AHR signalling (Dere et al., 2006; Flaveny et al., 2010).

## 12.2 Recruitment pattern of ERα following TCDD treatment

Our laboratory has shown that TCDD treatment induced the recruitment of ERα to the regulatory regions of *CYP1B1* and *CYP1A1* (Matthews et al., 2005). To elucidate whether this recruitment is a gene-selective event or if ERα is part of the activated AHR complex at all AHR target genes we completed ChIP-chip experiments after TCDD treatment and compared the genomic binding profile of AHR and ERα. Treatment with TCDD increased the overlapping genomic binding patterns of ERα and AHR, resulting in the identification of 110 regions or 27% of the $AHR_{TCDD}$ or 30% of the $ERα_{TCDD}$ regions (**Figure 9**). This result suggests that ERα is a gene specific modulator of AHR signalling. Sequential ChIPs (**Figure 10**) also confirmed that both factors were present at the same time in the subset of regions tested from the intersect group indicating a close relationship between the two factors. The large percentages are unlikely due to relaxed cutoffs, but they do indicate that co-binding of AHR and ERα was not absolute. For example, there were ERα binding events where AHR played no major part in ERα recruitment. At these regions, co-occupancy was achieved by TCDD-induced recruitment of AHR to regions already bound by ERα. This effect can be attributed to the culture conditions. All experiments were completed using 10% FBS (full serum) which contains growth factors as well as steroids such as estradiol which activate ERα. A comparison of the ERα-bound sites in the presence of full serum to those determined in MCF-7 cells treated with E2 revealed that about 30% of the binding sites overlapped (Carroll et al., 2006). This suggests that some of the ERα-bound sites determined from our study were due to the presence of estrogen in our plating medium. However, the overlap may have been affected by cell line specific differences (T-47D vs. MCF-

7), assay conditions, array platforms and data analysis strategies (Lo et al., 2010). In order to differentiate the TCDD-dependent and serum-dependent recruitment, ChIP-chip experiments should be repeated using reduced serum growth conditions. Experiments were not done under these conditions since reduced serum is not required to observe robust TCDD responses.

## 12.3 Significance of AHR and ERα co-occupancy

The molecular mechanism and physiological significance of the co-occupancy of AHR and ERα are unknown. TCDD does not directly bind to ERα (Matthews et al., 2007) nor does ERα interact with AHREs (Klinge et al., 1999). However, it has been observed that TCDD does activate ERE-driven luciferase reporter plasmids in the absence of AHR (Abdelrahim et al., 2006). We have previously suggested that the recruitment of ERα to AHR-regulated genes is a mechanism by which AHR inhibits ERα activity by diverting it away from estrogen target genes through facilitated recruitment to AHR target genes (Matthews et al., 2005). Alternatively, the recruitment of ERα to activated AHR might target ERα for AHR-mediated proteolytic degradation by an E3 ligase ubiquitination pathway and thus contribute to the well-documented inhibitory action of AHR on ERα activity (Safe and Wormke, 2003; Ohtake et al., 2007). However, expression-based microarray analysis has shown that AHR-mediated inhibition of estrogen-regulated genes occurs at some but not all estrogen-responsive genes (Boverhof et al., 2008). These findings coupled with the evidence presented here would argue against proteolytic degradation of ERα as the sole antiestrogenic activity of AHR since it would be expected to completely reduce estrogen activity but rather our results support a gene-specific inhibition of ER activity.

The presence of ERα within the activated AHR complex may also influence AHR signalling. ERα has been shown to directly interact with AHR via the AF1 and AF2 domains (Ohtake et al., 2003; Macpherson et al., 2009). Previous studies in our laboratory have shown that the AF2 domain of ERα was required for the TCDD-dependent recruitment of ERα to AHR targets, whereas the AF1 domain was important for mediating ERα-dependent enhancement of AHR target gene expression (Macpherson et al., 2009). The recruitment of ERα may influence the binding of other coregulatory proteins. A recent study has shown the co-occupancy of AHR and ERα affects coregulatory protein function (Madak-Erdogan and Katzenellenbogen, 2012). It has been suggested that the coactivator or corepressor function of RIP140 on AHR-mediated

transcription is related to the presence or absence of ERα at the regulatory region of target genes. They demonstrate that after TCDD treatment, RIP140 acts as a coactivator at regions occupied by AHR but not ERα, whereas at regions co-occupied by both AHR and ERα RIP140 acts as a corepressor (Madak-Erdogan and Katzenellenbogen, 2012). Interestingly, in the ERα knockdown study (**Figure 14**) we showed that recruitment of AHR was enhanced to the regulatory region of *CYP1B1* suggesting a gene-specific modulatory role for ERα in AHR signalling.

## 12.4 Transcription factor binding site analysis

TFBS analysis revealed that AHREs were enriched in the $AHR_{TCDD}$-isolated regions, although it was only present in ~30% of these regions using rather conservative thresholds in JASPAR. A study using ChIP combined with high-throughput southwestern chemistry-based enzyme-linked immunosorbent assay to identify TCDD-dependent AHR-bound sites in mouse hepatoma hepa-1c1c7 cells (Kinehara et al., 2008) isolated 77 sites with approximately half of them containing an AHRE. In addition, a study completed by our laboratory using whole genome promoter focused arrays in mouse liver also showed that AHREs were enriched in only 53% of regions after 2h TCDD treatment (Lo et al., 2011). Collectively, these findings demonstrate that TCDD-activated AHR binds to promoter regions that do not necessarily contain an AHRE. In support of this notion, a recent report demonstrates that AHR binds to a non-consensus AHRE in the regulatory region of the murine plasminogen activator-inhibitor 1 (Huang and Elferink, 2012). The occupancy of AHR at target promoters may also be mediated through tethering to other transcription factors and not necessarily through direct binding to DNA, which cannot be distinguished using the ChIP assay. AHR has been shown to tether to other transcription factors, such as the E2F, Sp1 and Rb protein (Ge and Elferink, 1998; Tsuchiya et al., 2003; Marlowe et al., 2004). Our analysis showed that Sp1 sites (**Figure 12**) were over-represented in the AHR data set suggesting that tethering may play a significant role in the recruitment of AHR after TCDD treatment in human breast cancer cells.

Alternatively, it is possible that AHRE- containing sequences were not present on our promoter-focused arrays. A recent study using whole genome tiling array analysis of AHR binding site in livers of TCDD-treated ovariectomized immature C57BL/6 mice revealed that only 32% of all AHR binding sites were situated 10 kb upstream from annotated genes (Dere et al., 2011).

Another ChIP-chip study using whole genome tiling arrays on murine lymphoma CH12.LX B-cells also showed that only 55% of their AHR-bound sites mapped to within 10kb of the transcriptional start site of target genes (De Abrew et al., 2010). These data show that the distribution of AHR binding extends beyond the coverage area of our promoter-focused array and that distal AHR-bound regions might also contribute to AHR mediated transcription. It may be that sites lacking AHREs have looped to distal AHRE containing sequences. Completion of the 3C chromosome assay will be important to determine if AHR uses long-range transcriptional mechanisms to regulate target gene expression as well as using whole-genome arrays.

## 12.5 AHR drives the recruitment of ERα in a gene-selective manner

TFBS analysis revealed that AHREs but not EREs were over-represented in the intersect group (**Figure 12**). This suggests that AHR is driving the recruitment of ERα. However, RNAi-mediated knockdown studies revealed that AHR was important for the recruitment of ERα to some but not all target genes (**Figure 14, 16**). It may be that binding of ERα occurred in an ERE-dependent manner to some of these regions. Alternatively, sites in which the recruitment of ERα was not dependent on AHR indicate that AHR was not the limiting factor in the activated ERα complex. However, there were genes in which AHR was required for the recruitment of ERα (**Figure 14**). These are CYP1B1, CCNG2, and ITPR1 which have also been determined to be estrogen target genes (Kirkwood et al., 1997; Tsuchiya et al., 2004b; Stossi et al., 2006). This effect supports the ability of AHR to regulate ERα signalling in a gene selective manner (Boverhof et al., 2008).

## 12.6 TCDD recruits AHR to ERα target genes

Our ChIP-chip study showed that AHR was recruited to ERα target genes (**Figure 9,10**). This recruitment was not limited to TCDD as similar effects were seen after 3MC treatment (Pansoy et al., 2010). The binding of AHR to ER-regulated genes is an important mechanism by which AHR inhibits estrogen- responsive gene expression (Wormke et al., 2003). It is thought to occur through direct competition for DNA binding with ERα at endogenous EREs, competition for DNA binding to GC-rich sites between AHR, AP-1 and/or Sp-1 transcription factors or AHR recruitment may interfere with the proper assembly of the pre-initiation complex (Gillesby et al., 1997; Klinge et al., 1999; Wang et al., 2001).

TCDD induced the recruitment of AHR to an AHRE located in the upstream regulatory region of ESR1 and caused a slight decrease in ERα mRNA levels, revealing ESR1 to be a direct AHR target gene (**Figure 11**). This result supports studies in rodents where TCDD treatment reduced ERα mRNA expression in the liver, ovary, and uterus of treated mice (Tian et al., 1998). AHR was also recruited to upstream regulatory regions of GREB1 and resulted in a slight reduction in GREB1 mRNA levels; however, an ERE but no AHREs were identified in this region (**Figure 11**). It is possible that AHR modulates ERα activity through distal regulatory regions of GREB1, which were not represented in our promoter-focused array. Genome-wide analysis of ERα-binding sites revealed that ERα regulates GREB1 through distal enhancer elements 100 kb upstream of the start site (Carroll et al., 2006). Recruitment of AHR to GREB1 but not ESR1 was dependent on ERα expression, suggesting that for some genomic sequences ERα influences the recruitment of AHR to those regions.

## 12.7 TCDD recruits ERα to AHR target genes

TCDD induced the recruitment of ERα to a subset of AHR target genes (**Figure 9**). ERα appeared to have a gene-specific modulatory role in AHR signalling since we observed that knockdown of ERα reduced the TCDD- dependent induction of CYP1B1 and CYP1A1, whereas the TCDD-dependent regulation of other AHR target genes were unaffected. Interestingly, this effect on CYP1B1 and CYP1A1 was not seen in MCF-7 transfected with siRNA against ERα (Wihlén et al., 2009). E2-dependent induction of CYP1A1 and CYP1B1 has also been observed (Frasor et al., 2004), and ERα has recently been shown to be an important factor in the elongation of RNA polymerase II at the CYP1B1 promoter (Kininis et al., 2007). In support of these findings, we observed reduced TCDD-mediated induction of CYP1B1 and CYP1A1 after siRNA-mediated knockdown of ERα. However, knockdown of AHR also significantly reduced recruitment of ERα to CYP1B1 in response to TCDD and AHR agonists strongly induce CYP1B1 expression in ERα-negative cell lines (Angus et al., 1999). Thus, the role of ERα in CYP1B1 expression is influenced by cell type, culture conditions, and AHR expression levels. However, knockdown of ERα did not affect the TCDD-dependent induction of target gene expression at the other loci examined. These findings suggest that interpretation of AHR-ERα crosstalk from the analysis of CYP1A1 and CYP1B1 regulation is not representative of all AHR-regulated genes. These data also show that recruitment of ERα to AHR-regulated target

genes does not necessarily equate to changes in AHR-mediated transcription.

# *13* AHR-dependent regulation of cyclin G2 requires FOXA1

## 13.1 Significance of TCDD-dependent CCNG2 upregulation

The regulatory region of *cyclin G2* was bound by both AHR and ERα in a TCDD-dependent manner in the initial ChIP-chip study (**Figure 10**). To our knowledge, we were the first to show CCNG2 is a direct genomic target of AHR. We decided to further characterize this gene because of the potential role it plays in proliferation and the AHR-mediated $G_1$ phase arrest. Most cyclins have been shown to facilitate growth by either promoting Go/$G_1$ to S phase or the $G_2$ to M phase transition. The G-type cyclins (cyclin G1, G2 and I) on the other hand are associated with cell cycle arrest (Bennin et al., 2002). Cyclin G1 is involved in $G_2$/M phase arrest, while cyclin I is thought to play a role in apoptosis (Griffin et al., 2006). Cyclin G2 has been shown to inhibit cell cycle progression by preventing $G_1$ to S phase transition (Horne et al., 1997; Bennin et al., 2002; Martinez-Gac et al., 2004). Transient transfection of CCNG2 into HEK 293 and CHO cells caused $G_1$ phase arrest but also the dysregulation of cellular division process leading to aberrant mitosis/cytokinesis (Bennin et al., 2002). Flow cytometry indicated that cyclin G2 over-expression accumulated cells in the $G_1$ phase, exhibited reduced CDK2 activity and DNA synthesis but maintained high levels of CDK4 activity (Bennin et al., 2002). These effects are consistent with mid-$G_1$ and $G_1$/S phase boundary arrest. Furthermore, CCNG2 was shown to interact with the protein phosphatase 2A (PP2A) C catalytic subunit (Bennin et al., 2002). It has been suggested that this interaction dephosphorylates Rb, a substrate of PP2A; sequestering E2F transcription factors thereby preventing progression out of the $G_1$ phase (Alberts et al., 1993; Bennin et al., 2002). Alternatively, the CCNG2-PP2A complex could also dephosphorylate CDK2 directly or indirectly through the activation of CDK2-activating phosphatase CDC25 leading to the $G_1$ phase arrest (Nilsson and Hoffmann, 2000). The presence of aberrant nuclei upon CCNG2 over-expression could also contribute to a $G_1$ phase arrest since aberrant cytokinesis has been shown to induce a $G_1$ phase arrest (Stewart et al., 1999; Andreassen et al., 2001; Bennin et al., 2002).

It is well documented that TCDD-dependent activation of AHR blocks cell cycle progression through the $G_1$ phase in several cell lines and under many different conditions, including mouse

hepatoma Hepa1c1c7 (Elferink et al., 2001), rat hepatoma 5L cells (Weiss et al., 1996), and in estrogen-induced MCF-7 cell proliferation (Wang et al., 1998). Potential mechanisms include the AHR-dependent induction of *p27*$^{Kip1}$ and *p21*$^{WAF1}$, inhibition of CDK function, reduced retinoblastoma phosphorylation, repression of E2F-regulated genes through interactions with Rb and displacement of the coactivator p300 (Puga et al., 2002a; Marlowe and Puga, 2005; Marlowe et al., 2008). AHR-dependent cell cycle arrest through p27$^{Kip1}$, a CDK inhibitor was reported to occur through increased mRNA expression in 5L rat hepatoma cells (Kolluri et al., 1999). The activation of p27$^{Kip1}$ then inhibits cyclin E-CDK2 or cyclin D-CDK4 complex consistent with preventing cell cycle progression at the $G_1$ phase. Similarly, p21$^{WAF1}$, another CDK inhibitor was shown to be upregulated inducing both a $G_1$ and $G_2$ phase arrest (Medema et al., 1998; Stewart et al., 1999; Ito et al., 2004). Unlike p27$^{Kip1}$, p21$^{WAF1}$ was not directly regulated by AHR (Ito et al., 2004). Instead, AHR was determined to upregulate GADD34 (Growth arrest and DNA damage-inducible protein 34) through an upstream AHRE which then phosphorylates p53 leading to enhanced p21$^{WAF1}$ expression (Ito et al., 2004). Another mechanism by which TCDD-activated AHR can cause cell cycle arrest is through its interaction with Rb (Ge and Elferink, 1998; Chan et al., 2001; Elferink et al., 2001). This interaction prevents the phosphorylation of Rb causing the inhibition of E2F-dependent genes leading to $G_1$ phase arrest. It also represses E2F-regulated genes by displacing the coactivator p300 at E2F target genes (Marlowe et al., 2004; Huang and Elferink, 2005). The TCDD-dependent increase in CCNG2 expression reported here provides another mechanism by which ligand activated AHR regulates cell cycle progression in the $G_1$ phase (Bennin et al., 2002). In support of our findings, it was previously shown that Jurkat T-cells stably expressing constitutively active AHR induced the expression of cyclin G2 and arrested in the $G_1$ phase (Ito et al., 2004). Furthermore, we report that the TCDD-dependent $G_1$ phase arrest was lost after knockdown of CCNG2 (**Figure 19**). This result highlights the importance of CCNG2 in mediating AHR-dependent $G_1$ phase arrest in breast cancer cells.

## 13.2 Role of FOXA1 in AHR signalling

FOXA1 has been implicated in ERα, GR and AR signalling (Gao et al., 2003; Yu et al., 2005; Lupien et al., 2008; Belikov et al., 2009; Hurtado et al., 2011). In ERα signalling, FOXA1 is responsible for almost all ER-chromatin interactions and gene expression changes by influencing genome-wide chromatin accessibility determined from RNAi-mediated FOXA1 knockdown studies followed by ChIP-seq (Hurtado et al., 2011). FOXA1 was determined to bind to histone

H3 lysine 4 dimethyl-rich sites, inducing an open chromatin state to facilitate ERα binding (Lupien and Brown, 2009). Although the RNAi-mediated knockdown of FOXA1 was reported to not affect ERα protein levels (Hurtado et al., 2011), we and others report that knockdown of FOXA1 decreases ERα mRNA expression and protein levels (Bernardo et al., 2010)(**Figure 25**). The reason for the discrepancies between the studies is unclear, but we observe that RNAi-mediated knockdown of FOXA1 reduces ERα protein levels in two different cell lines (T-47D and MCF-7, **Figure 25**) using two unique siRNA oligos targeting FOXA1. In support of our observations, FOXA1 binds to ten distinct regions in the regulatory region of *ESR1* suggesting that FOXA1 regulates ERα gene expression (Lupien et al., 2008). Using the chromatin immunoprecipitation assay, the binding of FOXA1 and RNA pol II was confirmed to one of the proximal regions in the absence of estradiol treatment (Bernardo et al., 2010). RNAi-mediated knockdown of FOXA1 reduced RNA pol II binding by 50% suggesting that FOXA1 directly regulates ERα expression which may explain our reduced protein levels (Bernardo et al., 2010).

In AR signalling FOXA1 is essential for prostate specific gene activation (Gao et al., 2003). Mutations to upstream forkhead recognition sites adjacent to AR response elements in two prostate specific genes (probasin and prostate-specific antigen) significantly reduced the maximal androgen induction of these genes (Gao et al., 2003). These effects were confirmed using the ChIP assay in LNCaP prostate cancer cells in which FOXA1 occupied the enhancer region of both genes (Gao et al., 2003). A physical interaction was also found between the DBD/hinge region of AR and the FKH domain of FOXA1 (Gao et al., 2003). In GR signalling, it was shown using the GR-regulated mouse mammary tumour virus (MMTV) promoter that FOXA1 binding creates an area of strongly remodeled chromatin structure adjacent to GR response elements enhancing GR binding and GR-dependent transcription (Belikov et al., 2009).

In our ChIP-chip study completed in T-47D cells (**Figure 12;**(Pansoy et al., 2010)) and mouse tissue (Lo et al., 2011) FKH sites are significantly enriched in AHR-bound regions supporting a possible role for forkhead proteins in AHR signalling. In line with these results, we show that FOXA1 is critical for the AHR-dependent induction of CCNG2 levels (**Figure 26**). We observe that FOXA1 is present at *CCNG2* in the absence of AHR activation consistent with its role as a pioneer factor (Lupien et al., 2008). Following AHR ligand treatment, the level of FOXA1, AHR, NCoA3 and H3K4Me2 were increased at *CCNG2* (**Figure 26, 27**), suggesting that FOXA1 primes the *CCNG2* for AHR recruitment and subsequent transcriptional activation. AHR

and FOXA1 interacted in Co-IP and re-chip experiments (**Figure 23**), demonstrating that they are part of the same multi-protein complex, which agrees with other reports showing that FOXA1 and FOXA2 interact with AR (Yu et al., 2005). Further studies are required to map the exact site of their interaction. FOXA1 may utilize both the direct interaction with AHR and altered chromatin structure to enhance AHR binding to *CCNG2*. We hypothesize that FOXA1 stabilizes the AHR activated complex at CCNG2 and therefore is required for maximal gene activation by AHR. Our results highlight that AHR acts like members of the nuclear receptor superfamily by requiring FOXA1 for AHR-dependent gene expression.

## 13.3 Role of Forkhead proteins in CCNG2 regulation

The regulation of CCNG2 by other members of the forkhead protein family has been previously reported. One group demonstrated that FoxO transcription factors increased CCNG2 expression in NIH 3T3 mouse embryonic fibroblasts (Martinez-Gac et al., 2004). They showed that the kinetics of CCNG2 expression resembled those of FoxO transcription factors, expression of an active FoxO increased CCNG2 mRNA levels, the CCNG2 mouse promoter contained forkhead binding sites which were bound by FoxO using the ChIP assay (Martinez-Gac et al., 2004). Recently, Nodal, a member of the transforming growth factor-β family was found to increase CCNG2 mRNA expression by increasing the expression of FOXO3a, which then forms a complex with Smad proteins at the *CCNG2* promoter region (Fu and Peng, 2011). They found that the more proximal FKH sites (FKH1 and FKH2, **Figure 20**) were required for FOXO3a-mediated induction of CCNG2, rather than the distal FKH sites (FKH3 and FKH4, **Figure 24**) that are required for AHR-dependent induction of CCNG2 reported here. Interestingly, the anti-proliferative effect of Nodal on ovarian cells was found to be partly mediated by CCNG2 (Xu et al., 2008). These findings demonstrate the important role of the forkhead protein family in the regulation of CCNG2, but reveal that the regulation of CCNG2 by FOXA1 or FOXO3a occurs via distinct FKH sites.

## 13.4 Gene-selective inhibition of ERα signalling

Results from my ChIP-chip study and those completed by others indicate that AHR affects ERα signalling in a gene-dependent manner (Astroff et al., 1990; 1991; Harper et al., 1994; Zacharewski et al., 1994; Krishnan et al., 1995; Lu et al., 1996; Wang et al., 2001; Boverhof et al., 2008; Ahmed et al., 2009). One function of ERα in mammary cells is to promote cell

proliferation in an estrogen dependent manner. This is facilitated by the binding of estrogen to ERα resulting in either increased expression of genes associated with proliferation or suppression of genes that block cell cycle progression. ERα has been previously shown to regulate CCNG2 in an estrogen dependent manner (Stossi et al., 2006; 2009). ERα represses CCNG2 expression in response to estrogen by recruiting a complex containing nuclear co-repressor (NCoR) and histone deacetylases to the *CCNG2* promoter region resulting in the displacement of RNA polymerase II (Stossi et al., 2006). Our experiments indicate that TCDD-activated AHR can overcome the repressive actions of ERα on *CCNG2* through the recruitment of NCoA3 and RNA pol II to mediate gene expression (**Figure 28**). The actions of AHR do not appear to be mediated by the blocking of ERα binding to EREs as TFBS analysis revealed that there were no EREs in close proximity to the active AHRE (**Figure 20**). Stossi et al., also suggested that the ERα-mediated repression of CCNG2 was facilitated by Sp1 factors which bind to GC-rich sites (Stossi et al., 2006). It may be that AHR binding (AHRE is also GC-rich) blocks Sp1 binding thereby indirectly inhibiting ERα recruitment. Further analysis of the reported repressive sequence bound by ERα (Stossi et al., 2006) indicates that the region bound by ERα was intronic (using Genome Browser). We were unable to detect ERα recruitment to the same region examined by Stossi et al., (2006). The reason for this is unclear and may have to do with cell-line specific differences as our studies were completed using T-47D cells while their studies were done in MCF-7 cells. Overall, our study demonstrates that the activation of AHR prevented ERα-dependent repression of CCNG2 providing another example where activation of the AHR pathway opposes the actions of ERα.

## 13.5 Implications for cell cycle progression and breast cancer

AHR has emerged as an important therapeutic target for breast cancer, since its activation has been reported to inhibit the growth of ER positive, ER negative and HER2 positive breast cancer cells (Wang et al., 1997; Safe et al., 1999; 2000; Zhang et al., 2009). In our study we report that ligand-activated AHR together with FOXA1 increases the expression of CCNG2 in ERα positive T-47D breast cancer cells. Our findings provide a new mechanism by which AHR can inhibit human breast cancer cell proliferation (**Figure 19**). The increase in CCNG2 expression by AHR further supports the notion that targeting AHR might be an effective therapy for breast cancer treatment (Safe et al., 1999; Reviewed in: Safe and McDougal, 2002). Although the clinical

importance of AHR-dependent activation of CCNG2 remains to be investigated, CCNG2 is upregulated in HER2 positive breast cells in response to the anti-HER2 antibody trastuzumab in a dose dependent manner (Le et al., 2007). CCNG2 levels are increased by trastuzumab in HER2 positive breast cancer cells (Le et al., 2005) which was validated in multiple HER2 positive breast cancer cell lines (Le et al., 2007). Le and colleagues also assessed the impact of CCNG2 expression on trastuzumab-dependent growth inhibition. Using RNAi-mediated knockdown of CCNG2 they showed that suppression of CCNG2 mRNA only modestly decreased trastuzumab-dependent growth inhibition suggesting that CCNG2 upregulation was not the limiting factor. However, there may have been compensatory mechanisms activated to counteract the lack of CCNG2 expression. This effect is in contrast to our results which showed CCNG2 is required to mediate the TCDD-dependent cell cycle arrest. Nonetheless, these findings suggest that modulating CCNG2 expression might be an important mechanism to inhibit cancer cell growth.

# *14* AHR knockout in MCF-7 and MDA-MB-231 affects ERα signalling, proliferation, and depletes CYP1B1 expression levels.

## 14.1 Zinc finger nucleases in molecular biology

Loss-of-function models are invaluable tools to assess the physiological significance of genes. The most widely used technique is RNAi-mediated gene knockdown but it is associated with multiple limitations including; incomplete knockdown, potential for off-target effects, and it is not permanent (Jackson and Linsley, 2010). Zinc finger nucleases overcome these limitations, since they allow for genetic mutations in immortalized cells resulting in gene knockout. My study is the first to use zinc finger nucleases for the targeted disruption of AHR in human breast cancer cells. Its ability to abolish AHR-mediated signalling was shown to be more efficient than RNAi-based methods (**Figure 33**). In Aim 1 of my thesis I used RNAi targeting AHR and although I observed reduced AHR protein levels, TCDD treatment still induced AHR target gene expression (Ahmed et al., 2009). RNAi-methods have also been used to generate stable cell lines to circumvent the transient nature of siRNA. I have employed this methodology and demonstrated that it still induces a functional AHR response (**Figure 30**). There are also two stable cell lines that have been created that are deficient in AHR function using non-RNAi based methods. These were generated by exposing either human MCF-7 breast cancer cells or the

murine Hepa1c1c7 hepatoma cells to low levels of benzo[*a*]pyrene for 6-9 months (MCF-7 $AH^{R100}$ and Hepa1 c12, and c19). However, it is unknown what prolonged exposure to B[*a*]P will do to other cellular functions. Another concern with RNAi-mediated knockdown is the potential for off-target effects (Qiu et al., 2005). Off-target effects are seen when the specificity of the RNAi sequence is low and causes the knockdown of random mRNA transcripts (Qiu et al., 2005). This compromises the experiment by creating confounding variables since we are unable to determine if the phenotypes seen are due to the knockdown of our protein of interest or other genes. Zinc finger nucleases have a much lower potential for off-target effects than RNAi sequences (Miller et al., 2007; Gutschner et al., 2011). However, if zinc finger domains are not specific enough, off-target cleavage may occur. This may lead to the production of double strand breaks, overwhelming the repair machinery and leading to chromosomal rearrangement or even cell death (Durai et al., 2005). Overall, the use of ZFNs to target AHR is a powerful tool to assess the role of AHR in ERα signalling and cell cycle control.

## 14.2 Role of AHR in ERα signalling

It was previously determined that AHR was recruited to estrogen target genes (**Figure 9;** (Ahmed et al., 2009)). However, the significance of this recruitment was not assessed in that study. To address this, I used MCF-7 cells that endogenously express both AHR and ERα as well as zinc finger-mediated *AHR*$^{-/-}$ MCF-7 cells to determine AHR function in ERα signalling. We focused on the genes *CYP1B1*, *TFF1*, and *GREB1*.

CYP1B1 is of interest since recent findings suggest it is a key enzyme involved in the metabolism of estrogen and is highly expressed in estrogen-related tissues such as the mammary, uterus, and ovary, indicating that it may be important in the localized control of estrogen levels (Hayes et al., 1996; Shimada et al., 1996; Hakkola et al., 1997). Metabolism of estrogen by CYP1B1 also leads to decreased estrogenic activity; however, the genotoxic 4-hydroxyestradiol metabolite which can undergo redox cycling inducing cellular damage is also produced (Han and Liehr, 1994). Interestingly, estrogen has been reported to induce the expression of CYP1B1 in MCF-7 cells mediated by the direct interaction of ERα with a half-site ERE on the *CYP1B1* gene (Tsuchiya et al., 2004b). Although CYP1B1 is a well-established AHR target gene, we have provided some evidence that ERα may play a role in mediating the AHR-dependent regulation (Ahmed et al., 2009). In this Aim I wanted to further characterize the role of AHR and ERα in

the regulation of *CYP1B1*. My results show that AHR-status was important for the recruitment of ERα to *CYP1B1* proximal and distal regions under all treatment conditions (**Figure 35**). These results are in contrast to a previous report which showed that mutations of AHREs known to be important for AHR-dependent regulation of CYP1B1 did not abolish E2-responsiveness of CYP1B1 reporter gene constructs (Tsuchiya et al., 2004). The discrepancies may be due to the fact that ERα recruitment in our study required chromatin remodeling which is not required for reporter genes. However, another report demonstrated that the E2-induced CYP1B1 expression requires AHR (Spink et al., 2003). They determined that elevated AHR levels elicits the E2-dependent increases in CYP1B1 expression. In that study AHR expression was significantly increased in response to estrogen stimulation and they speculated that this increase in AHR levels was responsible for the E2-dependent CYP1B1 regulation (Spink et al., 2003).

Another mechanism by which AHR may regulate ERα recruitment to and expression of CYP1B1 is through chromatin looping. AHR binding to distal AHREs might facilitate the binding of ERα to the proximal region. This may explain why the absence of AHR abolishes ERα recruitment to the proximal and distal *CYP1B1* regulatory regions. AHR may also cooperate with other transcription factors to regulate ERα recruitment. For *CYP1B1*, two Sp1 binding sequences have been reported near the putative half site ERE (Tsuchiya et al., 2004). AHR might facilitate the binding of Sp1 factors which then enhances ERα binding. Further studies will be important to clarify the role Sp1 factors play in the regulation of *CYP1B1*.

From the ChIP-chip study we showed that ERα was important in the TCDD-dependent increase in CYP1B1 expression levels (Ahmed et al., 2009). We attributed this finding to the role ERα plays in RNA pol II elongation, particularly at *CYP1B1* (Kininis et al., 2007). Our ChIP data in the MDA cells also confirm the role ERα plays in the AHR-dependent regulation of CYP1B1 (**Figure 36**). In the absence of ERα, recruitment of AHR to the proximal region was minimal but was significantly enhanced in the MDA cells stably expressing ERα. This suggests that ERα also affects AHR recruitment. In support of these findings, we also saw increased CYP1B1 expression in the MDA cells stably expressing ERα, which is in agreement with a previous report (Thomsen et al., 1994). In that report, transient transfection of *CYP1A1* reporter gene construct in the MDA cells did not induce TCDD-dependent CAT activity; however, in cells co-transfected with human ERα expression plasmid, TCDD was able to induce CAT activity

(Thomsen et al., 1994). Vickers and co-workers have also shown that expression of CYP1A1 correlates with ERα content in breast cancer cells (Vickers et al., 1989). Our study is the first to show the importance ERα plays at the *CYP1B1* regulatory region.

Although we observe E2-dependent recruitment of ERα to *CYP1B1* we did not see changes in mRNA levels in MCF-7 cells (**Figure 35**). This is in contrast to an earlier study which demonstrated that E2 causes a modest increase in CYP1B1 after 12h estrogen treatment (Tsuchiya et al., 2004). The discrepancies in our results may be attributed to the steroid deprivation protocol, which might not be sensitive enough to observe small changes in CYP1B1 levels. We used 5% DCC stripped fetal calf serum and incubated cells for 72h prior to estrogen treatment, whereas Tsuchiya et al. used 10% DCC stripped fetal bovine serum and incubated cells for 48h (Tsuchiya et al., 2004).

The estrogen-regulated gene *TFF-1* stimulates the migration of human breast cancer cells (Prest et al., 2002). Hormone therapies used to treat breast cancer have been shown to inhibit *TFF-1* (May and Westley, 1987; Johnson et al., 1989). Similarly, TCDD has also been shown to inhibit TFF-1 expression levels (Zacharewski et al., 1994; Gillesby et al., 1997; Labrecque et al., 2012). Zacharewski and colleagues first showed that TCDD inhibited E2-induced TFF-1 reporter gene constructs as well as protein levels (Zacharewski et al., 1994). Promoter analysis identified an AHRE approximately 100 base pairs upstream from an imperfect palindromic ERE required for E2-dependent regulation of TFF-1 (Gillesby et al., 1997). Using gel mobility shift assays it was shown that E2-responsiveness was dependent on interactions between ERα at the ERE and AP-1 factors at the upstream AP-1 site that overlapped with the AHRE. It was determined that the mechanism of AHR-dependent inhibition is due to AHR competing with AP-1 factors for binding leading to reduced responsiveness (Gillesby et al., 1997). Our results confirm the AHR-dependent inhibition (**Figure 37**). Interestingly, it appears that AHR is also involved in the constitutive regulation of *TFF-1*. In the *AHR*-null cells constitutive as well as E2-dependent ERα binding is significantly increased (**Figure 37**). The mechanism by which this occurs is not clearly understood but may be due to enhanced AP-1-ERα interactions. These data also suggest that AHR inhibits ERα signalling in the absence of exogenous ligand treatment. Western blot analysis confirms that this effect is not due to enhanced ERα protein levels, suggesting a

transcriptional mechanism of inhibition. Further studies are required to understand the basal regulation of *TFF-1*.

Unlike TFF-1, GREB1, a gene suggested to be involved in ERα-dependent proliferation (Rae et al., 2005), did not show enhanced basal binding of ERα but did exhibit increased E2-dependent recruitment to and expression of *GREB1* in the *AHR*$^{-/-}$ cells (**Figure 37**). The differences might be due to the location of AHRE. In the *TFF-1*, the active AHRE is proximal to the TSS and more likely to affect the pre-initiation complex while the GREB1 regulatory region was located distal to the TSS (approximately 24kb upstream from the start site). Analysis of ERα binding to the GREB1 regulatory region has demonstrated that there are multiple sites of binding spanning over 20kb (Deschênes et al., 2007). It may be that we did not capture an increased basal binding at the ERE we investigated. However, our findings are in agreement with previous siRNA-mediated knockdown of AHR studies showing that reduced AHR levels failed to affect the basal ERα binding at another GREB1 regulatory region isolated from our ChIP-chip study (Ahmed et al., 2009). Overall, our data indicate that AHR inhibits ERα signalling at TFF-1 and GREB1 at the transcriptional level. Further studies are required to investigate the role of AHR at other target genes. Microarray analysis is currently underway to further investigate the global effect of AHR loss on ERα transactivation.

## 14.3 Role of ARNT in ERα signalling

ARNT has been reported to be a potent coactivator of ERα signalling (Brunnberg et al., 2003; Labrecque et al., 2012). My study is the first to completely remove AHR allowing us to study the role of ARNT independent of AHR in human breast cancer cells. In the absence of AHR, ARNT is no longer recruited in a ligand-dependent manner but there is a significant increase in the constitutive binding of ARNT (**Figure 37**). Because of this increase in constitutive binding of ARNT we cannot conclusively say that ARNT does not play a role in ERα signalling. Furthermore, since the constitutive binding of both ARNT and ERα increased in the absence of AHR their interaction may contribute to the enhanced gene expression of TFF-1.

## 14.4 Significance of AHR-mediated CYP1B1 depletion

CYP1B1 is constitutively expressed in the mammary gland (Shimada et al., 1996). CYP1B1 mediates the metabolism of PAHs, aryl amines, and the metabolism of estrogen (Shimada et al.,

1996; Tsuchiya et al., 2005; Belous et al., 2007). CYP1B1 has received attention in the breast cancer field since it is overexpressed in tumours, metabolizes estrogen through hydroxylation at the C-4 position, and generates the genotoxic 4OH-E2 metabolite (Spink et al., 1998; Trombino et al., 2000; Belous et al., 2007). Association studies have also shown that polymorphisms in the CYP1B1 are associated with increased breast cancer risk (Watanabe et al., 2000). Constitutive CYP1B1 regulation has been linked to AHR activation where studies have demonstrated that AHR binding to enhancer AHREs increases CYP1B1 levels (Shehin et al., 2000; Tsuchiya et al., 2003; Roblin et al., 2004; Yang et al., 2008). Furthermore, AHR was shown to maintain high levels of CYP1B1 in human mammary epithelial cells which was inhibited after overexpression of AHRR or treatment with siAHR (Yang et al., 2008). Our results support these findings, where MCF-7 $AHR^{-/-}$ and MDA $AHR^{-/-}$ cells had greatly reduced CYP1B1 expression levels that are restored by transient over-expression of AHR (**Figure 34**). I also show that restoration of CYP1B1 expression required binding of AHR to *CYP1B1*. The significance of CYP1B1 in tumourigenesis was not assessed in our study, but the loss of constitutive expression may reduce the generation of mutagenic metabolites from both exogenous and endogenous substrates. It also suggests that AHR might act as a tumour promoter in breast cells through its ability to express CYP1B1.

## 14.5 Loss of AHR causes $G_1$ and $G_2$/M phase accumulation

The AHR has been implicated in cell cycle control. In the absence of exogenous ligand treatment, the presence of AHR facilitates cell cycle progression (Barhoover et al., 2010). This has been established in mouse hepa1 variants deficient in AHR (Ma and Whitlock, 1996), AHR-defective rat hepatoma BP8 (Weiss et al., 1996), and in HepG2 cells transiently transfected with siAHR (Abdelrahim et al., 2003). In all of these cases, cell lines lacking AHR had decreased proliferation indicating a growth-promoting role for AHR. Our results are in agreement in that both *AHR*-null MDA and MCF-7 cell lines showed decreased proliferation (**Figure 39 and 40**). Interestingly, MCF-7 cells that had AHR expression reduced by RNA interference showed enhanced $G_1$ to S phase transition indicating a growth-inhibitory role for AHR (Abdelrahim et al., 2003). The differences seen between our studies may be attributed to the serum conditions used to synchronize the cells, since my study used a 5% DCC stripped serum while their studies used serum-free conditions. They also did not validate their results with a second independent siRNA duplex to confirm their results were not due to off-target effects (Jackson and Linsley,

2010). The growth inhibitory effect of AHR was also seen in MCF7 $AH^{R100}$ cells which showed enhanced proliferation rates in the absence of AHR upon E2 stimulation (Spink et al., 2012). However, this cell line was exposed to B[*a*]P for extended periods of time which could have led to the dysregulation of the cell cycle independent of AHR.

Although both breast cancer cell lines showed decreased proliferation in the absence of AHR, the cell cycle phases affected were different between the two. The MCF-7 $AHR^{-/-}$ cells displayed a higher percentage of cells in both the $G_1$ phase and $G_2$/M (**Figure 39**). In contrast, the MDA $AHR^{-/-}$ cells had a lower percentage of cells in the $G_1$ phase while a much higher percentage in the $G_2$/M (**Figure 40**). A previous report also showed a decrease in $G_1$ with a concomitant increase in the $G_2$ in another MDA cell line MDA-MB-468 after RNAi-mediated AHR knockdown (Zhang et al., 2009). An increase in $G_1$ and $G_2$/M has been previously reported in $AHR^{-/-}$ mouse embryonic fibroblasts (Ma and Whitlock, 1996; Elizondo et al., 2000). This can be attributed to the interactions AHR has with the retinoblastoma protein as well as its effects on CDKs important for the $G_1$ to S and $G_2$ to M phase transitions (Ge and Elferink, 1998; Elizondo et al., 2000; Puga et al., 2000; Elferink et al., 2001; Barhoover et al., 2010). In the absence of an exogenous ligand, AHR is in complex with CDK4 and cyclin D1 serving as a scaffolding protein to bring the complex to Rb proteins leading to its phosphorylation, expression of E2F target genes, and $G_1$ to S phase transition (Barhoover et al., 2010). However, when AHR is removed, it no longer facilitates CDK4/CCND1 interaction with Rb leading to a hypophosphorylated state and $G_1$ phase arrest. Our results in the MCF-7 support this finding where we observe an accumulation of cells in the $G_1$ phase. The loss of AHR has also been associated with an accumulation of cells in the $G_2$/M phase (Elizondo et al., 2000). Using mouse embryonic fibroblasts derived from *Ahr*-null mice it was shown that AHR indirectly regulates the expression of two mitotic kinases CDK1 and Plk involved in $G_2$ to M phase transition. *Ahr*-null MEFs showed lower transcript and protein levels of both CDK1 and Plk but they were unable to show that they were direct targets of AHR (Elizondo et al., 2000). They attributed the down regulation of CDK1 and Plk to increased levels of latent and active TGF-β (Elizondo et al., 2000). TGF-β has been associated with diminished cell proliferation and elevated apoptosis (Jürgensmeier et al., 1994) and previous reports have shown that AHR can regulate TGF-β levels (Zaher et al., 1998; Elizondo et al., 2000; Santiago-Josefat et al., 2004). Overall, our results

suggest that AHR has a growth promoting role in both ERα negative and positive breast cancer cells but further studies in the both cell lines are required to determine the mechanisms utilized.

## 14.6 Loss of AHR affects E2-dependent cell proliferation

The proliferation of normal breast cells is dependent on estrogen (Laidlaw et al., 1995). The growth of luminal MCF-7 breast cancer cells mimics normal breast tissue requiring E2 to proliferate while the growth of the basal MDA-MB-231 breast cancer cells are E2-independent (Wiese et al., 1992; Mur et al., 1998). This characteristic was confirmed in our proliferation and FACS analysis where estrogen treatment induced both the growth and S phase entry of MCF-7 cells (**Figure 39**). Interestingly, the loss of AHR reduces the E2-dependent growth of MCF-7 cells and causes an accumulation in the S phase upon E2 treatment (**Figure 39**). In breast cancer cells, estrogen treatment through the actions of ERα upregulates cyclin D1, activates the cyclin E-CDK2 and cyclin D-CDK4/6 complexes increasing Rb phosphorylation, modulates the CDK inhibitor p21; all leading to $G_1$ to S phase transition (Foster and Wimalasena, 1996; Cicatiello et al., 2004). The mechanism of S phase accumulation in the MCF-7 *AHR*$^{-/-}$ cells is currently unknown. However, a recent report has shown that in human T-47D breast cancer cells exposed to extreme hypoxia a permanent S-phase arrest was initiated, which they attributed to reduced cyclin A levels. This mechanism may be utilized in the MCF-7 *AHR*$^{-/-}$ cells. Preliminary gene expression array analysis indicated that the expression of cyclin A2 was lower in *AHR*-null cells when compared to wildtype MCF-7 cells (unpublished findings). The ability of E2 to induce S phase entry indicates that the genes affected are related to S phase transition and not $G_1$ to S phase transition. A report has shown that cyclin A-CDK2 inactivation of E2F-1 binding activity is associated with orderly progression along the S phase and entrance into the $G_2$/M phase (Krek et al., 1995). Furthermore, inhibition of cyclin A expression or interaction with CDK2 leading to reduced phosphorylation of E2F has been associated with S-phase delay and subsequent apoptosis (Shan and Lee, 1994). It may be that AHR regulates cyclin A levels or interacts with cyclin A-CDK2 complexes important for the S phase transition. Further studies are required to show the importance of AHR regulation of cyclin A levels and E2F/Rb activities in the *AHR*-null MCF-7 cells.

## 14.7 Implications of AHR activation on ERα negative and positive breast cancer cell lines

AHR activators have been shown to inhibit the growth of both ERα positive and negative breast cancer cells (Safe et al., 1999; Safe and McDougal, 2002; Abdelrahim et al., 2003; Zhang et al., 2009; Hall et al., 2010). TCDD treatment decreases the E2-dependent proliferation and S phase progression of MCF-7 breast cancer cells (Abdelrahim et al., 2003). Our results confirm these effects and we show that they are AHR-dependent (**Figure 39**). In MDA-MB-231 cells, TCDD treatment protected against breast cancer cell invasiveness while in another study TCDD along with other AHR activator treatment was shown to inhibit the growth of multiple ERα negative cell lines (Zhang et al., 2009; Hall et al., 2010). In contrast, I show that TCDD treatment does not affect the proliferation or cell cycle progression of MDA-MB-231 cells (**Figure 40**). The reason for these discrepancies may be related to culture conditions and treatment length. Their study was completed using 2.5% DCC-stripped serum and were treated with TCDD for 6 days (Zhang et al., 2009). Our results were completed in 5% DCC-stripped serum. Although our proliferation data were analyzed at 6 days, our cell cycle analysis was completed after 48h treatment. It may be that longer treatment periods are required to see TCDD-dependent effects. Our results support the notion that AHR activation inhibits the growth of estrogen-dependent breast cancer cells. Investigating the role of AHR using other endpoints and increasing the treatment time may help clarify its role in ERα negative breast cancer.

# *15* Limitations and Recommendations

## 15.1 Aim 1: TCDD-activated AHR recruits ERα to a subset of genomic regions

Overall, this Aim showed that TCDD induces the recruitment of ERα to a subset of AHR target genes supporting the gene-specific modulatory role of ERα in AHR signalling (Matthews et al., 2005). However, the ChIP-chip experiments were done only at a single time point in one cell type using promoter focused microarrays limiting our analysis to the regions represented on the arrays. Emerging data indicate that complete genomic binding profiles for sequence-specific DNA-binding proteins cannot be obtained from one ChIP-chip experiment in a single cell line or tissue (John et al., 2008; Krum et al., 2008). For example, ligand-dependent recruitment of ERα and AHR exhibit oscillatory recruitment to their target regions (Shang et al., 2000; Wihlén et al.,

2009; Pansoy et al., 2010), which may not occur with the same kinetics for all ERα- and AHR-bound regions. Moreover, activation of AHR or ERα by different ligands that produce different receptor conformations of either receptor might produce a distinct set of receptor-bound regions from those identified in our study. A more comprehensive genomic binding profile for either of these factors will require genome-wide and temporal analysis in a variety of cell types.

Since the completion of this study, ChIP-seq has replaced ChIP-chip methodologies. ChIP-seq eliminates any biases due to the fixed position of tiled probes and eliminates the requirement of hybridization of ChIP fragments to arrays. Instead, all ChIP fragments isolated are sequenced. Completing our study using ChIP-seq methodologies would give us a better understanding of AHR and ERα binding. Furthermore, since our study was done using promoter focused arrays we were not able to see if AHR or ERα are recruited to distal enhancers which has been previously reported for both transcription factors (Carroll et al., 2006; Lupien et al., 2008; Dere et al., 2011).

Our study addressed the role of ERα in AHR signalling but we could not fully attribute the effects to TCDD treatment since all experiments were done using complete medium. To address this issue, using steroid deprived medium and treating with DMSO, TCDD, E2, and E2+TCDD followed by ChIP-seq and gene expression arrays will give us a better understanding of the role of ERα in AHR signaling and the reciprocal.

All of our studies were completed using human immortalized cell lines. It will be important to determine the biological significance of our *in vitro* findings using an *in vivo* model. Completion of experiments using immature ovariectomized C57BL/6 mice will help clarify AHR-ERα crosstalk *in vivo*. Treating mice with vehicle control, TCDD, E2, and E2+TCDD and then isolating the mammary gland for ChIP studies will address this problem. We will characterize the genomic binding profiles of both AHR and ERα in the mammary gland and compare it to the effects seen in our *in vitro* human breast cancer cells.

## 15.2 Aim 2: AHR-dependent regulation of cyclin G2 requires FOXA1

In this Aim we show that FOXA1 is required for the TCDD-dependent upregulation of CCNG2. Unlike what was observed for CCNG2, we have previously shown that RNAi-mediated

knockdown of ERα reduces the TCDD responsiveness of both CYP1A1 and CYP1B1 in T-47D cells (Ahmed et al., 2009). This suggests that for certain genes the reduced AHR transactivation following RNAi-mediated knockdown of FOXA1, may be due to reduced ERα levels and not reduced FOXA1 expression. Therefore, it will be important to distinguish the effects of FOXA1 knockdown on AHR transactivation compared to those mediated by ERα. Investigating the recruitment patterns of AHR and FOXA1 after RNAi-mediated knockdown of both FOXA1 and ERα or through zinc finger gene knockout approaches followed by ChIP-sequencing will be helpful in distinguishing the role of both transcription factors in AHR signalling. Also, we only determined the role of FOXA1 at a single gene. Completing the ChIP-seq experiments will help us identify whether FOXA1 is a general or gene-specific modulator of AHR signalling.

My studies did not address the mechanism by which FOXA1 impacts the activated AHR complex. I was able to show that AHR and FOXA1 are part of the same activated complex but using GST pull down assays with full length as well as truncations of each receptor will determine their exact sites of interactions. Furthermore, I cannot conclusively say that FOXA1 affects AHR signalling by creating an open chromatin state and facilitates AHR binding. Experiments that look at nucleosome structure will address this issue.

## 15.3 Aim 3: AHR knockout in MCF-7 and MDA-MB-231 affects ERα signalling, proliferation, and depletes CYP1B1 levels.

This Aim used zinc finger nucleases to knockout AHR to assess its role in ERα signalling and cell cycle regulation. Our results suggest that AHR inhibits ERα signalling at the regulatory regions of *TFF-1* and *GREB1*. To address the role of AHR at other ERα regulatory regions it will be important to complete ChIP-seq coupled with cDNA microarrays in the MCF-7 wildtype and $AHR^{-/-}$ cells. This will help clarify the function of AHR in ERα signalling. These experiments can also be coupled with an *in vivo* model. Using *Ahr*-null mice and following the same experimental protocol and isolation of the mammary gland will allow for comparison of *in vitro* and *in vivo* findings strengthening our conclusions.

The effects of AHR on cell cycle progression suggest that it is responsible for the $G_1$ to S phase transition and $G_2$ to M phase progression. However, my study did not address the mechanism by which this occurs. It will be important to look at Rb phosphorylation status, E2F target gene

expression, and CDKs activity in both the AHR$^{-/-}$ and MCF-7 and MDA cells (Ge and Elferink, 1998; Marlowe et al., 2004; Barhoover et al., 2010).

We attributed many of our findings to AHR status. However, our studies do not actually prove this. To address this issue, it will be important to knock back in AHR into MCF-7 and MDA AHR$^{-/-}$ cells to confirm our effects were directly related to AHR status. It is expected that this will restore CYP1B1 levels, ERα signalling, and cell cycle progression/proliferation rates. We can complete these experiments either transiently or using a stable cell line expressing AHR. A stable cell line would be a better model to use allowing for consistent AHR levels as transfection efficiency will not be a factor. Another limitation of our studies is that these experiments were completed using one clone of MCF-7 and MDA cells. Clonal selection is a problem with the generation of *in vitro* cell lines. To strengthen our results it will be important to repeat our experiments using another clone.

# *16* Summary of Findings and Significance

Despite many studies, the molecular mechanisms of reciprocal AHR-ERα crosstalk are not completely understood. Many studies have focused on a small subset of genes to describe their interplay. In my first Aim we set out to determine the role of ERα in AHR signalling and determined that it was only recruited to a subset of genes. This suggests that ERα is a gene-specific modulator of AHR signalling. Interestingly, we also showed that the most regions co-recruited by both factors contained an AHRE implying that AHR was driving the recruitment of ERα to these sites. To test this, we used RNA interference and determined that AHR was important for the recruitment of ERα to some but not all genes. TFBS analysis demonstrated that FOXA1 recognition sites were over-represented in our data set after TCDD treatment that led us to investigate its role in AHR signalling. We focused on the target gene *CCNG2*, a negative regulator of cell cycle known to be inhibited by ERα, but we show was up-regulated by TCDD in our ChIP-chip study. Using RNA interference, Co-IP, ChIP, and reporter gene constructs we demonstrated that FOXA1 was important in AHR-mediated and TCDD-dependent regulation of CCNG2. Moreover, we showed that TCDD treatment was able to overcome the E2-dependent negative regulation of CCNG2 implicating another ERα gene that can be inhibited by AHR activation. These experiments also identified a novel AHR target gene involved in the TCDD-dependent $G_1$ phase arrest.

To complement our first Aim, which studied the role of ERα in AHR signalling, we generated MCF-7 *AHR*$^{-/-}$ breast cancer cells to study the role of AHR in ERα signalling. We determined that AHR inhibited ERα signalling (*TFF-1, GREB1*) and E2-dependent growth. It appears that AHR regulates ERα signalling at the transcriptional and gene specific level and did not change protein levels. It will be important to then complete ChIP-seq studies in this cell line to supplement the current literature on the role of AHR in ERα signalling in the context of breast cancer.

We also investigated the role of AHR in ERα negative MDA-MB-231 cells where AHR expression was important in facilitating proper cell cycle progression but TCDD-activated AHR did not cause growth-inhibition. We also saw that AHR was important for normal cycling but did not cause the TCDD-dependent growth inhibition in the MCF-7 cells. Our data support the current literature that AHR facilitates cell cycle progression. It will be important to determine the mechanism by which this occurs.

Overall, the data I generated have helped elucidate the role of ERα in AHR signalling implicating it as a gene specific modulator. Furthermore, through the regulation of CCNG2 I have provided another ERα target gene that is also regulated by AHR highlighting the close relationship these two receptor systems have. I have also confirmed that AHR affects cell cycle progression, and the importance of FOXA1 in AHR-mediated gene expression. These findings support the current literature suggesting that AHR is not just a xenobiotic sensing protein and has more of a physiological function.

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# Appendices

**Table A1 Genomic coordinates of the AHR binding sites following TCDD treatment**

| Region | Chromosome | Start | End |
|---|---|---|---|
| AHR_1 | chr3 | 158017186 | 158018539 |
| AHR_2 | chr3 | 4733583 | 4734684 |
| AHR_3 | chr6 | 76051130 | 76052284 |
| AHR_4 | chr11 | 66551588 | 66552656 |
| AHR_5 | chr8 | 67198844 | 67199364 |
| AHR_6 | chr20 | 57940036 | 57940642 |
| AHR_7 | chr17 | 7321687 | 7322856 |
| AHR_8 | chr10 | 95507616 | 95508648 |
| AHR_9 | chr3 | 158012934 | 158013634 |
| AHR_10 | chr2 | 38157919 | 38158207 |
| AHR_11 | chr6 | 35850891 | 35851148 |
| AHR_12 | chr10 | 95184639 | 95184988 |
| AHR_13 | chr12 | 81600544 | 81601268 |
| AHR_14 | chr11 | 120826965 | 120827714 |
| AHR_15 | chr17 | 63804573 | 63805294 |
| AHR_16 | chr2 | 108701970 | 108702301 |
| AHR_17 | chr11 | 120032796 | 120033457 |
| AHR_18 | chr12 | 15259479 | 15261177 |
| AHR_19 | chr11 | 129221640 | 129222260 |
| AHR_20 | chr10 | 95231632 | 95232534 |
| AHR_21 | chr1 | 234745067 | 234745679 |
| AHR_22 | chr21 | 37365740 | 37366797 |
| AHR_23 | chr17 | 32922258 | 32922855 |
| AHR_24 | chr6 | 47091744 | 47092432 |
| AHR_25 | chr12 | 115197676 | 115198642 |
| AHR_26 | chr10 | 80497316 | 80497846 |
| AHR_27 | chr9 | 139888518 | 139889563 |
| AHR_28 | chr15 | 36330685 | 36331528 |
| AHR_29 | chr1 | 40612543 | 40613275 |
| AHR_30 | chr3 | 180271453 | 180272050 |
| AHR_31 | chrX | 106756502 | 106756994 |
| AHR_32 | chr16 | 84344286 | 84344517 |
| AHR_33 | chr6 | 35802343 | 35802981 |
| AHR_34 | chr19 | 63041856 | 63042390 |
| AHR_35 | chr12 | 108234008 | 108234362 |
| AHR_36 | chr13 | 38158173 | 38158600 |
| AHR_37 | chr4 | 78295835 | 78296684 |
| AHR_38 | chr16 | 27468004 | 27468407 |
| AHR_39 | chr4 | 6748397 | 6749516 |
| AHR_40 | chr11 | 59895048 | 59895375 |
| AHR_41 | chr20 | 20477209 | 20477818 |
| AHR_42 | chr14 | 76570148 | 76570393 |
| AHR_43 | chr3 | 4730584 | 4731240 |
| AHR_44 | chr16 | 84391389 | 84391696 |

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|---|---|---|---|
| AHR_45 | chr14 | 73321727 | 73322497 |
| AHR_46 | chr3 | 42513391 | 42513871 |
| AHR_47 | chr14 | 54580245 | 54580709 |
| AHR_48 | chr3 | 178398453 | 178398939 |
| AHR_49 | chr6 | 56921421 | 56921847 |
| AHR_50 | chr20 | 48777653 | 48777991 |
| AHR_51 | chr19 | 48444560 | 48445292 |
| AHR_52 | chr19 | 52104435 | 52104845 |
| AHR_53 | chr21 | 42349550 | 42349755 |
| AHR_54 | chr3 | 4509032 | 4509649 |
| AHR_55 | chr10 | 112666117 | 112666654 |
| AHR_56 | chr19 | 48255508 | 48255809 |
| AHR_57 | chr6 | 12827215 | 12828215 |
| AHR_58 | chr19 | 63016310 | 63016884 |
| AHR_59 | chr7 | 45084693 | 45086315 |
| AHR_60 | chr10 | 51213009 | 51213489 |
| AHR_61 | chr2 | 219433982 | 219434509 |
| AHR_62 | chr17 | 36028489 | 36028950 |
| AHR_63 | chr12 | 52658799 | 52659839 |
| AHR_64 | chr1 | 205556204 | 205557103 |
| AHR_65 | chr17 | 77651426 | 77652291 |
| AHR_66 | chr12 | 55197949 | 55198228 |
| AHR_67 | chr4 | 39876160 | 39876619 |
| AHR_68 | chr6 | 47120047 | 47120483 |
| AHR_69 | chr17 | 34114052 | 34114524 |
| AHR_70 | chr19 | 63080001 | 63080540 |
| AHR_71 | chr14 | 92455662 | 92456314 |
| AHR_72 | chr9 | 107045054 | 107045592 |
| AHR_73 | chr4 | 140806419 | 140806888 |
| AHR_74 | chr15 | 69175964 | 69176648 |
| AHR_75 | chr19 | 46988279 | 46988498 |
| AHR_76 | chr1 | 209678624 | 209678830 |
| AHR_77 | chr8 | 36858535 | 36858879 |
| AHR_78 | chr19 | 48359064 | 48359551 |
| AHR_79 | chr3 | 197109107 | 197109866 |
| AHR_80 | chr2 | 55308554 | 55309071 |
| AHR_81 | chr3 | 197122721 | 197123180 |
| AHR_82 | chr9 | 66079845 | 66080284 |
| AHR_83 | chrX | 129127453 | 129127918 |
| AHR_84 | chr12 | 51437055 | 51438402 |
| AHR_85 | chr8 | 103500319 | 103500612 |
| AHR_86 | chr17 | 72796511 | 72796720 |
| AHR_87 | chr16 | 69942842 | 69943390 |
| AHR_88 | chr12 | 94176837 | 94177534 |
| AHR_89 | chr8 | 133843563 | 133843922 |
| AHR_90 | chr10 | 95503209 | 95503764 |
| AHR_91 | chr11 | 75155931 | 75156364 |
| AHR_92 | chr11 | 59012370 | 59012714 |
| AHR_93 | chr3 | 188943149 | 188943627 |
| AHR_94 | chr11 | 129226997 | 129227575 |

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|---|---|---|---|
| AHR_95 | chr16 | 4464694 | 4464913 |
| AHR_96 | chr14 | 77158291 | 77158986 |
| AHR_97 | chr1 | 70591817 | 70592260 |
| AHR_98 | chr21 | 37368102 | 37368478 |
| AHR_99 | chr11 | 122936261 | 122936767 |
| AHR_100 | chr3 | 197107817 | 197108485 |
| AHR_101 | chr17 | 33705299 | 33705554 |
| AHR_102 | chr1 | 65225246 | 65225659 |
| AHR_103 | chr4 | 141663822 | 141664327 |
| AHR_104 | chr19 | 11498134 | 11498507 |
| AHR_105 | chr1 | 223394122 | 223394554 |
| AHR_106 | chr1 | 202461396 | 202461783 |
| AHR_107 | chr6 | 150223005 | 150224316 |
| AHR_108 | chrX | 23829353 | 23829697 |
| AHR_109 | chr11 | 116471753 | 116472124 |
| AHR_110 | chr8 | 105899 | 106279 |
| AHR_111 | chr15 | 72810229 | 72810603 |
| AHR_112 | chr17 | 43979738 | 43980150 |
| AHR_113 | chr1 | 143703936 | 143704356 |
| AHR_114 | chr19 | 63043290 | 63043726 |
| AHR_115 | chr15 | 88101129 | 88101418 |
| AHR_116 | chr21 | 39615268 | 39615830 |
| AHR_117 | chr17 | 53333639 | 53333985 |
| AHR_118 | chr14 | 51189314 | 51189756 |
| AHR_119 | chr17 | 52515182 | 52515383 |
| AHR_120 | chr14 | 36127693 | 36128091 |
| AHR_121 | chr18 | 14793051 | 14793585 |
| AHR_122 | chr18 | 14803938 | 14804472 |
| AHR_123 | chr11 | 8792652 | 8792997 |
| AHR_124 | chr3 | 11262856 | 11263082 |
| AHR_125 | chr10 | 104460329 | 104460841 |
| AHR_126 | chr22 | 48847857 | 48848471 |
| AHR_127 | chr11 | 93417127 | 93417536 |
| AHR_128 | chr15 | 48198588 | 48199028 |
| AHR_129 | chr5 | 168995798 | 168996001 |
| AHR_130 | chr1 | 151915510 | 151915743 |
| AHR_131 | chr6 | 3402884 | 3403405 |
| AHR_132 | chr11 | 9546873 | 9547285 |
| AHR_133 | chr13 | 27089716 | 27090589 |
| AHR_134 | chr20 | 48774913 | 48775464 |
| AHR_135 | chr6 | 17497506 | 17497686 |
| AHR_136 | chr2 | 238132465 | 238132923 |
| AHR_137 | chr19 | 107092 | 107364 |
| AHR_138 | chr11 | 3211706 | 3212365 |
| AHR_139 | chr14 | 88126251 | 88126454 |
| AHR_140 | chr11 | 30206589 | 30206958 |
| AHR_141 | chr10 | 104519135 | 104519639 |
| AHR_142 | chr1 | 43199806 | 43200268 |
| AHR_143 | chr11 | 65355405 | 65355810 |
| AHR_144 | chr9 | 42486832 | 42487232 |

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| AHR_145 | chr10 | 65605456 | 65605731 |
| AHR_146 | chr17 | 23677186 | 23677707 |
| AHR_147 | chr10 | 29232133 | 29232905 |
| AHR_148 | chr6 | 17209604 | 17209885 |
| AHR_149 | chr7 | 65609861 | 65610324 |
| AHR_150 | chr1 | 143050477 | 143050911 |
| AHR_151 | chr8 | 53798353 | 53798939 |
| AHR_152 | chr3 | 9976255 | 9976541 |
| AHR_153 | chr6 | 30827231 | 30827777 |
| AHR_154 | chr21 | 30668312 | 30668748 |
| AHR_155 | chr15 | 39571388 | 39571555 |
| AHR_156 | chr12 | 46500282 | 46500665 |
| AHR_157 | chr1 | 22136897 | 22137152 |
| AHR_158 | chr2 | 11360817 | 11361143 |
| AHR_159 | chr7 | 37925388 | 37925578 |
| AHR_160 | chr12 | 81603893 | 81604419 |
| AHR_161 | chr1 | 142533909 | 142534753 |
| AHR_162 | chrX | 102829205 | 102829567 |
| AHR_163 | chr4 | 37128253 | 37128819 |
| AHR_164 | chr10 | 115428322 | 115428848 |
| AHR_165 | chr2 | 220119276 | 220119706 |
| AHR_166 | chr21 | 44954097 | 44954886 |
| AHR_167 | chr1 | 147844270 | 147844802 |
| AHR_168 | chr15 | 20865733 | 20866001 |
| AHR_169 | chr16 | 31140213 | 31140773 |
| AHR_170 | chr20 | 31242965 | 31243359 |
| AHR_171 | chr3 | 63823692 | 63823975 |
| AHR_172 | chr8 | 11467988 | 11468222 |
| AHR_173 | chr3 | 128792994 | 128793567 |
| AHR_174 | chr6 | 161424588 | 161424899 |
| AHR_175 | chr14 | 51611685 | 51612154 |
| AHR_176 | chr14 | 34520302 | 34520735 |
| AHR_177 | chr1 | 146396532 | 146397340 |
| AHR_178 | chr17 | 16341788 | 16342000 |
| AHR_179 | chr12 | 73998245 | 73998462 |
| AHR_180 | chr6 | 123150762 | 123151070 |
| AHR_181 | chr9 | 83490612 | 83491814 |
| AHR_182 | chrX | 105174528 | 105174775 |
| AHR_183 | chr1 | 198646436 | 198646911 |
| AHR_184 | chr11 | 110678986 | 110679387 |
| AHR_185 | chr5 | 119820913 | 119821239 |
| AHR_186 | chr15 | 75778712 | 75779176 |
| AHR_187 | chr3 | 190354648 | 190354898 |
| AHR_188 | chr7 | 5518803 | 5519211 |
| AHR_189 | chr2 | 39201940 | 39202385 |
| AHR_190 | chr6 | 12826413 | 12826732 |
| AHR_191 | chr17 | 26174937 | 26175365 |
| AHR_192 | chr1 | 246867584 | 246868102 |
| AHR_193 | chr20 | 34810235 | 34810484 |
| AHR_194 | chr20 | 43956035 | 43956298 |

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| AHR_195 | chr11 | 76462 | 76877 |
| AHR_196 | chr9 | 37629458 | 37629724 |
| AHR_197 | chr15 | 31147335 | 31148415 |
| AHR_198 | chr8 | 103626160 | 103626331 |
| AHR_199 | chr15 | 83155466 | 83155805 |
| AHR_200 | chr1 | 149778555 | 149778877 |
| AHR_201 | chr5 | 179955342 | 179955730 |
| AHR_202 | chr1 | 220977682 | 220977952 |
| AHR_203 | chr8 | 30231735 | 30232121 |
| AHR_204 | chr6 | 152169903 | 152170367 |
| AHR_205 | chr7 | 5189143 | 5189478 |
| AHR_206 | chr2 | 177836151 | 177836519 |
| AHR_207 | chr15 | 56829945 | 56830111 |
| AHR_208 | chr1 | 114853692 | 114853907 |
| AHR_209 | chr10 | 92674264 | 92674579 |
| AHR_210 | chr12 | 15264778 | 15265316 |
| AHR_211 | chr1 | 199106748 | 199107073 |
| AHR_212 | chrX | 46965001 | 46965355 |
| AHR_213 | chr9 | 139480745 | 139481056 |
| AHR_214 | chr5 | 80572557 | 80572740 |
| AHR_215 | chr18 | 255715 | 256096 |
| AHR_216 | chr11 | 75064139 | 75064398 |
| AHR_217 | chr11 | 62076763 | 62077158 |
| AHR_218 | chr16 | 68009304 | 68009694 |
| AHR_219 | chr2 | 85666552 | 85666764 |
| AHR_220 | chr7 | 76589561 | 76589874 |
| AHR_221 | chr15 | 64586569 | 64586928 |
| AHR_222 | chr12 | 91851273 | 91851485 |
| AHR_223 | chr21 | 13979018 | 13979301 |
| AHR_224 | chr8 | 27230022 | 27230290 |
| AHR_225 | chr15 | 63263050 | 63263273 |
| AHR_226 | chr5 | 149861733 | 149861977 |
| AHR_227 | chr1 | 150398187 | 150398462 |
| AHR_228 | chr1 | 610772 | 610972 |
| AHR_229 | chr15 | 26135310 | 26135758 |
| AHR_230 | chr15 | 76892965 | 76893170 |
| AHR_231 | chr18 | 30326892 | 30327323 |
| AHR_232 | chr14 | 90822140 | 90822520 |
| AHR_233 | chr20 | 44525338 | 44525516 |
| AHR_234 | chr9 | 92450612 | 92450989 |
| AHR_235 | chr3 | 31998025 | 31998661 |
| AHR_236 | chr16 | 4607832 | 4608081 |
| AHR_237 | chr18 | 46060549 | 46060979 |
| AHR_238 | chr16 | 11270027 | 11270206 |
| AHR_239 | chr10 | 81448507 | 81448904 |
| AHR_240 | chr20 | 17499203 | 17499574 |
| AHR_241 | chr15 | 19261057 | 19261473 |
| AHR_242 | chr10 | 81128308 | 81128705 |
| AHR_243 | chr12 | 66931418 | 66931730 |
| AHR_244 | chr2 | 89610582 | 89610833 |

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|---|---|---|---|
| AHR_245 | chr3 | 153469876 | 153470115 |
| AHR_246 | chr5 | 67620350 | 67620535 |
| AHR_247 | chr9 | 68555260 | 68555472 |
| AHR_248 | chr4 | 159661474 | 159661828 |
| AHR_249 | chr11 | 16999433 | 16999708 |
| AHR_250 | chr7 | 7916166 | 7916489 |
| AHR_251 | chr1 | 152431889 | 152432330 |
| AHR_252 | chr2 | 11596942 | 11597344 |
| AHR_253 | chr8 | 110728219 | 110728503 |
| AHR_254 | chr13 | 32757450 | 32757824 |
| AHR_255 | chr1 | 146695458 | 146696290 |
| AHR_256 | chr17 | 38210374 | 38210772 |
| AHR_257 | chr17 | 69938289 | 69938607 |
| AHR_258 | chr8 | 11251966 | 11252300 |
| AHR_259 | chr3 | 131945915 | 131946125 |
| AHR_260 | chr15 | 26697224 | 26697674 |
| AHR_261 | chr6 | 52385245 | 52385559 |
| AHR_262 | chr6 | 50732 | 50967 |
| AHR_263 | chr10 | 62372657 | 62372906 |
| AHR_264 | chr5 | 167472256 | 167472574 |
| AHR_265 | chr15 | 87551980 | 87552192 |
| AHR_266 | chr1 | 154838134 | 154838309 |
| AHR_267 | chr10 | 95188343 | 95188680 |
| AHR_268 | chr1 | 213806890 | 213807174 |
| AHR_269 | chr14 | 102454471 | 102454991 |
| AHR_270 | chr17 | 4094596 | 4095002 |
| AHR_271 | chr3 | 194118667 | 194119009 |
| AHR_272 | chr22 | 17848173 | 17848449 |
| AHR_273 | chr12 | 119124348 | 119124591 |
| AHR_274 | chr18 | 54394846 | 54395053 |
| AHR_275 | chr1 | 207928399 | 207928582 |
| AHR_276 | chr12 | 122441383 | 122441667 |
| AHR_277 | chr13 | 99434026 | 99434365 |
| AHR_278 | chr9 | 139584574 | 139585010 |
| AHR_279 | chr1 | 150355689 | 150355976 |
| AHR_280 | chr8 | 124819307 | 124819525 |
| AHR_281 | chr20 | 34145124 | 34145479 |
| AHR_282 | chr16 | 46841931 | 46842463 |
| AHR_283 | chr6 | 84615449 | 84615643 |
| AHR_284 | chr15 | 89566092 | 89566385 |
| AHR_285 | chr9 | 69732841 | 69733053 |
| AHR_286 | chr10 | 88971718 | 88971929 |
| AHR_287 | chr10 | 88970513 | 88970943 |
| AHR_288 | chr1 | 16767318 | 16767830 |
| AHR_289 | chr19 | 19703413 | 19703568 |
| AHR_290 | chr9 | 134751268 | 134751467 |
| AHR_291 | chr15 | 18906896 | 18907459 |
| AHR_292 | chr9 | 90192114 | 90192328 |
| AHR_293 | chr12 | 2940021 | 2940324 |
| AHR_294 | chr5 | 168945532 | 168945722 |

| | | | |
|---|---|---|---|
| AHR_295 | chr10 | 52103484 | 52103948 |
| AHR_296 | chr3 | 199068343 | 199068647 |
| AHR_297 | chr11 | 129193328 | 129193534 |
| AHR_298 | chr11 | 3688546 | 3688729 |
| AHR_299 | chr11 | 122435896 | 122436224 |
| AHR_300 | chr1 | 26060429 | 26060768 |
| AHR_301 | chr11 | 113157848 | 113158534 |
| AHR_302 | chr10 | 13317586 | 13317892 |
| AHR_303 | chr9 | 126943471 | 126943759 |
| AHR_304 | chr15 | 88098899 | 88099365 |
| AHR_305 | chr8 | 37882065 | 37882286 |
| AHR_306 | chr15 | 88448153 | 88448339 |
| AHR_307 | chr8 | 23369154 | 23369515 |
| AHR_308 | chr2 | 29109207 | 29109467 |
| AHR_309 | chr10 | 5053560 | 5053982 |
| AHR_310 | chr17 | 64460674 | 64460878 |
| AHR_311 | chr16 | 65983822 | 65984211 |
| AHR_312 | chr15 | 61467443 | 61468013 |
| AHR_313 | chr21 | 39611910 | 39612298 |
| AHR_314 | chr1 | 147017051 | 147017418 |
| AHR_315 | chr1 | 169977088 | 169977483 |
| AHR_316 | chr6 | 111380743 | 111380924 |
| AHR_317 | chr21 | 39846619 | 39846905 |
| AHR_318 | chr14 | 52237770 | 52238017 |
| AHR_319 | chr1 | 233737068 | 233737241 |
| AHR_320 | chr9 | 124317246 | 124317730 |
| AHR_321 | chr1 | 791679 | 792333 |
| AHR_322 | chr8 | 55601079 | 55601279 |
| AHR_323 | chr17 | 23977218 | 23977474 |
| AHR_324 | chr16 | 79905242 | 79905454 |
| AHR_325 | chr1 | 240571844 | 240572219 |
| AHR_326 | chr1 | 212224854 | 212225143 |
| AHR_327 | chr6 | 30691463 | 30691835 |
| AHR_328 | chr17 | 8586399 | 8586695 |
| AHR_329 | chr1 | 233022080 | 233022304 |
| AHR_330 | chr1 | 226667562 | 226667808 |
| AHR_331 | chr19 | 62929788 | 62930276 |
| AHR_332 | chr17 | 68603007 | 68603269 |
| AHR_333 | chr17 | 40979581 | 40979826 |
| AHR_334 | chr9 | 42403935 | 42404198 |
| AHR_335 | chr6 | 167815072 | 167815433 |
| AHR_336 | chr3 | 36390560 | 36390740 |
| AHR_337 | chr1 | 246683999 | 246684283 |
| AHR_338 | chr7 | 93389327 | 93389616 |
| AHR_339 | chr3 | 54936589 | 54936907 |
| AHR_340 | chr17 | 33758417 | 33758625 |
| AHR_341 | chr5 | 68891886 | 68892308 |
| AHR_342 | chr16 | 67721091 | 67721419 |
| AHR_343 | chrX | 154372168 | 154372436 |
| AHR_344 | chr20 | 20146291 | 20146747 |

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|---|---|---|---|
| AHR_345 | chr3 | 33126022 | 33126286 |
| AHR_346 | chr10 | 65045690 | 65046149 |
| AHR_347 | chr9 | 129502220 | 129502488 |
| AHR_348 | chr16 | 74061525 | 74061699 |
| AHR_349 | chr7 | 95068738 | 95069099 |
| AHR_350 | chr5 | 136864608 | 136864873 |
| AHR_351 | chr3 | 13492572 | 13492919 |
| AHR_352 | chr2 | 73312999 | 73313217 |
| AHR_353 | chr1 | 222106376 | 222106736 |
| AHR_354 | chr2 | 121270151 | 121270341 |
| AHR_356 | chr1 | 142868899 | 142869326 |
| AHR_357 | chr1 | 148123382 | 148124117 |
| AHR_358 | chr3 | 197350242 | 197350420 |
| AHR_359 | chrX | 154234150 | 154234418 |
| AHR_360 | chr1 | 110733234 | 110733524 |
| AHR_362 | chr8 | 79836413 | 79836810 |
| AHR_363 | chr4 | 118231862 | 118232114 |
| AHR_364 | chr15 | 90735366 | 90735539 |
| AHR_365 | chr21 | 13716004 | 13716406 |
| AHR_366 | chr9 | 116201501 | 116201845 |
| AHR_367 | chr17 | 37680408 | 37680938 |
| AHR_368 | chr3 | 41769852 | 41770247 |
| AHR_369 | chr9 | 14310536 | 14310979 |
| AHR_370 | chr12 | 100614622 | 100615115 |
| AHR_371 | chr1 | 207469479 | 207469868 |
| AHR_372 | chr6 | 106884794 | 106885128 |
| AHR_373 | chr10 | 12213195 | 12213596 |
| AHR_374 | chr5 | 79613835 | 79614022 |
| AHR_375 | chr1 | 247121943 | 247122152 |
| AHR_376 | chr14 | 96034802 | 96035016 |
| AHR_377 | chr7 | 22729834 | 22730084 |
| AHR_378 | chr15 | 61576194 | 61576425 |
| AHR_379 | chr18 | 20072030 | 20072386 |
| AHR_380 | chr9 | 33442257 | 33442439 |
| AHR_381 | chr9 | 68794092 | 68794444 |
| AHR_382 | chr3 | 127167966 | 127168294 |
| AHR_383 | chr2 | 227958523 | 227958772 |
| AHR_384 | chr15 | 38773838 | 38774107 |
| AHR_385 | chr16 | 54470790 | 54470960 |
| AHR_386 | chr1 | 144021816 | 144022144 |
| AHR_387 | chr5 | 69747196 | 69747455 |
| AHR_388 | chr20 | 44425531 | 44425841 |
| AHR_389 | chr8 | 8082633 | 8082843 |
| AHR_390 | chr18 | 14460131 | 14460661 |
| AHR_391 | chr7 | 112196480 | 112196810 |
| AHR_392 | chr12 | 106010446 | 106010702 |
| AHR_393 | chr11 | 125257001 | 125257378 |
| AHR_394 | chr5 | 95321215 | 95321489 |
| AHR_395 | chr7 | 91913370 | 91913620 |
| AHR_396 | chr20 | 32870964 | 32871150 |

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|---|---|---|---|
| AHR_397 | chr21 | 45415967 | 45416324 |
| AHR_398 | chr17 | 5349721 | 5350021 |
| AHR_399 | chr4 | 100130954 | 100131246 |
| AHR_400 | chr17 | 41731894 | 41732155 |
| AHR_401 | chr1 | 159284948 | 159285310 |
| AHR_402 | chr6 | 155097409 | 155097624 |
| AHR_403 | chr2 | 218707576 | 218707980 |
| AHR_404 | chr5 | 157192265 | 157192623 |
| AHR_405 | chr18 | 10775160 | 10775427 |
| AHR_406 | chr1 | 245165974 | 245166254 |
| AHR_407 | chr10 | 43384494 | 43384924 |
| AHR_408 | chr11 | 116151991 | 116152243 |
| AHR_409 | chr3 | 130740840 | 130741094 |
| AHR_410 | chr12 | 48422052 | 48422416 |
| AHR_411 | chr2 | 74492205 | 74492382 |
| AHR_412 | chr14 | 80492895 | 80493210 |
| AHR_413 | chr20 | 57946590 | 57946843 |
| AHR_414 | chr17 | 39196235 | 39196454 |

**Table A2. Genomic coordinates of the ERα binding sites following TCDD treatment**

| Region | Chromosome | Start | End |
|---|---|---|---|
| ERa_1 | chr10 | 95184571 | 95184988 |
| ERa_2 | chr12 | 15259442 | 15261729 |
| ERa_3 | chr19 | 63041816 | 63042570 |
| ERa_4 | chr11 | 66551588 | 66552686 |
| ERa_5 | chr19 | 46118735 | 46119919 |
| ERa_6 | chr15 | 31146559 | 31149141 |
| ERa_7 | chr3 | 4733296 | 4734684 |
| ERa_8 | chr1 | 152429358 | 152430345 |
| ERa_9 | chr6 | 30827231 | 30827777 |
| ERa_10 | chr3 | 4730249 | 4731316 |
| ERa_11 | chr8 | 67198844 | 67199364 |
| ERa_12 | chr3 | 158012869 | 158013680 |
| ERa_13 | chr10 | 104459970 | 104461021 |
| ERa_14 | chr17 | 63803967 | 63805319 |
| ERa_15 | chr19 | 63016054 | 63016884 |
| ERa_16 | chr2 | 238132465 | 238133139 |
| ERa_17 | chr3 | 13492366 | 13492938 |
| ERa_18 | chr6 | 12826377 | 12828038 |
| ERa_19 | chr19 | 63079501 | 63080540 |
| ERa_20 | chr20 | 48777653 | 48778234 |
| ERa_21 | chr15 | 69175784 | 69176648 |
| ERa_22 | chr11 | 59895048 | 59895458 |
| ERa_23 | chr6 | 35850891 | 35851422 |
| ERa_24 | chr17 | 7321547 | 7322892 |
| ERa_25 | chr15 | 61466609 | 61468013 |
| ERa_26 | chr11 | 120032796 | 120033492 |
| ERa_27 | chr19 | 52104435 | 52105058 |

| | | | |
|---|---|---|---|
| ERa_28 | chr3 | 158017479 | 158018439 |
| ERa_29 | chr4 | 39876061 | 39876691 |
| ERa_30 | chr1 | 234745067 | 234745963 |
| ERa_31 | chr8 | 91065093 | 91065616 |
| ERa_32 | chr6 | 17497321 | 17497686 |
| ERa_33 | chr16 | 84344037 | 84344517 |
| ERa_34 | chr17 | 53333639 | 53334213 |
| ERa_35 | chr12 | 115197521 | 115198642 |
| ERa_36 | chr19 | 46187185 | 46189510 |
| ERa_37 | chr1 | 176961670 | 176962313 |
| ERa_38 | chr4 | 39868882 | 39869125 |
| ERa_39 | chr9 | 107044948 | 107045953 |
| ERa_40 | chr2 | 11589596 | 11590824 |
| ERa_41 | chr10 | 95507822 | 95508611 |
| ERa_42 | chr17 | 77655002 | 77655487 |
| ERa_43 | chr20 | 20477158 | 20477891 |
| ERa_44 | chr3 | 197350242 | 197350511 |
| ERa_45 | chr6 | 42302459 | 42303744 |
| ERa_46 | chr4 | 37132290 | 37133811 |
| ERa_47 | chr2 | 109906476 | 109906985 |
| ERa_48 | chr2 | 111053390 | 111053969 |
| ERa_49 | chr17 | 72793961 | 72796720 |
| ERa_50 | chr3 | 197122721 | 197123288 |
| ERa_51 | chr4 | 141390736 | 141391057 |
| ERa_52 | chr13 | 38156745 | 38158641 |
| ERa_53 | chr20 | 19211922 | 19212624 |
| ERa_54 | chr1 | 51559514 | 51560039 |
| ERa_55 | chr14 | 63084448 | 63084772 |
| ERa_56 | chr17 | 63970810 | 63971915 |
| ERa_57 | chr12 | 2775366 | 2776176 |
| ERa_58 | chr1 | 37276751 | 37277459 |
| ERa_59 | chr1 | 114320492 | 114321329 |
| ERa_60 | chr17 | 36944956 | 36945627 |
| ERa_61 | chr7 | 16886759 | 16887120 |
| ERa_62 | chr2 | 110771915 | 110772318 |
| ERa_63 | chr10 | 104519135 | 104519773 |
| ERa_64 | chr5 | 43452613 | 43452888 |
| ERa_65 | chr14 | 21811334 | 21811647 |
| ERa_66 | chr5 | 147665553 | 147666243 |
| ERa_67 | chr19 | 46095849 | 46096172 |
| ERa_68 | chr22 | 29262163 | 29262504 |
| ERa_69 | chr19 | 46988244 | 46988498 |
| ERa_70 | chr11 | 129221640 | 129222260 |
| ERa_71 | chr16 | 46826678 | 46827941 |
| ERa_72 | chr17 | 41334105 | 41334640 |
| ERa_73 | chr22 | 19600989 | 19601349 |
| ERa_74 | chr14 | 73845529 | 73845977 |
| ERa_75 | chr8 | 22026104 | 22026463 |
| ERa_76 | chr12 | 81600581 | 81601341 |
| ERa_77 | chr1 | 19795350 | 19796055 |

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|---|---|---|---|
| ERa_78 | chr11 | 120826925 | 120827685 |
| ERa_79 | chr15 | 27902172 | 27903237 |
| ERa_80 | chr21 | 13978171 | 13979451 |
| ERa_81 | chr15 | 19260981 | 19262197 |
| ERa_82 | chr18 | 14460131 | 14461422 |
| ERa_83 | chr1 | 9933051 | 9933421 |
| ERa_84 | chr10 | 115428076 | 115428997 |
| ERa_85 | chr6 | 76051366 | 76051951 |
| ERa_86 | chr16 | 88588622 | 88590053 |
| ERa_87 | chr14 | 87546318 | 87547383 |
| ERa_88 | chr10 | 62372488 | 62373107 |
| ERa_89 | chr6 | 20318557 | 20319118 |
| ERa_90 | chr3 | 42513424 | 42513871 |
| ERa_91 | chr10 | 79301622 | 79302335 |
| ERa_92 | chr1 | 226686222 | 226686397 |
| ERa_93 | chr1 | 205556106 | 205557212 |
| ERa_94 | chr21 | 37365740 | 37366797 |
| ERa_95 | chr2 | 11596942 | 11597561 |
| ERa_96 | chr5 | 179955198 | 179955985 |
| ERa_97 | chr2 | 108671688 | 108672306 |
| ERa_98 | chr19 | 63043290 | 63043762 |
| ERa_99 | chr4 | 37128296 | 37128819 |
| ERa_100 | chr4 | 78295903 | 78296684 |
| ERa_101 | chr6 | 35802343 | 35803199 |
| ERa_102 | chr3 | 185027026 | 185027616 |
| ERa_103 | chr6 | 47091744 | 47092611 |
| ERa_104 | chr21 | 39615305 | 39615901 |
| ERa_105 | chr17 | 73928737 | 73929531 |
| ERa_106 | chr19 | 63017220 | 63017700 |
| ERa_107 | chr2 | 38157968 | 38158207 |
| ERa_108 | chr4 | 6685829 | 6686162 |
| ERa_109 | chr12 | 94016095 | 94016736 |
| ERa_110 | chr10 | 61395155 | 61395713 |
| ERa_111 | chr20 | 57940036 | 57940460 |
| ERa_112 | chr9 | 127395796 | 127396706 |
| ERa_113 | chr7 | 142536809 | 142537021 |
| ERa_114 | chr10 | 112851647 | 112852264 |
| ERa_115 | chr16 | 82537766 | 82538362 |
| ERa_116 | chr1 | 145052434 | 145053458 |
| ERa_117 | chr10 | 104457096 | 104457443 |
| ERa_118 | chr6 | 152169903 | 152170519 |
| ERa_119 | chr6 | 167453788 | 167454334 |
| ERa_120 | chr19 | 52531915 | 52532440 |
| ERa_121 | chr20 | 31785331 | 31785883 |
| ERa_122 | chr11 | 113157625 | 113158281 |
| ERa_123 | chr3 | 168929147 | 168929618 |
| ERa_124 | chrX | 148435923 | 148436528 |
| ERa_125 | chr17 | 43979154 | 43980150 |
| ERa_126 | chr5 | 179956386 | 179956737 |
| ERa_127 | chr3 | 142143011 | 142143331 |

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|---|---|---|---|
| ERa_128 | chr17 | 32922258 | 32922613 |
| ERa_129 | chr21 | 42349515 | 42349755 |
| ERa_130 | chr11 | 71184277 | 71184946 |
| ERa_131 | chr12 | 52663225 | 52664138 |
| ERa_132 | chr18 | 489460 | 489870 |
| ERa_133 | chr1 | 213806792 | 213807218 |
| ERa_134 | chr19 | 41755415 | 41756044 |
| ERa_135 | chr17 | 77651426 | 77652291 |
| ERa_136 | chr10 | 104524065 | 104524572 |
| ERa_137 | chr15 | 48844251 | 48844921 |
| ERa_138 | chr15 | 72908204 | 72908507 |
| ERa_139 | chr5 | 68661571 | 68661870 |
| ERa_140 | chr20 | 11818344 | 11818624 |
| ERa_141 | chr11 | 60688817 | 60689336 |
| ERa_142 | chr1 | 55126405 | 55126901 |
| ERa_143 | chr14 | 105205554 | 105206080 |
| ERa_144 | chr14 | 73321297 | 73322570 |
| ERa_145 | chr9 | 131557776 | 131558341 |
| ERa_146 | chr10 | 95231893 | 95232435 |
| ERa_147 | chr3 | 197109107 | 197109866 |
| ERa_148 | chr8 | 8131321 | 8132177 |
| ERa_149 | chr14 | 93926663 | 93927163 |
| ERa_150 | chr2 | 190154552 | 190155148 |
| ERa_151 | chr16 | 46841931 | 46842761 |
| ERa_152 | chr13 | 92674753 | 92675376 |
| ERa_153 | chr11 | 64400535 | 64401428 |
| ERa_154 | chr17 | 36915634 | 36915932 |
| ERa_155 | chr11 | 9546873 | 9547285 |
| ERa_156 | chr8 | 11684454 | 11685234 |
| ERa_157 | chr1 | 51567423 | 51568489 |
| ERa_158 | chrX | 148851279 | 148851884 |
| ERa_159 | chr10 | 95188126 | 95188680 |
| ERa_160 | chr11 | 114877894 | 114878298 |
| ERa_161 | chr1 | 201324835 | 201325636 |
| ERa_162 | chr11 | 116471753 | 116472334 |
| ERa_163 | chr2 | 105386385 | 105387512 |
| ERa_164 | chr3 | 134944242 | 134944856 |
| ERa_165 | chrX | 106756502 | 106756994 |
| ERa_166 | chr11 | 100364080 | 100364288 |
| ERa_167 | chr4 | 6748518 | 6749479 |
| ERa_168 | chr8 | 127645492 | 127646182 |
| ERa_169 | chr7 | 142537614 | 142538182 |
| ERa_170 | chr16 | 11585284 | 11585815 |
| ERa_171 | chr10 | 104918122 | 104918656 |
| ERa_172 | chr1 | 239592119 | 239592540 |
| ERa_173 | chr1 | 16767043 | 16768102 |
| ERa_174 | chr8 | 87423109 | 87423712 |
| ERa_175 | chr6 | 18494524 | 18494997 |
| ERa_176 | chr17 | 36028445 | 36028985 |
| ERa_177 | chr12 | 123410621 | 123411169 |

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|---|---|---|---|
| ERa_178 | chr3 | 190374326 | 190374498 |
| ERa_179 | chr14 | 76570186 | 76570393 |
| ERa_180 | chr1 | 178464206 | 178464822 |
| ERa_181 | chr11 | 67320499 | 67321389 |
| ERa_182 | chr10 | 99197749 | 99198177 |
| ERa_183 | chr12 | 15264778 | 15265352 |
| ERa_184 | chr1 | 21677686 | 21678010 |
| ERa_185 | chr9 | 83490582 | 83491706 |
| ERa_186 | chr14 | 63770632 | 63771385 |
| ERa_187 | chr13 | 44936705 | 44936979 |
| ERa_188 | chr1 | 234747289 | 234747560 |
| ERa_189 | chr20 | 47860606 | 47861434 |
| ERa_190 | chr1 | 149247009 | 149247498 |
| ERa_191 | chr11 | 66772125 | 66773030 |
| ERa_192 | chr10 | 112666049 | 112666480 |
| ERa_193 | chr10 | 99087021 | 99087455 |
| ERa_194 | chr5 | 147525500 | 147526103 |
| ERa_195 | chr5 | 149863969 | 149864316 |
| ERa_196 | chr20 | 47862216 | 47862589 |
| ERa_197 | chr19 | 46095179 | 46095397 |
| ERa_198 | chr5 | 1657087 | 1657582 |
| ERa_199 | chr1 | 167344723 | 167345052 |
| ERa_200 | chr1 | 144751135 | 144752145 |
| ERa_201 | chr1 | 146048177 | 146049187 |
| ERa_202 | chr12 | 100616396 | 100616818 |
| ERa_203 | chr1 | 147017051 | 147017705 |
| ERa_204 | chr4 | 9309750 | 9310107 |
| ERa_205 | chr9 | 116201385 | 116201959 |
| ERa_206 | chr1 | 146695636 | 146696290 |
| ERa_207 | chr17 | 37679728 | 37681249 |
| ERa_208 | chr12 | 100614796 | 100615223 |
| ERa_209 | chr12 | 103131871 | 103132158 |
| ERa_210 | chr4 | 69386465 | 69386929 |
| ERa_211 | chr8 | 144168394 | 144168764 |
| ERa_212 | chr2 | 11588563 | 11588845 |
| ERa_213 | chr3 | 75558179 | 75558982 |
| ERa_214 | chr1 | 199124546 | 199124937 |
| ERa_215 | chr20 | 48774803 | 48775423 |
| ERa_216 | chr3 | 197498401 | 197499060 |
| ERa_217 | chr5 | 150518017 | 150518343 |
| ERa_218 | chr22 | 34348841 | 34349440 |
| ERa_219 | chr1 | 142868899 | 142869578 |
| ERa_220 | chr16 | 87520926 | 87521372 |
| ERa_221 | chr22 | 39970123 | 39970336 |
| ERa_222 | chr19 | 18418828 | 18419219 |
| ERa_223 | chr2 | 108701970 | 108702229 |
| ERa_224 | chr15 | 40350438 | 40350898 |
| ERa_225 | chr7 | 6941338 | 6942189 |
| ERa_226 | chr3 | 197107777 | 197108737 |
| ERa_227 | chr1 | 146854971 | 146855661 |

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|---|---|---|---|
| ERa_228 | chr1 | 6466339 | 6466524 |
| ERa_229 | chr1 | 31432157 | 31432885 |
| ERa_230 | chr4 | 185904659 | 185905271 |
| ERa_231 | chr13 | 32757450 | 32757982 |
| ERa_232 | chr6 | 35857885 | 35858612 |
| ERa_233 | chr21 | 42350069 | 42350876 |
| ERa_234 | chr7 | 92075386 | 92076013 |
| ERa_235 | chr12 | 53067955 | 53068241 |
| ERa_236 | chr3 | 8517061 | 8518031 |
| ERa_237 | chr9 | 90336773 | 90337320 |
| ERa_238 | chr18 | 75538623 | 75538838 |
| ERa_239 | chr19 | 18336768 | 18337122 |
| ERa_240 | chr14 | 63768868 | 63769333 |
| ERa_241 | chr15 | 38190289 | 38190733 |
| ERa_242 | chr7 | 93363519 | 93364103 |
| ERa_243 | chr7 | 47957690 | 47958124 |
| ERa_244 | chr5 | 149861806 | 149862299 |
| ERa_245 | chr6 | 3402848 | 3403258 |
| ERa_246 | chr20 | 17491393 | 17491744 |
| ERa_247 | chr6 | 150223176 | 150223933 |
| ERa_248 | chr3 | 50162861 | 50163339 |
| ERa_249 | chr3 | 127126104 | 127126668 |
| ERa_250 | chr17 | 23876266 | 23877060 |
| ERa_251 | chr16 | 82395049 | 82395500 |
| ERa_252 | chr12 | 46448487 | 46448950 |
| ERa_253 | chr19 | 48444560 | 48445292 |
| ERa_254 | chr1 | 143533654 | 143534528 |
| ERa_255 | chr10 | 126087 | 126473 |
| ERa_256 | chrX | 16799336 | 16799820 |
| ERa_257 | chr20 | 54397830 | 54398248 |
| ERa_258 | chr3 | 197749283 | 197749477 |
| ERa_259 | chr10 | 50417238 | 50417880 |
| ERa_260 | chrX | 13667972 | 13668255 |
| ERa_261 | chr3 | 131304159 | 131305064 |
| ERa_262 | chr5 | 354884 | 355149 |
| ERa_263 | chr5 | 355647 | 356165 |
| ERa_264 | chr1 | 172104499 | 172104998 |
| ERa_265 | chr10 | 91164114 | 91164335 |
| ERa_266 | chr19 | 49936820 | 49937319 |
| ERa_267 | chr15 | 61459101 | 61460198 |
| ERa_268 | chr6 | 42300257 | 42300534 |
| ERa_269 | chr22 | 42646383 | 42646912 |
| ERa_270 | chr5 | 172411313 | 172411623 |
| ERa_271 | chr5 | 43639623 | 43640081 |
| ERa_272 | chr2 | 96709735 | 96710293 |
| ERa_273 | chr8 | 119702318 | 119703123 |
| ERa_274 | chr8 | 22055779 | 22056218 |
| ERa_275 | chr5 | 177358275 | 177358705 |
| ERa_276 | chr1 | 178403421 | 178403805 |
| ERa_277 | chr11 | 60281107 | 60281489 |

| | | | |
|---|---|---|---|
| ERa_278 | chr20 | 52120528 | 52120976 |
| ERa_279 | chr12 | 10213805 | 10214331 |
| ERa_280 | chr14 | 77158470 | 77158908 |
| ERa_281 | chr7 | 20227284 | 20227757 |
| ERa_282 | chr5 | 149860695 | 149861047 |
| ERa_283 | chr6 | 100730951 | 100731307 |
| ERa_284 | chr17 | 68606872 | 68607429 |
| ERa_285 | chr8 | 109329651 | 109330229 |
| ERa_286 | chr12 | 52659038 | 52659804 |
| ERa_287 | chr1 | 144021816 | 144022467 |
| ERa_288 | chr3 | 197353573 | 197354174 |
| ERa_289 | chr3 | 47460178 | 47460531 |
| ERa_290 | chr8 | 12080329 | 12080759 |
| ERa_291 | chr8 | 12329572 | 12330002 |
| ERa_292 | chr18 | 58344442 | 58344871 |
| ERa_293 | chr1 | 146482909 | 146483704 |
| ERa_294 | chr17 | 7962413 | 7962770 |
| ERa_295 | chr4 | 173966957 | 173967303 |
| ERa_296 | chr16 | 29229431 | 29229793 |
| ERa_297 | chr8 | 102575951 | 102576491 |
| ERa_298 | chr16 | 70112989 | 70113464 |
| ERa_299 | chr1 | 152436064 | 152436430 |
| ERa_300 | chr22 | 22528935 | 22529162 |
| ERa_301 | chr10 | 52035065 | 52035490 |
| ERa_302 | chr7 | 72374313 | 72374583 |
| ERa_303 | chr8 | 102573969 | 102574519 |
| ERa_304 | chr17 | 63421538 | 63422277 |
| ERa_305 | chr3 | 155418798 | 155419658 |
| ERa_306 | chr12 | 47868178 | 47868483 |
| ERa_307 | chr20 | 61145609 | 61145828 |
| ERa_308 | chr4 | 981632 | 981946 |
| ERa_309 | chr12 | 48881985 | 48882260 |
| ERa_310 | chr15 | 88448153 | 88449044 |
| ERa_311 | chr19 | 16144909 | 16145089 |
| ERa_312 | chr15 | 36331094 | 36331478 |
| ERa_313 | chr1 | 232810419 | 232810673 |
| ERa_314 | chr3 | 9785574 | 9786160 |
| ERa_315 | chr14 | 93857291 | 93857852 |
| ERa_316 | chr19 | 12704026 | 12704239 |
| ERa_317 | chr11 | 129193263 | 129193534 |
| ERa_318 | chr10 | 3816046 | 3816417 |
| ERa_319 | chr20 | 23286215 | 23286442 |
| ERa_320 | chr4 | 1229037 | 1229825 |
| ERa_321 | chr10 | 95503245 | 95503695 |
| ERa_322 | chr19 | 48255508 | 48255809 |
| ERa_323 | chr14 | 64763390 | 64764004 |
| ERa_324 | chr14 | 105161839 | 105162454 |
| ERa_325 | chr19 | 50696146 | 50696365 |
| ERa_326 | chr3 | 155424918 | 155425193 |
| ERa_327 | chr4 | 68917167 | 68917666 |

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|---|---|---|---|
| ERa_328 | chr10 | 51212976 | 51213525 |
| ERa_329 | chr17 | 37202804 | 37203596 |
| ERa_330 | chrX | 12902551 | 12902926 |
| ERa_331 | chr19 | 4921781 | 4922395 |
| ERa_332 | chr2 | 88597790 | 88597963 |
| ERa_333 | chr3 | 185187444 | 185187940 |
| ERa_334 | chr14 | 105180584 | 105181075 |
| ERa_335 | chr3 | 4728320 | 4729467 |
| ERa_336 | chr1 | 205990959 | 205991335 |
| ERa_337 | chr11 | 119611508 | 119611891 |
| ERa_338 | chr16 | 82393880 | 82394229 |
| ERa_339 | chr12 | 51625507 | 51626498 |
| ERa_340 | chr11 | 20010025 | 20010208 |
| ERa_341 | chr15 | 69150384 | 69150823 |
| ERa_342 | chr11 | 76177066 | 76177508 |
| ERa_343 | chr1 | 149589258 | 149589565 |
| ERa_344 | chr3 | 4509270 | 4509598 |
| ERa_345 | chr1 | 108543434 | 108543868 |
| ERa_346 | chr6 | 38252561 | 38253122 |
| ERa_347 | chr6 | 26134708 | 26135333 |
| ERa_348 | chr19 | 1307147 | 1307505 |
| ERa_349 | chr3 | 33814663 | 33815012 |
| ERa_350 | chr11 | 110678844 | 110679688 |
| ERa_351 | chr11 | 3391651 | 3392533 |
| ERa_352 | chr11 | 35640265 | 35640594 |
| ERa_353 | chr14 | 95574556 | 95575059 |
| ERa_354 | chr12 | 2754986 | 2755321 |
| ERa_355 | chr19 | 2486528 | 2486816 |
| ERa_356 | chr2 | 11598193 | 11598451 |
| ERa_357 | chr19 | 23798840 | 23799085 |
| ERa_358 | chr7 | 93361722 | 93362198 |
| ERa_359 | chr3 | 188043548 | 188043831 |
| ERa_360 | chr11 | 107873486 | 107873834 |
| ERa_361 | chr6 | 31801755 | 31802107 |
| ERa_362 | chr15 | 73277506 | 73277881 |
| ERa_363 | chr19 | 43481426 | 43481934 |
| ERa_364 | chr14 | 105278891 | 105279417 |

**Table A3 A. Over-represented GO terms in the intersect set. P-values (Fishers Exact test) are corrected for multiple testing.**

| GO term | P-value | Description |
|---|---|---|
| GO:0005737 | 5.29e-05 | cytoplasm |
| GO:0032502 | 0.00225 | developmental process |

| | | |
|---|---|---|
| GO:0044424 | 0.00295 | intracellular part |
| GO:0005515 | 0.00543 | protein binding |
| GO:0043231 | 0.00543 | intracellular membrane-bound organelle |
| GO:0043227 | 0.00543 | membrane-bound organelle |
| GO:0005886 | 0.0168 | plasma membrane |
| GO:0044464 | 0.0168 | cell part |
| GO:0016020 | 0.0173 | membrane |
| GO:0051301 | 0.0232 | cell division |
| GO:0005792 | 0.0232 | microsome |
| GO:0042598 | 0.0232 | vesicular fraction |
| GO:0043229 | 0.0232 | intracellular organelle |
| GO:0045885 | 0.0232 | positive regulation of survival gene product activity |
| GO:0043226 | 0.0232 | organelle |
| GO:0022402 | 0.0232 | cell cycle process |
| GO:0007165 | 0.0271 | signal transduction |
| GO:0019221 | 0.0271 | cytokine and chemokine mediated signalling pathway |
| GO:0005634 | 0.0271 | nucleus |
| GO:0005622 | 0.0271 | intracellular |
| GO:0044425 | 0.029 | membrane part |
| GO:0048523 | 0.029 | negative regulation of cellular process |
| GO:0031349 | 0.029 | positive regulation of defense response |
| GO:0050729 | 0.029 | positive regulation of inflammatory response |
| GO:0007049 | 0.0306 | cell cycle |
| GO:0048519 | 0.0356 | negative regulation of biological process |
| GO:0007154 | 0.0404 | cell communication |
| GO:0043066 | 0.0465 | negative regulation of apoptosis |
| GO:0043069 | 0.0471 | negative regulation of programmed cell death |

| | | |
|---|---|---|
| GO:0048468 | 0.0471 | cell development |

**Table A3 B. Over-represented GO terms in the AHR-only set. P-values are corrected for multiple testing**

| GO term | P-value | Description |
|---|---|---|
| GO:0032502 | 9.77e-16 | developmental process |
| GO:0044424 | 3.25e-15 | intracellular part |
| GO:0044464 | 2.13e-14 | cell part |
| GO:0016043 | 5.55e-13 | cellular component organization and biogenesis |
| GO:0043229 | 6.93e-13 | intracellular organelle |
| GO:0043226 | 6.93e-13 | organelle |
| GO:0007267 | 3.28e-12 | cell-cell signalling |
| GO:0005622 | 6.12e-12 | intracellular |
| GO:0048518 | 1.55e-11 | positive regulation of biological process |
| GO:0032501 | 1.55e-11 | multicellular organismal process |
| GO:0048869 | 2.17e-11 | cellular developmental process |
| GO:0030154 | 2.17e-11 | cell differentiation |
| GO:0043231 | 4.55e-11 | intracellular membrane-bound organelle |
| GO:0043227 | 4.55e-11 | membrane-bound organelle |
| GO:0005515 | 9.41e-10 | protein binding |
| GO:0048522 | 1.3e-09 | positive regulation of cellular process |
| GO:0048856 | 3.48e-09 | anatomical structure development |
| GO:0019226 | 1.22e-08 | transmission of nerve impulse |
| GO:0007275 | 4.27e-08 | multicellular organismal development |
| GO:0005737 | 5.34e-08 | cytoplasm |
| GO:0065007 | 1.13e-07 | biological regulation |
| GO:0048468 | 1.54e-07 | cell development |

| | | |
|---|---|---|
| GO:0050789 | 2.31e-07 | regulation of biological process |
| GO:0009653 | 3.68e-07 | anatomical structure morphogenesis |
| GO:0005634 | 5.28e-07 | nucleus |
| GO:0007154 | 9.73e-07 | cell communication |
| GO:0044446 | 1.06e-06 | intracellular organelle part |
| GO:0044422 | 1.21e-06 | organelle part |
| GO:0008219 | 1.59e-06 | cell death |
| GO:0016265 | 1.59e-06 | death |

**Table A3 C. Over-represented GO terms in the ERα-only set. P-values are corrected for multiple testing**

| GO term | P-value | Description |
|---|---|---|
| GO:0043231 | 5.37e-17 | intracellular membrane-bound organelle |
| GO:0043227 | 5.37e-17 | membrane-bound organelle |
| GO:0044424 | 5.37e-17 | intracellular part |
| GO:0043229 | 2.18e-16 | intracellular organelle |
| GO:0043226 | 2.18e-16 | organelle |
| GO:0005737 | 6.91e-15 | cytoplasm |
| GO:0044444 | 6.96e-12 | cytoplasmic part |
| GO:0005622 | 8.97e-12 | intracellular |
| GO:0048856 | 1.51e-11 | anatomical structure development |
| GO:0044446 | 1.51e-11 | intracellular organelle part |
| GO:0044422 | 1.67e-11 | organelle part |
| GO:0044464 | 1.05e-10 | cell part |
| GO:0032502 | 2.53e-09 | developmental process |
| GO:0005515 | 1.24e-07 | protein binding |
| GO:0048523 | 1.95e-07 | negative regulation of cellular process |

| | | |
|---|---|---|
| GO:0009653 | 2.06e-07 | anatomical structure morphogenesis |
| GO:0048731 | 3.78e-07 | system development |
| GO:0009892 | 4.9e-07 | negative regulation of metabolic process |
| GO:0048519 | 7.45e-07 | negative regulation of biological process |
| GO:0007275 | 1.02e-06 | multicellular organismal development |
| GO:0031090 | 1.61e-06 | organelle membrane |
| GO:0065007 | 2.19e-06 | biological regulation |
| GO:0048513 | 3.72e-06 | organ development |
| GO:0008134 | 3.72e-06 | transcription factor binding |
| GO:0050789 | 5.22e-06 | regulation of biological process |
| GO:0045934 | 6e-06 | negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic |
| GO:0016481 | 8.1e-06 | negative regulation of transcription |
| GO:0032501 | 8.52e-06 | multicellular organismal process |
| GO:0050794 | 1.41e-05 | regulation of cellular process |
| GO:0031324 | 1.55e-05 | negative regulation of cellular metabolic process |

# *17* List of Publications and Abstracts

## 17.1 Referred Publications

- **Ahmed, S.,** Al-Saigh, S., & Matthews, J. (2012) FOXA1 is essential for the aryl hydrocarbon receptor-depedent regulation of cyclin G2. *Mol. Cancer Res.* **5,** 636-648

- **Ahmed, S.,** Valen, E., Sandelin, A., & Matthews J. (2009) Dioxin increases the interaction between aryl hydrocarbon receptor and estrogen receptor alpha at human promoters. *Toxicol. Sci.* **111,** 254-266
    - **Honourable mention for Paper of Year by the Society of Toxicology**

- Pansoy A., **Ahmed S.,** Valen E., Sandelin A., & Matthews J. (2010) 3-Muethylcholanthrene induces differential recruitment of aryl hydrocarbon receptor to human promoters. *Toxicol. Sci.* **117,** 90-100

- Lo R., Burgoon L., MacPherson L., **Ahmed S.,** & Matthews (2010) Estrogen receptor-dependent regulation of CYP2B6 in human breast cancer cells. *Biochim. Biophys. Acta*. **1799(5-6),** 469-479

- Wihlen B., **Ahmed S.,** Inzunza J., & Matthews J., (2009) Estrogen receptor subtype- and promoter-specific modulation of aryl hydrocarbon receptor-dependent transcription. *Mol. Cancer Res.* **7,** 977-86

- MacPherson L., Lo R., **Ahmed S.,** Pansoy A., & Matthews J. (2009) Activation function 2 mediates dioxin-induced recruitment of estrogen receptor alpha to CYP1A1 and CYP1B1. *Biochem. Biophys. Res. Commun.* **385,** 263-8

## 17.2 Referred Conference Abstracts

- **Ahmed, S.,** Lo, R., Celius, T., & Matthews, J., (2012) Zinc finger mediated knockout of the aryl hydrocarbon receptor in human breast cancer cells depletes constitutive cytochrome P450 1B1 levels. Supp. Toxicol Sci. 51$^{st}$ Annual SOT Meeting San Fransisco

- **Ahmed, S.,** Al-Saigh S., & Matthews J. (2011) FOXA1 is essential for the AHR-dependent regulation of cyclin G2. 7$^{th}$ Duesseldorf symposium on immunotoxicology-Biology of the Aryl Hydrocarbon receptor.

- **Ahmed, S.,** Al-Saigh S., & Matthews J. (2011) FOXA1 is essential for the AHR-dependent regulation of cyclin G2. *Supp. Toxicol. Sci.* 50$^{th}$ Annual SOT Meeting Washington D.C.

- **Ahmed, S.,** MacPherson, L. Pansoy, A. & Jason Matthews (2009) ChIP-chip analysis of TCDD activated aryl hydrocarbon receptor binding to human promoter tiling arrays identifies genomic binding signature and novel gene targets for AHR. *Supp. Toxicol. Sci.* **102**, 110

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| Title of your work | The molecular mechanisms of aryl hydrocarbon signal transduction and crosstalk with estrogen receptor alpha |
| Publisher of your work | n/a |
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