2,3,7,8-TETRACHLORODIBENZO-\(p\)-DIOXIN-INDUCIBLE POLY(ADP-RIBOSE) POLYMERASE IS A MONO-ADP-RIBOSYLTRANSFERASE AND A LIGAND-INDUCED REPRESSOR OF AHR TRANSACTIVATION

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Pharmacology and Toxicology
University of Toronto

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2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible poly(ADP-ribose) polymerase is a mono-ADP-ribosyltransferase and a ligand-induced repressor of AHR transactivation

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2014

Abstract

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible poly(ADP-ribose) polymerase (TiPARP/ARTD14) is a member of the ARTD family and is regulated by the aryl hydrocarbon receptor (AHR); however, little is known about TiPARP function. In this study we examined the catalytic function of TiPARP and determined its role in AHR transactivation. We observed that TiPARP exhibited auto-mono-ADP-ribosyltransferase activity and ribosylated core histones. RNAi-mediated knockdown of TiPARP in T47D breast cancer cells increased TCDD-dependent cytochrome P450 1A1 (CYP1A1) and CYP1B1 mRNA expression and recruitment of AHR to both genes. Overexpression of TiPARP reduced AHR-dependent increases in CYP1A1-reporter gene activity, which was restored by overexpression of AHR, but not ARNT. Deletion and mutagenesis studies showed that TiPARP-mediated inhibition of AHR required the zinc finger and catalytic domains. TiPARP and AHR co-localized in the nucleus, directly interacted and both were recruited to CYP1A1 in response to TCDD. Overexpression of TiPARP enhanced whereas RNAi-mediated knockdown of TiPARP reduced TCDD-dependent AHR proteolytic degradation. TCDD-dependent induction of AHR target genes was also enhanced in Tiparp−/− mouse embryonic fibroblasts compared to wildtype controls. Moreover, livers excised from
TCDD-treated Tiparp⁺/⁻ mice displayed significantly greater AHR target gene expression compared with wildtype or heterozygous mice. Comparison of TiPARP to a known negative regulator of AHR, AHR repressor (AHRR), revealed that TiPARP and AHRR had some notable similarities but also differences between their mechanisms of repression. Similar to TiPARP, AHRR was recruited to AHR target regulatory regions in response to TCDD and its overexpression repressed reporter gene activity. However unlike TiPARP, knockdown of the AHRR did not affect AHR transactivation or its proteasomal degradation. Despite some mechanistic similarities, our data suggest that TiPARP and AHRR independently repress AHR transactivation. Overall, our findings show that TiPARP is a mono-ADP-ribosyltransferase and a transcriptional repressor of AHR, revealing a novel negative feedback loop controlling AHR function.
Acknowledgments

There are many people who deserve recognition for their contributions to this thesis.

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<th>Description</th>
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<tbody>
<tr>
<td>3MC</td>
<td>3-methylcholanthrene</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ADPF</td>
<td>AHR degradation promoting factor</td>
</tr>
<tr>
<td>ARTD</td>
<td>ADP-ribosyltransferase diphtheria toxin-like</td>
</tr>
<tr>
<td>AHR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AHRE</td>
<td>aryl hydrocarbon receptor response element</td>
</tr>
<tr>
<td>AHRR</td>
<td>aryl hydrocarbon receptor repressor</td>
</tr>
<tr>
<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>ARH</td>
<td>ADP-ribosylhydrolase</td>
</tr>
<tr>
<td>B[a]P</td>
<td>benzo[a]pyrene</td>
</tr>
<tr>
<td>BAL</td>
<td>B-aggressive lymphoma</td>
</tr>
<tr>
<td>bHLH-PAS</td>
<td>basic Helix-Loop-Helix-Period, Aryl hydrocarbon receptor nuclear translocator Single minded</td>
</tr>
<tr>
<td>BRCT</td>
<td>BRCA1-carboxy terminus-like</td>
</tr>
<tr>
<td>Brg-1</td>
<td>brahma-related gene 1</td>
</tr>
<tr>
<td>CA-AHR</td>
<td>constitutively active aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>cytochrome P450 1A1-human</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>cytochrome P450 1B1-human</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<tr>
<td>e-MART</td>
<td>ecto-mono-ADP-ribosyltransferase</td>
</tr>
<tr>
<td>ERα</td>
<td>estrogen receptor α</td>
</tr>
<tr>
<td>ERβ</td>
<td>estrogen receptor β</td>
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<tr>
<td>FICZ</td>
<td>6-formalindolo-[3,2-b]carbazole</td>
</tr>
<tr>
<td>G6Pase</td>
<td>glucose-6-phosphatase</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
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<tr>
<td>HAH</td>
<td>halogenated aromatic hydrocarbon</td>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HES1</td>
<td>hairy and enhancer of split 1</td>
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<tr>
<td>Hsp90</td>
<td>90 kDa heat shock protein</td>
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<td>IC3</td>
<td>indole-3-carbinol</td>
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<td>ICZ</td>
<td>indolo[3,2-b]carbazole</td>
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<tr>
<td>LBD</td>
<td>ligand binding domain</td>
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<td>MART</td>
<td>mono-ADP-ribosyltransferase</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NASH</td>
<td>non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NCoA</td>
<td>nuclear co-activator</td>
</tr>
<tr>
<td>NCoR</td>
<td>nuclear receptor co-repressor</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export signal</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B-cells</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<td>NQO1</td>
<td>NAD(P)H: quinone oxidoreductase 1</td>
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<tr>
<td>NFE2L2</td>
<td>nuclear factor (erythroid-derived 2)-like 2</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>PAR</td>
<td>poly(ADP-ribose)</td>
</tr>
<tr>
<td>PARG</td>
<td>poly(ADP-ribose) glycohydrolase</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PCB</td>
<td>polychlorinated biphenyl</td>
</tr>
<tr>
<td>PCG1α</td>
<td>peroxisome proliferator-activated receptor gamma co-activator 1 alpha</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>RIP140</td>
<td>140 kDa receptor interacting protein</td>
</tr>
<tr>
<td>siRNA</td>
<td>small inhibitory RNA</td>
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<tr>
<td>SHP</td>
<td>short heterodimer partner</td>
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<tr>
<td>SIRT</td>
<td>sirtuin</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator of retinoid and thyroid hormone receptor</td>
</tr>
<tr>
<td>Sp1</td>
<td>stimulating protein 1</td>
</tr>
<tr>
<td>TAD</td>
<td>transcriptional activation domain</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TIF2</td>
<td>translation initiation factor 2</td>
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<tr>
<td>TiPARP</td>
<td>TCDD-inducible poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real-time PCR</td>
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<td>XAP2</td>
<td>hepatitis B virus X-associated protein 2</td>
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<tr>
<td>WWE</td>
<td>tryptophan-tryptophan-glutamate</td>
</tr>
<tr>
<td>ZAP</td>
<td>zinc finger antiviral protein</td>
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<tr>
<td>Zn</td>
<td>zinc finger</td>
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Chapter 1: Introduction

1 Statement of Research Problem

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible poly(ADP-ribose) polymerase (TiPARP) is a member of the diphtheria-like ADP-ribosyltransferase (ARTD) family of nicotinamide adenine dinucleotide (NAD⁺)-catabolizing enzymes, formerly known as the poly(ADP-ribose) polymerase (PARP) family. ARTD family members are best known for their ability to catalyze ADP-ribosylation reactions, a process that uses NAD⁺ as a substrate to add ADP-ribose onto target proteins. ADP-ribosylation has been implicated in numerous cellular processes including DNA damage responses, inflammation, apoptosis and transcriptional regulation. The function of ARTD1 (also known as PARP1), the founding ARTD, is well established; however, the potential function(s) of the majority of other ARTD members are just starting to be understood.

Currently, little is known about TiPARP/ARTD14 (will be referred to as TiPARP within this thesis). As a class 2 ARTD, TiPARP is predicted to be a mono-ADP-ribosyltransferase (MART); however, this has not been experimentally determined. As its name suggests TiPARP gene expression is induced by TCDD. The ligand-activated transcription factor, the aryl hydrocarbon receptor (AHR), is activated by TCDD to regulate its target genes. TiPARP expression is regulated by numerous AHR ligands in both in vitro and in vivo models, demonstrating it to be an important AHR regulated gene. Despite being an important AHR target gene, the potential function of TiPARP in AHR-mediated transcriptional regulation has not been investigated.

While the activation of AHR transcription has been extensively studied, its negative regulation has not received equal attention and is poorly understood. Proposed mechanisms of the negative regulation of AHR include the targeted proteasomal degradation of activated-AHR and induction of repressive factors such as the AHR repressor (AHRR) to down-regulate AHR-mediated transactivation; however, these models are controversial and not fully understood.

In the present study we set out to characterize TiPARP and determine its role in AHR transactivation.
2 ADP-ribosylation Reactions

2.1 Historical Overview

The presence of poly(ADP-ribose) was first described fifty years ago by Chambon et al. (1963) who discovered that the addition of nicotinamide adenine dinucleotide (NAD\(^+\)) to hen liver extracts stimulated the synthesis of polyadenylic acid which was later identified as poly(ADP-ribose) (Chambon, et al., 1963). This discovery launched the research of poly(ADP-ribose) metabolism. The structure of poly(ADP-ribose) (PAR) was later determined by three independent laboratories (Doly and Mandel, 1967; Reeder, et al., 1967; Sugimura, et al., 1967).

The enzyme responsible for synthesizing PAR was termed poly(ADP-ribose) polymerase or PARP. The gene encoding PARP (also referred to as poly-ADP-synthase or poly-ADP-ribosyltransferase) was isolated in the late 1980s (Alkhatib, et al., 1987; Kurosaki, et al., 1987; Uchida, et al., 1987). Today, this gene is known as PARP1 and for many years PARP1 was thought to be the only enzyme with ADP-ribosylation activity in mammalian cells. Fifteen years later, five different genes encoding PARP enzymes were identified indicating PARP1 belongs to a family of enzymes (Ame, et al., 1999; Johansson, 1999; Kaminker, et al., 2001; Kickhoefer, et al., 1999; Smith, et al., 1998). To date, eighteen human PARP genes have been cloned or described based on exhaustive searches of non-redundant protein databases using the PARP1 catalytic domain. However, the PAR synthesizing activity of only a few of these putative PARPs has been verified (Aguiar, et al., 2005; Aguiar, et al., 2000; Ame, et al., 2004; Ma, et al., 2001; Otto, et al., 2005; Yu, et al., 2004).

Not long after the structure of PAR was determined, mono-ADP-ribosylation reactions were discovered during studies of bacterial toxins such as diphtheria, pertussis and cholera toxins (Corda and Di Girolamo, 2003; Di Girolamo, et al., 2005; Holbourn, et al., 2006). These enzymes were found to be mono-ADP-ribosyltransferases (MARTs) (Gill, et al., 1969; Honjo, et al., 1968). Subsequently, extracellular MARTs or e-MARTs (or ecto-MARTs) were identified in mammalian cells and their enzymatic activities characterized (Haag and Koch-Nolte, 1998; Okazaki and Moss, 1996; Okazaki and Moss, 1999; Seman, et al., 2004). Several reports have predicted distinct families of mono-ADP-ribosylating enzymes with no obvious sequence similarity to e-MARTs (Corda and Di Girolamo, 2002; Corda and Di Girolamo, 2003; Seman, et al., 2004). Members of the sirtuin family (SIRTs) of NAD\(^+\)-dependent histone deacetylases were
reported to contain mono-ADP-ribosyltransferase activities and represent a potential family of intracellular mammalian MARTs (Frye, 1999; Garcia-Salcedo, et al., 2003; Haigis, et al., 2006; Liszt, et al., 2005; Tanny, et al., 1999). However, more recently twelve PARP family members have been predicted to be mono-ADP-ribosylating rather than poly-ADP-ribosylating enzymes, based on subtle differences within their catalytic domains (Hottiger, et al., 2010; Kleine, et al., 2008). These twelve PARP-like MARTs represent potential candidates of a family of intracellular MART and only recently are their enzymatic activities being verified. A new nomenclature for the PARP family has been proposed that reflects their true transferase rather than polymerase activity, renaming it to ADP-ribosyltransferase diphtheria toxin-like family or ARTD that takes into account their resemblance to bacterial mono-ADP-ribosylating enzymes (Hottiger, et al., 2010).

2.2 NAD⁺ Metabolism

In eukaryotes, NAD⁺ is best known for its essential role as a coenzyme/transmitter molecule in bioenergetic processes such as cellular metabolism and respiration (Magni, et al., 2004; Ziegler, 2000). The synthesis of adenosine triphosphate (ATP) and balance of redox potential are directly dependent on cellular NAD⁺ levels. NAD⁺ serves as an electron acceptor and its reduced form NADH as an electron donor in reactions catalyzed by enzymes of the mitochondrial electron transport chain leading to the generation of ATP through oxidative phosphorylation (Pollak, et al., 2007; Rich, 2003).

In addition to its vital role in energy metabolism, NAD⁺ is utilized by ADP-ribosyltransferases to transfer ADP-ribose to a substrate protein or to itself (Hassa, et al., 2006). In mono-ADP-ribosylation reactions, NAD⁺ is hydrolyzed by mono-ADP-ribosyltransferases and the ADP-ribose moiety is transferred to an acceptor protein (the enzyme itself or a target protein) and the vitamin nicotinamide is released (Figure 1). Poly-ADP-ribosylation is similar to mono-ADP-ribosylation but also includes an additional elongation reaction where the ADP-ribose units are linked by glycosidic ribose (1’-2’) ribose bonds creating long, irregular, branched polymers of ADP-ribose (Desmarais, et al., 1991; Kawaichi, et al., 1981; Mendoza-Alvarez and Alvarez-Gonzalez, 2004; Miwa, et al., 1981) (Figure 2). Polymers can reach 200-400 ADP-ribose units in vitro and in vivo and long polymers are branched with a frequency of approximately one branch point per linear section of 20-50 ADP-ribose units (Alvarez-Gonzalez and Jacobson,
1987; Juarez-Salinas, et al., 1982; Juarez-Salinas, et al., 1983; Kanai, et al., 1982; Miwa, et al., 1981). Poly-ADP-ribosylation is proposed to be the most important factor affecting the maintenance of the NAD$^+$ pool in cells (Elliott and Rechsteiner, 1975; Rechsteiner, et al., 1976; Williams, et al., 1985). The catabolism of NAD$^+$ in mammalian cells occurs primarily through poly-ADP-ribosylation reactions (Berger, et al., 2004). Activation of poly-ADP-ribosylation reactions, coinciding with increased poly-ADP-ribose polymer generation depletes intracellular NAD$^+$ levels to 10-20% of their normal levels within 5-15 min upon initiation of stimulus (Goodwin, et al., 1978; Skidmore, et al., 1979). Cellular NAD$^+$ depletion consequently results in ATP depletion, since NAD$^+$ is an essential coenzyme/transmitter for the generation of ATP through oxidative phosphorylation (Berger, 1985). Because NAD$^+$ connects bioenergetics to ADP-ribosylation reactions and cellular NAD$^+$ levels have important physiological consequences in the regulation of multiple cellular processes, the control of NAD$^+$-dependent enzymes must be tightly regulated.
Figure 1. Schematic representation of the mono-ADP-ribosylation reaction.

The chemical structure of the mono-ADP-ribosylation reaction to an acceptor protein is shown. Mono-ADP-ribosyltransferase (MART) catalyzes the addition of ADP-ribose to an acceptor protein using nicotinamide adenine dinucleotide (NAD\(^+\)) as a precursor resulting in the release of nicotinamide. ADP-ribosylhydrolases (ARH) remove the ADP-ribose moiety from an acceptor protein.
Figure 2. Metabolism of poly(ADP-ribose).

PARPs hydrolyse NAD\(^+\) and catalyze the successive addition of ADP-ribose units to acceptor proteins. Poly-ADP-ribose size and complexity is indicated by the x and y labels that represent values from 0 to >200. Poly-ADP-ribose-glycohydrolase (PARG) and ADP-ribosylhydrolase 3 (ARH3) can both hydrolyze poly-ADP-ribose at the indicated positions. ADE; adenine, NAM; nicotinamide, Rib; ribose. Modified from (Hakme, et al., 2008).
2.3 Mono-ADP-ribosylation

Mono-ADP-ribosylation of proteins is a phylogenetically ancient and reversible posttranslational modification. This modification was originally identified as an important aspect of bacterial physiopathology, catalyzed by several toxins including diphtheria, pertussis and cholera toxins (Corda and Di Girolamo, 2003; Di Girolamo, et al., 2005; Holbourn, et al., 2006). So far, six subclasses of bacterial MARTs have been identified based on amino acid sequence identity (Hassa, et al., 2006). The amino acid residues of host cell acceptor proteins modified by bacterial MARTs include: arginine, asparagine, glutamate, aspartate, cysteine and modified histidine (diphthamide) (Corda and Di Girolamo, 2002; Corda and Di Girolamo, 2003; Okazaki and Moss, 1996; Okazaki and Moss, 1999). Diphtheria toxin can be used as an example of the specificity of mono-ADP-ribosylation and the important physiological consequences of a single residue modification by a MART. Diphtheria toxin, is an exotoxin secreted by the bacterium Corynebacterium diphtheria, which mono-ADP-ribosylates host cell elongation factor 2 (eEF2) at diphthamide residue 699 blocking the interaction of eEF2 with other factors involved in translation and effectively preventing host cell protein synthesis (Collier, 2001).

Mono-ADP-ribosylation reactions in multicellular eukaryotes were later discovered. Endogenous MART activities that modify arginine, glutamate, cysteine, phosphoserine and potentially aspartate and asparagine on acceptor proteins have been detected in mammalian cells (Corda and Di Girolamo, 2003; Okazaki and Moss, 1996; Ord and Stocken, 1977; Seman, et al., 2004; Smith and Stocken, 1975). Intracellular mono-ADP-ribosylation in mammalian cells has been suggested to play important roles in processes such as: regulation of intracellular signalling cascades, transcriptional regulation, unfolded protein response, DNA repair, insulin secretion, immunity and cellular differentiation and proliferation (Corda and Di Girolamo, 2003; Di Girolamo, et al., 2005; Feijs, et al., 2013; Koch-Nolte, et al., 2008).

2.3.1 Mono-ADP-ribosyltransferase families

2.3.1.1 ecto-MARTs (e-MARTs)

Human and mouse e-MARTs that have been identified are glycosylphosphatidylinositol-anchored surface proteins or secretory proteins (Glowacki, et al., 2002; Koch-Nolte, et al., 2008; Okazaki and Moss, 1999). E-MARTs transfer ADP-ribose to target proteins within the
extracellular compartment and are involved in cell communication (Koch-Nolte, et al., 2008). Five e-MARTs (e-MART-1 to 5) have been identified in mammalian cells. Humans only express four e-MARTs due to a defective e-MART-2 gene while mice express six e-MARTs as a result of a duplication of the e-MART-2 gene (Seman, et al., 2004). e-MART-1, -2a, -2b and -5 transfer ADP-ribose to arginine residues of extracellular target proteins on cell surfaces, or circulating in body fluids (Corda and Di Girolamo, 2003; Glowacki, et al., 2002; Seman, et al., 2004). E-MART-3 is expressed on the surface of unstimulated human monocytes whereas e-MART-4 is only expressed on the cell surface once monocytes are stimulated by lipopolysaccharide (Grahnert, et al., 2002). Cell surface mono-ADP-ribosylated proteins on human monocytes are covalently modified at cysteine residues, suggesting that e-MART-3 and -4 may be cysteine-specific e-MARTs (Grahnert, et al., 2002).

2.3.1.2 Sirtuins

Silent information regulator SIR2-like proteins (sirtuins or SIRTs) are a conserved family of NAD⁺-dependent protein deacetylases (SIRT1 to 7) present in eukaryotes, prokaryotes and archaea (Blander and Guarente, 2004; Denu, 2005; Grubisha, et al., 2005). The SIRT family regulates a broad range of cellular processes including development, metabolism, DNA repair, DNA recombination, cellular differentiation, transcriptional regulation, apoptosis and lifespan (Blander and Guarente, 2004; Denu, 2005; Grubisha, et al., 2005). Most mammalian SIRTs are reported to exhibit in vivo and in vitro histone deacetylation activity (Blander and Guarente, 2004; Denu, 2005; Michishita, et al., 2005). Of the seven mammalian SIRTs, SIRTs 1, 2, 3 and 5 have been shown to have NAD⁺-dependent deacetylase activities in vitro (Blander and Guarente, 2004; Denu, 2005; Michishita, et al., 2005). SIRTs also have non-histone substrates and not all mammalian SIRTs localize to the nucleus.

The NAD⁺-dependent deacetylation reaction is an unusual mechanism performed by SIRTs where NAD⁺ is hydrolyzed into nicotinamide and ADP-ribose (Abdellatif, 2012). The ADP-ribose moiety is essential for the deacetylation reaction and serves as the acceptor of the acetyl group removed from the target protein forming the intermediates 2'-O-AADP-ribose and 3'-O-AADP-ribose (Smith and Denu, 2006; Zhao, et al., 2003; Zhao, et al., 2004). Several reports have suggested mammalian SIRT family members to contain mono-ADP-ribosyltransferase activities. SIRTs 1, 2 and 6 were reported to transfer mono-ADP-ribose to bovine serum albumin
and histones in vitro (Frye, 1999; Mostoslavsky, et al., 2006; Tanny, et al., 1999). SIRT6 has auto-mono-ADP-ribosylation (or self ribosylation) but does not possess deacetylase activity indicating enzymatic mechanisms of each SIRT may differ (Lisz, et al., 2005). Currently, the potential mono-ADP-ribosyltransferase activities of the different SIRT family members has not been fully examined and further investigation is required to determine if indeed the SIRTs are a class of bona fide intracellular mono-ADP-ribosyltransferases (Denu, 2005).

2.3.2 ADP-ribosylhydrolases

ADP-ribosylhydrolases (ARH) reverse mono-ADP-ribosylation reactions by catalyzing the removal of ADP-ribose from modified proteins (Moss, et al., 1992). The best characterized ADP-ribosylhydrolase is ADP-ribosylhydrolase 1 (ARH1), which specifically hydrolyzes ADP-ribose-arginine bonds, leading to the release of free mono-ADP-ribose and regeneration of the guanidino group of arginine (Moss, et al., 1986; Takada, et al., 1993). In mammalian cells, most ARH1 activities are cytosolic, although some are also located on the cell surface (Hassa, et al., 2006). Besides ARH1, additional unidentified proteins exhibiting ADP-ribose lyase activities have been reported, which are thought to function on glutamate, lysine or cysteine residues, releasing a deoxy form of ADP-ribose (Oka, et al., 1984; Okayama, et al., 1978; Tanuma and Endo, 1990). In silico screening revealed two ARH-like genes, ARH2 and ARH3 homologous to the human ARH1 gene (Glowacki, et al., 2002). ARH2 does not appear to possess hydrolase activity, but ARH3 was shown to hydrolyze poly(ADP-ribose) but not mono-ADP-ribose (see section 2.4.2.2)(Oka, et al., 2006).

Recently, macrodomain-containing proteins were discovered to catalyze the hydrolysis of mono-ADP-ribose from modified proteins (Jankevicius, et al., 2013; Rosenthal, et al., 2013; Sharifi, et al., 2013). Macrodomains are a family of evolutionarily conserved proteins that bind mono- or poly-ADP-ribose, polyadenylation or O-acetyl-ADP-ribose (Han, et al., 2011; Karras, et al., 2005; Kim, et al., 2012; Neuvonen and Ahola, 2009). Human proteins MacroD1, MacroD2 and C6orf130 hydrolyzed mono-ADP-ribose from modified proteins at acidic residues (Jankevicius, et al., 2013; Rosenthal, et al., 2013). MacroD-like hydrolases inactivated MART target proteins by removing mono-ADP-ribose and rendering them inactive and thus form the functional antagonists of intracellular MARTs (Rosenthal, et al., 2013). These findings define
macrodomain-containing proteins as a novel group of specific mono-ADP-ribosylhydrolases and establish mono-ADP-ribosylation as a fully reversible posttranslational modification.

### 2.4 Poly-ADP-ribosylation

Poly-ADP-ribosylation was discovered in multicellular eukaryotes and appears to be less widely used compared to mono-ADP-ribosylation (Hassa, et al., 2006). Poly-ADP-ribosylation is a rapid and reversible posttranslational modification in which linear and branched sequences of ADP-ribose units are covalently attached onto acceptor proteins (Otto, et al., 2005). DNA strand breakage was long considered to be the main trigger of poly-ADP-ribosylation of proteins; however, more recent studies suggest poly-ADP-ribosylation is important in a much broader spectrum of cellular and molecular functions including regulation of chromatin structure, transcriptional regulation, stress response, inflammation, cell proliferation and differentiation, apoptosis and maintenance of genome stability (Hakme, et al., 2008; Kraus, 2008; Krishnakumar and Kraus, 2010b; Luo and Kraus, 2012). Poly-ADP-ribosylation reactions are mediated by some members of the ARTD family that catalyze the transfer of ADP-ribose units from NAD$^+$ to form a polymer of ADP-ribose units onto glutamate, aspartate and lysine residues of acceptor proteins (Hakme, et al., 2008). ARTD1 (formerly known as PARP1), the founding and best-studied ARTD family member, was first thought to be the only enzyme in mammalian cells to generate poly(ADP-ribose) polymers. However in recent years, *in silico* studies have identified seventeen other proteins that share a catalytic PARP domain with PARP1 and that may contain poly-ADP-ribosylation activity (Ame, et al., 2004; Otto, et al., 2005).

#### 2.4.1 ADP-ribosyltransferase diphtheria toxin-like (ARTD) family

##### 2.4.1.1 Structural features and nomenclature

ARTDs are a family of ADP-ribosyltransferases that consists of eighteen members all encoded by different genes. All ARTD family members contain a conserved catalytic domain homologous to the ARTD1 catalytic domain (Ame, et al., 2004). Besides the catalytic domain, a wide spectrum of modular domains is present in the different ARTD family members (*Figure 3*), suggesting that these members are involved diverse physiological responses (Kleine, et al., 2008). The catalytic domain is located at the C-terminus of the protein (except in ARTD4) other modular domains are involved in DNA or RNA binding, protein-protein interactions or cell...
signalling (Schreiber, et al., 2006). The catalytic domain binds NAD\(^+\) via a protein fold termed the ‘PARP signature’ that shares homology with bacterial mono-ADP-ribosylating exotoxins, such as diphtheria toxin (Gibson and Kraus, 2012). Based on both structural homology of the diphtheria toxin in complex with NAD\(^+\) and \textit{in silico} characterization of ARTDs, histidine and tyrosine (H862 and Y896 in ARTD1) are critical for the proper positioning the ribose moiety of NAD\(^+\) for ADP-ribosylation (Bell and Eisenberg, 1996; Otto, et al., 2005). In poly-ADP-ribosylating ARTDs, a catalytic glutamate (E988 in ARTD1) is essential for the catalysis of additional ADP-ribose units or poly-ADP-ribose chain elongation onto acceptor residues (Marsischky, et al., 1995; Rolli, et al., 1997; Ruf, et al., 1998). These three residues, histidine-tyrosine-glutamate, make up the catalytic ‘HYE’ triad that appears in all \textit{bona fide} poly-ADP-ribosylating ARTDs (Gibson and Kraus, 2012). The absence of the catalytic glutamate of the ARTD catalytic domain restricts activity to mono-ADP-ribosylation (Kleine, et al., 2008; Marsischky, et al., 1995; Rolli, et al., 1997; Ruf, et al., 1998). Other structural features such as the loop length between \(\beta\)-4 and \(\beta\)-5 sheets of the catalytic core have been correlated with lack of poly-ADP-ribosylation activity (Han and Tainer, 2002; Kleine, et al., 2008; Ruf, et al., 1998; Sun, et al., 2004). Because the HYE triad of ARTDs is most similar to that of diphtheria toxin, PARPs were renamed diphtheria-like ADP-ribosyltransferases, abbreviated ARTDs, which better reflects their transferase rather than polymerase activity (Hottiger, et al., 2010). Of the eighteen ARTD family members only six contain the catalytic glutamate in the HYE triad, while in the remaining twelve the catalytic glutamate is replaced by isoleucine, leucine, threonine, valine or tyrosine residues, suggesting that they will exhibit mono- rather than poly-ADP-ribosyltransferase activity (Otto, et al., 2005). ARTD9 and ARTD13 lack both the catalytic glutamate as well as the histidine of the HYE triad and are predicted to be catalytically inactive (Otto, et al., 2005). Thus ARTDs can be divided into three classes based on their catalytic activities (\textbf{Table 1}). Class 1 ARTDs are poly-ADP-ribosyltransferases, class 2 are mono-ADP-ribosyltransferases and class 3 are catalytically inactive.
<table>
<thead>
<tr>
<th>Class</th>
<th>Transferase Name</th>
<th>PARP Name</th>
<th>Alternative Name</th>
<th>Triad Motif</th>
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<td>PARP1</td>
<td>HYE</td>
<td>P &amp; B</td>
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</tr>
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<td>P &amp; B</td>
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<td>PARP3</td>
<td>HYE</td>
<td></td>
<td>M (P predicted)</td>
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<td>P &amp; O</td>
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<td>HYI</td>
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<td>I</td>
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</tbody>
</table>

*Known or predicted enzymatic activity: mono- (M), oligo- (O) or poly-ADP-ribosylation (P) or branching (B) or inactive (I). (Adapted from (Gibson and Kraus, 2012))
**Figure 3. Domain architecture of the 18 human ARTD family members.**

Schematic comparison of the domain architecture of the human ARTD (PARP) family (adapted from Hottiger, et al., 2010). The following domains are indicated: The ART domain is the catalytic core required for ART activity. Within each ART domain, the region that is homologous to the PARP signature (residues 859–908 of ARTD1) as well as the equivalent of the ARTD1 catalytic E988 is shaded. The PARP regulatory domain (PRD) might be involved in regulation of the ADP-ribose branching activity. The WGR domain named after a conserved central motif (W-G-R) is also found in a variety of polyA polymerases and in proteins of unknown function. The BRCT domain (BRCA1 carboxy-terminal domain) is found within many DNA damage repair and cell cycle checkpoint proteins. The sterile alpha motif (SAM), a domain found within many proteins, can mediate homo- or hetero-dimerization. The ankyrin repeat domains (ARD) mediate protein–protein interactions. The vault protein interalphatrypsin (VIT)
and von Willebrand type A (vWA) domains are conserved domains found in all inter-alpha-
trypsin inhibitor family members. Both of these domains are presumed to mediate protein–
protein interactions. The WWE domain is named after three conserved residues (W-W-E), and is
predicted to mediate specific protein–protein interactions. The Macro domains can serve as
ADPr or O-acetyl-ADP-ribose binding module. ZFD: zinc finger domains. SAP:
SAF/Acinus/PIAS-DNA-binding domain, MVP-ID: Major-vault particle interaction domain,
NLS: nuclear localization signal. CLS: centriole-localization signal. HPS: histidine-proline-
serine region. RRM is an RNA-binding/recognition motif. UIM: ubiquitin interaction motif.
MVP-ID: M-vault particle interaction domain. TPH: Ti-PARP homologous domain. GRD:
glycine-rich domain. TMD: transmembrane domain.

2.4.1.1.1 Class 1 ARTDs

**ARTD1/PARP1** is involved in a number of cellular activities including DNA repair, apoptosis,
chromatin remodeling and transcriptional regulation (D'Amours, et al., 1999; Kim, et al., 2005;
Kraus and Lis, 2003; Schreiber, et al., 2006; Tulin and Spradling, 2003). ARTD1 accounts for
approximately 85-90% of mammalian cell poly-ADP-ribosylation activity (Shieh, et al., 1998).
ARTD1 is a nuclear enzyme that is characterized mainly by three modular domains: an N-
terminal DNA binding domain consisting of three zinc finger domains, an automodification
domain and a C-terminal catalytic ADP-ribosyltransferase domain containing the conserved
HYE motif (**Figure 4**)(D'Amours, et al., 1999). The DNA binding domain plays a critical role in
the recognition of DNA strand aberrations and concurrent activation of ARTD1 (Benjamin and
Gill, 1980; D'Amours, et al., 1999; Ikejima, et al., 1990). The automodification domain is
comprised of a BRCA1-carboxy terminus-like module that mediates several protein-ARTD1 and
DNA-ARTD1 interactions and is also auto-ADP-ribosylation domain (de Murcia and Menissier
de Murcia, 1994; de Murcia, et al., 1994; Mazen, et al., 1989; Schreiber, et al., 1995; Smith,
2001). ARTD1 is best known for its function in the base excision repair pathway during DNA
damage (D'Amours, et al., 1999; de Murcia and Menissier de Murcia, 1994). ARTD1 is a DNA
nick-sensor that is activated by single- and double-strand DNA breaks, DNA hairpins, cruciform
DNA binding by ARTD1 leads to a conformational change of the enzyme, followed by extensive
auto-ADP-ribosylation and hetero-ADP-ribosylation of histones H1 and H2B (Langelier, et al.,
ARTD1-dependent ADP-ribosylation has two effects. One, the poly(ADP-ribose) chains displace histones leading to decondensation of chromatin (Poirier, et al., 1982; Zahradka and Ebisuzaki, 1982) and two, the poly(ADP-ribose) chains function as a binding platform for DNA repair enzymes (Dantzer, et al., 2000; El-Khamisy, et al., 2003; Leppard, et al., 2003). The accumulation of negative charges added to ARTD1 through auto-ADP-ribosylation leads to an electrostatic repulsion from the DNA and subsequent loss of activity (D'Amours, et al., 1999; Schreiber, et al., 2006). Poly(ADP-ribose) chains are then degraded through hydrolysis by poly(ADP-ribose) glycohydrolase (PARG) (D'Amours, et al., 1999; Zahradka and Ebisuzaki, 1982). Apart from its role in DNA repair ARTD1 is also involved in apoptosis, inflammation and transcriptional regulation (Altmeyer, et al., 2010; Kraus, 2008; Kraus and Lis, 2003; Liaudet, et al., 2002).

**Figure 4. ARTD1 domain architecture.**

ARTD1 uses NAD⁺ to form polymers of ADP-ribose onto acceptor proteins or to itself (automodification). Major domains include: DNA-binding domain, a nuclear localization signal (NLS), an automodification domain containing a BRCT (BRCA1-carboxy terminus-like) motif and a catalytic ADP-ribosyltransferase domain containing the conserved ‘PARP signature’ NAD⁺ fold. Adapted from (David, et al., 2009).

**ARTD2/PARP2** was discovered as a result of the residual DNA-dependent PARP activity in embryonic fibroblast derived from **Artd1**-deficient mice (Ame, et al., 1999; Shieh, et al., 1998).
The ARTD2 catalytic domain displays the highest sequence homology (69% similarity) to that of ARTD1 (Ame, et al., 2004). Similar to ARTD1, ARTD2 is a nuclear protein that is activated by DNA nicks leading to its auto-ADP-ribosylation and synthesis of long branched chains of poly(ADP-ribose) (Ame, et al., 1999; Schreiber, et al., 2002). Both Artd1- and Artd2-deficient mice display sensitivity to DNA damaging agents associated with defects in DNA damage processing and cell cycle progression but to different extents. Artd1-deficient mice and cell lines are more sensitive to high-dose ionizing radiation and alkylating agents, whereas Artd2-deficient mice display hyper-radiosensitivity to low-dose irradiation, demonstrating that ARTD1 and ARTD2 functions are complementary but do not fully overlap (Chalmers, et al., 2004). Double mutant Artd1- and Artd2-deficient mice are embryonically lethal highlighting a critical role in early embryogenesis and underlying partial functional redundancy in maintaining genome integrity (Menissier de Murcia, et al., 2003; Schreiber, et al., 2002). In addition to its role in maintenance of genomic stability and DNA repair, ARTD2 has been implicated in various differentiation processes, including spermatogenesis, adipogenesis and immune cell development (Bai, et al., 2007; Dantzer, et al., 2006; Robert, et al., 2009; Yelamos, et al., 2006).

ARTD3/PARP3 was initially identified as a core component of the centrosome preferentially located at the daughter centriole throughout the cell cycle (Augustin, et al., 2003). ARTD3 overexpression interfered with the G1/S phase cell cycle progression and it was described to interact with ARTD1 at the centrosome (Augustin, et al., 2003). A later study disputed the centrosomal localization of ARTD3 and suggested that ARTD3 localized to the nucleus and associated with polycomb group proteins involved in gene silencing and DNA repair networks including DNA protein kinases, DNA ligase III and IV, Ku70 and Ku80 and ARTD1 (Boehler, et al., 2011; Rouleau, et al., 2007). ARTD3-depleted cells and Artd1−/− and Artd3−/− double knockout mice showed increased hypersensitivity to ionizing radiation suggesting a functional synergy of ARTD3 and ARTD1 in the cellular response to DNA damage (Boehler, et al., 2011). These studies suggested that ARTD3 is an important partner in the maintenance of genomic stability. Auto-ADP-ribosylation and hetero-ADP-ribosylation activities of ARTD3 were initially described as mono-ADP-ribosyltransferase activity (Loseva, et al., 2010); however, a later report has described ARTD3 to possess poly-ADP-ribosyltransferase activity (Boehler, et al., 2011). ARTD3 was described to poly-ADP-ribosylate the mitotic factor NuMA directly and indirectly through ARTD5, suggesting that ARTD3 was required for mitotic spindle integrity during
mitosis (Boehler, et al., 2011). Collectively, these reports implicate ARTD3 in the maintenance of genomic integrity, mitotic spindle integrity and transcriptional repression.

**ARTD4/PARP4/VPARP** is the largest member of the ARTD family and was originally identified as a component of mammalian cytoplasmic ribonucleoprotein complexes called vault particles that have been proposed to be involved in multidrug resistance of human tumours and to function in intracellular transport (Kickhoefer, et al., 1999). ARTD4 associates with two essential proteins of the vault particle, major vault protein (MVP) and telomerase-associated protein (TEP1) (Kickhoefer, et al., 1999). ARTD4 is also present in the nucleus and at the mitotic spindle suggesting that it may play multiple roles not yet identified (Kickhoefer, et al., 1999). The structure of ARTD4 is unusual in that it is the only ARTD member to have its catalytic domain located at the N-terminal portion of the protein. Despite this unique feature, ARTD4 is catalytically active and poly-ADP-ribosylates MVP and as well as itself (Kickhoefer, et al., 1999).

**ARTD5/PARP5a/Tankyrase 1 and ARTD6/PARP5b/Tankyrase 2** are two closely related ARTD family members that share 83% sequence identity (Kaminker, et al., 2001; Kuimov, et al., 2001; Smith, et al., 1998). ARTD5 and ARTD6 have been implicated in a diverse range of functions including telomere maintenance, WNT signalling, mitosis and mediation of insulin stimulated glucose uptake (Chi and Lodish, 2000; Cook, et al., 2002; Dynek and Smith, 2004; Guo, et al., 2012; Hsiao and Smith, 2008; Huang, et al., 2009; Smith and de Lange, 2000; Smith, et al., 1998). ARTD5 was first discovered as a factor that regulated telomere length by binding the negative regulator of telomere length telomeric repeat binding factor 1 (TRF1) (Smith, et al., 1998). ARTD5 was originally named tankyrase 1 due to its interaction with TRF1, the presence of 24 consecutive ankyrin repeats which make up the major portion of the enzyme, and the presence of ARTD catalytic domain (Smith, et al., 1998). ARTD5 catalyzed auto-poly-ADP-ribosylation and poly-ADP-ribosylation of TRF1, implicating ARTD5 in telomere elongation (Smith, et al., 1998). A careful analysis of ARTD5 auto-ADP-ribosylation revealed that ARTD5 synthesized ADP-ribose polymers with an average length of 20 ADP-ribose units but polymers lacked branching (Rippmann, et al., 2002). ARTD6 was also reported to associate with and poly-ADP-ribosylate TRF1, indicating a potential redundant role of ARTD5 and ARTD6 in telomere regulation (Cook, et al., 2002; Kaminker, et al., 2001). ARTD6 also associates with ARTD5 and both enzymes share most of their protein partners including insulin-responsive aminopeptidase
(IRAP), NuMA and 182 kDa tankyrase-binding protein (TAB182) (Kaminker, et al., 2001; Sbodio and Chi, 2002; Sbodio, et al., 2002; Seimiya and Smith, 2002). Artd5- and Artd6-deficient mice do not display observed defects in development; however, the Artd5⁻/⁻ and Artd6⁻/⁻ double knockout was embryonic lethal (Chiang, et al., 2008).

2.4.1.1.2 Class 2 ARTDs

ARTD7/PARP15/BAL3 and ARTD8/PARP14/BAL2 were originally identified as two genes closely related to ARTD9/PARP9/BAL1 (B-aggressive lymphoma 1, see section 2.4.1.1.3) (Aguiar, et al., 2005; Aguiar, et al., 2000). The encoded proteins both contain N-terminal macrodomains and a C-terminal PARP catalytic domain (Aguiar, et al., 2005). Macrodomains are protein domains known to bind mono- and poly-ADP-ribose (Forst, et al., 2013; Han and Tainer, 2002; Kleine and Luscher, 2009; Welsby, et al., 2012). Recently, macrodomains 2 and 3 of ARTD8 were reported to recognize and read mono-ADP-ribosylated ARTD10 and substrates of ARTD10 (Forst, et al., 2013). Both ARTD7 and ARTD8 demonstrated auto-mono-ADP-ribosylation activity (Aguiar, et al., 2005). ARTD7 was reported to have a transcriptionally repressive function through its N-terminal macrodomains and its auto-ADP-ribosylation activity was suggested to counteract the repressive effect of the macrodomains (Aguiar, et al., 2005). Whereas ARTD7 remains poorly characterized, ARTD8 is better understood and has been implicated in STAT6 (Signal Transducer and Activator of Transcription 6)-dependent transcriptional control and cytokine-regulated control of cellular metabolism (Cho, et al., 2011; Goenka, et al., 2007). ARTD8 potentiated interleukin 4 (IL4)-induced STAT6 transactivation via its macrodomains and catalytic activity (Goenka and Boothby, 2006; Goenka, et al., 2007). ARTD8 was also recently identified as a downstream effector of the Jun N-terminal kinase 2 (JNK2)-dependent pro-survival signal by binding to and inhibiting JNK1 pro-apoptotic activity promoting the survival of myeloma cells (Barbarulo, et al., 2012).

ARTD10/PARP10 is the founding member of the class 2 ARTDs and was discovered through in silico screening ARTD family members (Ame, et al., 2004). The domain structure of ARTD10, with the exception of the catalytic domain is unique from the other ARTDs. The N-terminus contains a putative RNA recognition motif (RRM) followed by a glycine-rich region, which is present in other RNA-binding proteins (Burd and Dreyfuss, 1994). The C-terminus contains a functional nuclear export signal (NES) within its glutamate-rich region and two functional
ubiquitin-interaction motifs (UIMs) (Yu, et al., 2005). ARTD10 is a MART that auto-ADP-ribosylates itself as well as each of the four core histones (Kleine, et al., 2008; Yu, et al., 2005). ARTD10 was also reported to be an interacting partner of the oncoprotein MYC (Yu, et al., 2005). MYC/Ha-RAS- and E1A/Ha-RAS-dependent transformation of rat embryo fibroblasts was inhibited by ARTD10 (Yu, et al., 2005). Inhibition was independent of ARTD10 catalytic activity and neither MYC nor its heterodimerization partner MAX were ADP-ribosylated by ARTD10 (Yu, et al., 2005). Recently, ARTD10 has also been reported to be a regulator of the NF-κB pathway by mono-ADP-ribosylating NF-κB essential modulator, reducing its poly-ubiquitination and activation of NF-κB (Verheugd, et al., 2013). Unlike the regulation of MYC, the regulation of NF-κB was dependent on ARTD10 catalytic activity and the UIMs (Verheugd, et al., 2013).

**ARTD12/PARP12/ZC3HDC1** has been implicated in inflammatory processes in relation to various pathogens. During infection by the alphavirus, Venezuelan equine encephalitis virus (VEEV), the long isoform (containing the catalytic domain) of ARTD12 was up-regulated (Atasheva, et al., 2012). ARTD12 exhibited inhibitor effects on replication of VEEV as well as other alphaviruses and RNA viruses (Atasheva, et al., 2012). Interferon stimulation up-regulated ARTD12 gene expression to counteract infections which was suggested as being a cellular defence against invading viral pathogens, although this was not mechanistically determined (Schoggins, et al., 2011). In addition to its role in viral pathogen infection inhibition, ARTD12 has been implicated in post-transcriptional gene expression (Leung, et al., 2011). In that study, overexpressed ARTD12 assembled in cytoplasmic mRNA-protein complexes called stress granules and alleviated microRNA (miRNA)-mediated repression (Leung, et al., 2011). ARTD12 exhibited auto-mono-ADP-ribosylation activity and associated with both long and short isoforms of ARTD13 (short isoform is missing catalytic domain), ARTD5 and ARTD7 within stress granules (Leung, et al., 2011).

**ARTD14/PARP7/TiPARP** (will be referred to as TiPARP) was first identified as a 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin)-induced gene under the control of TCDD-activated aryl hydrocarbon receptor (AHR) (Ma, et al., 2001). TiPARP is broadly expressed in murine tissues, suggesting that TiPARP may be involved in diverse physiological functions (Ma, et al., 2001). In its initial study, TiPARP exhibited enzymatic ADP-ribosylation capacity but it
was not determined whether it undergoes auto- or hetero-ADP-ribosylation and whether this was mono-ADP-ribosylation activity (Ma, et al., 2001). A more recent report has identified TiPARP as a mediator the TCDD-induced suppression of hepatic glucose production (Diani-Moore, et al., 2010). This study proposed the TCDD-induced expression of TiPARP contributed to NAD$^+$ depletion by TiPARP catalytic activity, which decreased SIRT1 activity leading to decreased gluconeogenic gene expression (Diani-Moore, et al., 2010). TCDD-induced TiPARP gene expression and NAD$^+$ depletion has also been implicated in the deactivation of SIRT3 which leads to decreased superoxide dismutase 2 activity resulting in heightened sensitivity to non-alcoholic steatohepatitis (He, et al., 2013). Both proposed mechanisms of TiPARP-mediated NAD$^+$ depletion were derived from observation that TCDD induces TiPARP gene expression and decreases NAD$^+$ levels. The enzymatic activity of TiPARP was not properly investigated in these studies and neither study confirmed the mono-ADP-ribosyltransferase activity of TiPARP and/or if this was required for the observed outcomes.

**ARTD15/PARP16** was the first ARTD member found to associate with the endoplasmic reticulum (ER) (Di Paola, et al., 2012). ARTD15 was the only ARTD member to contain a predicted C-terminal transmembrane domain and was found to be an ER-tailed anchored enzyme that associated with the nuclear transport factor karyopherin-β1/importin-β1 (Kapβ1) at the ER and the nuclear envelope (Di Paola, et al., 2012). Kapβ1 was selectively mono-ADP-ribosylated by ARTD15 and this modification was hypothesized to control nuclear transport (Di Paola, et al., 2012). ARTD15 also demonstrated auto-mono-ADP-ribosylation that could be inhibited with canonical PARP inhibitors (Di Paola, et al., 2012; Karlberg, et al., 2012). Mono-ADP-ribosylation was reported to not occur at arginine, glutamate or cysteine residues and could not be reverted by ARH1 or ARH3 (Di Paola, et al., 2012). A recent report has described that ARTD15 was required for activation of the ER stress response kinases PERK and IREα during the unfolded protein response (UPR), an ER stress signal that ultimately leads to apoptosis (Hetz, 2012; Jwa and Chang, 2012). During ER stress, ARTD15 was found to auto-ADP-ribosylate itself and hetero-ADP-ribosylate PERK and IREα (Jwa and Chang, 2012). ADP-ribosylation of PERK and IREα increased their kinase activities and the endoribonuclease activity of IREα, which appeared necessary for proper execution of the UPR (Jwa and Chang, 2012).
**ARTD17/PARP6** was recently described as a negative regulator of cell proliferation and proposed tumour suppressor involved in colorectal cancer development (Tuncel, et al., 2012). ARTD17 overexpression was reported to arrest cells in S-phase and was dependent on the presence of the catalytic domain (Tuncel, et al., 2012). The catalytic activity of ARTD17 has not been evaluated and its potential activity in other cellular functions has not been fully determined.

**ARTD11/PARP11, ARTD16/PARP8 and ARTD18/TPT1** (the single member of the NAD+-dependent tRNA 2’-phosphotransferase family) have no known domains outside of their catalytic domains (with the exception of the single WWE domain of ARTD11) and their functions have not been determined.

### 2.4.1.1.3 Class 3 ARTDs

**ARTD9/PARP9/BAL1** is a nucleocytoplasmic shuttling protein that has been identified as a risk-related gene product in aggressive diffuse large B-cell lymphoma (DLBCL) (Aguiar, et al., 2000; Juszczynski, et al., 2006). ARTD9 contains two prototypical macrodomains within the N-terminus, which can bind mono- and poly-ADP-ribose (Karras, et al., 2005). ARTD9 was described to possess transcriptional repressive activity that was dependent on interaction through its macrodomains but independent of its catalytic activity (Aguiar, et al., 2005). Overexpression of ARTD9 promoted lymphocyte migration, indicating a tumour-promoting role in high-risk DLBCL (Aguiar, et al., 2000). Promotion of lymphocyte migration by ARTD9 has been suggested through modulation of interferon gamma (IFNγ) signalling-related gene expression (Juszczynski, et al., 2006). ARTD9 was identified as a novel co-repressor of transcription of interferon response factor 1 (IRF1), a tumour suppressor (Camicia, et al., 2013). ARTD9 directly interacted with STAT1β (signal transducer and activator of transcription 1 isoform β) to inhibit IRF1 expression, repressing the anti-proliferative and pro-apoptotic INFγ-STAT1-IRF1-p53 complex (Camicia, et al., 2013). ARTD9 has also been linked to the DNA damage response pathway (Yan, et al., 2013). In response to DNA strand breaks, ARTD9 and its partner BBAP (B-lymphoma and BAL-associated protein), an E3 ligase were recruited to DNA damage sites and co-localized with ARTD1 and its product poly(ADP-ribose) (Yan, et al., 2013). ARTD9 and BBAP at DNA damage sites mediate the specific recruitment of the adaptor protein RAP80 and checkpoint mediators 53BP1 and BRCA1 through BBAP-mediated ubiquitylation, which limits early and delayed DNA damage and enhances cellular viability (Yan, et al., 2013).
ARTD13/PARP13/ZAP/ZC3HAV1 is a type 1 interferon-inducible host factor that regulates viral RNA transcripts and was initially identified as zinc finger antiviral protein (ZAP) in a screen for host factors that confer resistance to the retrovirus murine leukemia virus (MLV) infection (Bick, et al., 2003; Gao, et al., 2002; Muller, et al., 2007). ARTD13 antiviral activities were later confirmed in other retroviruses including HIV-1 and other RNA virus families, including alphaviruses and filoviruses (Bick, et al., 2003; Muller, et al., 2007; Zhu, et al., 2011). ARTD13 binds directly to specific viral mRNAs through its N-terminal zinc finger domains and recruits cellular mRNA degradation factors to promote degradation of the target viral mRNA and the inability of the virus to replicate efficiently (Guo, et al., 2004; Guo, et al., 2007; Zhu, et al., 2011; Zhu and Gao, 2008). These antiviral properties are not due to ADP-ribosylation since full-length ARTD13 is not catalytically active nor is its short isoform lacking the PARP catalytic domain (Kleine, et al., 2008; Leung, et al., 2011). Both isoforms of ARTD13 were recently reported to localize to cytoplasmic stress granules along with ARTD5, ARTD7 and ARTD12 (Leung, et al., 2011). Interestingly, when overexpressed, both isoforms decreased miRNA-mediated silencing (Leung, et al., 2011).

2.4.2 Poly(ADP-ribose) Catabolism

2.4.2.1 Poly(ADP-ribose) glycohydrolase (PARG)

The removal of poly(ADP-ribose) chains from modified proteins is catalyzed by poly-ADP-ribose glycohydrolase (PARG), which is the primary enzyme responsible for poly(ADP-ribose) degradation in vivo (Alvarez-Gonzalez and Althaus, 1989; Davidovic, et al., 2001; Jonsson, et al., 1988). PARG has both exo- and endoglycosidase activities that hydrolyze the glycosidic bond between ADP-ribose units that generate large amounts of free ADP-ribose (Desnoyers, et al., 1995; Heeres and Hergenrother, 2007; Oka, et al., 1984). The structure of a bacterial PARG revealed that the PARG catalytic domain is a distant member of the macrodomain family suggesting a potential role in the regulation by poly-ADP-ribosylation (Slade, et al., 2011). The single mammalian PARG gene encodes several PARG proteins that localize to various cellular compartments that also contain ARTDs (Meyer-Ficca, et al., 2004). Four PARG isoforms have been identified so far, but the two predominant isoforms include PARG-110/111 (110 kDa), the full-length and very active nuclear isoform and PARG-59/60 (65 kDa), a shorter isoform (Brochu, et al., 1994; Meyer-Ficca, et al., 2004). PARG-102 (102 kDa), a splice variant that lacks exon 1 and PARG-99 (99 kDa), a splice variant that lacks exons 1 and 2 are cytoplasmic
but their roles are not fully understood (Meyer-Ficca, et al., 2004). Because of its low abundance in eukaryotic cells and its extreme sensitivity to proteases, PARG has been difficult to study (Bonicalzi, et al., 2005). Furthermore, a detailed analysis of the biological role of PARG has been precluded by the lack of specific and selective inhibitors (Schreiber, et al., 2006).

Much of the current understanding of the biological role of PARG is largely based on gene disruption studies and data obtained from Parg-deficient mice (Burkle and Virag, 2013). Targeted deletion of exon 4 resulted in complete deletion of all PARG isoforms in Parg-deficient mice (Koh, et al., 2004). Mice lacking all PARG isoforms arrest during development at embryonic day 3.5 as a result of apoptosis that was induced by poly(ADP-ribose) accumulation (Koh, et al., 2004). In vitro gene knockdown studies have revealed that PARG, like ARTD1, is required for efficient DNA repair of single- and double-strand breaks and mitotic spindle checkpoints since knockdown of either PARG or ARTD1 sensitized cells to apoptotic cell death following DNA damage (Ame, et al., 2009; Erdelyi, et al., 2009; Fisher, et al., 2007; Keil, et al., 2006). Mice that are deficient of full-length PARG (PARG110/111, Parg110-deficient) are viable with no obvious phenotype and normal poly(ADP-ribose) metabolism (Cortes, et al., 2004). However, Parg110-deficient mice are hypersensitive to ionizing radiation and alkylating agents, similar to Artd1-deficient mice (Cortes, et al., 2004). Data obtained from Parg110-deficient embryonic fibroblasts are similar to those reported for ARTD1 inhibition/knockout studies, including defects in DNA repair, genomic instability and chromosomal aberrations (Min, et al., 2010). These observations support a model in which a balance between poly(ADP-ribose) synthesis and degradation is necessary for efficient repair of DNA strand breaks and cell survival.

2.4.2.2 ADP-ribosylhydrolase 3 (ARH3)

ADP-ribosylhydrolase 3 (ARH3) is a member of the family of dinitrogenase reductase-activating glycohydrolase-related proteins and shares very little similarity with the PARG catalytic domain sequence (Mueller-Dieckmann, et al., 2006; Oka, et al., 2006). ARH3 localizes to the mitochondria but is also present in the nucleus and cytosol (Niere, et al., 2008; Oka, et al., 2006). ARH3 catalyzes the hydrolysis of both poly(ADP-ribose) and O-acetyl-ADP-ribose (OAADPR), a metabolite that results from the activity of sirtuins (Imai and Guarente, 2010; Oka, et al., 2006; Ono, et al., 2006). ARH3 was shown to degrade poly(ADP-ribose) in vitro and in cell but its
specific activity was significantly less than that of PARG (Niere, et al., 2008; Oka, et al., 2006). Embryonic fibroblasts from \textit{Arh3}-deficient mice lacked most of the mitochondrial poly(ADP-ribose) degrading activity and ARH3 appears to be the only identified enzyme to degrade PAR in the mitochondrial matrix (Niere, et al., 2012). Because of its localization to the cytosol and nucleus as well as its poly(ADP-ribose) degrading activity, ARH3 has also been proposed to assist PARG in degrading poly(ADP-ribose) in the nucleus and cytosol (Niere, et al., 2012).

2.5 Regulation of chromatin structure and transcription by ARTDs

The disruption of chromatin structure by ADP-ribosylation of histones and destabilization of nucleosomes were some of the earliest characterized effects of ARTD1 (Huletsky, et al., 1989; Mathis and Althaus, 1987; Poirier, et al., 1982). The ability of ARTD1 to modulate chromatin structure and function underlies its contribution to transcriptional regulation (Krishnakumar and Kraus, 2010b). Early studies reported that ARTD1 activity is preferentially associated with regions of chromatin that are actively transcribed (De Lucia, et al., 1996; Levy-Wilson, 1981; Mullins, et al., 1977). ARTD1 was identified as a factor that could increase specificity of RNA polymerase transcriptional initiation, supporting ARTD activity to directly regulate transcription (Matsui, et al., 1980; Slattery, et al., 1983). Transcriptional regulation by ARTD activity has been proposed to occur by at least two mechanisms that are not mutually exclusive: (1) modification of histones to alter chromatin structure and (2) functioning as part of the regulatory region (enhancer/promoter) binding complexes in conjunction with other DNA-binding factors and co-activators (Kraus and Lis, 2003).

2.5.1 Modulation of chromatin structure by ARTD1 and poly(ADP-ribose)

Poly-ADP-ribosylation of chromatin protein can cause profound effects on nucleosome architecture and on the stability of individual nucleosomes (D'Amours, et al., 1999). Histones H1 and H2B show the most poly-ADP-ribosylation \textit{in vivo} and are the preferred targets of ARTD1 \textit{in vitro}, although all histones are modified to some extent (Adamietz and Rudolph, 1984; D'Amours, et al., 1999; Huletsky, et al., 1989; Poirier, et al., 1982). As seen directly by electron microscopy, native polynucleosomes when poly-ADP-ribosylated by purified ARTD1 led to complete decondensation and mimicked the effects of linker histone H1 depletion from higher-order chromatin (Poirier, et al., 1982). Further studies suggested polyanionic poly(ADP-ribose),
attached to target proteins or as a free polymer acts as an attractive core histone binding matrix that can act as a histone acceptor to further destabilize nucleosomes (Mathis and Althaus, 1987; Realini and Althaus, 1992). Moreover, ARTD1-dependent accumulation of poly(ADP-ribose) at decondensed, transcriptionally active loci in native chromatin has been reported (Tulin and Spradling, 2003). Collectively, these data support a model of transcriptional regulation whereby ARTD1 promotes the decondensation of chromatin by causing the disassembly of nucleosomes through poly-ADP-ribosylation of H1 and core histones, causing destabilization of nucleosomes (D'Amours, et al., 1999; Kraus and Lis, 2003; Rouleau, et al., 2004; Tulin and Spradling, 2003).

More recent biochemical studies have demonstrated that in the absence of NAD$^+$ or significant auto-ADP-ribosylation, ARTD1 binds to nucleosomes and promotes the condensation of chromatin by bringing together neighbouring nucleosomes (Kim, et al., 2004; Wacker, et al., 2007). Addition of NAD$^+$, which led to considerable auto-ribosylation of ARTD1, promoted the release of ARTD1 from chromatin, leading to nucleosome decondensation and the restoration of transcription (Kim, et al., 2004; Wacker, et al., 2007). These results suggest a new NAD$^+$-dependent model of modulation of chromatin structure and transcription whereby ARTD1 can regulate chromatin structure without modifying histones or promoting disassembly of nucleosomes (Kim, et al., 2004; Wacker, et al., 2007).

2.5.2 Enhancer-binding of actions of ARTD1

In addition to modulating transcription through alterations in chromatin structure, ARTD1 can regulate transcription by functioning as a direct enhancer-binding factor. Many of the initial studies describing the effects of ARTD on transcriptional regulation of target genes focused on the binding of ARTD1 to specific DNA sequences or structures (Akiyama, et al., 2001; Butler and Ordahl, 1999; Huang, et al., 2004; Nirodi, et al., 2001; Zhang, et al., 2002). Although no consensus DNA binding sequence for ARTD1 has been established, the ability of ARTD1 to bind DNA in a sequence-specific manner has been demonstrated where ARTD1 preferentially bound to DNA oligonucleotides containing the 5’-TGTTG-3’ nucleotide sequence motif (Huang, et al., 2004; Simbulan-Rosenthal, et al., 2003). Two independent studies demonstrated ARTD1 binds to specific sequences immediately upstream of chemokine (CXC motif) ligand 1 (CXCL1) and in the first intron of B-cell lymphoma 6 (BCL6) to repress transcription (Ambrose, et al., 2007; Amiri, et al., 2006).
2.5.3 Promoter-binding and transcriptional co-regulation by ARTD1

Genomic studies have revealed that ARTD1 localizes to as many as 90% of expressed Pol II-transcribed promoters in vitro which suggested that it plays a role in promoting the formation of chromatin structures that are receptive to transcription (Krishnakumar, et al., 2008). The enrichment of ARTD1 at these promoters correlated with the depletion of linker histone H1, and a high ARTD1/H1 ratio specified genes that are actively transcribed (Krishnakumar, et al., 2008). These findings indicated that ARTD1 localized to sites of ongoing transcription, where it may exert stimulatory or inhibitory effects (Krishnakumar, et al., 2008). Roles of ARTD1 as a promoter-specific co-regulator (either co-activator or co-repressor) of different sequence specific DNA-binding transcriptional factors have been reported (Table 2). In some cases, the catalytic activity of ARTD1 is required, while in other cases it is not (Ju, et al., 2004; Olabisi, et al., 2008; Zaniolo, et al., 2007; Zhang, et al., 2013). These studies highlight the diverse mechanisms of ARTD1 co-regulation, which are likely to vary in a gene- or promoter-specific manner.

Table 2. Transcription factors co-regulated by ARTD1.

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Co-regulatory effect</th>
<th>Catalytic function required?</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>AP2</td>
<td>stimulatory</td>
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<td>(Kannan, et al., 1999)</td>
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<tr>
<td>b-MYC</td>
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<td>(Cervellera and Sala, 2000)</td>
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<tr>
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<td>(Cohen-Armon, et al., 2007)</td>
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<td>stimulatory</td>
<td>yes</td>
<td>(Zhang, et al., 2013)</td>
</tr>
<tr>
<td>HES1</td>
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<td>yes</td>
<td>(Ju, et al., 2004)</td>
</tr>
<tr>
<td>HTLV Tax1</td>
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<td>(Anderson, et al., 2000)</td>
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<td>(Mendoza-Alvarez and Alvarez-Gonzalez, 2001)</td>
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<td>(Pavri, et al., 2005)</td>
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<tr>
<td>Sp1</td>
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<td>(Zaniolo, et al., 2007)</td>
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<tr>
<td>YY1</td>
<td>inhibitory</td>
<td>n/d</td>
<td>(Oei, et al., 1997)</td>
</tr>
</tbody>
</table>

AP2; activating protein 2, Elk-1; ETS domain-containing protein, ERα; estrogen receptor α, HES1; hairy and enhancer of split 1, HTLV; human T-cell leukemia virus type 1, NFAT; nuclear factor of activated T-cells, NF-κB; nuclear factor kappa-light-chain-enhancer of activated B-cells, Oct-1; octamer transcription factor 1, RAR; retinoic acid receptor, Sp1; specificity protein
1, TEF-1; transcriptional enhancer factor 1, TR/RXR; thyroid hormone receptor/retinoid X receptor heterodimer, YY1; yin ying 1. n/d; not determined.

2.5.4 Transcriptional regulation by other ARTDs

Studies have emerged examining the regulation of transcription by other ARTD members, including, ARTD2, ARTD8, ARTD9 and ARTD10 (Aguiar, et al., 2005; Camicia, et al., 2013; Szanto, et al., 2012; Yu, et al., 2005).

Similar to ARTD1, ATRD2 has been demonstrated to co-regulate DNA-binding transcription factors (Bai, et al., 2011; Bai, et al., 2007; Maeda, et al., 2006). The activity of the peroxisome proliferator-activated receptor γ (PPARγ) was modulated by ARTD2 (Bai, et al., 2007). ARTD2 demonstrated direct interaction and co-occupancy with PPARγ/RXR heterodimers at promoter regions of PPARγ target genes and acts as a co-activator of transcription leading to triglyceride accumulation (Bai, et al., 2007). Estrogen receptor α (ERα) but not ERβ-dependent transcription was decreased by ARTD2 knockdown suggesting a co-activator role of ARTD2 in ERα-dependent transcription (Bai, et al., 2007; Szanto, et al., 2012). ARTD2 along with ARTD1 have also been reported to serve as co-activators of thyroid transcription factor 1 (TTF1) and regulate expression of surfactant protein B in lung (Maeda, et al., 2006). ARTD2 has been reported to be a potent transcriptional repressor of the SIRT1 promoter and the reduction or knockout of ARTD2 expression induced SIRT1 mRNA expression, protein and activity (Bai, et al., 2011).

The mono-ADP-ribosyltransferase ARTD8 was reported to activate a STAT6-regulated reporter gene, which was dependent on its macro- and catalytic domains (Goenka and Boothby, 2006; Goenka, et al., 2007). ARTD8 acted as a transcriptional switch, where under non-stimulating conditions ARTD8 was bound to STAT6-responsive promoters and recruited histone deacetylase 2 (HDAC2) and HDAC3 to keep genes silent (Mehrotra, et al., 2011). Upon IL4 stimulation ARTD8 catalyzed the ADP-ribosylation of itself, HDAC2 and HDAC3 leading to their dissociation from the promoter thereby allowing STAT6 dimers binding (Mehrotra, et al., 2011).

ARTD10 may possibly play a role in MYC-induced transcription. ARTD10 was identified as an interacting partner of MYC and this interaction was demonstrated to occur in the nucleus (Kleine, et al., 2012; Yu, et al., 2005). ARTD10 has also recently been reported to negatively regulate NF-κB signalling in response to IL-1β and TNFα stimulation by inhibiting NF-κB
target gene and protein expression (Verheugd, et al., 2013). ARTD10-mediated inhibition of NF-κB signalling was dependent on its catalytic activity and mono-ADP-ribosylation of NF-κB essential modulator by ARTD10 reduced its poly-ubiquitination, leading to reduced expression of NF-κB target genes (Verheugd, et al., 2013). ARTD10 also prevented the nuclear translocation of the NF-κB transcription factor p65 (Verheugd, et al., 2013).

Recently, ARTD9 has been identified as a novel co-repressor of transcription of IRF1, a tumour suppressor (Camicia, et al., 2013). ARTD9 directly interacted with STAT1β through its macrodomains mediated by trans-mono-ADP-ribosylation to inhibit IRF1 expression (Camicia, et al., 2013). This was shown to repress the anti-proliferative and pro-apoptotic INFγ-STAT1-IRF1-p53 complex and mediated proliferation, survival and chemo-resistance in DLBCL (Camicia, et al., 2013).

The transcriptional regulatory roles of the remaining ARTD members, including TiPARP have not been evaluated. The expression of TiPARP is regulated by aryl hydrocarbon receptor (AHR), a ligand activated transcription factor, but its potential function in AHR transactivation has not been evaluated.

3 Aryl hydrocarbon receptor

The AHR is a ligand-activated transcription factor and a member of the basic-helix-loop-helix Per (Period)-ARNT (aryl hydrocarbon receptor nuclear translocator)-SIM (single-minded) (bHLH/PAS) family of heterodimeric transcriptional regulators (Burbach, et al., 1992). Other members of the bHLH/PAS family of transcription factors include ARNT, hypoxia factor 1α (HIF1α), SIM, Clock, Per, and the p160 family of co-activators (Hankinson, 1995). bHLH/PAS family of transcription factors are involved in the control of a variety of physiological processes which include: circadian rhythms, organ development, neurogenesis, metabolism and the stress response to hypoxia (Crews, 1998; Fernandez-Salguero, et al., 1997; Gonzalez and Fernandez-Salguero, 1998; Whitlock, 1999). Although AHR plays an important role in physiology it is most well-known for mediating the toxic effects of environmental contaminants (Abbott, et al., 1995; Hahn, 2002; Lahvis, et al., 2000; Lin, et al., 2001; Mimura, et al., 1997; Schmidt, et al., 1996; Van den Berg, et al., 1998).
3.1 Structure

The human AHR is composed of several modular domains with distinct functions (Figure 5A). AHR shares similar domain architecture to other bHLH/PAS proteins, with a highly conserved N-terminal bHLH motif, adjacent PAS region, and loosely conserved C-terminal transactivation or transrepression regions (Figure 5B and C) (Kewley, et al., 2004). The basic region of the bHLH motif mediates DNA binding and the HLH domain is involved in dimerization (Kikuchi, et al., 2003). Within the N-terminus of AHR is a bipartite nuclear localization signal (NLS), which overlaps with the basic region of the bHLH (Ikuta, et al., 1998). Moreover, AHR also contains a leucine-rich NES with helix 2 of the bHLH motif (Ikuta, et al., 1998). The PAS region of AHR contains two tandem PAS domains designated PAS A and PAS B and both PAS domains along with the bHLH motif contribute to dimerization with AHR obligatory interaction partner ARNT and interaction with chaperone protein HSP90 (heat shock protein 90 kDa) (Lindebro, et al., 1995; Pongratz, et al., 1992). The PAS A domain controls dimerization specificity and stability, and strengthens DNA binding (Chapman-Smith, et al., 2004; Lindebro, et al., 1995; Pongratz, et al., 1998). The PAS B domain contains the ligand binding domain and deletion of this domain results in a constitutively active receptor (McGuire, et al., 2001).

The C-terminal half of AHR contains a modular transactivation domain (TAD) that is composed of three distinct subdomains: acidic, glutamine-rich (Q-rich) and proline-serine-threonine-rich (P/S/T) (Jain, et al., 1994; Rowlands, et al., 1996; Sogawa, et al., 1995). Individually, each of the three subdomains exhibits low levels of transactivation; however, any combination of two subdomains can synergistically activate transcription (Rowlands, et al., 1996). The Q-rich subdomain is critical for transcriptional activation and its deletion completely inactivates the receptor (Kumar, et al., 2001). Additionally the Q-rich subdomain also appears to be required for interaction with co-regulatory proteins nuclear co-activator 1 (NcoA1) and receptor interacting protein 140 (RIP140) (Kumar and Perdew, 1999; Kumar, et al., 2001; Kumar, et al., 1999).
Figure 5. Schematic of the modular domains of three bHLH-PAS family members. (A) Aryl hydrocarbon receptor (AHR), (B) Aryl hydrocarbon nuclear translocator (ARNT), and the (C) Aryl hydrocarbon receptor repressor (AHRR). bHLH (basic helix-loop-helix), PAS (Per/ARNT/Sim), P/S/T (Proline/Serine/Threonine).

3.2 AHR ligands

AHR is a promiscuous receptor that can bind and be activated by a wide variety of structurally diverse exogenous and natural compounds that produce a spectrum of the biological and toxic effects (Denison and Heath-Pagliuso, 1998; Denison and Nagy, 2003; Denison, et al., 2011; Nguyen, et al., 1999; Poland and Knutson, 1982; Safe, 1990). AHR ligands can be divided into two major categories: exogenous and naturally occurring.
3.2.1 Exogenous AHR ligands

The majority of the high affinity AHR ligands that have been identified and characterized are exogenous, which include planar hydrophobic halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) and related compounds (Figure 6) (Denison and Heath-Pagliuso, 1998; Gillner, et al., 1993; Poland and Knutson, 1982; Safe, 1990). Many HAHs and PAHs are environmental contaminants that are by-products of chlorine chemistry, waste incineration, metal production and fossil fuel or wood burning (Denison and Nagy, 2003; DeVito, et al., 1994; Hankinson, 2005; Kulkarni, et al., 2008; Safe, 1986; Van den Berg, et al., 1998). The HAHs represent the most potent class of AHR ligands and include chlorinated and brominated dibenzo-p-dioxins, dibenzofurans and biphenyls. HAHs are metabolically stable and have binding affinities in the picomolar to nanomolar range (Denison and Nagy, 2003). PAHs include: 3MC, B[a]P, benzoanthracenes and benzoflavones (Denison and Nagy, 2003; Poland and Knutson, 1982). PAHs are metabolically less stable than HAHs and tend to have lower binding affinities usually within the nanomolar to micromolar range. Structure-activity relationship analysis of many HAHs and PAHs congeners has demonstrated toxicity is positively correlated to the affinity of the congener for AHR (Kafafi, et al., 1993; Mhin, et al., 2002; Poland and Knutson, 1982; Safe, 1986; Tuppurainen and Ruuskanen, 2000; Waller and McKinney, 1995). The HAH, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) has the highest affinity for AHR and is the most toxic of the known AHR ligands and remains the prototype AHR ligand for which all subsequent ligands are compared (Nebert, et al., 2004).
3.2.2 Naturally occurring AHR ligands

The greatest source of human exposure to AHR ligands (exogenous and naturally occurring) comes from the diet (Denison and Nagy, 2003; Jeuken, et al., 2003). A variety of naturally occurring dietary constituents are AHR ligands (Bjeldanes, et al., 1991; MacDonald, et al., 2001; Wattenberg and Loub, 1978). Indole-3-carbinol (IC3) is found in cruciferous vegetables and is hydrolyzed under the acidic conditions of the gastrointestinal tract to many products including 3,3’-diindolylmethane (DIM) and indolo[3,2-b]carbazole (ICZ) (Bjeldanes, et al., 1991; Grose and Bjeldanes, 1992). DIM is a partial agonist for AHR while ICZ is a potent activator of AHR activity (Broadbent and Broadbent, 1998; Chen, et al., 1996; Kleman, et al., 1994). ICZ has high
affinity for AHR but it does not cause toxicity (Pohjanvirta, et al., 2002). Naturally occurring AHR ligands can also be antagonists and numerous have been previously described as such. Resveratrol (3,5,4’-trihydroxystilbene), a polyphenol present in grapes, peanuts and berries and kaempferol (3,4’,5,7-tetrahydroxyflavone), a flavonol found in teas, broccoli, grapefruit and other plant sources are two potent AHR antagonists (Casper, et al., 1999; Ciolino, et al., 1999; MacPherson and Matthews, 2010).

To date no definitive endogenous AHR ligand has been identified; however, numerous endogenous chemicals have been identified that bind to AHR and/or activate AHR-dependent gene expression (Denison and Nagy, 2003). Bilirubin, tryptophan metabolites, arachidonic acid metabolite lipoxin A4, several prostaglandins, retinoids, indigo and indirubin are some examples of endogenous compounds known to activate AHR (Adachi, et al., 2001; Bittinger, et al., 2003; Gambone, et al., 2002; McMillan and Bradfield, 2007; Phelan, et al., 1998; Schaldach, et al., 1999; Seidel, et al., 2001; Sugihara, et al., 2004). FICZ (6-formylindolo[3,2-b]carbazole), a tryptophan photoproduct has been reported to be the most potent and highest affinity candidate endogenous AHR ligand known to date, capable of competitively displacing TCDD (Fritsche, et al., 2007; Helferich and Denison, 1991; Rannug, et al., 1987; Wincent, et al., 2009). Femtomolar levels of FICZ were reported to sufficiently induce AHR target mRNA and enzyme activity (Wincent, et al., 2012). Unlike TCDD, however, FICZ is a substrate for Phase I enzymes cytochrome P450 1A1, CYP1A2 and CYP1B1 and is readily metabolized (Wincent, et al., 2009).

### 3.3 AHR-mediated toxicity

Most of what is known about the toxicity of TCDD and related compounds come from studies in experimental animals, but effects are also observed in humans (Birnbaum, 1994; Birnbaum and Tuomisto, 2000; Pohjanvirta and Tuomisto, 1994; Sewall and Lucier, 1995; Tuomisto, 2005; White and Birnbaum, 2009). One of the most dramatic effects from a single TCDD exposure in experimental animals is the lethal wasting syndrome that is characterized by progressive weight loss, hypophagia and gluconeogenic suppression (Pohjanvirta and Tuomisto, 1994; Seefeld, et al., 1984; Seefeld and Peterson, 1984; Stahl, et al., 1993). While wasting accompanies death, wasting per se is not likely the sole cause of death since maintenance of body weight by parenteral nutrition does not prevent mortality (Gasiewicz, et al., 1980). Lethality of exposed
animals is delayed and does not ensue until 1-8 weeks after exposure with the time course depending on the sensitivity of the animal (Birnbaum and Tuomisto, 2000; Poland and Knutson, 1982). A wide range of other short-term toxic effects following TCDD exposure are known, including thymic atrophy, hepatic necrosis or hypertrophy, lipid accumulation within hepatocytes, a variety of endocrine imbalances and immunosuppression (Birnbaum and Tuomisto, 2000; Pohjanvirta and Tuomisto, 1994). Ahr-null mice challenged with TCDD are resistant to TCDD-induced toxicities as well as to TCDD-induced transcriptional alterations (Boutros, et al., 2009; Fernandez-Salguero, et al., 1995; Mimura, et al., 1997; Schmidt, et al., 1996; Tijet, et al., 2006). Mice expressing a mutated form of AHR that is unable to translocate to the nucleus or a DNA binding mutant are also essentially refractory to TCDD-mediated toxicity (Bunger, et al., 2008; Bunger, et al., 2003). Furthermore, mice hypomorphic for ARNT are phenotypically non-responsive to dioxins (Walisser, et al., 2004). Collectively, these genetic manipulations indicate that TCDD toxicities are dependent on the transcriptional regulatory function and DNA-binding capacity of AHR.

### 3.4 AHR transactivation

In its latent state, AHR is found in the cytosol where it is stably associated with a multi-protein complex consisting of two molecules of chaperone protein HSP90, hepatitis B virus X-associated protein 2 (XAP2) and a 23 kDa co-chaperone protein referred to as p23 (Figure 7)(Carver, et al., 1994; Kazlauskas, et al., 1999; Ma and Whitlock, 1997; Meyer, et al., 1998; Perdew, 1988; Pollenz, et al., 1994). HSP90 interacts with AHR via both the bHLH motif and the PAS B domain and is an essential component of the unliganded AHR complex as its presence is necessary for the high affinity ligand binding conformation of AHR and retention of AHR in the cytosol by masking its NLS sequence (Antonsson, et al., 1995a; Antonsson, et al., 1995b; Dolwick, et al., 1993; Pongratz, et al., 1992; Whitelaw, et al., 1993; Whitelaw, et al., 1995). XAP2 has a multifunctional role which includes maintaining proper cytosolic folding of AHR, improving chaperone complex stability, subcellular localization and ligand binding ability of AHR and repressing AHR transcriptional activity (Kazlauskas, et al., 2000; LaPres, et al., 2000; Meyer and Perdew, 1999; Meyer, et al., 1998; Petrulis, et al., 2000). Co-chaperone p23 stabilizes the chaperone complex and is important for nuclear import of AHR (Kazlauskas, et al., 1999).
Most AHR ligands are highly lipophilic and therefore can enter the cell through simple diffusion (Delescluse, *et al.*, 2000; Goryo, *et al.*, 2007). Ligand binding induces a conformational change in AHR that exposes its NLS (Henry and Gasiewicz, 2003; Ikuta, *et al.*, 2004; Whitlock, 1999). Liganded AHR translocates into the nucleus where it dissociates from its chaperone protein complex and then heterodimerizes with ARNT and is converted to its high affinity DNA conformation (Hankinson, 2005; Okey, *et al.*, 1980; Tomita, *et al.*, 2000). The AHR/ARNT heterodimer recognizes a DNA element designated aryl hydrocarbon receptor response element (AHRE) located within the regulatory regions of target genes such as cytochrome P450 1A1 (CYP1A1) (Mimura and Fujii-Kuriyama, 2003; Whitlock, 1999). The AHRE is composed of two half-sites, TnGC and GTG recognized by AHR and ARNT, respectively (Denison, *et al.*, 1988; Swanson, *et al.*, 1995). Once bound to an AHRE the AHR/ARNT complex interacts with multiple co-regulator proteins to regulate transcription (Hankinson, 2005). A list of co-regulator proteins known to interact and/or modulate AHR/ARNT activity is provided in Table 3.

**Table 3. Partial list of proteins and co-regulator proteins that interact with AHR/ARNT to modulate transcription.**

<table>
<thead>
<tr>
<th>Factors</th>
<th>Response</th>
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<tbody>
<tr>
<td><strong>Co-activators</strong></td>
<td></td>
</tr>
<tr>
<td>Brg-1/Brm1</td>
<td>enhances transcription mediated by AHR/ARNT (Hankinson, 2005)</td>
</tr>
<tr>
<td><strong>Co-repressors</strong></td>
<td></td>
</tr>
<tr>
<td>SMRT, SHP</td>
<td>inhibits transcriptional activity of AHR/ARNT complex (Jepsen and Rosenfeld, 2002; Nguyen, <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td><strong>General Transcription Factors</strong></td>
<td>key components of the general transcription machinery that are directly involved in gene activation by AHR/ARNT (Hankinson, 2005; Harper, <em>et al.</em>, 2006)</td>
</tr>
</tbody>
</table>

For a complete list of AHR co-regulators see reference (Hankinson, 2005).
Figure 7. General mechanism of AHR transactivation.

Ligands bind (TCDD) to AHR in the cytosol and triggers translocation into the nucleus. AHR is released from its chaperone complex and then binds with its dimerization partner ARNT. The AHR/ARNT complex binds AHREs located within the regulatory region of target genes. Once bound to the AHRE, AHR/ARNT associates with co-activators to activate transcription.

3.5 Negative regulation of AHR transactivation

There are several mechanisms by which AHR transactivation can be attenuated (Figure 8). One mechanism involves ligand-dependent degradation of AHR protein through a proteasomal pathway (Pollenz, 2002; Wentworth, et al., 2004). The decrease in AHR protein leads to reduced AHR-dependent transcription (Pollenz, et al., 1998). Other proposed mechanisms involve an autoregulatory feedback loop created by induction of AHR-dependent transcriptional repressor. The aryl hydrocarbon receptor repressor (AHRR) is induced by activated-AHR and is a proposed transcriptional repressor of AHR (Mimura, et al., 1999). Another and more hypothetical mechanism combines ideas from the two previous mechanisms where a yet to be identified AHR-dependent labile factor promotes the proteolytic degradation of AHR protein and the attenuation of transcription (Ma, 2002; Ma and Baldwin, 2002).
Figure 8. Three proposed mechanisms of negative regulation of AHR transactivation.

(A) Targeted proteolytic degradation of AHR protein through the ubiquitin 26S proteasome pathway. (B) Induction of the transcriptional repressor AHRR. (C) Induction of a labile factor that promotes the proteolytic degradation of AHR.

3.5.1 Proteolytic Degradation of AHR

Numerous studies have established AHR ligand binding leads to rapid and sustained reduction in the level of endogenous AHR protein in both in vitro and in vivo models (Pollenz, 2002). The ligand-induced degradation of AHR has been observed in many cell lines of different tissue lineages, although the time-course and range of maximal degradation varied amongst cell lines (Pollenz and Buggy, 2006). AHR degradation has also been observed in vivo. For example, AHR protein concentration was reduced in liver, spleen, thymus and lung from Sprague-Dawley rats given an acute dose of TCDD (Pollenz, et al., 1998). The ubiquitin-proteasome pathway has been implicated in the ligand-induced degradation of AHR due to the ability of the proteasome inhibitors, MG-132 and lactacystin, two specific inhibitors of the 26S proteasome, to completely block degradation (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000). Inhibitors of calpains,
serine/cysteine, lysosomal and serine proteases failed to block AHR degradation (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Roberts and Whitelaw, 1999; Wormke, et al., 2000). In addition, AHR degradation is inhibited in cells that contain a temperature-sensitive mutation in the E1 ubiquitin conjugating enzyme (Ma and Baldwin, 2000). The inhibition of AHR degradation resulted in sustained levels of AHR in the nucleus over time and significantly increased in the concentration of DNA-bound AHR/ARNT heterodimers (Ma and Baldwin, 2000; Roberts and Whitelaw, 1999). These studies also demonstrated that AHR degradation required the C-terminal transactivation domain and DNA-binding activity, as AHR lacking the C-terminus and a DNA-binding mutant were more resistant to degradation than wildtype AHR (Ma and Baldwin, 2000). TCDD induced the accumulation of high molecular weight AHR that contained ubiquitin; however, specific ubiquitination sites are yet to be identified (Ma and Baldwin, 2000). Taken together, these studies provide compelling evidence that AHR is degraded via the 26S proteasome.

The ligand-induced degradation of AHR was connected to down-regulation of AHR target gene expression by analyzing the duration and magnitude of gene expression following proteasomal inhibition. AHR-regulated reporter gene experiments demonstrated pretreatment of cells with MG-132 resulted in significantly greater reporter gene activity following TCDD treatment compared to cells treated with TCDD alone (Davarinos and Pollenz, 1999). Inhibition of AHR degradation also resulted in higher endogenous AHR target mRNA induction compared to non-inhibited cells when exposed to TCDD (Ma and Baldwin, 2000). Reporter genes were also used to assess whether down-regulation of endogenous AHR rendered cells non-responsive. Cell lines propagated in the presence of TCDD for 12 days and then re-challenged with TCDD demonstrated reductions in AHR protein and the absolute level of reporter gene activity (Pollenz, et al., 1998). Collectively, these results correlate ligand-induced gene expression increases with AHR degradation inhibition.

3.5.2 Aryl hydrocarbon receptor repressor (AHRR)

The aryl hydrocarbon receptor repressor (AHRR) is a bHLH-PAS protein that was discovered because of its similarity to AHR (Mimura, et al., 1999). The AHRR shares high amino acid identity with the AHR in the N-terminal region containing the bHLH (81%) and PAS A domain (60%) but diverges significantly towards the C-terminus and does not contain a ligand binding
(PAS B) and transactivation domain (Figure 5C) (Mimura, et al., 1999). Due to the lack of a ligand binding domain, the AHRR does not bind AHR ligands and appears to function in a ligand-independent manner (Karchner, et al., 2009). Like AHR, the AHRR can heterodimerize with ARNT and bind AHREs through its HLH-PAS A domains and basic region respectively (Mimura, et al., 1999). Consistent with this, the initial mechanism of AHRR-mediated repression was hypothesized to involve competition between AHR and the AHRR for ARNT heterodimerization and competition between AHR/ARNT and AHRR/ARNT heterodimers for AHRE binding (Mimura, et al., 1999). AHRR/ARNT binding to AHREs do not transcriptionally activate due to the AHRR lacking a transactivation domain (Mimura, et al., 1999). Four putative AHREs have been identified in the 5’ proximal to the promoter of the AHRR gene, and not surprisingly AHR ligands have been reported to induce AHRR gene expression (Baba, et al., 2001; Evans, et al., 2005; Karchner, et al., 2002; Mimura, et al., 1999; Tsuchiya, et al., 2003; Yamamoto, et al., 2004). Induction of the AHRR by AHR agonists and AHRR-mediated repression of AHR transactivation implicates that the AHRR forms a negative feedback loop with AHR.

AHRR mRNA is constitutively expressed in a variety of tissues with the testis, lung, spleen, heart and kidneys expressing the highest levels (Bernshausen, et al., 2006; Korkalainen, et al., 2004; Nishihashi, et al., 2006; Tsuchiya, et al., 2003; Yamamoto, et al., 2004). In some tissues that express high AHR levels such as the liver, constitutive AHRR mRNA is low and only becomes measurable after induction with potent AHR ligands such as TCDD, B[a]P or 3MC (Bernshausen, et al., 2006; Korkalainen, et al., 2005; Korkalainen, et al., 2004; Mimura, et al., 1999). Conversely, constitutively high AHRR mRNA levels have been attributed to the lack of AHR ligand inducibility of targets in primary cells and some cell lines (Gradin, et al., 1999; Tsuchiya, et al., 2003). Reduction of AHRR expression by siRNA can in some cases restore inducibility of AHR targets, but low constitutive expression of AHRR does not always correlate with high AHR target inducibility (Haarmann-Stemmann, et al., 2007; Tsuchiya, et al., 2003; Yamamoto, et al., 2004). For instance, constitutive AHRR mRNA is decreased in the heart and brain tissue of Ahr−/− mice (Bernshausen, et al., 2006). Additionally, B[a]P induces significant CYP1A1 mRNA in murine kidney tissue but does not induce detectable levels of AHRR mRNA (Bernshausen, et al., 2006). The ability of AHR to transactivate the AHRR gene and the ability of AHRR to repress AHR activity is likely to be tissue-, cell- or context-specific.
The AHRR has been demonstrated to be an effective transcriptional repressor of AHR activity; however, its repressive activity on AHR transactivation has been largely based on in vitro overexpression reporter gene experiments (Evans, et al., 2008; Evans, et al., 2005; Karchner, et al., 2002; Karchner, et al., 2009; Mimura, et al., 1999; Oshima, et al., 2007). Overexpression experiments using GFP-tagged AHRR were used to determine GFP-AHRR and presumably endogenous AHRR localize primarily to the nucleus in MCF7, Hepa1c1c7 and COS7 cells (Evans, et al., 2008; Kanno, et al., 2007; Mimura, et al., 1999). Using a series of GFP-tagged AHRR truncations the AHRR was found to contain a functional NLS and NES and was shown to undergo nucleocytoplasmic shuttling that favoured nuclear localization (Kanno, et al., 2007). Overexpression experiments determined that the C-terminus of the AHRR was required for repression, suggesting the presence of a ‘transrepression domain’ (Oshima, et al., 2007). These observations support the hypothesis that AHRR is a nuclear transcriptional repressor. The exact molecular mechanisms by which the AHRR represses AHR transactivation is currently subject to debate. The initial mechanism proposed for AHRR-mediated repression involved competition between the AHRR and AHR for heterodimerization with ARNT; however, subsequent studies have reported that overexpression of ARNT had no effect on AHRR-mediated repression, demonstrating that competition for ARNT is not the primary mechanism of repression (Evans, et al., 2008; Karchner, et al., 2009). Moreover, a DNA-binding point mutant of the AHRR effectively repressed AHR activity indicating that AHRE binding was not required for repression (Evans, et al., 2008). Furthermore, interaction experiments demonstrated the AHRR interacted with ARNT and AHR in a ligand-independent manner (Karchner, et al., 2009). These studies illustrate the complexity of AHRR-mediated repression and the mechanism by which the AHRR represses requires further clarification.

### 3.5.3 Induction of a labile AHR degradation promoting factor (ADPF)

A third proposed mechanism by which AHR transactivation can be terminated is through a hypothetical labile factor that promotes the proteasomal degradation of AHR, termed the AHR degradation promoting factor (ADPF) (Ma, 2007; Ma and Baldwin, 2002; Ma, et al., 2000). The existence of a potential labile factor was first proposed based on the observation that protein synthesis inhibition of liver cells increased the AHR ligand-induced target gene expression well above levels of AHR ligand treatment alone (Whitlock and Gelboin, 1973). Cycloheximide, a potent protein synthesis inhibitor, enhanced the TCDD-induced CYP1A1 mRNA to levels
greater than the sum of each compound alone, a phenomenon termed superinduction (Ma and Baldwin, 2002; Ma, et al., 2000). Superinduction results from increased transcriptional rate and subsequent generation of AHR target mRNA and protein but not from increased mRNA or protein stability (Israel, et al., 1985; Israel and Whitlock, 1983). Superinduction requires substantial (>90%) protein synthesis inhibition and functional AHR and ARNT suggesting that the synthesis of a repressor protein, which is labile and induced during AHR target induction is inhibited (Lusska, et al., 1992). CHX co-treatment was later found to fully inhibit the proteasomal degradation of AHR that correlated with superinduction in a time- and dose-dependent manner (Ma, et al., 2000). Similarly, proteasomal inhibition with MG-132 also superinduced AHR targets following AHR agonist treatment further supporting the notion that increasing the stability of AHR by decreasing its degradation superinduced target gene expression (Ma and Baldwin, 2000; Ma, et al., 2000). Together these observations establish the hypothesis that a repressor induced by agonist-activated AHR and sensitive to CHX mediates the proteasomal degradation of AHR, this unknown factor is referred to as the ADPF (Ma, et al., 2000). Two possible mechanisms, which are not mutually exclusive, have been proposed for the ADPF based on results from inhibitor studies. One is an autoregulatory mechanism in which the ADPF is an AHR target protein that is induced by AHR agonists (Ma and Baldwin, 2002). Blockage of the induction of the ADPF by protein synthesis disrupts the autoregulation (Ma and Baldwin, 2002). The second is the ADPF is a labile factor that requires constant protein synthesis for function and therefore sensitive to cycloheximide (Ma and Baldwin, 2002). Despite the evidence supporting its existence, an ADPF has not yet been identified.

3.5.4 In vivo models used to study negative regulation of AHR

3.5.4.1 Ahrr-deficient mice

Ahrr-deficient mice were generated to study the function of the AHRR in AHR transactivation in vivo (Hosoya, et al., 2008). The AHR-dependent induction of CYP1A1 was expected to be greater in Ahrr-deficient mice than wildtype mice following 3MC treatment; however, this pattern of induction was highly tissue-specific and limited to only the heart, spleen and skin (Hosoya, et al., 2008). Tissues with high AHR expression including the liver, lung, kidney and thymus did not display significant increases in 3MC-treated Ahrr-deficient mice compared with treated wildtype mice nor did the brain or stomach (Hosoya, et al., 2008). Constitutive expression of CYP1A1 in Ahrr-deficient mice was not significantly different than wildtype mice.
for all the tissues examined (Hosoya, et al., 2008). Ahrr-deficient mice treated with subcutaneous B[α]P showed a significant delay in skin tumour formation than wildtype (Hosoya, et al., 2008). It was suggested that the increased CYP1A1 expression in the skin shifted the balance of metabolic activity of CYP1A1 in favour of detoxification and this attributed to the delay in skin carcinogenesis (Hosoya, et al., 2008). B[α]P-treated Ahrr-deficient mice demonstrated a slightly lower mortality rate trend compared with treated-wildtype mice (Hosoya, et al., 2008).

3.5.4.2 Constitutively active AHR mice

Deletion of the entire PAS B domain of AHR results in a protein that does not interact with HSP90, shows constitutive nuclear localization, binds ARNT and exhibits potent transactivation in the absence of ligand i.e. TCDD, a constitutively active AHR (CA-AHR) (McGuire, et al., 2001). Transgenic mice expressing CA-AHR were generated and used to study the in vivo consequences of lack of AHR transactivation termination (Andersson, et al., 2002; Andersson, et al., 2003; Brunnberg, et al., 2006; Brunnberg, et al., 2011; McGuire, et al., 2001; Moennikes, et al., 2004; Wejheden, et al., 2010). Transgenic CA-AHR animals expressed CA-AHR in lymphatic organs including thymus, spleen, lymph nodes, enriched T and B cells and bone marrow as well as in lung, liver, muscle, skin, brain, heart, kidney and throughout the gastrointestinal tract (Andersson, et al., 2002). All tissues that showed CA-AHR expression also demonstrated induced CYP1A1 mRNA expression at varying levels (Andersson, et al., 2002). Additionally, homozygous CA-AHR mice demonstrated significantly greater CYP1A1, CYP1A2 and AHRR mRNA expression in the liver compared with wildtype animals (Andersson, et al., 2002). CYP1A1 mRNA induction in homozygous CA-AHR mice was similar to that of low to mid-dose TCDD-treated wildtype mice in the liver, thymus and stomach (Andersson, et al., 2002). CA-AHR mice also showed several symptoms that are similar to TCDD toxicity such as thymic atrophy and liver enlargement (Andersson, et al., 2002). CA-AHR mice showed a significantly reduced lifespan where mice began dying as early as 6 months and often without symptoms (other than body weight loss a day before death), only a few animals survived after 12 months (Andersson, et al., 2002). Another distinguishing feature of CA-AHR mice was the development of invasive tumours of the glandular stomach at an early age that correlated with increased mortality (Andersson, et al., 2002). Other findings in these CA-AHR mice included: decreased population of B lymphocytes in the peritoneal cavity, enlarged heart and kidneys and
increased hepatocarcinogenesis (Andersson, et al., 2003; Brunnberg, et al., 2006; Brunnberg, et al., 2011; Moennikes, et al., 2004).

3.6 AHR target genes

The up-regulation of a battery of six enzymes involved in biotransformation of xenobiotics and endogenous compounds in response to TCDD and related AHR ligands has been well established and studied in several species (Nebert, 1989). This battery includes cytochrome P450 1A1 (CYP1A1), CYP1A2, glutathione S-transferase Ya (GSTYa), aldehyde-3-dehydrogenase (ALDH-3) and NAD(P)H: quinone oxidoreductase (NQO1) (Nebert, et al., 1990; Nebert, et al., 2000; Schrenk, 1998). A third cytochrome P450 enzyme, CYP1B1 was later identified to be up-regulated by AHR and added to the gene battery (Savas, et al., 1994; Shen, et al., 1993; Sutter, et al., 1994). CYP1A1, CYP1A2 and CYP1B1 are NADPH-dependent monooxygenases that mediate phase I metabolism rendering end-products more reactive for subsequent reactions. The other four enzymes mediate phase II metabolism by conjugating charged groups onto xenobiotics to facilitate their elimination. Many AHR ligands are metabolized by these enzymes and the idea of induction of these enzymes as an adaptive response to AHR ligand exposure has been proposed (Gu, et al., 2000; Nebert, et al., 2004; Okey, 1990). The induction of these enzymes, however, cannot explain the plethora of toxic effects that are associated with TCDD exposure. TCDD is poorly metabolized and thus causes chronic and sustained induction of cytochrome P450 enzymes, leading to oxidative stress, formation of reactive oxygen species, lipid peroxidation and DNA damage (Hassoun, et al., 2002; Hassoun, et al., 2001; Nebert, et al., 2000; Shertzer, et al., 1998; Slezak, et al., 2000).

Several different methodological approaches have been used to discover new AHR target genes, such as microarrays, differential display, representational difference analysis and serial analysis of gene expression (Boverhof, et al., 2006; Dong, et al., 1997; Fletcher, et al., 2005; Frericks, et al., 2006; Frueh, et al., 2001; Hayes, et al., 2007; Kurachi, et al., 2002; Ohbayashi, et al., 2001; Oikawa, et al., 2002; Puga, et al., 2000; Svensson and Lundberg, 2001; Tijet, et al., 2006; Zeytun, et al., 2002). These studies revealed that AHR ligands induce the expression of a number of genes that do not encode metabolizing enzymes. Some of these genes have been shown to be ‘true’ AHR target genes, being transcriptionally regulated via a genomic AHR pathway involving AHRE binding within the gene regulatory region while others are affected by a non-
AHRE binding or non-genomic pathway (Dere, et al., 2011; Lo, et al., 2011; Lo and Matthews, 2012). TiPARP is an example of a novel AHR target gene that encodes a non-xenobiotic metabolizing enzyme and is regulated via the genomic AHR pathway (Hao, et al., 2012; Ma, et al., 2001).

3.6.1 TCDD-inducible poly(ADP-ribose) polymerase (TiPARP): a novel AHR target

TCDD-inducible poly(ADP-ribose) polymerase (TiPARP) was first identified in mouse hepatoma cells by differential display as a TCDD-induced mRNA (Ma, et al., 2001). The human orthologue of the mouse Tiparp gene was later identified and contains six exons and human TiPARP protein displayed 91.8% total amino acid identity with mouse TiPARP (Katoh and Katoh, 2003). Induction of TiPARP gene expression by TCDD is mediated via the AHR (Ma, 2002; Ma, et al., 2001). TiPARP induction by TCDD demonstrated characteristics similar to other AHR target genes such as superinduction in the presence of proteasomal and protein synthesis inhibitors (Ma, 2002). Subsequent microarray studies have shown that TiPARP mRNA is consistently up-regulated by TCDD both in vitro and in vivo (Dere, et al., 2006; Forgacs, et al., 2013; Frericks, et al., 2006; Henry, et al., 2010; Lo, et al., 2011; Tijet, et al., 2006). Additionally, TiPARP gene regulation by other AHR ligands both exogenous and naturally occurring as well as AHR antagonists has been previously observed (de Waard, et al., 2008; Hao, et al., 2012; Ito, et al., 2006; Pansoy, et al., 2010; Wahl, et al., 2008). Together these studies support TiPARP as an AHR target gene.

3.6.1.1 AHR regulation of TiPARP expression

TiPARP induction by TCDD has been reported to be rapid reaching maximal levels after 1.5 h to 2 h of treatment leading to TiPARP sometimes being referred to as an immediate early gene (Diani-Moore, et al., 2010; Hao, et al., 2012; Ma, 2002; Ma, et al., 2001). Currently, the regulation of TiPARP by activated-AHR is not fully understood. Previous studies have reported regions upstream of the Tiparp transcriptional start that are highly bound by ligand activated-AHR suggesting the presence of an AHRE-containing regulatory region (Ahmed, et al., 2009; Hao, et al., 2012; Lo, et al., 2011; Lo and Matthews, 2012; Pansoy, et al., 2010). There are a total of 24 AHRE core elements (GCGTG) located 10 kb upstream of the mouse Tiparp transcriptional start site (Hao, et al., 2012). Six of these AHREs are clustered together within a
45 bp fragment including four tandem repeats of AHRE core elements arranged in a concatemer configuration (Hao, et al., 2012). This AHRE concatemer was bound by activated-AHR and when expressed as a reporter gene displayed activity, although the level of ligand-induced activity was not substantial due to high constitutive activity (Hao, et al., 2012).

3.6.1.2 TiPARP Structure

TiPARP encodes a protein of 657 amino acid residues that contains three predicted modular domains: a C-terminal PARP catalytic domain, a central tryptophan-tryptophan-glutamate (WWE) domain and a single CX₈CX₅CX₅-type zinc-finger (Figure 9) (Schreiber, et al., 2006). The PARP catalytic domain shares 43% sequence homology with the catalytic domains of ARTDs (Ma, et al., 2001). The catalytic triad of the catalytic domain is H-Y-I and TiPARP is predicted to possess mono-ADP-ribosyltransferase (MART) activity (Otto, et al., 2005). During its initial characterization, TiPARP was reported to be a catalytically active enzyme; however, its potential mono-ADP-ribosyltransferase activities were not evaluated and remain to be determined (Ma, et al., 2001).

The WWE domain is composed of two conserved tryptophan residues and one glutamic acid residue and is the putative protein-protein interaction motif of TiPARP that also occurs in classes of proteins associated with ubiquitination (Aravind, 2001). Other ARTD family members including ARTD8, ARTD11, ARTD12 and ARTD13 contain at least one WWE domain and it was recently reported that the WWE domain of ARTD11 binds ADP-ribose (He, et al., 2012). The role of the TiPARP WWE domain is currently unknown.

The CCCH-type zinc finger is a putative RNA binding motif characterized by three cysteines and one histidine, which together coordinate a zinc ion to form a local peptide structure (Blackshear, 2002; Liang, et al., 2008). ARTD13 contains four CCCH-type zinc fingers in tandem and has demonstrated to bind specific viral mRNA sequences (Gao, et al., 2002). CCCH-type containing proteins are best known for their RNA binding ability but they also bind single-stranded DNA (Bai and Tolias, 1996; Collart, et al., 2005). The functionality and nucleic acid binding potential of CCCH-type zinc finger of TiPARP have not been determined.
3.6.1.3 Potential role of TiPARP in TCDD-mediated toxicity

TiPARP has been implicated in the suppression of hepatic gluconeogenesis, a prominent feature of the lethal wasting syndrome seen in experimental animals exposed to TCDD (Diani-Moore, et al., 2010). Phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are two key gluconeogenic genes known to be down-regulated by TCDD (Fan and Rozman, 1994; Hsia and Kreamer, 1985; Stahl, et al., 1993; Weber, et al., 1991). Because TCDD treatment of chick embryo livers reduced NAD<sup>+</sup> levels, increased total cellular PARP activity and TiPARP expression is induced by TCDD, it was hypothesized that TiPARP was responsible for NAD<sup>+</sup> and NADH depletion (Diani-Moore, et al., 2010). The depletion of NAD<sup>+</sup> was suggested to decrease the activity of SIRT1, which is dependent on NAD<sup>+</sup>. PEPCK and G6Pase are transcriptionally regulated by peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α), which is activated by deacetylation by SIRT1 (Diani-Moore, et al., 2010). The decreased SIRT1 activity would lead to decreased PGC1α activity and decrease PEPCK and G6Pase expression levels. TiPARP overexpression resulted in decreased glucose output, NAD<sup>+</sup> levels, PEPCK mRNA, PGC1α protein levels and increased acetylated PGC1α (Diani-Moore, et al., 2010). Knockdown of TiPARP resulted in increased glucose output and NAD<sup>+</sup> levels (Diani-
Moore, et al., 2010). It appeared that TiPARP mediated the TCDD-induced suppression of gluconeogenesis and its overexpression mimicked the effects of TCDD in a chick embryo model.

Similarly, TiPARP has been recently suggested to be involved in heightened sensitivity to non-alcoholic steatohepatitis (NASH) through depletion of NAD$^+$ (He, et al., 2013). Activation of AHR has been reported to lead to spontaneous fatty liver disease, lipid peroxidation and oxidative stress, all of which are risk factors for NASH (Dalton, et al., 2002; Lee, et al., 2010; Lu, et al., 2011). The increase in reactive oxygen species (ROS) production in mouse liver that is associated with activated-AHR was proposed to be a result of lowered superoxide dismutase 2 (SOD2) activity and compromised clearance of ROS (He, et al., 2013). In another elaborated model, TiPARP gene induction by TCDD was connected to a decrease in NAD$^+$ levels by catalytic consumption, this in turn deactivated mitochondrial SIRT3, which reduced SOD2 deacetylation decreasing its activity (He, et al., 2013). The decreased activity of SOD2 led to increased ROS production and increased sensitivity to NASH (He, et al., 2013).

These two studies based their proposed models of TiPARP-mediated hepatic depletion of NAD$^+$ to TiPARP gene induction by TCDD. Both studies failed to measure increased TiPARP protein levels following TCDD treatment or increased catalytic activity of TiPARP, making models highly presumptuous. Additionally, ARTD1 when activated accounts for 90% cellular poly(ADP-ribose) formation leading one to question whether TiPARP, a predicted MART could be solely responsible for NAD$^+$ depletion via catalytic consumption (Schreiber, et al., 2002).
Chapter 2: Aims of the present study

The ARTD superfamily consists of eighteen members and their potential activities in diverse cellular processes are just now being explored. TiPARP, the seventh ARTD family member to be identified, has received little attention since its discovery over ten years ago. TiPARP is better known as an AHR target gene that is induced by a vast array of AHR ligands in both in vivo and in vitro models. Despite being an apparently important AHR target gene, the potential role of TiPARP in AHR-mediated transcriptional regulation is not known. Moreover, the predicted functional domains of TiPARP also give little insight into a potential role in AHR transcriptional regulation. The TiPARP catalytic triad motif predicts that TiPARP is a class 2 ARTD with mono-ADP-ribosyltransferase activity rather than a class 1 poly-ADP-ribosylating ARTD; however, this has not been experimentally confirmed. Because TiPARP is an important AHR target and a potential mono-ADP-ribosyltransferase, we were interested in whether TiPARP and mono-ADP-ribosylation were involved in the AHR-mediated transcription.

The repression of AHR-mediated transcription is an aspect that is less understood than transactivation. Three mechanisms of negative regulation have been proposed: the targeted proteasomal degradation of activated-AHR via the 26S proteasome, the AHRR and an autoregulatory feedback loop involving an unidentified labile factor that promotes the proteolytic degradation of AHR. Because TiPARP expression is induced by activated-AHR we examined its potential as negative regulator of AHR transactivation.

The overall objective of this thesis research was to understand TiPARP and its potential role in AHR transactivation. Specific aims were:

- Demonstrate TiPARP is a mono-ADP-ribosyltransferase
- Determine role of TiPARP in AHR transactivation and understand its mechanism
- Compare TiPARP- and aryl hydrocarbon receptor repressor (AHRR)-mediated regulation of AHR transactivation
Chapter 3: Materials and Methods

4 Materials

4.1 Chemicals and biological reagents

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was purchased from Wellington Laboratories (Guelph, ON, Canada). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO). Cell culture reagents: Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 1 g/L, high glucose (4.5 g/L glucose) DMEM, 1:1 mixture of DMEM and Ham’s F-12 nutrient mixture (DMEM/F-12), RPMI-1640 medium, fetal bovine serum (FBS), penicillin/streptomycin antibiotic mixture, 0.25% Trypsin-EDTA, Phosphate Buffered Saline, were all purchased from Sigma-Aldrich (St. Louis, MO). Protein A-Agarose Fast Flow 50% (v/v) was purchased from Sigma-Aldrich. Lipofectamine LTX with Plus reagent was purchased from Life Technologies (Burlington, ON) and FuGENE HD tranfection reagent was purchased from Roche (Laval, QC).

Antibodies used for chromatin immunoprecipitation (ChIP), co-immunoprecipitation (IP), indirect immunofluorescence (IF) or Western blot (WB) experiments were: anti-AHR (H-211, ChIP, IF, WB), anti-AHR (N-19, WB), anti-ARNT (H-172, ChIP), anti-ARNT (C-19, WB), anti-rabbit immunoglobulin (IgG, sc-2027, ChIP, IP) (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-PARP7 (Abcam, 84664, Cambridge, MA, IF), anti-AHRR (HPA019614, ChIP, WB) and anti-β-actin (A2228, WB) both from Sigma-Aldrich, anti-AHR (SA-210, Biomol International Inc., Plymouth Meeting, PA), anti-GFP (632460, ChIP) and anti-GFP (JL-8, WB) both from Clontech (Mountain View, CA). Conjugated secondary antibodies used were: Horseradish Peroxidase (HRP)-conjugated anti-rabbit IgG whole antibody (NA934, GE Healthcare; Buckinghamshire, UK), HRP-conjugated normal anti-mouse IgG (sc2954) and HRP-conjugated normal anti-goat IgG (sc2741) all from Santa Cruz, and HRP-conjugated Clean Blot IP Reagent (ThermoScientific) and Alexa 568 Fluor-conjugated anti-rabbit IgG (Life Technologies, Burlington, ON, Canada). ECL Advance Western blotting detection system and ECL Plus both from GE Healthcare and SuperSignal West Dura (ThermoScientific) were used to visualize proteins for both Western blots and Co-IP experiments. For purification of PCR products Buffer PB (Qiagen, Toronto, ON) and EZ-10 Spin Column PCR Purification Kit (Bio
Basic Inc. Markham, ON) were used. For RNA extraction and cDNA synthesis, Aurum™ Total RNA Mini kit (Bio-Rad, Hercules, CA) and Superscript III reverse transcriptase (Life Technologies) were used. *Pfu Ultra* DNA polymerase (Agilent, Mississauga, ON) and *Pfx50* DNA polymerase (Life Technologies) were used for site-directed mutagenesis. High Fidelity PCR enzyme mix (ThermoScientific) was used for amplification of PCR products. QIAquick Gel Extraction Kit and HiSpeed Plasmid Maxi Kit were purchased from Qiagen. SsoFast EvaGreen® Supermix (Bio-Rad) was used for all quantitative real-time PCR reactions. ONE-glo™ Luciferase Assay System, TNT® Coupled Reticulocyte Lysate System and pGEM®-T Easy Vector System were purchased from Promega (Madison, WI). ^{35}S-radio-labeled L-methionine and ^{32}P-radio-labeled β-NAD^+ were purchased from Perkin-Elmer (Woodbridge, ON). All primers used for real-time PCR and for the generation of expression constructs were synthesized by Integrated DNA Technologies (IDT, Coralville, IA).

### 4.2 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 flat bottom plates used for luciferase assays were purchased from Corning (Lowell, MA). The 1.5 ml (Axygen) and 1.7 ml low-binding (Progene) microcentrifuge tubes were purchased from Utldent Scientific (St. Laurent, QC). 96-well non-skirted PCR plates were purchased from D-Mark Biosciences (Toronto, ON).

### 4.3 Instruments

For cell culture, HERAcell® 150 incubators (Kendro, Langenselbold, Germany) were used. The water bath (model # 1228, Sheldon manufacturing, Cornelius, OR) was purchased from VWR International (Plainfield, NJ). Cells were centrifuged with Centrifuge 5702 (Eppendorf, Hamburg, Germany). All centrifuge steps in PCR Purification and RNA extraction processes used Centrifuge 5415D (Eppendorf). Locator JR Cryo Biological Storage System (Thermolyne) was used to freeze, and store human immortalized cells was purchased from VWR International (Plainfield, NJ). To count cells, a Bright-Line Hemocytometer (Hausser Scientific, Horsham, PA) and Vistavision Light Microscope (VWR) were used. The purity and concentration of RNA and protein were measured with an Ultrospec 2100 pro Spectrophotometer (Biochrom, Cambridge,
England). Branson Digital Sonifier 450 (Danbury, CT) was used to sonicate cells. The rotator used for the ChIP assay and Western blotting was from Mini LabRoller (Labnet Inc., Edison, NJ). Real-time PCR amplification of ChIP DNA and cDNA was performed on Chromo4 Real-Time PCR detector (Bio-Rad). A DNAEngine Peltier Thermal Cycler (Bio-Rad) was used for site-directed mutagenesis and cDNA synthesis of extracted RNA. Protein and β-galactosidase levels were determined using a ThermoScientific 96 well Multiskan EX Photometer (Waltham, MA). Luciferase levels from reporter gene constructs were measured using GLO-max 96-well microplate luminometer (Promega). Indirect immunofluorescence images were viewed with Imager.Z1 epifluorescence microscope and Axiovision software (Zeiss, Toronto, ON) or an Olympus Fluoview 1000 confocal microscope (Center Valley, PA). Mouse livers were homogenized on a VWR PowerMax Advanced Homogenizing System 200 (VWR).

5 Methods

5.1 Maintenance of HuH7, COS7, TREx-FLP-IN 293, T47D, NCI-N87 and MCF7 cell lines

HuH7 human hepatoma, T47D human breast carcinoma, MCF7 human breast carcinoma and NCI-N87 human gastric carcinoma cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). TREx-FLP-IN 293 human embryonic kidney cells were purchased from Life Technologies. COS7 African Green Monkey kidney cells were a generous gift from Dr. Stephane Angers (Faculty of Pharmacy, University of Toronto). All cell lines were cryopreserved and stored in liquid nitrogen. All cells were ~80% confluent before freezing, cells were trypsinized and neutralized with fresh medium and centrifuged for 2 min at 4000 rpm. The cell pellet was re-suspended in a 90% FBS containing 10% DMSO (v/v) solution and aliquoted into labelled cryovials. Cryovials were immediately frozen at -80°C and then transferred to liquid nitrogen for long-term storage. Before use, HuH7, COS7 and TREx-FLN cells were rapidly thawed at 37°C and transferred to a T-75 tissue culture flask containing high glucose DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (PEST). Once cell lines were at least 80% confluent they were sub-cultured 1:6 to 1:12 twice a week. T47D cells were thawed and transferred to a T-25 tissue culture flask containing DMEM/F12 medium supplemented with 10% FBS and 1% PEST. Once cells were at least 80% confluent, cells were sub-cultured 1:2 to 1:3 twice a week. NCI-N87 cells were thawed and transferred to T-25 tissue
culture flask containing RPMI-1640 supplemented with 10% FBS and 1% PEST. MCF7 cells were thawed and transferred to T-25 tissue culture flask containing low glucose DMEM containing 10% FBS and 1% PEST. Once cells were at least 80% confluent they were sub-cultured 1:2 to 1:3 twice a week. All cell lines were sub-cultured as follows: medium was aspirated from culture flask and cells were washed once with 5-10 ml of sterile PBS. PBS was aspirated and 2-4 ml of pre-warmed trypsin-EDTA was added to the flask. The flask was placed back into incubator for 1-5 min until cells detached completely. An equal volume of pre-warmed completed medium was added to cell suspension in flask to neutralize the activity of trypsin. Cell suspension was vigorously pipetted up and down until homogenous then 1-4 ml of the cell solution was transferred to a T-175 flask containing 40 ml of completed media. All cell lines were maintained at 37°C and 5% CO₂.

5.2 Generation of Expression Constructs

All full-length human TiPARP expression constructs, TiPARP N-terminal truncation expression constructs and the C-terminal truncation 1-234 were created by PCR amplification of TiPARP cDNA from pCMV6-XL4-hTiPARP (Origene, Rockwood, MD). Two-hundred micrograms of pCMV6-XL4-hTiPARP was added to a reaction mixture containing 0.2 μM of desired forward primer containing Kozak sequence and/or EcoRI restriction site and reverse primer containing SalI restriction cut site (Table 4), 0.2 mM dNTPs and High Fidelity PCR enzyme mix (ThermoScientific) and amplified using the DNAEngine Peltier Thermal Cycler (Bio-Rad). PCR products were loaded on a 1% agarose gel and separated by gel electrophoresis. PCR products were excised from gel and purified using QIA quick Gel Extraction Kit following manufacturer’s instructions. One hundred nanograms of purified PCR product was ligated into 50 ng pGEM-T Easy vector with T4 DNA ligase (New England Biolabs, Whitby, ON) in a 10 μl reaction overnight at room temperature. Ligation reactions were transformed into 100 μl chemically competent DH5α strain E. coli. Bacteria were plated on LB-ampicillin agar plates containing 40 μg/ml X-gal and incubated overnight at 37°C. Individual white colonies were selected and grown in 3 ml LB-ampicillin overnight at 37°C with constant shaking. The following morning, plasmid DNA from selected colonies was purified using GenElute Plasmid Miniprep kit (Sigma) according to the manufacturer’s instructions. Purified plasmids were digested with EcoRI and SalI restriction enzymes for 1 h and digestions were loaded on a 1% agarose gel and separated by
gel electrophoresis. Inserts were excised from gel and purified using QIAquick Gel Extraction Kit (Qiagen). Inserts were ligated into pcDNA3.1 (Life Technologies) using EcoRI and XhoI restriction sites, pEGFP-C2 (Clontech) using EcoRI and SalI restriction sites and pGEX-4T1 (GE Healthcare) using EcoRI and XhoI restriction sites overnight at room temperature. The following day ligations were transformed into chemically competent DH5α strain *E. coli* and grown on LB-ampicillin plates for pcDNA3.1 and pGEX-4T1 ligations or LB-kanamycin plates for pEGFP-C2 ligations overnight at 37°C. The following day individual colonies were selected and grown in LB-ampicillin or LB-kanamycin overnight at 37°C with constant shaking. Plasmids were purified using GenElute Plasmid Miniprep kit (Sigma) according to the manufacturer’s instructions. pcDNA3.1 and pGEX-4T1 constructs were screened by restriction digests using EcoRI and XhoI restriction enzymes and pEGFP-C2 constructs were digested using EcoRI and SalI. Digests were loaded onto a 1% agarose gel and separated by gel electrophoresis. All plasmids that screened positive were sent to The Centre for Applied Genomics DNA Sequencing and Synthesis Facility (Medical and Related Sciences (MaRS) building, Toronto, ON, Canada) to verify sequence and framing. Mouse Tiparp expression constructs were generated by PCR amplification of mTiPARP cDNA from pCMV6-kan/neo-mTiPARP (Origene) using forward primer containing EcoRI restriction site and reverse primer containing XhoI restriction site (*Table 4*). Chicken TiPARP expression vectors were generated by PCR amplification of chicken TiPARP cDNA from total *Gallus gallus* liver RNA (Zyagen, San Diego, CA) using forward primer containing EcoRI restriction site and reverse primer containing SalI site (*Table 4*). Both chicken and mouse TiPARP inserts were cloned into EcoRI and XhoI sites of pcDNA3.1 and pGEX-4T1 and EcoRI and SalI sites of pEGFP-C2.

pEGFP-C2-AHRR-FL, pEGFP-C2-AHRRΔ8 (delta exon 8), pAcGFP-N1-AHRR-FL and pAcGFP-N1- AHRRΔ8 were generated by sub-cloning from pcDNA3.1-AHRR-FL and pcDNA-AHRRΔ8, (both gracious gifts from Dr. Mark Hahn, Woods Hole Oceanographic Institute, Woods Hole, MA). PCR products were amplified using forward primer containing EcoRI restriction site and reverse primer containing stop codon and XhoI restriction site or without stop codon and BamHI restriction site (*Table 4*). Both AHRR inserts were cloned into EcoRI and SalI sites of pEGFP-C2 or EcoRI and BamHI sites of pAcGFP-N1 (Clontech).
Table 4. PCR primers used for generation of TiPARP and AHRR inserts.

<table>
<thead>
<tr>
<th>Primer Description</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiPARP EcoRI 5’</td>
<td>CAAA GAATTC ATGGGAATGGAAACCACCCGAACC</td>
</tr>
<tr>
<td>TiPARP Sall 3’</td>
<td>CAAA GTGCAC TCAAATGGAAACGATGTGTAAGC</td>
</tr>
<tr>
<td>EcoRI TiPARP 33 5’</td>
<td>CAAA GAATTC ATCACTCCATGAAGACTGTTTTAAG</td>
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<tr>
<td>EcoRI Kozak TiPARP 33 3’</td>
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</tr>
<tr>
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</tr>
<tr>
<td>EcoRI Kozak TiPARP 53 5’</td>
<td>CAAA GAATTC ACCATG CTGAGGTCTTTTGAGGCAATATT</td>
</tr>
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</tr>
<tr>
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<td>CAAA GAATTC TCACGAAACTTTTACCTCGAAACCTT</td>
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<tr>
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<tr>
<td>Sall TiPARP 234 3’</td>
<td>CAAA GTGCAC TCAAGTGTTGACTCGAAGGGTCTGAC</td>
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<tr>
<td>EcoRI mouse TiPARP 5’</td>
<td>CAAA GAATTC ATGGAAATGGAAACCACCCGAACC</td>
</tr>
<tr>
<td>XhoI mouse TiPARP 3’</td>
<td>CAAA CTGGAC TCAATGGAAACCACCCGAACC</td>
</tr>
<tr>
<td>EcoRI chicken TiPARP 5’</td>
<td>CAAA GTGCAC TCAATGGAAACCACCCGAACC</td>
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<tr>
<td>Sall chicken TiPARP 3’</td>
<td>CAAA GTGCAC TCAATGGAAACCACCCGAACC</td>
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<tr>
<td>EcoRI hAHRR 5’</td>
<td>CAAA GAATTC ATGCGAGGAGCTGATCCCC</td>
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<tr>
<td>XhoI hAHRR stop 3’</td>
<td>CAAA CTGGAC TCAATGGAAACCACCCGAACC</td>
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<tr>
<td>BamHI AHRR no stop 3’</td>
<td>CAAA GAATTC CCG TGGCGAGGAGCTGATCCCC</td>
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5.3 Site-directed mutagenesis

Catalytic, zinc finger, nuclear localization point mutants, double alanine point mutants and C-terminal 1-448 truncation were generated by site-directed mutagenesis. Fifty nanograms of pcDNA-TiPARP, pEGFP-C2-TiPARP and pGEX-4T1-TiPARP was added to a reaction mixture containing 0.2 mM dNTPs, 0.2 μM forward and reverse primers containing desired mutation
(Table 5), Pfu Ultra DNA polymerase (Agilent) or Pfx50 DNA polymerase (Life Technologies) with 0.5 mM MgCl₂ and amplified using the DNAEngine Peltier Thermal Cycler (Bio-Rad). PCR products were digested by 1 μl DpnI restriction enzyme (New England BioLabs) for 1 h at 37°C and 10 μl of digested PCR product was transformed into chemically competent DH5α E. coli. Bacteria were plated on LB-ampicillin or LB-kanamycin agar plates and incubated overnight at 37°C. The following day colonies were selected and grown overnight at 37°C under constant agitation. The following morning plasmid DNA from selected colonies was purified using GenElute Plasmid Miniprep kit (Sigma) according to the manufacturer’s instructions. Purified plasmid DNA was sent to The Centre for Applied Genomics DNA Sequencing and Synthesis Facility (Medical and Related Sciences (MaRS) building, Toronto, ON, Canada) to verify mutations.

Table 5. PCR primers used for site-directed mutagenesis of TiPARP.

<table>
<thead>
<tr>
<th>TiPARP</th>
<th>Primer 1</th>
<th>Primer 2</th>
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<tr>
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<td>TiPARP K42A; K43A 3’</td>
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<tr>
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<td>TiPARP H532A 3’</td>
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<tr>
<td>TiPARP Y564A 5’</td>
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<tr>
<td>TiPARP I631A 5’</td>
<td>TTGTCTTCTGAGCTCAAGGCTGTTTTGTGCTGTTAATG</td>
<td>TiPARP I631A 3’</td>
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<tr>
<td>TiPARP I631E 5’</td>
<td>AGGAAAAAGTGAAGAGGCTCGTTTTGACGCTGTTAATG</td>
<td>TiPARP I631E 3’</td>
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<td>TiPARP 449 stop 5’</td>
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<td>TiPARP 449 stop 3’</td>
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<td>TiPARP A218; S219A 5’</td>
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<td>TiPARP A218; S219A 3’</td>
</tr>
<tr>
<td>TiPARP L220A; D221A 5’</td>
<td>AGGAAAAAGTGAAGAGGCTCGTTTTGCTGAGCTG</td>
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<tr>
<td>TiPARP L222A; V223A 5’</td>
<td>GTGAAGAGGCTTCTCGAGTGCCTGCTGAGCTG</td>
<td>TiPARP L222A; V223A 3’</td>
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</tbody>
</table>
5.4 Transient transfection of HuH7 cells for RNA expression, Western blot and ChIP assays

HuH7 cells were seeded in six-well plates (35 mm diameter) at a density of 2.0 x 10^5 cells/well. Following 24 h cells were transfected with 3 μg pcDNA or pcDNA-TiPARP or pEGFP or pEGFP-C2-TiPARP using 6 μl Lipofectamine LTX and 6 μl Plus reagent. Medium was changed the following day and cells were treated with 10 nM TCDD or DMSO (0.1%) for 6 h and RNA isolation was performed (see below). For ChIP assays transfected cells were treated with 10 nM TCDD or DMSO for 45 min and cells were harvested for ChIP assays (see below). For Western blots, HuH7 were transfected with 2 μg pEGFP-TiPARP or pEGFP-TiPARP point mutant or truncation construct with 4 μl Lipofectamine LTX + 4 μl Plus reagent. Following 24 h medium was changed and proteins were overexpressed for another 24 h and cells were prepared for protein extraction.

5.5 Transient transfection of T47D cells for Western blot

T47D cells were plated in six-well plates at a density of 3.0 x 10^5 cells/well. Following 24 h cells were transfected with 3 μg pcDNA or pcDNA-TiPARP using 6 μl Lipofectamine LTX and 6 μl
Plus reagent. Medium was changed the following day and cells were treated for 0-24 h with 10 nM TCDD or DMSO and cells were harvested for protein extraction.

5.6 Transient transfection of HuH7 cells and reporter gene assays

HuH7 cells were plated in 12-well plates (22 mm diameter) at a density of 1.2 x 10^5 cells/well. The following day cells were transfected with 50-1500 ng pcDNA-TiPARP and/or 250-750 ng pRc-CMV-hAHR (a generous gift from Dr. Patricia Harper) and/or 250-750 ng pcDNA4-hARNT (a generous gift from Dr. Kevin Gardner) or 200 ng pcDNA-mTiparp, pcDNA-chTiPARP, 200-1500 ng pCMV-XL-ARTD1 or pCMV-XL-ARTD12 (Origene) or 200 ng pcDNA-TiPARP truncation or point mutant plasmids or 5-100 ng pcDNA-AHRRΔ8 or 500-100 ng pcDNA-AHRR-FL and 200 ng pCYP1A1-luc or pGL3-CYP1B1-luc plasmid using 2-4 μl Lipofectamine LTX and 2-4 μl Plus reagent. All reactions included 50 ng of pCH110-β-Gal (GE Healthcare) to normalize for transfection efficiency. pcDNA3.1 was used as carrier DNA for all transfection experiments. After 24 h cells were treated with TCDD or DMSO for 24 h and cells were washed once with 1 ml PBS and lysed with 250 μl 1X Passive Lysis Buffer (Promega) for 15 min with gentle rocking. Twenty-five microliters of lysate were loaded in duplicate into a 96-well black flat bottom plate and 25 μl ONE-Glo luciferase substrate (Promega) were added to each well and luciferase activity was determined using a GLO-max luminometer (Promega). For β-galactosidase activity 10 μl of lysate were loaded into a 96-well clear flat bottom plate in duplicate and 100 μl β-galactosidase buffer (0.6 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 1 mM MgSO₄, 100 mM β-mercaptoethanol) and 2.5 mM ONPG (ortho-Nitrophenyl-β-galactoside) were added to lysate and plate was incubated at 37°C for 30 min and reaction was stopped addition of 160 mM Na₂HCO₃ to reaction and 420 nm absorbance was measured using Multiskan EX Photometer (ThermoScientific). Luciferase activity was normalized to corresponding β-galactosidase activity.

5.7 Transient transfection and RNAi

T47D and NCI-N87 cells were plated in 6-well plates at a density of 3.0 x 10^5 cells/well. HuH7 cells were plated in in 6-well plates at a density of 2.0 x 10^5 cells/well. MCF7 cells were plated in 6-well plates at a density of 3.5 x 10^5 cells/well. Twenty-four hours after plating T47D, HuH7 and NCI-N87 cells were transfected with 100 nM SMARTpool: ON-TARGETplus TiPARP
siRNAs (siTiPARP; L-013948-00) or ON-TARGETplus Non-targeting Pool (NTP; D-0011810-10) siRNAs with 4 μl DharmaFECT1 (Dharmacon, Lafayette, CO). T47D cells were transfected with 100 nM ON-TARGETplus TiPARP (siTiPARP-12; J-013948-12, siTiPARP-13; J-013948-13) siRNAs or ON-TARGETplus non-targeting siRNA #2 (NT; D-0018100-02) (Dharmacon) with 4 μl DharmaFECT1. MCF7 cells were transfected with 50 nM MISSION AHRR siRNAs (siAHRR1; SASI_Hs02_00353810, siAHRR2; SASI_Hs01_00213384, Sigma-Aldrich) or NT2 (Dharmacon) with 2 μl DharmaFECT1. MCF7 cells were transfected with 50 nM MISSION AHRR siRNAs (siAHRR1; SASI_Hs02_00353810, siAHRR2; SASI_Hs01_00213384, Sigma-Aldrich) or NT2 (Dharmacon) with 2 μl DharmaFECT1. Medium of all cell lines was changed 24 h following transfection. TiPARP was knocked down for 48 h post-transfection and dosed with 10 nM TCDD or DMSO for 1.5, 3 h and 24 h and cells were harvested for Western blot, ChIP and RNA expression assays. AHRR was knocked down for 24 h post-transfection and dosed with 10 nM TCDD or DMSO for 24 h and cells were harvested for protein and RNA expression assays.

5.8 mRNA time-course

T47D cells were seeded in 6-well plates at a density of 3.0 x 10^5 cells/well. Following 24 h cells were treated with 1 μg/ml actinomycin D (Sigma) for 1 h to 24 h or 10 nM TCDD for 0.25 h to 24 h. MCF7 cells were seeded in 6-well plates at a density of 3.5 x 10^5 cells/well. Following 24 h cells were treated with 10 nM TCDD or DMSO for 0.25 h to 24 h.

5.9 Tiparp-null mice and generation of mouse embryonic fibroblasts (MEF)

Tiparp^{-/} mice (strain B6;129S4-Tiparp^{Gt(ROSA)79Sor}) were purchased from Jackson Laboratory (Bar Harbor, ME) and housed and bred at the Division of Comparative Medicine (University of Toronto) under the management of our lab technician, Debbie Brenneman. Six to eight week old male and female wildtype (Tiparp^{+/+}), knockout (Tiparp^{-/-}) and heterozygous (Tiparp^{+/}) mice were treated with a single dose of 30 μg/kg b.w. TCDD or corn oil (control) by intraperitoneal injection. Mice were sacrificed after 6 h of treatment and livers excised and snap frozen in liquid nitrogen and transferred to a -80ºC freezer. Four mice of each group were tested.

Wildtype, Tiparp-deficient (Tiparp^{-/-}) and Tiparp^{+/} fibroblasts were prepared from E14.5 embryos derived from matings of mice heterozygous for disruption of the Tiparp allele. Primary fibroblasts from wildtype, Tiparp^{-/-} and Tiparp^{+/} siblings were immortalized at passage 2 after transfection with Simian virus large T antigen (SV40gp6) in pSG5 (Stratagene) and a puromycin
resistance plasmid, and selected in puromycin-containing medium. The genotypes of the MEFs were verified by PCR. Once immortalized, all MEF cell lines were plated into 10 cm dishes at a density of 2.0 million cells/dish. Twenty-four hours after plating cells were treated with 10 nM TCDD or DMSO for 45 min for ChIP assays and 24 h for Western blots. For RNA expression experiments MEFs were seeded in 6-well plates at a density of 1.5 x 10⁵ cells/well and treated 24 h following plating with 10 nM TCDD or DMSO for 6 h or 24 h or pre-treated with 10 μg/ml cycloheximide (CHX, Sigma) for 1 h followed by 10 nM TCDD for 6 h or pre-treated with 10 μM MG132 (Sigma) for 30 min followed by 10 nM TCDD for 4 h. Following all treatments cells were harvested for RNA isolation.

5.10 RNA isolation and cDNA synthesis

5.10.1 Mammalian cells
For RNA isolation of all transfected and/or treated cells, medium was aspirated and cells were washed once with PBS. Three hundred and fifty microliters of Total RNA Lysis Solution (Bio-Rad) containing 1% β-mercaptoethanol was added to each well to lyse cells. Following 10 min cell lysates were scraped and transferred to microcentrifuge tubes. An equal volume of 70% ethanol was added to each tube and was mixed by gentle pipetting to bind RNA. RNA was purified using Aurum™ Total RNA Mini Kit (Bio-Rad) following the manufacturer’s protocol. Briefly, RNA was bound to spin columns by centrifugation at 13,000 rpm for 30 s. The column membranes were washed once with Total RNA Wash Low Stringency solution and 80 μl DNase I solution (DNase I stock solution diluted 1:40 in DNase I Dilution Solution) was added directly to column membranes and allowed to digest for 15 min at room temperature. DNase I was removed by addition of Total RNA Wash High Stringency solution followed by centrifugation at 13,000 rpm for 30 s. Column membranes were then washed with Total RNA Wash Low Stringency solution and centrifuged at 13 000 rpm for 1 min. Columns were dried by centrifugation at 13,000 rpm for 2 min prior to elution. To elute, columns were transferred to new microcentrifuge tubes and 40 μl of Total RNA Elution Solution was placed directly onto column membrane and allowed to incubate for 1 min. Tubes were centrifuged at 13,000 rpm for 2 min. RNA concentration was measured using the spectrophotometer (Biochrom) and all samples were set to 50 ng/μl concentrations by addition of DNAase/RNase-free distilled water.
5.10.2 Mouse liver

Approximately 40 mg of snap frozen liver was thawed on ice and re-suspended in 700 μl Total RNA Lysis Solution (Bio-Rad) containing 1% β-mercaptoethanol and homogenized for 20 s using a VWR PowerMax AHS homogenizer. Homogenates were centrifuged at 13,000 rpm for 3 min and supernatant was transferred to a new microcentrifuge tube. An equal volume of 70% ethanol was added to supernatant and mixed by gentle pipetting until homogenous. RNA was purified using Aurum™ Total RNA Mini Kit (Bio-Rad) following the manufacturer’s protocol.

5.10.3 cDNA synthesis

For cDNA synthesis, 500 ng of extracted RNA was reversed transcribed using 2500 U SuperScriptIII (Life Technologies) in a total volume of 20 μl containing 2.5 μM random hexamers and 1 μM dNTPs. cDNA was synthesized for 1 h at 50°C then heated to 70°C for 15 min to inactivate enzyme. After synthesis cDNA samples were diluted with 60 μl DNAse/RNAse-free distilled water. Real-time PCR (qPCR) was performed on 1 μl of cDNA synthesis sample using SsoFast EvaGreen® SYBR Supermix (Bio-Rad) and primers to amplify CYP1A1, CYP1B1, TiPARP, AHRR, NFE2L2, NQO1 and β-actin mRNAs (Table 6) using Chromo4 Real-Time PCR detector (Bio-Rad) and analyzed using Opticon Monitor 3 (Bio-Rad). All target gene transcripts were normalized to β-actin mRNA and run in triplicate. Fold change was calculated using the difference between cycle threshold (Ct) value of treatment and control using the formula comparative method. ΔCt sample - ΔCt control = ΔΔCt. The expression level of each gene was then calculated using 2-ΔΔCt and compared to DMSO or 0 h treatment (=1). PCR was performed using specific primers (Table 6). Cycling conditions consisted of an initial denaturation step of 95°C for 3 min followed by 45 cycles of 95 °C for 15 s, and the 60°C annealing temperature for 30 s.

Table 6. List of qPCR primers used in RNA expression assays.

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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>hCYP1B1 mRNA</td>
<td>CCTATGTCCCTGGCCCTTCTTTT</td>
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<tr>
<td>hCYP1B1 mRNA</td>
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</tr>
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<td>hAHRR mRNA 3’</td>
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<td>mCYP1A1 mRNA 5’</td>
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<tr>
<td>mCYP1A1 mRNA 3’</td>
<td>TCCCCAAATCATTGCTAGAT</td>
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<tr>
<td>mCYP1B1 mRNA 5’</td>
<td>CCAGATCCGGCTGCTTACA</td>
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<td>mTiPARP mRNA 5’</td>
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<tr>
<td>mNQO1 mRNA 3’</td>
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### 5.11 Chromatin Immunoprecipitation (ChIP)

For ChIP assays, all three MEF cell lines were plated in 10 cm dishes at a density of 2.0 million cells/dish. MCF7 cells were plated in 10 cm dishes at a density of 3.5 million cells/dish. Cells were grown for 24 h and treated with 10 nM TCDD or DMSO for 45 min. Treated HuH7 cells overexpressing EGFP-TiPARP or EGFP and treated T47D cells with TiPARP knockdown (from sections 5.4 and 5.7 respectively) were used for ChIP assays. Following treatment protein-DNA complexes were cross-linked with 1% formaldehyde (Sigma) for 10 min and then quenched with 125 mM glycine for 5 min. The medium was aspirated and cells were washed twice with 2 ml PBS. Five hundred microliters of PBS + 0.1% (v/v) Tween 20 (PBS/T) was added to each well and cells were scraped and centrifuged for 5 min at 7 500 rpm at 4°C. Supernatant was aspirated off and cells were re-suspended in ice cold 1 ml Buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl2, 10 mM KCl, 0.05% NP-40) containing 1X protease inhibitor cocktail (PIC, Sigma) and rotated at 4°C for 15 min to remove cytosol. Nuclei were pelleted by centrifugation at 7,500 rpm for 5 min and supernatant containing cytosolic fraction was aspirated. Nuclei were re-suspended in 400 μL TSE I buffer (50 mM Tris-Base [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing 1X PIC and incubated on ice for 10 min. Nuclei were sonicated on ice for 10 times for 10 sec each time, resulting in the shearing of chromatin to an average size of ~500 bp. Soluble chromatin was collected by centrifugation at 13 000 rpm for 10 min at 4°C and supernatant transferred to a new microcentrifuge tube. A volume of 30 μl of Protein A-Agarose (50% slurry) was added to chromatin and incubated at 4°C under gentle rotation for 2 h.
aliquot of chromatin was stored at -20°C for the 5% total input sample. Agarose beads were pelleted by centrifugation and chromatin was aliquoted into fresh microcentrifuge tubes containing 1 μg of antibody, 0.5 μg/μl BSA and 0.05 μg/μl salmon sperm DNA in TSE I buffer. Chromatin was immunoprecipitated overnight at 4°C under gentle rotation. The following morning Protein A-Agarose (25 μl of 50% slurry) was added and chromatin was incubated for another 1.5 hours at 4°C under gentle rotation. Protein A beads were pelleted by centrifugation and washed successively for 10 min in 3 X 1 ml of TSE I, 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), 1 ml of LiCl buffer (20 mM Tris-Base [pH 8.0], 250 mM LiCl, 1 mM EDTA, 1% NP-40, 1% Na-deoxycholate), and 2 X 1 ml of TE (10 mM Tris-Base [pH 8.0], 1 mM EDTA). Protein-DNA complexes were eluted in 110 μl of elution buffer (TE + 1% SDS) for 30 min under constant rotation, and the cross-links were reversed by overnight incubation at 65°C. The following morning, eluted DNA was bound using 5X volume of Buffer PB (Qiagen) and purified using EZ-10 Spin Column PCR Purification Kit (Bio Basic) according to manufacturer’s instructions with slight modification. Briefly, bound DNA was added to column and centrifuged at 11 000 rpm for 1 min. Columns were washed twice with wash solution (containing 95% ethanol) and dried by centrifugation. DNA was eluted from column using 100 μl of elution buffer. ChIP DNA was quantified by qPCR using SsoFast EvaGreen® SYBR Supermix (Bio-Rad) and primers (Table 7) to amplify human CYP1A1 and CYP1B1 enhancer regions and mouse Cyp1a1 and Cyp1b1 enhancer regions and downstream negative binding regions of each gene. ChIP DNA samples were run in triplicate on Chromo4 Real-Time PCR detector (Bio-Rad) and analyzed using Opticon Monitor 3 (Bio-Rad). Results were normalized to 5% total input and reported as percent recruitment relative to 100% total input.

Table 7. List of qPCR primers used for ChIP assays.

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<th>Primers</th>
<th>Sequence</th>
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<td>hCYP1B1 enhancer 3’</td>
<td>GCCGAACCTTTATCGGGTTGA</td>
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<tr>
<td>hCYP1A1 5’782 bp downstream 5’</td>
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### Table

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### 5.12 Western blot

All transfected and/or treated cells were washed once with PBS and scraped in 350-750 μl PBS/T and cells were pelleted by centrifugation at 7500 rpm for 5 min at 4°C and supernatants were aspirated and pellets were frozen at -80°C. For detection of AHR, cell pellets were thawed on ice and re-suspended in 200 μl cold Cell Lysis Buffer (50 mM HEPES [pH 7.5], 10% glycerol, 100 mM KCl, 2 mM EDTA, 0.1% NP-40) containing 1X PIC and 2 mM DTT. Cell suspensions were incubated on ice for 10 min then sonicated for 15 s at 30% amplitude to lyse cells. Cell lysates were placed back on ice and incubated for 10 min and soluble proteins were quantified by Bradford assays. Fifty micrograms of cell lysates were resolved by SDS-8%-PAGE and transferred to a nitrocellulose blotting membrane (Pall Life Sciences) in 25 mM Tris base (pH 8.3) containing 19.2 mM glycine and 20% (v/v) methanol. The membranes were blocked in 2% fat-free milk PBS/T for 2 h at room temperature with constant rocking and then immunoblotted with a 1:5000 dilution of anti-AHR (H-211, Santa Cruz) or 1:10 000 AHR (SA-210, Biomol International Inc.) antibody in 2% fat-free milk PBS/T overnight at 4°C with constant rocking. The membrane was then washed with PBS/T for 30 min and then incubated with ECL Anti-rabbit IgG, Horseradish Peroxidase linked antibody (NA934, GE Healthcare) for 1 h at room temperature with constant rocking. Proteins were visualized using ECL Select Western Blotting Detection System (GE Healthcare) according to the manufacturer’s instructions. The membranes were exposed to an autoradiography film for 1 to 5 min. β-actin was used as a loading control. AHR protein levels were normalized to β-actin levels and quantified using ImageJ analysis software (NIH).

For detection of AHRR, ARNT or GFP-TiPARP fusions, frozen cell pellets were thawed on ice and then re-suspended in 100 μl cold TENG buffer (50 mM Tris-Base [pH 8.0], 2 mM EDTA, 150 mM NaCl, 5% glycerol, 1% NP-40) containing 2.5X PIC and 2 mM DTT. Cell suspensions were incubated on ice for 10 min and then frozen at -80°C for 10 min to lyse cells. Lysates were thawed on ice for 10 min and then centrifuged at 13 000 rpm for 10 min at 4°C to pellet cell
debris. Supernatants were collected and transferred to new microcentrifuge tubes and solubilized proteins were quantified by Bradford assays. For AHRR, ARNT and GFP blots 200 μg whole cell lysates were resolved on by SDS-8%-PAGE transferred to a nitrocellulose blotting membrane as described above. For AHRR immunobLOTS, membranes were blocked for 2 h at room temperature in 2% fat-free milk TNT buffer (100 mM Tris-Base [pH 7.5], 150 mM NaCl, 0.1% (v/v) Tween 20) and then immunoblotted with 1:2000 anti-AHRR antibody (HPA019614, Sigma) in 2% milk TNT overnight at 4°C with constant rocking. For ARNT immunoblots membranes were blocked with 5% milk TNT for 2 h at room temperature then immunoblotted with 1:200 anti-ARNT antibody (C-19, Santa Cruz) in 5% milk TNT overnight at 4°C with constant rocking. The membranes were washed with TNT for 30 min and then membranes were incubated with ECL Anti-rabbit IgG, Horseradish Peroxidase linked antibody (GE Healthcare) for AHRR immunobLOTS or HRP-conjugated normal anti-goat IgG (sc2741, Santa Cruz) for ARNT immunobLOTS for 1 h at room temperature with constant rocking. Proteins were visualized using SuperSignal West Dura (Thermo Scientific) and ECL Plus Western Blotting Detection System (GE Healthcare) for AHRR and ARNT immunobLOTS respectively, according to the manufacturers’ instructions. The membranes were exposed to an autoradiography film for 1 to 5 min. For GFP immunobLOTS membranes were blocked in 5% fat-free milk PBS/T for 2 h and immunoblotted for GFP-fusion proteins using 1:2000 anti-EGFP antibody (JL-8, Clontech) in 2.5% milk PBS/T overnight at 4°C with constant rocking. The following morning membranes were washed with PBS/T for 30 min and membranes were incubated with HRP-conjugated normal anti-mouse IgG (sc2954) in 2.5% milk PBS/T for 1 h at room temperature with constant rocking. Proteins were visualized using SuperSignal West Dura (Thermo Scientific) according to manufacturer’s instructions. Membranes were exposed to autoradiography film for 1 to 5 min.

5.13 Co-immunoprecipitation

TREx-FLP-IN 293 cells were seeded in 6-well plates at a density of 4.0 x 10^5 cells/well were transfected with 650 ng pRc-CMV-AHR, pRc-CMV-AHR-1-425, pCMV-AHR-TAD (464-848) (all generous gifts from Dr. Patricia Harper), 200 ng pcDNA4-hARNT and 650 ng pEGFP-TiPARP-FL or 650 ng pEGFP-TiPARP truncation with 3 μl FuGENE HD (Roche) according to the manufacturer’s instructions. Twenty-four hours following transfection cells were treated with 10 nM TCDD or DMSO for 1.5 h and cells were scraped in PBS/T and centrifuged at 7 500 rpm for 5 min at 4°C. Whole cell extracts were prepared by two freeze-thaw cycles using 200 μl ice
cold TENG buffer containing 1X PIC and 2 mM DTT as described above. Two micrograms of anti-AHR antibody (H-211) or anti-GFP (632460, Clontech) 1:200 dilution were incubated with whole cell extracts for 1 h in 4°C with constant rotation followed by incubation with protein-A beads for 1 h. Beads were washed five times with NP-40 buffer (20 mM Tris Base [pH 8.0], 150 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA) and eluted in 1X sample buffer, separated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were exposed to anti-GFP (JL-8, Clontech), anti-AHR (H-211) or anti-AHR (N-19) antibodies. All membranes were then washed and incubated with the appropriate secondary antibodies for 1 h at room temperature. After washing, bands were visualized using SuperSignal West Dura substrate or ECL Select or ECL Plus.

5.14 *In vitro* translation and \(^{35}\)S P ARP assays

All pcDNA-TiPARP constructs were *in vitro* translated and labelled with \(^{35}\)S-methionine using the TNT® Coupled Reticulocyte Lysate System according to manufacturer’s instructions to verify expression. Briefly, one microgram of pcDNA-TiPARP construct was added to rabbit reticulocyte lysate containing 1X TNT buffer, 1 mM amino acid mixture minus methionine, 40 U RNAsOUT (Invitrogen), T7 RNA polymerase and \(^{35}\)S-methionine (Perkin-Elmer) and reactions were incubated at 30°C for 90 min. Five percent of each reaction was diluted in TENG buffer and 1X loading dye was added to each tube. Proteins were resolved by SDS-8%-12% PAGE and gels were incubated in KODAK™ ENLIGH TNING™ Rapid Autoradiography Enhancer (Perkin-Elmer) for 30 min with gentle rocking. Gels were then incubated in gel drying solution (10% (v/v) ethanol and 5% acetic acid) for 20 min with gentle rocking and gels were dried at 80°C for 2 h. Proteins were visualized by autoradiography.

\(^{35}\)S P ARP assays were carried out in a 30 \(\mu\)l reaction volume at 30°C in 1X P ARP buffer (5 mM Tris Base [pH 8.0], 0.2 mM DTT, 4 mM MgCl\(_2\), 1% BSA) or 500 \(\mu\)M \(\beta\)-NAD\(^+\) (Sigma) or 1 \(\mu\)g activated DNA (Sigma) with 5% *in vitro* translated proteins. Reactions were stopped with 1X sample buffer and resolved by SDS-8%-12% PAGE.

5.15 Indirect immunofluorescence

Glass cover slips (VWR No. 1, 22 mm x 22 mm) were sterilized in 70% ethanol for 20 min in the cell culture hood. Cover slips were transferred to 6-well cell culture plates and allowed to dry
against well wall for 15 min. HuH7 and COS7 cells were seeded onto sterile cover slips at a density of 2.0 \times 10^5 \text{ cells/well} and MCF7 cells were seeded onto cover slips at a density of 2.0 \times 10^5 \text{ cells/well} and cells were allowed to adhere overnight. Cells were transfected with 700 ng pEGFP-TiPARP constructs and/or 500 ng pRc-CMV-AHR and 300 ng pcDNA4-ARNT with 3 \mu l Lipofectamine LTX or 100 ng pEGFP-AHRR constructs or 500 ng pAcGFP-AHRR constructs and all proteins were allowed to overexpress for 24 h. Cells co-transfected with pEGFP-TiPARP, pRc-CMV-AHR and pcDNA4-ARNT were treated with 10 nM TCDD for 1.5 h. Cover slips were washed once with PBS and fixed in 4\% paraformaldehyde for 10 min. Cells were PBS washed three times for 5 min each and then incubated with PBS + 0.4\% Triton-X in PBS for 20 min to permeabilize nuclei. Cover slips were washed three times with PBS for 5 min and mounted with Vectashield containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

For cells co-overexpressed with GFP-TiPARP constructs, AHR and ARNT coverslips were blocked in 1\% goat serum/0.2\% Triton-X in PBS for 1 h and incubated with 1:100 anti-AHR antibody (H-211, Santa Cruz) in 1\% goat serum/0.2\% Triton-X in PBS overnight at 4^\circ C. The following morning cover slips were then incubated with Alexa 568-conjugated secondary antibody (Life Technologies) for 45 min and mounted with Vectashield containing DAPI.

For detection of endogenous TiPARP, HuH7 cells were fixed with methanol/acetone for 20 min/1 min at -20^\circ C. Cells were permeabilized with 0.4\% Triton-X in PBS for 20 min and then blocked with 1\% goat serum/0.2\% Triton-X in PBS for 1 h. Cover slips were incubated overnight with anti-TiPARP (84664, Abcam) diluted 1:100 in 1\% goat serum/0.2\% Triton-X in PBS overnight at 4^\circ C. The following morning cover slips were then incubated with Alexa 568-conjugated secondary antibody for 45 min and mounted with Vectashield containing DAPI.

All images were acquired using an Imager.Z1 epifluorescence microscope and Axiovision software (Zeiss) or an Olympus Fluoview 1000 confocal microscope.

### 5.16 GST purification

pGEX-4T1-TiPARP constructs were transformed into BL21 stain E. coli. The following day single colonies were selected and cultured overnight in 10 ml LB. Overnight cultures were transferred to 500 ml LB-ampicillin and cultured at 30^\circ C under constant shaking. Once cultures
reached OD$_{600}$ = 0.8-1.0 GST fusion protein expression was induced with 0.1 mM isopropyl-β-D-thio-galactoside (IPTG, BioShop) for 3-4 h at 37°C with shaking. Bacteria were pelleted at 8 000 rpm for 10 min at 4°C and frozen overnight at -20°C. Pellets were thawed on ice and re-suspended in 10 ml GST lysis buffer (20 mM Tris Base [pH 7.6], 10 mM MgCl$_2$, 1 mM MnCl$_2$, 1 mM EDTA, 150 mM NaCl, 5% Glycerol) containing 1X PIC and then incubated with 1 mg/ml lysozyme (BioShop), 1 μg/ml DNAse I (Life Technologies), 10 mg/ml RNAse A (BioShop) for 15 min at room temperature. Extracts were sonicated for 2 min at 45% amplitude and rotated at 4°C for 30 min. Extracts were centrifuged at 14 000 rpm for 15 min and soluble protein were transferred to pre-cooled 50 ml conical tubes. One milliliter of equilibrated Glutathione Sepharose 4 Fast Flow beads (50% slurry in GST lysis buffer) were added to extracts and rotated overnight at 4°C. The following morning beads were loading into 20 ml Econo-Column Chromatography Columns (Bio-Rad). Beads were consecutively washed at 4°C with cell lysis buffer (20X bed volume) 3X and NETN buffer (50 mM Tris Base [pH 7.6], 100 mM NaCl, 1 mM MgCl$_2$, 10% glycerol, 0.5% NP-40) 3X. GST fusion proteins were eluted from beads in 2 ml 10 mM glutathione for 30 min and concentrated and purified using Amicon Ultra-0.5 ml Centrifugal Filters 50 kDa cut off (Millipore).

5.17 $^{32}$P-NAD$^+$ auto-ADP-ribosylation and hetero-ADP-ribosylation assays

Purified ARTD1 was purchased from Trevigen (Gaithersburg, MD). Auto-ribosylation assays were carried out in a 30 μl reaction volume at 30°C in 1X PARP buffer (5 mM Tris Base [pH 8.0], 0.2 mM DTT, 4 mM MgCl$_2$, 0.1 μg/ml BSA), 2 μCi $^{32}$P-NAD$^+$ (Perkin-Elmer, Woodbridge, ON) and/or 500 μM β-NAD$^+$ (Sigma), 1 μg activated DNA (Sigma) or RNA. Approximately 5 μg of GST fusion partially purified protein or 1 μg ARTD1 were used. For hetero-ribosylation experiments 8 μg of core histones (H2A, H2B, H3, H4) (New England Biolabs) were added to the reaction. Reactions were incubated for 30 min and stopped by addition of 1X sample buffer, separated by SDS-PAGE and visualized by autoradiography.

5.18 Statistical Analysis

All real-time PCR results are expressed as means ± standard error of the means (SEM). Statistical analysis was calculated using GraphPad Prism statistical software (San Diego, CA).
One-way Analysis of Variance (ANOVA) followed by Tukey’s multiple comparison tests were used to assess statistical significance ($P<0.05$).
Chapter 4: Results

6 TiPARP is a mono-ADP-ribosyltransferase

6.1 Auto-ADP-ribosylation

The catalytic activities of ARTDs are dependent on a well-conserved glutamate residue within the catalytic active site of the HYE triad sequence (Holbourn, et al., 2006; Marsischky, et al., 1995). Sequence alignment of the ARTD catalytic core sequences of the 18 ARTD family members showed that H532, Y564 and I631 of TiPARP are equivalent to the ARTD catalytic HYE triad. Since TiPARP has an isoleucine in place of the catalytic glutamate and a shortened connecting loop, it is predicted to possess MART rather than poly-ADP-ribosyltransferase activity (Kleine, et al., 2008); however, this has not been experimentally determined. To examine the catalytic activity of TiPARP, we compared the enzymatic properties of TiPARP to that of ARTD1 using an auto-ADP-ribosylation assay. ARTD1 demonstrated auto-ADP-ribosylation with the incorporation of $^{32}$P-NAD$^+$ and a marked shift in mobility and smearing pattern which was indicative of poly(ADP-ribose) polymer formation (Figure 10A). Partially purified GST-TiPARP also showed evidence of auto-modification but no apparent change in mobility, suggesting that TiPARP exhibits mono-ADP-ribosyltransferase rather than poly(ADP-ribose) polymer synthase activity (Figure 10B). This activity was competed away with unlabelled $\beta$-NAD$^+$. We cannot exclude the possibility that TiPARP generates short oligo-ADP-ribose units since they would not alter the migration of the protein. Because ARTD1/PARP1 activity is stimulated by its interaction with DNA (D'Amours, et al., 1999) and TiPARP contained a zinc-finger domain with putative RNA binding capacity (Kelly, et al., 2007), we tested whether the presence of DNA or RNA modified TiPARP auto-ribosylation activity. Addition of activated DNA or DNAse-treated total RNA did not alter the migration pattern of GST-TiPARP (Figure 10B). We next tested mutants of the TiPARP catalytic triad. GST-TiPARP-H532A and GST-TiPARP-Y564A did not show any evidence of auto-ribosylation or shift in mobility (Figure 10B). GST-TiPARP-I631A demonstrated similar auto-ribosylation activity to that of wildtype, suggesting that this residue was not required for enzymatic activity (Figure 10B).

Next we evaluated the catalytic activity of mouse and chicken (Gallus gallus) TiPARP as GST fusion proteins. GST-mouse TiPARP (GST-mTiPARP) demonstrated auto-ribosylation that
could be mono-ADP-ribosyltransferase activity due to the lack of shift in mobility (Figure 10B). GST-chicken TiPARP (GST-chTiPARP) demonstrated weak auto-ribosylation activity compared to human, despite the loading of similar amounts of protein (Figure 10B). Addition of activated DNA or DNAse-treated RNA did not alter the migration pattern or auto-ribosylation ability of GST-mTiPARP or GST-chTiPARP (Figure 10C).

To determine if mutation of isoleucine 631 to a glutamate would convert TiPARP MART activity to poly-ADP-ribosyltransferase activity, we analyzed the auto-ribosylation activity of GST-TiPARP-I631E mutant. The auto-ribosylation activity of GST-TiPARP-I631E was similar to that of wildtype (Figure 10D), exhibiting MART but not polymer synthase activity.

To further examine TiPARP catalytic activity we used an *in vitro* 35S-labelled protein shift assay, which allowed us to directly compare mobility shift of modified and unmodified ARTD proteins. *In vitro* translated 35S-labelled ARTD1 (35S-ARTD1) incubated with β-NAD⁺ (and activated DNA) resulted in a marked shift in its mobility compared to unmodified 35S-ARTD1 in the absence of β-NAD⁺ (Figure 11A). Conversely, only a small change in mobility was observed for 35S-TiPARP when incubated with β-NAD⁺, which may represent mono-ADP-ribosylation of multiple residues or short oligo-ADP-ribose units. None of the 35S-labelled TiPARP catalytic point mutants demonstrated altered mobility compared to wildtype TiPARP (Figure 11B). The absence of a modest mobility shift of 35S-labelled TiPARP-I631A in the presence of β-NAD⁺ is in contrast to results obtained from 32P-NAD⁺ PARP assays with GST-TiPARP I631A (Figure 11B). This discrepancy could be due to differences in sensitivity between the two assays. The I631A mutant may have reduced auto-ADP-ribosylation activity that could be below the threshold of sensitivity of the 35S protein shift assay. A similar mono-ribosylation pattern was observed for mouse 35S-TiPARP but not chicken 35S-TiPARP (Figure 11C). 32P-NAD⁺ catalytic assays performed on GST-chTiPARP demonstrated very weak auto-ribosylation that may not have been detected by the 35S protein shift assay. Taken together, these data revealed that TiPARP was a mono-ADP-ribosyltransferase.

We next used the 35S-labelled *in vitro* protein shift assay to assess the catalytic activity of the N-terminal truncations of TiPARP. Deletion mutants from amino acid residue 33 to residue 275 showed a slight migration or shift comparable to full-length TiPARP when incubated with β-
NAD\(^+\) (Figure 11D). However, this shift was not apparent in N-terminal deletion mutants 328-657 and 445-657, suggesting that the auto-modification domain of TiPARP is between residues 275 to 328.
Figure 10. TiPARP is a mono-ADP-ribosyltransferase with auto-ribosylation activity.

(A) Auto-ADP-ribosylation of ARTD1, purified ARTD1 was incubated with $^{32}$P-NAD$^+$ and 1 μg activated DNA then analyzed the SDS-PAGE and autoradiography.

(B) Auto-ADP-ribosylation
of GST-TiPARP, GST-TiPARP catalytic mutants (GST-TiPARP-H532A, –Y564A and –I631A), GST-mouse TiPARP (GST-mTiPARP) and GST-chicken TiPARP (GST-chTiPARP). Auto-ribosylation reactions were carried out with semi-purified GST-TiPARP fusion proteins and $^{32}\text{P}$-NAD$^+$ or co-incubated 500 μM β-NAD$^+$ or 1 μg activated DNA or RNA. Automodified GST-TiPARP fusion proteins were analyzed by SDS-PAGE and autoradiography. (C) Auto-ADP-ribosylation of mouse TiPARP (mTiPARP) and chicken TiPARP (chTiPARP) incubated with DNA or RNA. Mouse GST-tagged TiPARP (GST-mTiPARP) and chicken GST-tagged TiPARP (GST-chTiPARP) were incubated with $^{32}\text{P}$-NAD$^+$ and activated DNA or DNase-treated RNA and analyzed by SDS-PAGE and autoradiography. (D) Comparison of auto-ADP-ribosylation of GST-TiPARP-I631E (GST-I631E) with wildtype GST-TiPARP (GST-wt). All figures represent data from three independent experiments. Closed arrow denotes mobility shifted ARTD1.
Figure 11. Putative auto-ribosylation domain of TiPARP is between residues 275 and 328.

(A) Mobility shift comparisons of modified and unmodified $^{35}$S-labelled ARTD1 and TiPARP. In vitro translated $^{35}$S-labelled ARTD1 and TiPARP were incubated with or without 500 μM β-NAD$^+$, activated DNA or RNA and analyzed by SDS-PAGE and autoradiography. (B) TiPARP
catalytic point mutants are inactive. (C) Auto-ribosylation of mouse TiPARP and chicken TiPARP. \textit{In vitro} translated $^{35}$S-labelled TiPARP, catalytic point mutants, mouse and chicken TiPARP were incubated with or without 500 $\mu$M $\beta$-NAD$^+$ and analyzed by SDS-PAGE and autoradiography. (D) TiPARP automodification domain is between residues 275 and 328. \textit{In vitro} translated $^{35}$S-labelled TiPARP and N-terminal truncations were incubated with or without 500 $\mu$M $\beta$-NAD$^+$ and analyzed by SDS-PAGE and autoradiography. These figures represent data from three independent experiments. Closed arrow denotes shifted ARTD1.
6.2 Hetero-ADP-ribosylation

We then compared the ability of GST-TiPARP and ARTD1 to hetero-ribosylate core histones. In agreement with a previous report, ARTD1 hetero-ADP-ribosylated each of the core histones (Figure 12A)(Messner, et al.). GST-TiPARP also mono-ADP-ribosylated each of the core histones as evidenced by the lack of a marked mobility shift in molecular weight of the histones (Figure 12B). No ADP-ribosylation of the core histones was observed in the presence of the catalytic mutant GST-TiPARP-H532A (Figure 12C). Similar to wildtype, GST-TiPARP-I631A also ribosylated core histones in the presence of $^{32}$P-NAD$^+$ (Figure 12D). These results demonstrated TiPARP hetero-ribosylation activity was mono-ADP-ribosyltransferase.
Figure 12. TiPARP is a mono-ADP-ribosyltransferase with heteroribosylation activity.

(A) Hetero-ribosylation of core histones by ARTD1. Purified ARTD1 was incubated with 2 μCi $^{32}$P-NAD$^+$, individual or mixed core histones (core) and hetero-ribosylation was analyzed by SDS-PAGE followed by autoradiography. 

(B) Hetero-ribosylation of core histones by GST-TiPARP. GST-TiPARP was incubated with $^{32}$P-NAD$^+$ and histones H2A, H2B, H3 or H4 and analyzed by SDS-PAGE and autoradiography. 

(C) Catalytic mutant GST-TiPARP-H532A did not hetero-ribosylation core histones. GST-TiPARP-H532A (H532A) or wildtype GST-TiPARP (wt) were incubated with mixed core histones and hetero-ribosylation was determined by SDS-PAGE followed by autoradiography. 

(D) Hetero-ribosylation of core histones by TiPARP catalytic mutant I631A. GST-TiPARP-I631A was incubated with $^{32}$P-NAD$^+$ and histones H2A, H2B, H3 or H4 and analyzed by SDS-PAGE and autoradiography.
Identification of TiPARP as a novel negative regulator of AHR

7.1 Temporal induction of TiPARP mRNA by TCDD

TiPARP was first reported to be a TCDD-responsive gene that was regulated by AHR in mouse hepatoma Hepa1c1c7 cell line (Ma, et al., 2001); however, little is known about the regulation of TiPARP in human cell lines. With this in mind, we determined the temporal changes in TiPARP expression after treatment of T47D human breast carcinoma cells with TCDD. The results were compared to those obtained for CYP1A1. TCDD-dependent increases in TiPARP mRNA levels peaked at 1.5 h, whereas CYP1A1 mRNA levels increased over the 24 h time course (Figure 13A). Similarly, TiPARP mRNA induction peaked at 1.5 h TCDD treatment in Hepa1c1c7 cells (Figure 13B) (Ma, 2002). However, the TiPARP mRNA declined less rapidly compared to T47D cells, suggesting possible cell type differences in TiPARP regulation or mRNA stability. Similar kinetic differences between TCDD-induced TiPARP and CYP1A expression levels were observed in chick embryo hepatocytes (Diani-Moore, et al., 2010), demonstrating that these regulatory differences between TiPARP and CYP1A genes are conserved across species.
Figure 13. Temporal induction of TiPARP mRNA in T47D and Hepa1c1c7 cell lines treated with TCDD.

(A) Temporal analysis of TCDD-induced TiPARP and CYP1A1 mRNA expression levels. T47D cells were treated with 10 nM TCDD at the times indicated. (B) Temporal analysis of TCDD-induced TiPARP mRNA expression levels in Hepa1c1c7 cells. Hepa1c1c7 cells were treated with 10 nM TCDD for the times indicated. Data are presented as means ± S.E.M. of three independent replicates.
7.2 TiPARP knockdown increased TCDD-induced AHR transactivation

To test whether TiPARP modulates AHR activity, we used RNAi-mediated knockdown of TiPARP using pooled siTiPARP siRNA (containing four different siTiPARP sequences) in human gastric carcinoma NCI-N87 cells, human hepatoma HuH7 cells and human breast carcinoma T47D cells and examined TCDD-dependent changes in CYP1A1 and CYP1B1 mRNA levels. TiPARP mRNA levels were reduced to approximately 25-38% compared to control cells from the cell lines with a non-targeting pool (NTP) of siRNA (Figure 14A). Endogenous TiPARP protein levels could not be assessed by immunoblot due to the lack of a suitable antibody. RNAi-mediated knockdown of TiPARP resulted in significantly greater TCDD-dependent induction of CYP1A1 and CYP1B1 mRNA expression levels compared to NTP control cells after 24 h exposure (Figure 14B and C). Next we verified these results by using single siTiPARP sequences (siTiPARP12 and siTiPARP13) to knockdown TiPARP in T47D cells. TiPARP mRNA levels were reduced to approximately 23% and 34% compared to non-targeting (NT) control cells using siTiPARP12 and siTiPARP13 sequences, respectively (Figure 15A). Due to the lack of suitable antibody to detect endogenous TiPARP protein, we instead co-transfected GFP-tagged TiPARP with siTiPARP sequences to demonstrate knockdown of overexpressed GFP-TiPARP protein with anti-GFP antibody (Figure 15B). Single-strand RNAi-mediated knockdown of TiPARP resulted in significantly greater TCDD-dependent induction of CYP1A1 and CYP1B1 mRNA expression levels compared to NT control cells after 24 h exposure which is in agreement with that we observed with pooled siTiPARP knockdown (Figure 15C). We next tested how soon after treatment TCDD-induced gene expression was increased in T47D cells following 48 h knockdown of TiPARP. T47D cells were treated with TCDD for 1.5 h and 3 h. Increased TCDD-dependent induction of CYP1A1 and CYP1B1 mRNA levels following TiPARP knockdown was observed after 3 h but not 1.5 h after treatment (Figure 15D and E).

To examine the TiPARP-dependent mechanism of AHR repression we performed ChIP assays in T47D cells after RNAi-mediated TiPARP knockdown of TiPARP and treatment with TCDD for 1 h. In agreement with increased CYP1A1 and CYP1B1 mRNA levels, knockdown of TiPARP resulted in significantly greater recruitment of AHR and ARNT to the CYP1A1 and CYP1B1 enhancer regions compared to the NT control (Figure 16A and C). No significant differences in
AHR or ARNT recruitment to downstream genomic control regions for both genes were observed (Figure 16B and D).

We next determined the effect of TiPARP knockdown on ligand-induced AHR protein degradation. Cells with TiPARP knocked down trended toward increased constitutive AHR protein levels (Figure 16E). TiPARP knockdown cells treated with TCDD for 24 h demonstrated reduced AHR degradation compared with TCDD-treated non-targeting cells, which is consistent with elevated AHR-regulated gene expression and AHR recruitment results (Figure 16E). These data suggested TiPARP mechanism of AHR repression to potentially involve reduced proteolytic degradation of AHR.
Figure 14. TiPARP knockdown in NCI-N87, HuH7 and T47D cells increased TCDD-induced AHR transactivation.

Cell lines were transfected with siRNA pool (containing four different siRNAs) targeting against TiPARP for 48 h. Cells were then treated with 10 nM TCDD and DMSO (control) for 24 h and RNA was isolated and reverse transcribed as described in the Materials and Methods. Changes in (A) TiPARP; (B) CYP1A1; and (C) CYP1B1 mRNA expression was then determined using qPCR. Data were normalized to non-targeting pool (NTP) DMSO (control) and to β-actin levels of each cell line. Results shown are means ± S.E.M for at least four independent experiments. mRNA expression levels for each cell line significantly ($P < 0.05$) different than NTP transfected cells are denoted with an asterisk.
Figure 15. TiPARP knockdown increased TCDD-induced AHR transactivation.

(A) TiPARP mRNA expression levels in T47D cells following 48 h knockdown with single siRNAs against TiPARP (siTP12 and siTP13) and non-targeting control (NT). Expression levels significantly \( (P < 0.05) \) lower than NT were denoted with asterisks. (B) Western blot analysis of GFP-TiPARP protein overexpression following RNAi-mediated TiPARP knockdown in TREX-FLP-IN 293 cells. (C) TiPARP knockdown increased TCDD-induced CYP1A1 and CYP1B1 mRNA expression levels in T47D cells following 24 h treatment. Gene expression levels significantly \( (P < 0.05) \) greater than NT were denoted with asterisks. (D) TiPARP knockdown did not affect CYP1A1 and CYP1B1 mRNA levels following 1.5 h TCDD treatment. Asterisks denote TiPARP mRNA levels significantly lower than NT. (E) TiPARP increased TCDD-induced CYP1A1 and CYP1B1 mRNA levels following 3 h treatment. Gene expression levels significantly \( (P < 0.05) \) different than NT were denoted with asterisks.
Figure 16. TiPARP knockdown increased AHR/ARNT recruitment to *CYP1A1* and *CYP1B1* regulatory regions and reduced TCDD-induced degradation of AHR.

TiPARP knockdown increased TCDD-induced AHR and ARNT recruitment to *CYP1A1* (A-B) and *CYP1B1* (C-D). AHR and ARNT to *CYP1A1* regulatory region (A), *CYP1A1* distal downstream region (B), *CYP1B1* regulatory region (C) and *CYP1B1* distal downstream region (D). T47D cells with TiPARP knocked down were treated with 10 nM TCDD for 1 h and harvested for ChIP assays. Percent recruitment significantly greater (*P* < 0.05) than NT was denoted with an asterisk. (E) TiPARP knockdown reduced TCDD-induced AHR degradation in T47D cells. T47D cells following TiPARP knockdown were treated with TCDD (T) or DMSO (D) for 24 h and AHR protein expression was determined by Western blot. Left panel; representative Western blot from three independent experiments, right panel; Quantification of AHR protein levels, densitometry was performed using ImageJ analysis software. AHR protein levels were normalized to pcDNA-transfected and DMSO treated cells. Asterisks denoted AHR protein levels significantly (*P* < 0.05) greater than treatment-matched non-targeting-transfected cells. All data were presented as means ± S.E.M for three independent experiments. Statistical analysis was determined by two-tailed Student’s *t*-test.
7.3 Overexpression of TiPARP repressed AHR-mediated transactivation

Because the RNAi experiments suggested that TiPARP might act as a negative regulator of AHR transactivation, we investigated the ability of TiPARP overexpression to repress AHR-dependent gene expression in HuH7 cells transiently transfected with a CYP1A1-regulated luciferase reporter plasmid (CYP1A1-luc). Transient transfection of increasing amounts of TiPARP resulted in a dose-dependent reduction of TCDD-induced reporter gene activity (Figure 17A). Similar results were observed using a CYP1B1-regulated luciferase reporter plasmid (CYP1B1-luc) (Figure 17B). To examine whether TiPARP-mediated repression was limited to CYP1 regulatory regions we used an artificial 3XAHRE-luc reporter (3X repeat of Hairy and Enhancer of Split-1 (HES1) AHRE sequence) and determined TCDD-induced reporter gene activity. Overexpression of increasing amounts of TiPARP dose-dependently repressed 3XAHRE-luc reporter gene activity, which was similar to the CYP1-luc reporter constructs (Figure 17C). Transfection of up to one microgram of the catalytic inactive mutant TiPARP-H532A plasmid did not repress CYP1A1-luc reporter gene activity but rather demonstrated dominant negative activity, indicating catalytically active TiPARP is required for repression (Figure 17D). We next tested mouse TiPARP, which shares 92.8% sequence homology with human TiPARP and demonstrated it to repress induction of CYP1A1-luc activity similar to human TiPARP (Figure 17E). Chicken TiPARP, which only exhibits 68.5% sequence homology with human TiPARP did not repress reporter gene activity. The lack of repression of chicken TiPARP may have been due the use of mammalian cells and/or reporter gene in our model. Similar findings were observed after transfection with GFP-tagged TiPARP fusion proteins (Figure 17F). Transfection of equal amounts of GFP-TiPARP plasmid DNA resulted in similar protein expression levels of human and mouse GFP-tagged TiPARP, whereas higher protein levels of chicken TiPARP were observed (Figure 17G). Collectively these data showed that TiPARP was a negative regulator of AHR transactivation, but this effect may exhibit species-specificity.

To examine if TiPARP overexpression inhibited endogenous CYP1A1 and CYP1B1 mRNA expression, we transiently transfected HuH7 cells with TiPARP, treated them with TCDD for 24 h prior to isolating RNA and performing qPCR. We observed a significant repression of TCDD-dependent increases in CYP1A1 and CYP1B1 mRNA levels in cells overexpressing TiPARP, supporting our reporter gene assay results (Figure 18A and B).
We then determined if TiPARP was recruited to AHR target gene enhancer regions using ChIP assays. We transiently transfected HuH7 cells with GFP-tagged TiPARP and ChIP assays were performed with an anti-GFP antibody. HuH7 cells transfected with GFP-TiPARP revealed a small but significant TCDD-dependent increase in GFP-TiPARP recruitment to CYP1A1 after 45 min of TCDD treatment (Figure 18C), but not to a downstream chromatin region that was not bound by AHR (Figure 18D). Interestingly, reduced AHR occupancy at CYP1A1 was also observed in cells overexpressing GFP-TiPARP, which supported the increased AHR and ARNT recruitment we observed with TiPARP knockdown (Figure 18C). We next examined if TiPARP overexpression affected AHR protein levels. Time course studies showed a reduction in AHR protein levels in cells overexpressing TiPARP in the presence or absence of TCDD (Figure 18E), which was in support of the increased AHR protein levels observed after RNAi-mediated knockdown of TiPARP (Figure 16E).
Figure 17. TiPARP overexpression reduced AHR-regulated reporter gene activity.
(A-C) Concentration-dependent reduction of TCDD-induced CYP1A1-luc, CYP1B1-luc and 3xAHRE/HEs1-luc reporter gene activity with TiPARP overexpression. (D) Dose-response of TiPARP catalytic mutant H532A in HuH7 cells. (E-F) Mammalian TiPARP inhibited TCDD-induced CYP1A1-luc reporter gene activity. Cells were co-overexpressed with reporter gene and increasing amounts of pcDNA-TiPARP (50 ng to 200 ng), pcDNA-TiPARP-H532A (500 ng to 1000 ng), 200 ng pcDNA-chicken TiPARP (chTiPARP), 200 ng pcDNA-mouse TiPARP (mTiPARP), pEGFP-hTiPARP, pEGFP-chTiPARP or pEGFP-mTiPARP. All transfected cells were treated with TCDD or DMSO for 24 h and reporter gene activity was determined. Values
were normalized to 100% TCDD treatment. Data are represented as means ± S.E.M. of three independent experiments. Statistical significance was analyzed by one-way ANOVA and Tukey’s multiple comparisons test. Reporter gene activity significantly different than \( P < 0.05 \) TCDD-treated empty vector was denoted with an asterisk. (G) Western blot of overexpressed GFP-hTiPARP, GFP-chTiPARP and GFP-mTiPARP in HuH7 cells.
Figure 18. Overexpression of TiPARP repressed TCDD-induced AHR transactivation.

(A-B) TiPARP overexpression repressed TCDD induction of CYP1A1 (A) and CYP1B1 (B) mRNA in HuH7 cells. Asterisks denote mRNA expression significantly lower ($P < 0.05$, two-tailed Student’s $t$-test) than treatment-matched empty vector transfected cells. (C) Overexpressed GFP-TiPARP recruitment to CYP1A1 regulatory region was induced by TCDD and repressed AHR recruitment. (D) Recruitment of GFP-TiPARP and AHR to CYP1A1 distal downstream region. HuH7 cells were transfected with pEGFP empty vector or pEGFP-TiPARP and dosed with TCDD for 45 min and cell were harvested for ChIP assays. Percent recruitment of GFP-TiPARP of significantly greater ($P < 0.05$, two-tailed Student’s $t$-test) than transfection-matched DMSO-treated cells was denoted with an asterisk. Percent recruitment of AHR significantly lower ($P < 0.05$, two-tailed Student’s $t$-test) than treatment-matched GFP-transfected cells is denoted with a pound sign. Data shown are representative of three independent experiments. (E) TiPARP overexpression increased TCDD-induced AHR proteasomal degradation. T47D cells
were transfected with vector or pcDNA-TiPARP and dosed with TCDD for the indicated times and AHR protein levels were determined by Western blot. Left panel; representative Western blots from three independent experiments. Right panel; quantification of AHR protein levels, densitometry was performed using ImageJ analysis software (NIH). Asterisks denote AHR protein levels significantly ($P < 0.05$, two-tailed Student’s $t$-test) lower than treatment-matched vector-transfected cells.

7.4 Tiparp knockout increased TCDD-dependent AHR target transactivation and AHR protein levels

To investigate the impact of TiPARP loss on AHR transactivation and protein expression we created immortalized mouse embryonic fibroblasts (MEFs) from wildtype, Tiparp$^{-/-}$ and Tiparp$^{+/+}$ siblings (strain B6;129S4). As expected, TiPARP mRNA levels were induced in wildtype and Tiparp$^{+/+}$ cells after 24 h treatment with TCDD, but no TiPARP was detected in Tiparp$^{-/-}$ cells (Figure 19A). TCDD treatment of Tiparp$^{-/-}$ MEFs resulted in greater increases in CYP1A1, CYP1B1 and AHRR mRNA levels compared to wildtype and Tiparp$^{+/+}$ cells, while GAPDH mRNA levels were unaffected by TCDD treatment or by genotype (Figure 19A and B). Ectopic expression of TiPARP into Tiparp$^{-/-}$ MEFs reduced TCDD-dependent AHR transactivation compared to vector control (Figure 19C). ChIP assays revealed significantly greater TCDD-induced AHR recruitment to Cyp1a1 and Cyp1b1 in Tiparp$^{-/-}$ compared to wildtype or Tiparp$^{+/+}$ cells (Figure 19D and E). Because we observed increased AHR transactivation in Tiparp$^{-/-}$ compared to wildtype or Tiparp$^{+/+}$ MEFs, we examined the relative AHR protein levels and ligand-induced AHR degradation in the different MEF lines. Tiparp$^{-/-}$ cells had higher constitutive AHR protein levels compared to wildtype and Tiparp$^{+/+}$ cells (Figure 19F). TCDD-induced degradation of AHR was reduced in Tiparp$^{-/-}$ compared to wildtype and Tiparp$^{+/+}$ cells (Figure 19F). TCDD treatment for 24 h caused a 72% and 81% reduction in AHR protein levels in wildtype and Tiparp$^{+/+}$ cells, respectively, but only a 53% reduction in Tiparp$^{-/-}$ MEFs (Figure 19F). In contrast to increased AHR protein levels, small but significant TCDD-dependent decreases in AHR mRNA levels were detected in Tiparp$^{-/-}$ cells (Figure 19G).
Figure 19. Tiparp knockout fibroblasts exhibited increased TCDD-induced AHR transactivation.

(A) Wildtype (Tiparp\(^{+/+}\)), Tiparp\(^{-/-}\) and Tiparp\(^{+/+}\) immortalized mouse embryonic fibroblast (MEF) lines were treated with 10 nM TCDD for 24 h and gene expression determined. Gene expression was normalized relative to DMSO treated wildtype cells. Gene expression levels significantly (\(P < 0.05\)) different than treatment-matched wildtype cells are denoted with an asterisk, and gene expression levels significantly (\(P < 0.05\)) different than treatment-matched Tiparp\(^{+/+}\) (+/-) cells are denoted with a pound sign. (B) Expression of GAPDH mRNA in immortalized mouse embryonic fibroblast lines. (C) Genetic complementation of Tiparp\(^{-/-}\) MEFs with mouse TiPARP overexpression. Tiparp\(^{-/-}\) MEFs were transfected with 2 \(\mu\)g pcDNA-mTiPARP or pcDNA (vector). Following 24 h transfected cells were treated with TCDD for 24 h and CYP1A1, CYP1B1 and AHRR mRNA levels were determined. Data presented were representative of means ± S.E.M. of three independent experiments. mRNA expression significantly (\(P<0.05\)) different than vector-transfected cells was denoted with an asterisk. (D-E) Tiparp\(^{-/-}\) cells exhibit increased TCDD-induced AHR recruitment to Cyp1a1 and Cyp1b1. (D) AHR recruitment to Cyp1a1 regulatory region and distal downstream control region. (E) AHR recruitment to Cyp1b1 regulatory region and distal downstream control region. MEF lines were treated with 10 nM TCDD for 45 min and harvested for ChIP assays. Percent recruitment significantly greater (\(P < 0.05\)) than treatment-matched wildtype cells was denoted with an asterisk, and percent recruitment significantly greater (\(P < 0.05\)) than treatment-matched Tiparp\(^{+/+}\) (+/-) cells was denoted with a pound sign. (F) Tiparp\(^{-/-}\) fibroblasts have reduced TCDD-induced AHR degradation. MEF lines were treated with TCDD (T) or DMSO (D) for 24 h and AHR protein expression was determined by Western blot. Left panel; representative Western blot from three independent experiments. Right panel; quantification of AHR protein levels, densitometry was performed using ImageJ analysis software (NIH). Asterisks denoted AHR protein levels significantly (\(P < 0.05\), two-tailed Student’s \(t\)-test) greater than treatment-matched wildtype cells. Pound sign denotes AHR protein levels significantly (\(P < 0.05\), two-tailed Student’s \(t\)-test) less than genotype-matched DMSO-treated cells. (G) AHR mRNA expression levels of MEF cell lines treated with DMSO or TCDD for 24 h. Asterisks denoted AHR mRNA levels significantly (\(P < 0.05\), two-tailed Student’s \(t\)-test) greater than treatment-matched wildtype cells. Pound sign denoted AHR mRNA levels significantly (\(P < 0.05\), two-tailed Student’s \(t\)-test) less than genotype-matched DMSO-treated cells.
7.5 *Tiparp* knockout increased AHR-mediated gene expression *in vivo*

To determine if TiPARP was a negative regulator of AHR transactivation *in vivo* we treated male and female 6-8 week old *Tiparp*^{+/+}, *Tiparp*^{+/−} and *Tiparp*^{−/−} mice (strain B6;129S4- *Tiparp^{Gr(Rosa)79Sor}* with 30 μg/kg b.w. TCDD or corn oil (vehicle control) for 6 h. Mice were sacrificed, livers excised and AHR-regulated gene expression was determined. As expected TiPARP expression was detected in hepatic tissues from wildtype and *Tiparp*^{+/−} mice but not in *Tiparp*^{−/−} mice (*Figures 20A and 21A*). Following 6 h TCDD treatment both female and male *Tiparp*^{−/−} mice demonstrated significantly greater AHR target gene CYP1A1, CYP1B1, AHRR, nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) and NAD(P)H dehydrogenase (quinone) 1 (NQO1) mRNA expression compared to wildtype mice (*Figures 20B-F and Figure 21B-F*). These data supported our TiPARP knockdown and *Tiparp*^{−/−} MEF data and indicated that TiPARP is a negative regulator of AHR transactivation *in vivo*. 
Figure 20. Female Tiparp<sup>−/−</sup> mice demonstrated increased TCDD-induced AHR transactivation.

Six week old female Tiparp<sup>+/+</sup>, Tiparp<sup>+/−</sup> and Tiparp<sup>−/−</sup> mice were treated with corn oil (CO) or 30 μg/kg b.w. TCDD (TCDD) for 6 h and livers were excised, RNA was isolated and mRNA reversed transcribed. Gene expression was normalized relative to CO-treated wildtype mice. Each bar represented means ± S.E.M. from four animals. Data were analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Gene expression levels significantly (P < 0.05) different than treatment-matched wildtype mice were denoted with an asterisk and gene expression levels significantly (P < 0.05) different than treatment-matched Tiparp<sup>+/−</sup> (+/−) mice were denoted with a pound sign.
Figure 21. Male Tiparp\textsuperscript{+/-} mice demonstrated increased TCDD-induced AHR transactivation.

Six week old male Tiparp\textsuperscript{+/+}, Tiparp\textsuperscript{+/-} and Tiparp\textsuperscript{-/-} mice were treated with corn oil (CO) or 30 μg/kg b.w. TCDD (TCDD) for 6 h and livers were excised, RNA was isolated and mRNA reversed transcribed. Gene expression was normalized relative CO-treated wildtype mice. Each bar represented the means ± S.E.M. from four animals. Data were analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Gene expression levels significantly (\(P < 0.05\)) different than treatment matched wildtype mice were denoted with an asterisk and gene expression levels significantly (\(P < 0.05\)) different than treatment- matched Tiparp\textsuperscript{+/+} (+/-) mice were denoted with a pound sign.
8 Understanding the mechanism of TiPARP-mediated repression of AHR transactivation

8.1 TiPARP selective repression of AHR activity

Because ARTD1 and other ARTD family members can function as transcriptional repressors or activators (Kraus, 2008), we examined the ability of ARTD1 and ARTD12 to modulate AHR transactivation. ARTD1 was selected since it is the most studied ARTD, whereas ARTD12 was chosen because it shared the greatest sequence homology (46.8%) with TiPARP compared to other members of the ARTD family (Schreiber, et al., 2006). We co-transfected 200 ng and 1500 ng of ARTD1 and ARTD12 with CYP1A1-luc into HuH7 cells and determined reporter gene activity after 24 h TCDD treatment. Transfection of HuH7 cells with 200 ng of TiPARP caused a ~50% reduction in TCDD-dependent induction of CYP1A1-luc reporter gene activity, whereas transfection with the same amount of ARTD1 or ARTD12 had no effect (Figure 22). Increasing the amount of transfected plasmid DNA to 1500 ng caused a greater than 85% reduction in CYP1A1-luc reporter gene activity by TiPARP, but only a ~40% reduction by ARTD1 or ARTD12. These results suggested that TiPARP exhibited selective repression of AHR transactivation compared to other ARTD family members.
Figure 22. CYP1A1-luc reporter gene activity was preferentially inhibited by TiPARP. HuH7 cells were co-overexpressed with 200 ng or 1500 ng of pcDNA-TiPARP, pCMV-ARTD1 or pCMV-ARTD12 and pCYP1A1-luc and treated with TCDD. Reporter gene activity significantly lower ($P < 0.05$) than empty vector transfected cells was denoted with an asterisk. Reporter gene activity significantly ($P < 0.05$) greater than TiPARP (1500 ng) transfected cells was denoted with a pound sign. All luciferase data were presented as means ± S.E.M from three independent experiments and statistical significance analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Western blots confirming the presence of increased ARTD1 and ARTD12 protein expression following transient transfection are presented to the right of bar graph.
8.2 Ectopic AHR but not ARNT prevented TiPARP-mediated inhibition of AHR transactivation

The AHRR negatively regulates AHR transactivation by competing with AHR for ARNT dimerization and binding to the AHRE (Mimura, et al., 1999), but also through ARNT-independent mechanisms (Evans, et al., 2008; Karchner, et al., 2009). To evaluate the role of ARNT in TiPARP-mediated repression of AHR transactivation we assessed the ability of TiPARP to repress AHR transactivation in the presence of increasing amounts of AHR or ARNT. Transient co-transfection of AHR but not ARNT rescued the TiPARP-dependent repression of CYP1A1-regulated reporter gene induction (Figure 23A and B).
Figure 23. Overexpression of AHR but not ARNT prevented TiPARP-mediated inhibition of AHR transactivation.

HuH7 cells were co-transfected with pCYP1A1-luc, TiPARP (200 ng) and (A) AHR (250-750 ng) or (B) ARNT (250-750 ng). All transfected cells were treated with 10 nM TCDD for 24 h and reporter gene activity was determined. Results were shown as means ± S.E.M from three independent experiments and significance analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Reporter gene activity of cells co-overexpressing TiPARP significantly lower than \(P < 0.05\) transfection- and treatment-matched control cells were denoted with an asterisk. Western blots confirming the presence of increased AHR and ARNT protein expression following transient transfection were presented to the right of respective bar graphs.
8.3 Identification of a putative AHR repressor domain of TiPARP

To determine the mechanism of TiPARP-dependent repression of AHR, we created a series of TiPARP truncations and site-directed point mutants, and determined their ability to repress AHR-dependent reporter gene activity in HuH7 cells. The expression of each truncated product was confirmed by *in vitro* translation (Figure 24). N-terminal truncation constructs of TiPARP expressing amino acids 33-657 to 218-657 repressed CYP1A1-regulated reporter gene activity similar to full length TiPARP, with the exception of 200-657, which exhibited greater repressor activity (Figure 25A). The 225-657 truncation construct exhibited a significantly reduced ability to repress reporter gene activity, whereas overexpression of 245-657 to 445-657 truncations did not repress AHR-regulated reporter gene activity. A similar pattern of repression was observed with the CYP1B1-luc reporter (Figure 25B). We created GFP-tagged versions of all N-terminal truncations to verify expression in transfected cells by Western blot with anti-GFP antibody. We first tested the ability of the GFP-tagged N-terminal truncations to repress CYP1A1- and CYP1B1-luc. The GFP-tagged N-terminal truncations displayed a similar pattern of repression of both reporter genes to their untagged counterparts (Figure 26A and B). Western blot analysis of the GFP-TiPARP N-terminal truncations showed that the lack of repression was not due to reduced protein expression of the different GFP-TiPARP truncations since the protein expression of all N-terminal truncations was greater than full-length GFP-TiPARP despite transfecting and loading equal amounts (Figure 27).

Because loss of AHR repressive ability was lost between residues 218 and 245, we were then interested in whether this region of TiPARP was a putative AHR repressor domain that contained specific residues pertinent to its ability to repress AHR transactivation. We performed an alanine scan study whereby we mutated every two consecutive residues to alanines between 218 and 241 and determined the ability of each double alanine point mutant to repress TCDD induction of CYP1A1- and CYP1B1-luc reporter gene activity. Endogenous glycines were not mutated. All double point mutants significantly repressed CYP1A1- and CYP1B1-luc activity to varying degrees except for the Y232A; H233A mutant which failed to repress induction of both reporter genes (Figure 28A and B). These findings indicated that Y232 and H233 were important for the ability of TiPARP to repress AHR transactivation. Point mutations between residues 220 and 235 significantly reduced the AHR repressive activity of TiPARP indicating these residues may contribute to repression of AHR as well.
Figure 24. *In vitro* expression of $^{35}$S-labelled N-terminal TiPARP truncations.

N-terminal pcDNA-TiPARP constructs were *in vitro* translated with $^{35}$S-methionine and detected by SDS-PAGE and autoradiography.
Figure 25. Overexpression of N-terminal TiPARP truncations on AHR-regulated reporter gene activity.

Reporters gene activity of CYP1A1-luc (A) and CYP1B1-luc (B). HuH7 cells were transiently co-transfected with pcDNA-TiPARP N-terminal truncation construct and pCYP1A1-luc or pCYP1B1-luc reporter genes. Cells were treated with DMSO or TCDD for 16 h and reporter gene activity was determined. Values were normalized to 100% TCDD treatment. Data are represented as means ± S.E.M. of three independent experiments. Statistical significance was analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Reporter gene activity significantly different than \((P < 0.05)\) TCDD-treated empty vector was denoted with an asterisk. Reporter gene activity significantly different than \((P < 0.05)\) TCDD-treated full-length TiPARP (1-657) was denoted with a pound sign.
Figure 26. Expression of N-terminal GFP-tagged TiPARP truncations on AHR-regulated reporter gene activity.

Reporter gene activity of CYP1A1-luc (A) and CYP1B1-luc (B). HuH7 cells were transiently co-transfected with pEGFP-TiPARP N-terminal truncation construct and pCYP1A1-luc or pCYP1B1-luc reporter genes. Cells were treated with DMSO or TCDD for 16 h and reporter gene activity was determined. Values were normalized to 100% TCDD treatment. Data are represented as means ± S.E.M. of three independent experiments. Statistical significance was analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Reporter gene activity significantly different than \( P < 0.05 \) TCDD-treated empty vector was denoted with an asterisk. Reporter gene activity significantly different than \( P < 0.05 \) TCDD-treated full-length TiPARP (1-657) was denoted with a pound sign.
Figure 27. Overexpression of GFP-tagged TiPARP N-terminal truncations.

HuH7 cells were overexpressed with pEGFP-TiPARP N-terminal truncation constructs for 48 h and proteins were detected by Western blot. GFP-TiPARP fusions were detected with anti-GFP antibody. β-actin was used as a loading control.
Figure 28. Effects of TiPARP double alanine point mutants on AHR-regulated reporter gene activity.

Reporter gene activity of CYP1A1-luc (A) and CYP1B1-luc (B). HuH7 cells were transiently co-transfected with pcDNA-TiPARP double alanine point mutant and pCYP1A1-luc or pCYP1B1-luc. Cells were treated with DMSO or TCDD for 16 h and reporter gene activity was determined. Values were normalized to 100% TCDD treatment. Data were represented as means ± S.E.M. of three independent experiments. Statistical significance was analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Reporter gene activity significantly different than \( P < 0.05 \) TCDD-treated empty vector was denoted with an asterisk. Reporter gene activity significantly different than \( P < 0.05 \) TCDD-treated wildtype TiPARP was denoted with a pound sign.
8.4 Repression of AHR by TiPARP required zinc finger function

Because the zinc-finger domain (residues 237-264) overlapped with the putative repressor domain (residues 218-245), we tested the importance of the zinc finger domain in TiPARP-mediated repression by creating single point mutants of the cysteine residues of CCCH-type zinc-finger motif. We verified the expression of all zinc finger mutants by in vitro translation with $^{35}\text{S}$-methionine (Figure 29A). All three TiPARP cysteine point mutants failed to inhibit TCDD-induced CYP1A1-luc and CYP1B1-luc reporter gene activity (Figure 29B and C). We created GFP-tagged versions of all three zinc finger point mutants to test expression in transiently transfected HuH7 cells. We compared the ability of the GFP-tagged zinc finger point mutants to untagged. All GFP-tagged point mutants failed to repress induction of CYP1A1- and CYP1B1-luc, which was similar to the untagged mutants (Figure 29D and E). Expression of GFP-tagged zinc finger point mutants was greater than wildtype GFP-TiPARP despite equal loading and transfection of the same amount. These observations demonstrated that the lack of repression of the zinc finger point mutants was not due to lack of expression (Figure 29F). Zinc finger function was required for TiPARP-mediated repression of AHR transactivation.
Figure 29. Repression of AHR transactivation required zinc finger function.

(A) *In vitro* translated $^{35}$S-labelled TiPARP zinc finger point mutants. pcDNA-TiPARP zinc finger point mutant constructs were *in vitro* translated with $^{35}$S-methionine and proteins were detected by SDS-PAGE and autoradiography. (B-C) CYP1A1-luc and CYP1B1-luc reporter gene activity was not repressed by TiPARP zinc finger point mutants. HuH7 cells were transiently co-transfected with pcDNA-TiPARP zinc finger point mutant constructs and pCYP1A1-luc or pCYP1B1-luc. Cells were treated with DMSO or TCDD for 16 h and reporter gene activity was determined. (D-E) CYP1A1-luc and CYP1B1-luc reporter gene activity was not repressed by GFP-tagged TiPARP zinc finger point mutants. HuH7 cells were transiently co-transfected with pEGFP-TiPARP zinc finger point mutant constructs and pCYP1A1-luc or pCYP1B1-luc. Cells were treated with DMSO or TCDD for 16 h and reporter gene activity was determined. All luciferase data were normalized to TCDD treatment, which was set to 100%. Data were represented as means ± S.E.M. of three independent experiments. Statistical significance was analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Reporter gene activity significantly different than ($P < 0.05$) TCDD-treated empty vector was denoted with an asterisk. Reporter gene activity significantly different than ($P < 0.05$) TCDD-treated wildtype TiPARP was denoted with a pound sign. (F) Western blot of GFP-tagged TiPARP zinc finger mutants. pEGFP-TiPARP zinc finger point mutant constructs were overexpressed in HuH7 cells for 48 h. Proteins were detected by Western blot using anti-GFP antibody. Representative Western blot of three independent replicates. β-actin was used as a loading control.
8.5 Repression of AHR transactivation by TiPARP required catalytic domain and function

To evaluate the importance of TiPARP catalytic domain in mediating the repression of AHR transactivation, we created C-terminal truncation and catalytic point mutants, and evaluated them in our transient transfection reporter gene assay. We in vitro translated both C-terminal truncations of catalytic mutants constructs with $^{35}$S-methionine to confirm expression (Figures 30A and 31A). Deletion of the TiPARP C-terminus containing the catalytic domain (residues 449-657) abolished the ability of TiPARP to repress AHR-dependent reporter gene activity (Figure 30B). The ability of TiPARP to repress CYP1A1- and CYP1B1-mediated reporter gene activity was prevented with the H532A and Y564A point mutants, but not I631A (Figure 31B), which retained catalytic activity (Figure 10B). Similar results were observed with GFP-tagged TiPARP mutants and immunoblotting confirmed that the lack of repression was not due to lack of protein expression (Figures 30C-D and 31C-D). Together these data demonstrated TiPARP-mediated repression of AHR transactivation required catalytic activities.
Figure 30. Repression of AHR transactivation required C-terminal catalytic domain.

(A) In vitro translated $^{35}$S-labelled TiPARP C-terminal truncations. pcDNA-TiPARP C-terminal truncation constructs were in vitro translated with $^{35}$S-methionine and proteins were detected by SDS-PAGE and autoradiography. (B-C) CYP1A1-luc and CYP1B1-luc reporter gene activity was not repressed by TiPARP C-terminal truncations. HuH7 cells were transiently co-transfected with pcDNA-TiPARP C-terminal truncation constructs and pCYP1A1-luc or pCYP1B1-luc. Cells were treated with DMSO or TCDD for 16 h and reporter gene activity was determined. (D-E) CYP1A1-luc and CYP1B1-luc reporter gene activity was not repressed by GFP-tagged TiPARP C-terminal truncations. HuH7 cells were transiently co-transfected with pEGFP-TiPARP C-terminal truncation constructs and pCYP1A1-luc or pCYP1B1-luc. Cells were treated with DMSO or TCDD for 16 h and reporter gene activity was determined. All luciferase data were normalized to 100% TCDD treatment. Data are represented as means ± S.E.M. of three independent experiments. Statistical significance was analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Reporter gene activity significantly different than ($P < 0.05$) TCDD-treated empty vector was denoted with an asterisk. (F) Western blot of GFP-tagged TiPARP C-terminal truncations. pEGFP-TiPARP C-terminal truncation constructs were overexpressed in HuH7 cells for 48 h. Proteins were detected by Western blot using anti-GFP antibody. Representative Western blot of three independent replicates. β-actin was used as a loading control.
Figure 31. Repression of AHR transactivation required catalytic function.

(A) *In vitro* translated $^{35}$S-labelled TiPARP catalytic point mutants. pcDNA-TiPARP catalytic point mutant constructs were *in vitro* translated with $^{35}$S-methionine and proteins were detected by SDS-PAGE and autoradiography. (B-C) CYP1A1-luc and CYP1B1-luc reporter gene activity was not repressed by TiPARP catalytic point mutants. HuH7 cells were transiently co-transfected with pcDNA-TiPARP catalytic mutant constructs and pCYP1A1-luc or pCYP1B1-luc. Cells were treated with DMSO or TCDD for 16 h and reporter gene activity was determined. (D-E) CYP1A1-luc and CYP1B1-luc reporter gene activity was not repressed by GFP-tagged TiPARP catalytic point mutants. HuH7 cells were transiently co-transfected with pEGFP-TiPARP catalytic point mutant constructs and pCYP1A1-luc or pCYP1B1-luc. Cells were treated with DMSO or TCDD for 16 h and reporter gene activity was determined. All luciferase data were normalized to 100% TCDD treatment. Data are represented as means ± S.E.M. of three independent experiments. Statistical significance was analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Reporter gene activity significantly different than ($P < 0.05$) TCDD-treated empty vector was denoted with an asterisk. (F) Western blot of GFP-tagged TiPARP catalytic point mutants. pEGFP-TiPARP catalytic point mutant constructs were overexpressed in HuH7 cells for 48 h. Proteins were detected by Western blot using anti-GFP antibody. Representative Western blot of three independent replicates. β-actin was used as a loading control.
8.6 TiPARP interaction with AHR

To examine direct interactions between AHR and TiPARP we performed co-localization and co-immunoprecipitation experiments. We first determined the subcellular localization of TiPARP. Endogenous TiPARP localized as small nuclear foci in HuH7 cells (Figure 32A, panel i). Overexpressed GFP-TiPARP exhibited focal nuclear expression patterns similar to endogenous TiPARP (Figure 32A, panel ii). GFP-tagged zinc finger mutant C243A displayed cytosol foci, suggesting the zinc finger domain is important for nuclear localization (Figure 32A, panel iii). The GFP-tagged catalytic mutant H532A displayed a diffuse nuclear expression pattern, suggesting catalytic function is important for foci organization (Figure 32A, panel iv). GFP-tagged mouse TiPARP expressed a similar nuclear focal pattern to human (Figure 32A, panel v), while GFP-tagged chicken TiPARP diffusely expressed in the cytosol (Figure 32A, panel vi).

We then tested whether AHR co-localized with GFP-TiPARP in transfected HuH7 cells treated with TCDD 1.5 h. In co-transfected cells, a fraction of the nuclear AHR was enriched in nuclear foci containing GFP-TiPARP (Figure 32B). To verify interaction, experiments were repeated in COS7 cells to score co-localization (Figure 32C). COS7 were selected for scoring because they contain low levels of endogenous AHR (Xu, et al., 2007) and cells overexpressing AHR could be easily distinguished from untransfected cells. GFP-TiPARP and AHR co-localization was scored based on co-transfection and AHR focal staining. COS7 cells transfected with only AHR and ARNT and stained for AHR were scored for focal staining. We observed AHR focal staining in 75.6% of GFP-TiPARP and AHR and ARNT co-transfected cells, whereas only 5.5% of cells expressing AHR and ARNT demonstrated focal staining (Table 8).

Table 8. Frequency of co-localization of GFP-TiPARP and AHR in COS7 cells

<table>
<thead>
<tr>
<th>Transfection</th>
<th>Total number of cells</th>
<th>AHR focal staining</th>
<th>No AHR focal staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-TiPARP + AHR/ARNT</td>
<td>113</td>
<td>90 (75.6%)</td>
<td>23 (24.4%)</td>
</tr>
<tr>
<td>AHR/ARNT</td>
<td>238</td>
<td>13 (5.5%)</td>
<td>225 (94.5%)</td>
</tr>
</tbody>
</table>
Co-immunoprecipitation experiments were then performed using TREx FLP-IN 293 cells transfected with GFP-TiPARP, AHR and ARNT and treated with DMSO or TCDD for 1.5 h to support interaction. Similar to co-localization experiments GFP-TiPARP and AHR co-immunoprecipitated (Figure 33A). Deletion analysis revealed that AHR interacted with residues 275-448 of TiPARP (Figure 33B). Reciprocal co-immunoprecipitation experiments demonstrated that both the N-terminus (residue 1-425) and C-terminal transactivation domain (residue 464-848) of AHR co-immunoprecipitated with GFP-TiPARP (Figure 33C and D).

We then tested the ability of the catalytically inactive and non-repressive point mutant GFP-TiPARP H532A to interact with AHR. COS7 cells co-overexpressed with GFP-TiPARP H532A and AHR and ARNT demonstrated co-localization within the nucleus (Figure 33E). Co-immunoprecipitation experiments demonstrated interaction between GFP-TiPARP H532A and AHR in the presence and absence of TCDD treatment (Figure 33F). Similarly, GFP-TiPARP C243A, a mutant that did not repress AHR-dependent reporter gene activity, co-immunoprecipitated with AHR in the presence and absence of TCDD (Figure 33F). Interestingly, the repressive N-terminal truncation, GFP-TiPARP 200-657, which co-immunoprecipitated with AHR (Figure 33B) localized within the cytosol and co-localized with cytosolic AHR (Figure 33G). These observations suggested that TiPARP interaction with AHR is not dependent on subcellular localization or the repressive function of TiPARP.
Figure 32. TiPARP interacts with AHR in the nucleus.

(A) Nuclear localization of endogenous TiPARP using anti-PARP7 (84664) (panel i), overexpressed GFP-TiPARP (panel ii), zinc finger mutant GFP-TiPARP-C243A (panel iii), catalytic mutant GFP-TiPARP-H532A (panel iv), GFP-mouse TiPARP (GFP-mTiPARP, panel v) and GFP-chicken TiPARP (GFP-chTiPARP, panel vi) in HuH7 cells. Images were acquired using an Olympus Fluoview 1000 confocal microscope.

(B-C) Co-localization of overexpressed GFP-TiPARP and AHR in HuH7 (B) and COS7 cells (C). For co-localization with AHR cells were treated with 10 nM TCDD for 1.5 h and fixed, immunostained for AHR, counterstained with DAPI and mounted using Vectashield. Images were acquired using an Imager.Z1 epifluorescence microscope and Axiovision software (Zeiss) following deconvolution.
Figure 33. TiPARP truncation and point mutant interaction with AHR.
(A) Reciprocal co-immunoprecipitation experiments of overexpressed GFP-TiPARP and AHR. TREx-FLP-IN 293 cells were transfected and treated with TCDD (T) or DMSO (D) for 1.5 h. (B) Co-immunoprecipitation experiments of AHR and GFP-TiPARP truncations in TREx-FLP-IN 293 cells (upper panel). Co-immunoprecipitation experiments with AHR and GFP in TREx-FLP-IN 293 cells (lower panel). AHR (H-211) antibody was used for immunoprecipitation and GFP (JL-8) was used to probe. (C-D) Co-immunoprecipitation experiments of GFP-TiPARP and AHR truncations in TREx-FLP-IN 293 cells. GFP (632460) antibody was used for immunoprecipitation and AHR (N-19 or H-211) antibodies were used to probe. (E) Co-localization of overexpressed GFP-tagged TiPARP catalytic mutant H532A (GFP-H532A) and AHR in COS7 cells. (F) Co-immunoprecipitation experiments of GFP-TiPARP catalytic (H532A) and zinc finger (C243A) point mutants with AHR in TREx-FLP-IN 293 cells. (G) Co-localization of overexpressed GFP-tagged TiPARP N-terminal truncation 200-657 (GFP-200-657) with AHR. For all co-localization experiments transfected cells were treated with 10 nM TCDD for 1.5 h and fixed, immunostained for AHR, counterstained with DAPI and mounted using Vectashield. Images were acquired using an Imager.Z1 epifluorescence microscope and Axiovision software (Zeiss).
8.7 Identification of nuclear localization signal of N-terminus of TiPARP.

Our co-localization observations suggested the presence of a nuclear localization signal (NLS) within the N-terminus of TiPARP between residues 1 and 200. We then examined the expression patterns of the N-terminal and C-terminal GFP-TiPARP truncations. We transiently transfected GFP-tagged TiPARP truncations into HuH7 cells and observed the subcellular localization. We first overexpressed GFP-tagged N-terminal truncation 33-657 in HuH7 cells and observed expression patterns similar to full-length GFP-TiPARP (Figure 34A panels i and ii). GFP-TiPARP 53-657 expressed in the cytosol as foci within the perinuclear region (Figure 34A panel iii), whereas GFP-TiPARP 103-657 expressed within the perinuclear region with less defined foci (Figure 34A panel iv). Both GFP-TiPARP 235-657 and GFP-TiPARP 445-657 truncations expressed in less defined pattern in the perinuclear region (Figure 34A panels v and vi). We then overexpressed the C-terminal truncations in HuH7 cells and both GFP-TiPARP 1-448 and 1-234 expressed diffusely and exclusively in the nucleus (Figure 34A panels vii and viii). These data suggested the presence of a nuclear localization signal between residues 33 and 53 of TiPARP. Between residues 33 and 53 we identified a short sequence consisting of basic amino acids (KKKDQKR), which is consistent with a putative NLS sequence (Marfori, et al., 2011). We created a triple mutant of the three tandem lysines (K41A; K42A; K43A) as well as a single mutant of the first lysine (K41A) and examined their cellular localization in HuH7 cells. Both the triple and single NLS mutants expressed in the cytosol suggesting that these residues are part of a functional NLS sequence (Figure 34A panels ix and x).

We next tested the ability of TiPARP NLS mutants to repress AHR transactivation. Using reporter gene assays we determined both the triple and single NLS mutants retained the ability to repress AHR-regulated reporter gene activity similar to wildtype (Figure 34B and C). TiPARP 53-657 also retained the ability to repress reporter gene activity similar to wildtype, which was expected based on our previous findings that TiPARP truncations 33-657 through 225-657 repressed reporter gene activity. Despite the expression of the NLS mutants and N-terminal truncations, 53-657 through 225-657, in the cytosol, AHR transactivation was still repressed. These findings suggested that nuclear localization is not required for TiPARP-mediated repression of AHR transactivation.
Figure 34. Identification of putative nuclear localization signal (NLS) within TiPARP N-terminus.

(A) Localization of EGFP-TiPARP N-terminal and C-terminal truncations and nuclear localization single point mutants in HuH7 cells. Panel i: Full-length GFP-TiPARP (1-657), N-terminal truncations- panel ii: GFP-TiPARP 33-657, panel iii: GFP-TiPARP 53-657, panel iv: GFP-TiPARP 103-657, panel v: GFP-TiPARP 235-657, panel vi: GFP-TiPARP 445-657, C-terminal truncations- panel vii: GFP-TiPARP 1-448, panel viii: GFP-TiPARP 1-234, NLS point mutants- panel ix: GFP-TiPARP K41A; K42A; K43A, panel x: GFP-TiPARP K41A. All GFP fusions were overexpressed for 24 h and then fixed in 4% paraformaldehyde. Images were acquired using an Olympus Fluoview 1000 confocal microscope. (B-C) TiPARP 53-657 truncation and NLS mutants repressed CYP1A1-luc reporter gene activity. HuH7 cells were transiently co-transfected with pcDNA-TiPARP truncation or point mutant expression constructs (B) or pEGFP-TiPARP truncation or point mutant expression constructs (C). Cells were treated with DMSO or TCDD for 16 h and reporter gene activity was determined. All luciferase data were normalized to 100% TCDD treatment. Data are represented as means ± S.E.M. of three independent experiments. Statistical significance was analyzed by one-way ANOVA and Tukey’s multiple comparisons test. Reporter gene activity significantly different than (P < 0.05) TCDD-treated empty vector was denoted with an asterisk.
8.8 TiPARP is a labile factor that negatively regulates AHR transactivation

TCDD-induced proteolytic degradation of AHR has been proposed to be under the control of a labile factor, referred to as an AHR degradation promoting factor (ADPF) that when inhibited increases the expression of AHR target genes, such as CYP1A1 (Ma, 2007; Ma and Baldwin, 2000). In support of these findings, pre-treatment with cycloheximide (CHX) resulted in enhanced TCDD-dependent CYP1A1 mRNA levels (Ma, 2007; Ma and Baldwin, 2000). Because we observed that TiPARP was a negative regulator of AHR, we were interested to determine if TiPARP was a labile factor regulating AHR protein levels. To this end, we tested the stability of TiPARP mRNA by treating T47D cells with actinomycin D and isolating RNA at the time indicated (Figure 35A). We observed a 50% loss in TiPARP mRNA after 1 h actinomycin D treatment (Figure 35A), which was in agreement with a previous report (Ma and Baldwin, 2002). Due to the lack of a suitable antibody to detect endogenous TiPARP protein levels, we examined the stability of TiPARP by overexpressing GFP-tagged TiPARP in HuH7 and treating with the 26S proteasome inhibitor, MG-132. After 6 h MG-132 treatment we observed increased GFP-TiPARP expression compared to transiently transfected but untreated cells (Figure 35B).

These data suggested that TiPARP was a candidate for the labile regulator of AHR protein levels and responsible for the increased expression of AHR target genes after pre-treatment with CHX (Ma, 2007; Ma and Baldwin, 2000). To test this notion, we pre-treated wildtype, Tiparp^{+/−} and Tiparp^{−/−} MEFs for 1 h with CHX prior to treatment with 6 h TCDD. Because we were interested in determining the effect of CHX on TCDD-dependent target gene expression, we also determined the fold increase of TCDD+CHX over TCDD alone for each genotype (Figure 35C and D). CYP1A1 mRNA levels increased approximately 480- and 300-fold following TCDD+CHX compared to TCDD alone in wildtype and Tiparp^{−/−} MEFs, respectively. CYP1B1 and AHRR mRNA levels were also significantly increased following TCDD+CHX compared to TCDD alone, but the fold increases were an order of magnitude lower than those observed for CYP1A1 (Figure 35C). For Tiparp^{−/−} cells, treatment with CHX+TCDD resulted in significant but modest increases of approximately 2-fold for each of AHR target gene examined compared to TCDD alone (Figure 35C). A similar pattern of CYP1A1 and CYP1B1 mRNA
superinduction after TCDD treatment was observed after MG-132 pre-treatment, although the
magnitude of induction was lower than that observed with CHX (Figure 35D). AHRR mRNA
was not significantly induced when co-treated with MG-132 for all MEF lines (Figure 35D).
These data suggested TiPARP was an important factor regulating the CHX- and MG-132-
dependent increases in ligand-activated AHR transactivation.
Figure 35. TiPARP is a labile negative regulator of AHR.

(A) TiPARP mRNA is rapidly degraded after actinomycin D treatment. T47D cells were treated with 1 μg/ml actinomycin D for the times indicated and TiPARP mRNA expression levels determined by qPCR. (B) GFP-TiPARP overexpression is increased after proteasome inhibition. HuH7 cells were transfected with pEGFP-TiPARP, pcDNA (vector) and pEGFP for 24 h and treated with 25 μM MG-132 for 6 h and GFP-TiPARP protein levels were determined by Western blot using anti-GFP antibody. The data presented were from a representative Western blot from three independent experiments. (C) AHR target gene induction in MEF lines pre-treated with 10 μg/ml cycloheximide (CHX). MEF cell lines were pre-treated with 10 μg/ml CHX for 1 h then treated with TCDD for 6 h and gene expression was determined. Data were normalized to wildtype DMSO. Fold changes between TCDD alone and CHX+TCDD (CHX+T) were provided for each gene. Gene expression results were shown as means ± S.E.M for three independent experiments and significance analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Gene expression levels significantly different ($P < 0.05$) than TCDD alone within each genotype were denoted with an asterisk. (D) MEFs lines were pre-treated with 25 μM MG-132 (MG) for 30 min then treated with TCDD for 4 h and gene expression was determined. Data were normalized to wildtype DMSO. Fold changes between TCDD alone and MG-132+TCDD (MG+T) were provided for each gene. Gene expression results were shown as means ± S.E.M for three independent experiments and significance analyzed by One-way ANOVA and Tukey’s multiple comparisons test. Gene expression levels significantly different ($P < 0.05$) than TCDD alone within each genotype were denoted with an asterisk.
9 Comparison of TiPARP- and AHRR-mediated negative regulation of AHR transactivation

Much of our understanding of the negative regulation of AHR comes from studies of the aryl hydrocarbon receptor repressor (AHRR), which is a part of a negative feedback loop regulating AHR transactivation (Evans, et al., 2008; Karchner, et al., 2009; Mimura, et al., 1999). Similar to TiPARP, AHRR expression is induced by ligand-activated AHR (Mimura, et al., 1999). The AHRR has been proposed to negatively regulate AHR transactivation by competing with AHR for ARNT heterodimerization and AHRE binding (Mimura, et al., 1999). However, we have observed in both Tiparp⁻/⁻ MEFs and Tiparp⁻/⁻ mouse liver tissue the TCDD-mediated induction of AHRR mRNA was increased. This observation contradicts the proposed mechanism of AHRR-mediated repression of AHR transactivation since greater expression of the AHRR in Tiparp⁻/⁻ mice should result in greater repression of AHR target transcription rather than greater induction. To better understand the mechanism(s) of AHR repression we compared TiPARP- and AHRR-mediated repression of AHR transactivation.
9.1 Kinetics of AHRR induction by TCDD

Because both TiPARP and AHRR gene expression are induced by TCDD we first compared the temporal changes in TiPARP and AHRR mRNA following TCDD treatment in MCF7 breast carcinoma cells. TCDD-dependent increases in TiPARP mRNA levels were rapid and peaked after 1.5 h similar to T47D cells, whereas AHRR mRNA an increased over 24 h (Figure 36A). We detected AHRR protein after 24 h TCDD treatment (Figure 36B); due to lack of suitable antibody for TiPARP we were unable to detect TiPARP protein expression following TCDD treatment. We next examined AHRR recruitment to CYP1A1 and CYP1B1 enhancer regions. Because we detected AHRR protein after 24 h TCDD treatment we treated cells up to 24 h and then performed ChIP assays. We observed recruitment of AHRR to the CYP1A1 and CYP1B1 regulatory regions only after 24 h TCDD treatment (Figure 36C). AHR/ARNT recruitment peaked after 45 min TCDD and declined following 6 h, similar to our previous reports (Figure 36D) (Ahmed, et al., 2009; Pansoy, et al. 2010). We previously observed recruitment of overexpressed GFP-TiPARP to the CYP1A1 enhancer region following TCDD treatment (Figure 18C), indicating that both TiPARP and AHRR are present at AHR regulatory regions in response to TCDD.
Figure 36. AHRR is induced by TCDD and recruited to AHR-regulated genes.
(A) Temporal induction of AHRR mRNA and TiPARP mRNA in MCF7 cells. MCF7 cells were treated with TCDD for the times indicated. (B) AHRR protein expression following 24 h TCDD or DMSO treatment. (C) Recruitment of AHRR to the CYP1A1 and CYP1B1 regulatory regions. MCF7 cells were treated with TCDD for the times indicated and AHRR recruitment was determined by ChIP. (D) Recruitment of AHR and ARNT to the CYP1A1 and CYP1B1 regulatory regions. MCF7 cells were treated with TCDD for the times indicated and AHR and ARNT recruitment was determined.
9.2 AHRR knockdown did not affect AHR transactivation or protein levels

We have demonstrated that RNAi-mediated knockdown of TiPARP resulted in increased TCDD-induced CYP1A1 and CYP1B1 mRNA expression and we were then interested in the effects of AHRR knockdown on CYP1A1 and CYP1B1 mRNA levels. We transfected MCF7 cells with three siAHRR strands for 24 h and treated cells with DMSO or TCDD for 24 h and determined AHRR mRNA knockdown. All three siAHRR strands reduced AHRR mRNA levels by 38%-48% following DMSO treatment (Figure 37A). AHRR mRNA levels were also reduced following TCDD treatment (Figure 37B). Because AHRR protein was not detected in non-induced (DMSO) MCF7 cells (Figure 36B), we induced AHRR expression by treating transfected cells with TCDD 24 h post-transfection to determine AHRR protein knockdown. We observed substantial knockdown of TCDD-induced AHRR protein by all three siAHRR sequences tested (Figure 37B). We chose to use siAHRR2 and siAHRR3 strands for all subsequent experiments because they showed greatest AHRR knockdown. We then examined the effect of AHRR knockdown on AHR target gene expression. AHRR knockdown did not significantly affect the TCDD-mediated induction of CYP1A1, CYP1B1 or TiPARP mRNA levels (Figure 37C). We repeated TiPARP knockdown experiments in MCF7 cells to compare the effects of TiPARP knockdown to AHRR knockdown. Similar to our previous data (Figure 15C) TiPARP knockdown increased TCDD-induced CYP1A1, CYP1B1 and AHRR mRNA levels (Figure 37D), indicating the mechanism of AHRR-mediated repression of AHR transactivation may differ from that of TiPARP.

We were then interested in whether AHRR knockdown affected AHR protein levels. Knockdown of AHRR did not increase constitutive AHR protein levels (DMSO) nor was TCDD-induced AHR proteasomal degradation reduced, which is in contrast to the effects of TiPARP knockdown on AHR protein levels (Figure 38A). These results were, however, in agreement with previous reports demonstrating AHRR overexpression did not alter AHR protein levels (Evans, et al., 2008). Since ARNT has been previously proposed to play an important role in AHRR-mediated repression of AHR (Mimura, et al., 1999) we tested ARNT protein levels following AHRR knockdown. Basal ARNT protein levels following TCDD treatment were not affected by AHRR knockdown (Figure 38A). TiPARP knockdown increased constitutive AHR
and ARNT levels and decreased TCDD-induced degradation of both proteins, similar to that observed in T47D cells (Figure 38B). Both basal and TCDD-induced AHRR protein levels were not affected by TiPARP knockdown despite elevated TCDD-induced AHRR mRNA levels (Figure 38B). A potential explanation for the discrepancy between AHRR mRNA and protein levels could be that the increase in AHRR mRNA levels may not be sufficient to observe increases in AHRR protein.
Figure 37. Effects of AHRR knockdown and TiPARP knockdown on AHR transactivation. 

(A) AHRR mRNA and (B) protein knockdown in MCF7 cells. MCF7 cells were transfected with non-targeting (NT) or three siAHRR strands, following 24 h knockdown cells were treated with DMSO (D) or TCDD (T) for 24 h and Western blots were performed. 

(C) Effects of AHRR knockdown on AHR target gene induction. AHRR was knocked down in MCF7 cells, treated with DMSO or TCDD for 24 h and AHR target mRNA expression determined. 

(D) TiPARP mRNA knockdown in MCF7 cells 

(E) Effect of TiPARP knockdown on AHR target gene induction. TiPARP was knocked down in MCF7 cells for 48 h and cells were treated with DMSO or TCDD for 24 h. Gene expression data were presented as means ± S.E.M. of three independent replicates. Statistical significance was determined by One-way ANOVA and Tukey’s multiple comparisons test. mRNA significantly (P < 0.05) different than treatment-matched non-targeting (NT) was denoted with an asterisk.
Figure 38. AHRR, AHR and ARNT protein levels following AHRR (A) and TiPARP (B) knockdown.

AHRR and TiPARP were knockdown in MCF7 cells. Cells were transfected with non-targeting (NT), siAHRR2 and siAHRR3 or siTiPARP12 and siTiPARP13. Following knockdown cells were treated with DMSO (D) or TCDD (T) for 24 h and protein levels determined by Western blot.
9.3 AHRR overexpression repressed AHR-regulated reporter gene activity

We next complemented the AHRR knockdown studies with overexpression studies. Based on a previous report identifying two isoforms of the AHRR we tested both full-length AHRR (AHRR\textsubscript{719}) and the predominantly expressed active form of the AHRR, which lacks exon 8 (AHRR\textsubscript{Δ8}) (Karchner, et al., 2009). We overexpressed increasing amounts of both forms of AHRR into HuH7 cells and measured the ability of each form to repress TCDD-induced CYP1A1-luc activity. Similar to a previous report, overexpression of increasing amounts of AHRR\textsubscript{719} did not affect CYP1A1-luc reporter gene activity, whereas AHRR\textsubscript{Δ8} potently inhibited activity (Figure 39A and B) (Karchner, et al., 2009). We next tested the ability of AHR and ARNT overexpression to rescue AHRR\textsubscript{Δ8} repression. Increasing amounts of AHR but not ARNT prevented the AHRR\textsubscript{Δ8}-mediated repression of CYP1A1-luc reporter gene activity as described previously (Karchner, et al., 2009). These results were also similar to rescue experiments with TiPARP where AHR but not ARNT rescued TCDD-dependent induction of CYP1A1-luc reporter gene activity (Figure 39C and D).
Figure 39. Overexpression of AHRRΔ8 repressed AHR-regulated reporter gene activity.
HuH7 cells were co-transfected with pCYP1A1-luc and pcDNA-AHRR719 (A) or pcDNA-AHRRΔ8 (B) at the amounts indicated. Cells were treated with TCDD for 16 h and reporter gene activity was determined. Transfection efficiency was normalized to β-galactosidase activity. Data are presented as means ± S.E.M. of three independent replicates. Co-overexpression of AHR (C) but not ARNT (D) rescued CYP1A1-luc activity. HuH7 cells were co-transfected with 5 ng pcDNA-AHRRΔ8 construct and 100-500 ng pRc-CMV-AHR or pcDNA4-ARNT. Transfected cells were treated with DMSO or TCDD for 16 h and CYP1A1-luc reporter gene activity was determined. Transfection efficiency was normalized to β-galactosidase activity. Data are presented as means ± S.E.M. of three independent replicates. Reporter gene activity significantly different than AHRRΔ8 alone was denoted with an asterisk.
9.4 Expression patterns of the AHRR

To examine the cellular localization patterns of the AHRR we created both N- and C-terminal GFP-tagged expression constructs of AHRRΔ8 and AHRR719. We overexpressed all constructs in HuH7, COS7 and MCF7 cells. Unexpectedly, both GFP-tagged AHRRΔ8 and AHRR719 expressed in the cytosol in HuH7 and COS7 cells (Figure 40). The location (N- or C-terminally linked) of the GFP tag on either AHRRΔ8 or AHRR719 did not affect their expression or localization pattern. We then repeated overexpression of GFP-tagged AHRR constructs in MCF7 cells, GFP-tagged AHRRΔ8 localized to the nucleus, whereas the GFP-tagged AHRR719 localized diffusely to both the nucleus and cytosol (Figure 40). GFP-tagged TiPARP, when overexpressed in MCF7 cells, appeared as nuclear foci, similar to that observed in HuH7 and COS7 cells (Figure 32A). Despite the differences in expression patterns of the AHRR in the three cell lines tested the AHRR significantly repressed AHR-regulated reporter gene activity in all three cell lines (Karchner, et al., 2009).
Figure 40. Expression of N-terminal and C-terminal GFP-tagged AHRR in MCF7, HuH7 and COS7 cells.

MCF7, HuH7 and COS7 cells were transfected with pEGFP-C2-AHRRΔ8, pEGFP-C2-AHRR719, pAcGFP-N1-AHRRΔ8, pAcGFP-N1-AHRR719 or pEGFP-C2-TiPARP. All GFP fusions were overexpressed for 24 h then fixed with 4% paraformaldehyde. Images were acquired using an Olympus Fluoview 1000 confocal microscope.
Chapter 5: Discussion

10 TiPARP is a mono-ADP-ribosyltransferase

Class 2 ARTDs are predicted to be mono-ADP-ribosyltransferases based on the sequence and structural features of their catalytic domains (Otto, et al., 2005). The presence of a glutamate within the catalytic HYE triad motif, which is essential for poly(ADP-ribose) chain elongation is absent from class 2 ARTD members and restricts their enzymatic activities to mono-ADP-ribosylation (Marsischky, et al., 1995; Rolli, et al., 1997; Ruf, et al., 1998). In agreement with this notion, the class 2 ARTDs, ARTD7, 8, 10, 12 and 15 have been shown to exhibit mono-ADP-ribosyltransferase activity (Aguiar, et al., 2005; Di Paola, et al., 2012; Kleine, et al., 2008; Leung, et al., 2011). TiPARP (ARTD14) has previously demonstrated to be a catalytically active enzyme; however, whether this catalytic activity was mono-ADP-ribosylation was not determined (Ma, et al., 2001). We demonstrated that TiPARP is a mono-ADP-ribosyltransferase that ADP-ribosylates itself and core histones (section 6). Our results indicate the catalytic activities of TiPARP are dependent on the catalytic histidine and tyrosine of its HYI motif, because mutation of these residues abolished auto- and hetero-ADP-ribosylation activities. Auto-ADP-ribosylation of TiPARP is not affected by the presence of activated DNA or RNA, which is similar to previous reports for ARTD7 and ARTD10 (Aguiar, et al., 2005; Kleine, et al., 2008). These results suggest TiPARP is a nucleic acid-independent mono-ADP-ribosyltransferase.

We used two assays to examine the auto-ribosylation activity of TiPARP, the $^{32}$P-NAD$^+$ assay and the $^{35}$S protein shift assay. The $^{32}$P-NAD$^+$ assay uses purified TiPARP incubated with $^{32}$P-labelled NAD$^+$ is an established methodology to detect catalytic activity of ARTDs by indicating substrate incorporation and shift in mobility by SDS-PAGE (Shah, et al., 2011). The $^{35}$S protein shift assay, uses in vitro translated $^{35}$S-labelled TiPARP to detect changes in mobility of $^{35}$S-TiPARP following incubation with or without NAD$^+$ by SDS-PAGE. This small shift in mobility (or smearing) that we observed for $^{35}$S-TiPARP when incubated with $\beta$-NAD$^+$ could be indicative of short oligo-ADP-ribose polymers, dimers or trimers, or mono-ADP-ribose on multiple residues of TiPARP (Lindahl, et al., 1995; Satoh, et al., 1994). The actual polymer sizes of auto-ADP-ribosylated TiPARP can be analyzed by sequencing gel (Alvarez-Gonzalez and Jacobson, 1987; Tanaka, et al., 1978).
For all variants and species of TiPARP as well as conditions tested (with nucleic acids) results from both $^{32}\text{P-}\text{NAD}^+$ and $^{35}\text{S}$ protein shift assays were in agreement except for the catalytic isoleucine mutant (I631A) and chicken TiPARP. Results from the $^{32}\text{P-}\text{NAD}^+$ assay demonstrated I631A mutant to be catalytically active while the $^{35}\text{S}$ protein shift assay did not show a mobility shift when incubated with NAD$^+$. This discrepancy may be due to sensitivity differences between the two assays with the $^{32}\text{P-}\text{NAD}^+$ assay being more sensitive than the $^{35}\text{S}$ protein shift assay. A mutation of the catalytic isoleucine of ARTD10 to a glutamate greatly reduced auto-ADP-ribosylation activity but did not abolish it (Kleine, et al., 2008). The $^{35}\text{S}$ protein shift assay may not be sensitive enough to detect a reduction in the level of TiPARP catalytic activity. This hypothesis may also explain the discrepancy between the two assays with respect to chicken TiPARP. We observed weaker auto-ADP-ribosylation activity of chicken TiPARP than mammalian TiPARPs by the $^{32}\text{P-}\text{NAD}^+$ assay but no apparent activity in the $^{35}\text{S}$ protein shift assay, suggesting that sensitivity may be limited in this assay.

We used the $^{35}\text{S}$ protein shift assay to determine whether TiPARP contains an auto-ribosylation domain. We observed auto-ADP-ribosylation activity was greatly reduced between residues 275 and 328 of TiPARP suggesting the presence of an auto-ribosylation domain. The BRCT (BRCA1 C-terminus-like) domain of ARTD1 is generally referred to as the auto-ribosylation (or auto-modification) domain (Loeffler, et al., 2011). Careful dissection of the ARTD1 auto-ribosylation domain revealed three specific residues that are modified (one aspartate and two glutamates) (Tao, et al., 2009). Other reports have described lysines within the auto-ribosylation and DNA binding domain to be target residues for modification (Altmeyer, et al., 2009). A recent study identified 12 auto-ribosylation sites of ARTD1, namely glutamates, aspartates and lysines, 8 of these auto-ribosylation sites were found outside of the so-called auto-ribosylation domain (Chapman, et al., 2013). Based on these findings auto-ribosylation of TiPARP may not be restricted to a specific domain but is rather dispersed throughout the enzyme on multiple residues. It has been suggested that there are preferred sites of auto-ribosylation of ARTD1 and when these preferred or primary sites are unavailable secondary sites become prevalent (Tao, et al., 2009). Results from our $^{35}\text{S}$ mobility shift assay may suggest TiPARP may contain multiple primary and secondary sites of auto-ribosylation throughout the protein. Currently it is not clear which residues of ARTD family members serve as modification sites. Glutamate and aspartate residues were reported to be auto-ribosylation sites on ARTD10 (Kleine, et al., 2008; Rosenthal,
et al., 2013). Other residues that have been reported to be modified by eukaryotic MARTs include arginine, cysteine, phosphoserine and asparagine and together with glutamate and aspartate represent potential auto-ribosylated sites on TiPARP (Corda and Di Girolamo, 2003; Okazaki and Moss, 1996; Ord and Stocken, 1977; Seman, et al., 2004; Smith and Stocken, 1975). Our laboratory is currently determining specific sites of auto-ADP-ribosylation of TiPARP by mass spectrometry. We have identified 6 distinct peptides that are mono-ADP-ribosylated. These findings confirm the mono-ADP-ribosyltransferase activity of TiPARP, but we have not mapped or identified the modified amino acid residues. We have initiated electron dissociation transfer mass spectrometry to specifically identify the ribosylated residues.

We have demonstrated that TiPARP possesses hetero-ADP-ribosylation activity and ADP-ribosylates core histones. ARTD1, 2, 3 and 10 have also been described so far to ADP-ribosylate histones (Kleine, et al., 2008; Messner, et al., 2010; Rulten, et al., 2011). Histone ribosylation links ADP-ribosylation to other modifications such as acetylation, methylation and phosphorylation, which constitute an epigenetic code for histones (Messner and Hottiger, 2011). The contribution of histone ribosylation to the histone code and epigenetic regulation is not well known. Moreover, ribosylated histones only constitute a small fraction (< 1%) of total histones (Boulikas, 1989). Recently, macrodomain-containing proteins such as ARTD8, ARTD9, MacroD1, MacroD2 and C6orf130 have been reported to interact with ADP-ribosylated target proteins and represent potential ‘readers’ of ADP-ribosylation (Jankevicius, et al., 2013; Rosenthal, et al., 2013; Sharifi, et al., 2013). MacroD1, MacroD2 and C6orf130 have been identified as mono-ADP-ribosylhydrolases that bind mono-ADP-ribose and mediate its removal from target proteins (Rosenthal, et al., 2013). Potential ‘readers’ and mono-ADP-ribosylhydrolases of ADP-ribosylated histones by TiPARP have not been determined, but would be an interesting future direction as it will help expand our understanding of the function of histone ribosylation.

11 TiPARP is a novel negative regulator of AHR transactivation

We determined that TiPARP is a novel negative regulator of AHR transactivation. Our studies using human cell lines and MEFs isolated from wildtype and Tiparp-deficient mice reveal that TiPARP modulates ligand-induced proteolytic degradation of AHR and functions as a repressor
of AHR transactivation. In this model, TCDD-activated AHR induces TiPARP expression, TiPARP is recruited to AHR target genes either through direct DNA binding or tethering through AHR, increased levels of TiPARP enhance the proteolytic degradation of AHR, by either direct ribosylation or through the ribosylation of unidentified intermediary factor(s) to repress AHR target gene transcriptional activation (Figure 41).

Figure 41. Proposed model of repression of AHR transactivation by TiPARP.

The AHR mediates the TCDD-induced expression of TiPARP (1). TiPARP is recruited to the AHR/ARNT-bound AHRE of targets to repress transcription (CYP1A1 in this example) (2A). TiPARP represses its own transcription and the regulation of other AHR target genes by binding to AHR/ARNT-bound AHRE or potentially through direct DNA binding, creating a negative feedback loop (2B) in which AHR dissociates from AHRE, translocates from the nucleus and is proteolytically degraded by the 26S proteasome (26S) (3). BTF; basal transcription factors, Co-act; co-activators.
11.1 Potential co-repression by ADP-ribosylation of AHR

The ability of TiPARP to repress AHR transactivation requires its catalytic domain and the direct mono-ADP-ribosylation of AHR by TiPARP is an obvious mechanism to consider. Previous studies have reported that ARTDs act as co-regulators (co-activators or co-repressors) for a number of different transcriptional regulators (see section 2.5.4 for further information). In some cases, enzymatic activity is required while in others it is not. ADP-ribosylation of Oct-1 and Sp1 electrostatically repels them from DNA resulting in altered transcript expression profiles (Gagne, et al., 2008; Nie, et al., 1998; Smith, 2001). ADP-ribosylation of p53 and NF-κB prevents their association with other nuclear factors, which also affected gene expression (Kanai, et al., 2007; Zerfaoui, et al., 2010). It is possible that mono-ADP-ribosylation of AHR by TiPARP prevents AHR binding to AHREs or its association with co-regulatory proteins. AHR that is not complexed with ARNT is susceptible to degradation which could explain the changes in AHR protein levels we observe when TiPARP expression altered (Davarinos and Pollenz, 1999; Heid, et al., 2000; Kazlauskas, et al., 2000; Shetty, et al., 2003). The impact of mono-ADP-ribosylation on transcription factor activity is not well understood and it is unclear whether mono-ADP-ribosylation can alter transcription factor function by the same mechanism or extent as poly(ADP-ribose). However it should be noted that mono-ADP-ribosylation of a single amino acid residue by bacterial toxins, such as diphtheria and cholera toxins, can drastically alter target protein function leading to severe physiological consequences (Collier, 2001).

The potential that ADP-ribosylation represents a novel posttranslational modification of AHR is extremely exciting. AHR is subject to posttranslational modifications including SUMOylation, ubiquitination and phosphorylation (Chen and Tukey, 1996; Pongratz, et al., 1991; Xing, et al., 2012). Phosphorylation of specific serine and tyrosine residues of AHR is important for DNA-binding and maximal transactivation, demonstrating modification of AHR affects its function (Chen and Tukey, 1996; Park, et al., 2000; Pongratz, et al., 1991). Therefore ADP-ribosylation of AHR could alter many processes involved in the AHR transactivation such as ARNT dimerization, DNA-binding, association with co-regulatory or basal transcription factors, nuclear import/export and transactivation. Because the structure and function of the modular domains of AHR are well understood, mapping of potential mono-ADP-ribosylation sites by TiPARP will be very informative in determining the effects ADP-ribosylation has on its function. We are
currently optimizing purification of AHR protein to determine if it is hetero-ribosylated by TiPARP and if so identify ADP-ribosylation using in vitro assays and mass spectrometry.

11.2 Potential targeting of histones and other co-regulatory factors

Core histones, linker histone H1 and chromatin-associated proteins are all targets of ADP-ribosylation by ARTD1 (Krishnakumar and Kraus, 2010b). ADP-ribosylation (mono- or poly-) of H1, H2A and H2B by ARTD1 may play a role in the regulation of chromatin structure, although the general extent of histone modification to transcriptional regulation remains to be clarified (D'Amours, *et al.*, 1999). ARTD1 can displace the H1 by ADP-ribosylation and block binding of H1 to promoter chromatin creating a structure permissive to transcription (Ju, *et al.*, 2004; Kim, *et al.*, 2004; Krishnakumar, *et al.*, 2008). The hetero-ADP-ribosylation of core histones by TiPARP is a potential mechanism by which TiPARP modulates chromatin structure at active AHR regulatory regions to repress transcription. TiPARP could also target co-regulatory proteins with histone-modifying activities that are known to interact with AHR/ARNT (see Table 3) altering their intrinsic histone-modifying or chromatin-remodelling activities by ADP-ribosylation. Many of the known AHR co-activators have histone acetyltransferase (HAT) activity such as NCoA1, 2, 3 and p300 (Hankinson, 2005; Harper, *et al.*, 2006). Histone ADP-ribosylation by TiPARP could reduce co-activator activity by competing with or blocking histone acetylation and thus reducing transactivation. Poly-ADP-ribosylation of histone lysine demethylase KDM5B has been shown to block binding of KDM5B to chromatin and inhibit its demethylase activity supporting the hypothesis that hetero-ADP-ribosylation by TiPARP may reduce AHR co-activator function (Krishnakumar and Kraus, 2010a). TiPARP could also modulate other chromatin-associated proteins to alter chromatin to architecture more favourable for repression. ARTD1 also hetero-ribosylates DEK, a chromatin-associated protein and promotes its release from chromatin suggesting TiPARP may potentially modulate other chromatin-associated proteins besides co-activators to alter chromatin to architecture in favour of repression (Gamble and Fisher, 2007; Kappes, *et al.*, 2008). Proteomics will be very useful to identify interacting proteins with TiPARP and provide insights into the mechanism of TiPARP-mediated repression.
The auto-ribosylation activity of TiPARP may also be important for its repressive function. Auto-ribosylation accounts for approximately 90% of ARTD1 total catalytic activity indicating the enzyme itself is the primary target (D'Amours, et al., 1999). Auto-ribosylated ARTD1 provides a binding platform for DNA repair enzymes and scaffolding proteins (Gagne, et al., 2008; Hegde, et al., 2008). Therefore it is possible that auto-ribosylated TiPARP recruits co-repressor proteins including known AHR co-repressors SMRT, SHP or AHRR to promote transcriptionally repressive complex assembly at the AHREs of target genes (Harper, et al., 2006; Mimura, et al., 1999; Nguyen, et al., 1999). Alternatively, it is equally possible that auto-ribosylated TiPARP at AHREs could also prevent the association of AHR/ARNT or basal transcription factors with co-activators. Identification of potential auto-ribosylation sites on TiPARP will help clarify their importance in mediating the repressive function of TiPARP.

11.3 TiPARP is a candidate AHR degradation promoting factor (ADPF)

Our proposed model of TiPARP-mediated repression (Figure 41) is reminiscent of the ADPF hypothesis which proposes the induction of a labile factor that is sensitive to proteasomal and protein synthesis inhibition and promotes the proteasomal degradation of AHR (Ma and Baldwin, 2000; Ma and Baldwin, 2002; Ma, et al., 2000). We show by use of transcriptional and proteasomal inhibitors that TiPARP mRNA and protein is rapidly degraded indicating TiPARP is labile. Protein synthesis inhibition of Tiparp−/− MEFs did not result in superinduction of AHR target genes when cells were treated with TCDD which suggested newly generated TiPARP represses AHR transactivation. We also observed TiPARP knockdown or knockout increases AHR protein levels and transactivation mimicking the effects of protein synthesis and proteasomal inhibition (Ma and Baldwin, 2000). Overall, our data support TiPARP as a candidate labile ADPF.

The ubiquitin-proteasome pathway plays an important role in TCDD-mediated degradation of AHR (Ma and Baldwin, 2000; Pollenz, 2007). ARTD1 plays a role in the reactive oxygen-induced activation of the proteasome (Ullrich, et al., 1999). During oxidative stress auto-ribosylated ARTD1 interacts with and activates nuclear proteasome pathways leading to the rapid up-regulation of 20S proteasome activity to remove damaged histones more efficiently (Ullrich, et al., 1999). Inhibition of ARTD5 and ARTD6 activity prevents the degradation of...
axin through the ubiquitin-proteasome pathway and this increased half-life of axin promotes the 
\( \beta \)-catenin degradation (Mayer-Kuckuk, *et al.*, 1999). These findings demonstrate there is 
interplay between the nuclear proteasome proteins and poly-ADP-ribosylation. It is therefore 
tempting to hypothesize TiPARP activates components of the proteasome to enhance the TCDD-
mediated AHR degradation. It is also equally possible that TiPARP directly ADP-ribosylates 
AHR, targeting it for degradation. Iduna is a poly(ADP-ribose)-dependent E3 ubiquitin ligase 
that binds and ubiquitinates both poly-ADP-ribosylated and poly(ADP-ribose)-binding proteins 
labelling these proteins for ubiquitin proteasomal degradation and provides an example of how 
ADP-ribosylation can be used to target proteins for degradation (Kang, *et al.*, 2011).

### 11.3.1 TiPARP and the nuclear proteasome

TiPARP localizes to the nucleus and exhibits a focal expression pattern, which can be described 
as nuclear bodies (von Mikecz, 2006). It has been established that proteasomes occur in the 
nucleus and are associated with nuclear bodies including PML bodies, nucleoplasmic speckles 
and focal clusters throughout the nucleoplasm representing proteolytic centres in the nucleus 
by proteasomal inhibition resulting in its accumulation in the nucleus (data not shown) and 
suggests that TiPARP is subject to proteolytic degradation via the 26S proteasome. All TiPARP 
catalytic mutants and cytosolic N-terminal truncations and point mutants (both zinc finger and 
NLS mutants) are more highly expressed compared with wildtype indicating that components 
within the nucleus and the catalytic activity of TiPARP are responsible for its instability.

Because TiPARP affects AHR protein levels and AHR interacts with TiPARP within the nuclear 
foci it is reasonable to hypothesize that TiPARP associates with nuclear bodies containing 
proteasomes to target AHR for proteasomal degradation. The catalytic mutant TiPARP-H532A 
focal expression pattern was greatly altered but still interacted with AHR. This suggests that 
catalytic activity of TiPARP is required for association with proteolytic centres in the nucleus to 
degrade AHR, repressing its transactivation. The effects of overexpression of TiPARP catalytic 
mutants on AHR protein levels will help clarify whether catalytic activity is required for the 
proteolytic degradation of AHR. Together, our findings indicate TiPARP is associated with the 
nuclear proteasome and this association is dependent upon its catalytic activity. Currently, we are
determining which nuclear body types are associated with TiPARP to provide evidence that TiPARP is associated with the nuclear proteasome.

11.3.2 Connecting nuclear localization with repression of AHR transactivation

We identified a functional NLS of TiPARP within its N-terminus between residues 41-47 (Figure 42). The zinc finger domain is also important for nuclear localization of TiPARP. Zinc finger motifs have been identified as the NLS of several zinc finger proteins (Kuwahara, et al., 2000; Matheny, et al., 1994; Racca, et al., 2011; Spittau, et al., 2007). Mutation or removal of either the NLS or zinc finger results in the cytosolic localization of TiPARP. The purpose of having two different NLSs is currently not known. One significant difference between the NLS and zinc finger is the NLS mutants still repress AHR transactivation whereas zinc finger mutants do not. These findings suggest repression is not dependent on nuclear localization but rather zinc finger function. Zinc finger mutants still interact with AHR presumably in the cytosol indicating the zinc finger is important for repression and potential proteasomal degradation of AHR. Zinc finger domains of proteins are known to play a role in protein-protein interactions and thus the zinc finger domain of TiPARP could play a role in the association with the cytosolic proteasome rather than with AHR (Kang, et al., 2004; Zhang, et al., 2011). Determining interacting partners by proteomics and AHR protein levels following overexpression of zinc finger TiPARP mutants can be used to test this hypothesis.

All C-terminal truncations of TiPARP are nuclear but do not repress AHR transactivation. In constrast, N-terminal truncations 53-657 to 225-657 are cytosolic and still repress AHR activity. TiPARP truncation 200-657 which is cytosolic and functional and interacts with AHR in the cytosol. Because AHR exists in the nucleus and cytosol this observation is not completely unexpected. It is possible the TiPARP 200-657 truncation interacts with the cytosolic proteasome to degrade AHR and repress transactivation, representing an alternative pathway to nuclear AHR degradation. Determining AHR protein levels in cells overexpressed with TiPARP 200-657 will clarify this alternative pathway. The association with AHR and repression of transactivation by TiPARP 200-657 may also be independent of proteasomal degradation and instead TiPARP 200-657 prevents the nuclear translocation of AHR. As far as we know all the truncations and mutants we created do not exist endogenously in cells and their activity, or lack thereof, is observed experimentally using overexpression but may not occur biologically. During apoptosis
ARTD1 is cleaved into 24 and 89 kDa fragments and each fragment is functional (Chaitanya, et al., 2010). The 24 kDa fragment containing two of the zinc fingers localizes to the nucleus and binds DNA irreversibly and the 89 kDa contains the auto-modification and catalytic domains and retains its catalytic activity but localizes to the cytosol (D'Amours, et al., 2001; Soldani, et al., 2001). Therefore it is also not completely unexpected that truncated cytosolic TiPARP variants repress AHR and that potential endogenous TiPARP truncations may exist and exhibit a similar ability to repress AHR.

Figure 42. Updated schematic of the structure of TiPARP.

NLS; nuclear localization signal, Zn; CCCH-type zinc finger domain, WWE; tryptophan-tryptophan-glutamate.

11.4 Identification of an AHR repressive domain

The use of N-terminal truncations identified a putative AHR repressive domain between residues 218 and 245 (Figure 42). Alanine scan results narrowed this region to between residues 220 and 235. Alanine scanning also identified tyrosine 232 (Y232) and histidine 233 (H233) to be important for repression of AHR transactivation. The exact function and contribution of Y232 and H233 to repression is not clear and we can only speculate their roles. Both tyrosine and histidine residues are phosphorylated which can alter protein function and conformation (Miller, 2012; Steeg, et al., 2003). For example, AHR phosphorylation at tyrosine residues is important for its AHRE-binding activity (Minsavage, et al., 2003; Park, et al., 2000). Therefore it is possible that phosphorylation of Y232 and/or H233 is important for TiPARP-mediated repression of AHR transactivation. Tyrosines are commonly sulfated which strengthens protein-protein interactions, Y232 may also be important for stabilizing interactions with intermediary
proteins involved in the repression of AHR (Seibert and Sakmar, 2008). It is unlikely that Y232 or H233 are important for interaction with AHR since the TiPARP 275-657 truncation interacted with AHR. Given the proximity of H233 to the zinc finger domain (four residues away) and the fact that histidine is commonly involved in coordinating the zinc ion of zinc fingers, H233 could be involved with zinc finger function and its loss resembles a zinc finger mutation (Alberts, et al., 1998).

12 Comparing TiPARP- with AHRR-mediated transrepression

Much of our understanding of AHR repression comes from studies of AHRR, which is also part of negative feedback loop regulating AHR function (Evans, et al., 2008; Karchner, et al., 2009; Mimura, et al., 1999). Despite its discovery more than ten years ago and several reports from independent laboratories, the precise mechanism of AHRR-mediated transrepression of AHR is not well understood. Conflicting reports between independent laboratories has been a major contributing factor for our lack of understanding of the AHRR. We compared the AHR repressive functions of TiPARP to the AHRR to better understand the mechanisms of negative regulation of AHR transactivation.

12.1 Gene expression and recruitment

As expected, both TiPARP and the AHRR gene expression are induced by TCDD treatment. The AHRR expression gradually increased over 24 h, whereas TiPARP induction peaked after 1.5 h and declined thereafter. In line with gene expression kinetics, we detected endogenous AHRR protein and its recruitment to endogenous AHRE-containing regulatory regions after 24 h TCDD treatment. This recruitment can be supported by previous reports that used in vitro methods to determine AHRR interaction with AHRE-containing sequences (Evans, et al., 2008; Mimura, et al., 1999). Recruitment of the AHRR to AHR target regulatory regions coincides with reduced AHR/ARNT recruitment, suggesting that AHRR displaces AHR/ARNT binding which is similar to a previous study that describes the AHRR recruitment to an AHRE-containing regulatory region of AHRR correlated with AHR/ARNT recruitment loss (Haarmann-Stemmann, et al., 2007). Similarly, we observed TCDD-dependent recruitment of GFP-TiPARP and AHRR to the CYP1A1 regulatory region suggesting that both TiPARP and the AHRR can influence the AHR/ARNT complexes at AHREs. The mechanism of how the AHRR and TiPARP alter
AHR/ARNT complexes at AHREs may differ. TiPARP alters AHR protein levels and the enhanced AHR proteolytic degradation which could lead to reduced AHR recruitment to its target genes. The AHRR does not affect the proteolytic degradation of AHR therefore the reduced AHR/ARNT recruitment we observed after 6 h and 24 h is not likely due AHR degradation (Evans, et al., 2008).

The direct AHRE-binding of the AHRR is not involved in the mechanism of repression of the AHRR. This is supported by studies using an AHRR DNA-binding mutant that localizes to the nucleus and still substantially represses AHR transactivation (Evans, et al., 2008). Direct AHRE-binding is also not essential for TiPARP-mediated repression as TiPARP NLS mutants and N-terminal truncations from residue 53 to residue 225 still repress AHR-regulated reporter gene activity despite localizing to the cytosol. Both TiPARP and the AHRR can directly interact with AHR; therefore, it is possible that TiPARP and the AHRR directly interact with AHRE-bound as well as unbound AHR to repress transactivation.

12.2 Overexpression of ARNT and co-regulators

Overexpression of AHR but not ARNT significantly reduces repression by AHRR or TiPARP, suggesting that AHR rather than ARNT is the primary target of both repressors (Evans, et al., 2008; Karchner, et al., 2009). The lack of involvement of ARNT does not support the original model AHRR-mediated repression, which proposed the AHRR competes with AHR for ARNT heterodimerization and AHRE binding (Mimura, et al., 1999). Additionally, overexpression of many known AHR co-activators did not rescue the inducibility of AHR-mediated reporter gene activity indicating competition with co-activators is not involved in the AHRR mechanism of repression. Histone deacetylases 4 and 5 (HDAC4 and HDAC5) are co-repressors that are associated with the AHRR perhaps recruitment of co-repressors is a mechanism by which the AHRR can negatively repress AHR transactivation (Oshima, et al., 2009; Oshima, et al., 2007).

12.3 Subcellular localization

Previous reports have described that the AHRR is a nuclear protein with an active NLS located within its N-terminus (Evans, et al., 2008; Kanno, et al., 2007). However we observe GFP-tagged AHRR constructs to localize to the nucleus in a cell context-dependent manner. Only in MCF7 cells did we observe the nuclear localization of the AHRR, AHRR localized to the cytosol
in HuH7 and COS7 cells. TiPARP localized to the nucleus in all three cell lines. The reason for the different localization patterns of the AHRR among the cell lines is currently not known. ARNT expression has been proposed to influence the localization of the AHRR (Kanno, et al., 2007). The expression of an AHRR NLS mutant in COS7 cells was shifted from the cytosol to the nucleus when ARNT was co-overexpressed suggesting ARNT expression can influence AHRR localization (Kanno, et al., 2007). We observed that AHRR localizes to the cytosol in both COS7 cells which have low levels of ARNT and in HuH7 cells which express ARNT, so it does not appear that ARNT expression influences the localization of the AHRR. However, we did not co-overexpress ARNT with the AHRR in COS7 and HuH7 cells therefore it is possible that overexpressed ARNT influences subcellular localization of the AHRR. Despite the difference in localization of the AHRR, the AHRR is a potent repressor of AHR transactivation in all three cell lines (Evans, et al., 2008; Karchner, et al., 2009). This observation is similar to that observed with the TiPARP NLS mutants and N-terminal truncations from residue 53 up to residue 225 that repress AHR transactivation but localize to the cytosol. The AHRR has previously been reported to not alter nuclear translocation of AHR by comparing the nuclear translocation of AHR when AHRR was co-overexpressed although these reports did not examine AHR and AHRR co-localization. Although the subcellular localization of endogenous AHRR has not yet been determined, our recruitment findings suggest that a proportion of endogenous AHRR is nuclear.

12.4 Knockdown and knockout models

Our knowledge on the AHRR function is largely based on overexpression models, and when overexpressed the AHRR is an effective repressor of AHR-regulated reporter gene activity. However, findings from AHRR overexpression studies are not fully supported by knockout models (Hosoya, et al., 2008; Tigges, et al., 2013). Previous studies that have reported that cell lines that have low AHR ligand-dependent inducibility of CYP1A1 have high basal levels of AHRR and knockdown of the AHRR increases basal CYP1A1 expression and AHR ligand inducibility (Haarmann-Stemmann, et al., 2007; Oshima, et al., 2007). We observed the constitutive levels of CYP1A1 mRNA in MCF7 cells were not significantly affected by AHRR knockdown. Our results are supported by a recent report that describes a non-causal relationship between AHRR expression and CYP1A1 induction and activity human skin fibroblasts (Tigges, et al., 2013). Reports using fibroblasts isolated from Ahrr-deficient mice demonstrated
impressive CYP1A1 induction by AHR ligands which suggest that the knockdown levels we achieved in MCF7 cells were not sufficient to significantly increase inducibility of AHR target genes (Hosoya, et al., 2008; Tigges, et al., 2013). Alternatively, given that the increased CYP1A1 expression levels by AHR ligand treatment was limited to the heart, spleen and skin of Ahrr-deficient mice our choice of cell type (MCF7 breast carcinoma) may have limited results. TiPARP knockdown in all cell types that we examined which includes two human breast carcinoma, hepatoma and gastric carcinoma lines and the Tiparp-deficient MEFs displayed significantly greater AHR target inducibility which suggests TiPARP to be a general negative regulator of AHR transactivation and the AHRR to be a tissue/cell type-specific repressor. Interestingly, Ahrr-deficient MEFs demonstrated significantly greater constitutive AHR mRNA levels compared to wildtype MEFs (Tigges, et al., 2013). In contrast, Tiparp-deficient MEFs do not display significantly different basal AHR mRNA levels and altered transcriptional expression of the AHR represents a potential difference between the modes of repression of TiPARP and AHRR.

12.5 Potential overlap of TiPARP and the AHRR repressive pathways

A comparison of what is known about the AHR repressive functions of TiPARP and the AHRR reveals similarities but also some notable differences. Similarities between TiPARP- and AHRR-mediated inhibition of AHR transactivation include: (1) both genes are induced by TCDD and regulated by AHR; (2) both interact with AHR; and (3) increased ARNT expression fails to prevent AHRR- or TiPARP-dependent repression of AHR transactivation (Evans, et al., 2008; Karchner, et al., 2009; Lo, et al., 2011; Mimura, et al., 1999). In contrast, AHRR repression of AHR action does not involve increased AHR degradation, direct binding to AHREs, or inhibition of the AHR nuclear translocation (Evans, et al., 2008; Hahn, et al., 2009). These similarities and differences suggest there could be pathway overlap and interplay between TiPARP and the AHRR.

The kinetic profiles of TCDD-regulated expression of TiPARP and AHRR suggest that TiPARP induction could be an immediate mechanism to repress AHR transactivation, while the AHRR is a more long-term mechanism of repression. We observe the expression and recruitment of TiPARP after 45 min of TCDD treatment, whereas the expression and recruitment of the AHRR
occurs after 24 h TCDD treatment. The rapid induction and recruitment of TiPARP can be a primary response to repress AHR and the more gradual induction of AHRR expression and recruitment to AHR target genes may represent a secondary mechanism of repression. TiPARP knockdown and knockout increased TCDD-induced expression of AHRR mRNA and other AHR target genes. Initially this might seem counterintuitive since greater expression of AHRR should decrease AHR target expression rather than enhance it; however, this could indicate that AHRR requires TiPARP for optimal repression. Because in the absence of TiPARP the increase of AHRR expression alone is not sufficient to repress AHR target induction this suggests TiPARP and the AHRR function in tandem or co-operatively. Both TiPARP and AHRR can directly interact with AHR and overexpression of AHR can restore induction of AHR targets when co-overexpressed with either TiPARP or AHRR suggesting that AHR is a common target of both repressors (Karchner, et al., 2009). Our finding that TCDD induction of AHR targets in Tiparp-deficient MEFs with protein synthesis inhibition was approximately 2-fold greater than TCDD treatment alone indicates that TiPARP induction is not solely responsible for the observed repression and that inhibition of AHRR synthesis might also contribute to the increases. Double knockout of TiPARP and AHRR will be very helpful in determining potential co-operatively between TiPARP and the AHRR to repress AHR transactivation.

13 TiPARP is an in vivo negative regulator of AHR transactivation

Hepatic tissue from Tiparp-deficient mice show significantly greater TCDD-induced AHR target gene expression compared with tissue isolated from wildtype mice, supporting that TiPARP is an in vivo negative regulator of AHR transactivation. This AHR target induction profile was not observed in hepatic tissue from 3MC-treated Ahrr-deficient mice (Hosoya, et al., 2008). Increased CYP1A1 induction of Ahrr-deficient mice was only observed in spleen, heart and skin tissues, which indicates that AHRR is a tissue-specific repressor (Hosoya, et al., 2008). Preliminary results indicate that kidney and spleen tissue from 6 h TCDD-treated Tiparp-deficient mice show a similar AHR target induction profile to hepatic tissue suggesting TiPARP to potentially be a more wide-spread repressor than the AHRR (data not shown). The tissue distribution and increased induction CYP1A1 of Tiparp-deficient mice is similar to CA-AHR transgenic mice which display heightened CYP1A1 mRNA in many tissues including liver, kidney and spleen (Andersson, et al., 2002; Brunnberg, et al., 2006; Moennikes, et al., 2004).
However it should be noted that beyond CYP1A1, the gene induction profile of hepatic tissue from CA-AHR mice is very different from hepatic tissue isolated from TCDD-treated wildtype mice and most of the TCDD-responsive genes, including TiPARP, were not detected in CA-AHR mouse livers (Dere, et al., 2006; Kurachi, et al., 2002; Moennikes, et al., 2004; Zeytun, et al., 2002). TCDD-treated Tiparp-deficient mice demonstrate significantly greater induction of many TCDD-responsive genes indicating that uninhibited AHR transactivation by the lack of repression of AHR is different from continuous AHR activation as observed in the CA-AHR mouse model.

13.1 Role of TiPARP in TCDD-mediated toxicity

13.1.1 Purported effects of TiPARP on the NAD$^+$ pool and SIRT activity

Our knowledge of a role of TiPARP in TCDD-mediated toxicity has been limited to studies that use chicken (Gallus gallus) TiPARP to examine the effects of TiPARP on the suppression of gluconeogenesis that is associated with wasting syndrome (Diani-Moore, et al., 2010; Diani-Moore, et al., 2013). Chicken TiPARP was identified as a mediator of the TCDD-induced suppression of gluconeogenesis that consumes hepatic NAD$^+$, lowering SIRT activity, repressing gluconeogenic genes G6Pase and PEPCK, and reducing glucose output (Diani-Moore, et al., 2010). TiPARP induction was also purported to be involved in AHR-mediated sensitivity to NASH by depleting NAD$^+$ levels which lowers SIRT3 activity leading decreased SOD2 activity and less superoxide scavenging (He, et al., 2013). Conclusions from both of these studies were largely based observations that TCDD treatment increases TiPARP gene expression and decreases NAD$^+$ levels suggesting that NAD$^+$ levels control gene expression (Diani-Moore, et al., 2010; He, et al., 2013). We considered whether lower NAD$^+$ levels rather than TiPARP directly could be responsible for the repression of AHR transactivation; however, addition of nicotinamide, a NAD$^+$ precursor also represses induction of AHR targets indicating that NAD$^+$ levels and TiPARP-mediated repression of AHR targets are independent (Diani-Moore, et al., 2010).

TiPARP was found to contribute to TCDD-mediated suppression of gluconeogenesis because TCDD treatment induces TiPARP mRNA and decreases gluconeogenic gene expression and subsequently reduces glucose output (Diani-Moore, et al., 2010). TiPARP overexpression reduces PEPCK mRNA and glucose output, mimicking the effects of TCDD, leading the authors
to conclude that TiPARP mediates TCDD-induced suppression of gluconeogenesis (Diani-Moore, et al., 2010). If TiPARP is a mediator of TCDD toxicity, Tiparp-deficient mice should be more resistant to TCDD-induced gluconeogenesis suppression and wasting; however, preliminary studies suggest that Tiparp-deficient mice are more sensitive to TCDD suggesting TiPARP plays a protective rather than a mediator role in TCDD-mediated toxicity (data not shown). Toxicity studies with Tiparp-deficient mice are currently ongoing.

Chicken TiPARP is reported to be a poly-ADP-ribosylating ARTD that is able to deplete the cellular NAD$^+$ pool (Diani-Moore, et al., 2010; Diani-Moore, et al., 2013). We have demonstrated using catalytic assays that chicken TiPARP is a mono-ADP-ribosyltransferase, which agrees with the structural prediction of its catalytic activity (Otto, et al., 2005). The choice of assay to demonstrate catalytic activity is most likely the reason for this discrepancy. We use purified GST-tagged chicken TiPARP incubated with $^{32}$P-NAD$^+$, the generally accepted method to examine catalytic activity of a specific ARTD whereas Diani-Moore et al. used an indirect method by immunoprecipitating poly(ADP-ribose) from whole cell extracts transfected with TiPARP or treated with TCDD as an indirect means to study TiPARP catalytic activity (Diani-Moore, et al., 2010; Diani-Moore, et al., 2013). The anti-poly(ADP-ribose) antibodies are able to detect six or more ADP-ribose units and cannot detect mono-ADP-ribose accurately, therefore it is unclear why an anti-poly(ADP-ribose) antibody is able to detect catalytic activity directly from TiPARP (Kleine, et al., 2008). It is still unclear how TiPARP, which is a MART, is capable of significantly depleting the NAD$^+$ pool. ARTD1 accounts for 85-90% of cellular ADP-ribose synthesis suggesting that TiPARP is not the primary consumer of cellular NAD$^+$ (Shieh, et al., 1998). The reported depletion of cellular NAD$^+$ could be due to stress caused by transfection of TiPARP which leads to the activation of ARTD1 and increase in poly(ADP-ribose) activity and thus decreases cellular NAD$^+$ levels. Additionally, we observe chicken TiPARP is less catalytically active than mammalian TiPARPs making TiPARP less likely of a candidate in chicken cells to substantially deplete the NAD$^+$ pool.

Another difference between chicken TiPARP and mammalian TiPARPs is when transfected into mammalian cells chicken TiPARP did not repress AHR transactivation or form nuclear foci. The lack of activity could be due to cell context, since we transfected chicken TiPARP into immortalized human cell lines. These findings suggest that TiPARP exhibits species- or model-specific differences. Chicken TiPARP is also less catalytically active than mammalian TiPARPs
illustrating another species difference between mammalian and chicken TiPARP. Since chickens are more sensitive to TCDD than mice (LD$_{50} < 25$ μg/kg bw for chickens versus 182 μg/kg bw for B6 strain mice), it is possible that the lower TiPARP activity could be a potential contributing factor to the higher TCDD sensitivity reported in chickens (Chapman and Schiller, 1985; Greig et al., 1973).

14 TiPARP-mediated regulation of other transcription factors

The ability of TiPARP to regulate transcription is most likely not limited to AHR. ARTD1 and ARTD2 regulate many transcriptional factors acting as co-activators and co-repressors in different pathways (section 2.5.4) (Krishnakumar and Kraus, 2010b; Szanto et al., 2012). Despite its name, the AHRR has also shown activity toward transcription factors other than AHR including hypoxia inducible factor 1 alpha (HIF-1α) (Karchner et al., 2009). TiPARP is regulated by other transcription factors including estrogen receptor alpha (ERα) and glucocorticoid receptor supporting the possibility that TiPARP could be involved in these pathways (Kininis et al., 2007; Reddy et al., 2009). Ongoing studies in our laboratory reveal that TiPARP interacts with ERα and negatively regulates ERα-mediated transactivation, whereas TiPARP co-activates liver X receptor (LXR)-mediated transactivation (data not shown). All together our results indicate that TiPARP is a multifunctional transcription factor co-regulator.

15 Limitations and future considerations

15.1 TiPARP antibody

Our studies of TiPARP function were highly dependent on transient transfection to overexpress or knockdown TiPARP. We were unable to verify TiPARP protein overexpression, knockdown of endogenous TiPARP or TCDD-induced endogenous TiPARP due to the lack of suitable commercially available antibody able to detect TiPARP. To verify knockdown we resorted to overexpressing GFP-tagged TiPARP with siTiPARP sequences and using an anti-GFP antibody to detect GFP-TiPARP protein levels. Similarly, we overexpressed GFP-TiPARP to perform ChIP assays and test GFP-TiPARP protein stability. Plasmid-derived overexpressed protein is not identical to endogenous protein, which is why a high quality anti-TiPARP antibody is critical.
for studying the endogenous TiPARP protein behaviour. An anti-TiPARP antibody would also be extremely useful to detect TCDD-induced TiPARP expression, recruitment to AHR target regulatory regions and co-immunoprecipitation with endogenous AHR. Due to the lack of a suitable antibody we were also unable to confirm complete knockout in the MEFs isolated from Tiparp-deficient mice at the protein level, leaving open the possibility Tiparp-deficient mice to express a truncated version of TiPARP. To date the only evidence we have of endogenous TiPARP protein are confocal images of putative TiPARP staining in the nuclei of HuH7 cells by a commercially available anti-TiPARP antibody that in our hands only works for immunofluorescence. The specificity of the antibody was tested with overexpressing GFP-TiPARP and staining for TiPARP (data not shown). We are currently purifying TiPARP protein for generation of our own antibody. Once generated, we intend to use the antibody to detect and confirm TiPARP protein expression in all our models. We also plan to use the antibody to perform ChIP-sequencing to determine TiPARP recruitment and potential overlapping recruitment with AHR and ARNT. A proper anti-TiPARP antibody will certainly be an important tool for our laboratory.

15.2 ARTD catalytic activity assays

The in vitro ADP-ribosylation assays, which involve incubation of purified ARTD protein and $^{32}$P-NAD$^+$ are relatively simple and is a well-accepted method to demonstrate ARTD activity. Although catalytic activity assays provide insights into auto- and hetero-ADP-ribosylation of ARTDs, they do not identify specific sites/residues of ADP-ribosylation. Furthermore, although ARTD catalytic assays do illustrate extensive poly-ADP-ribosylation with the reduced mobility of modified protein by SDS-PAGE they do not provide information about polymer length. Because the molecular weight of a single ADP-ribose moiety is quite small (540 Da) mono-ADP-ribosylated and short oligo-ADP-ribosylated proteins are visually indistinguishable (Laing, et al., 2011). Sequencing gels of modified proteins can be used to illustrate the lengths of ADP-ribose chains. We used catalytic activity assays to demonstrate TiPARP has auto- and hetero-ADP-ribosylation activities and TiPARP is most likely a mono-ADP-ribosyltransferase. However, we did not quantify the amount of ADP-ribose units TiPARP is able to synthesize leaving open the possibility that TiPARP can synthesis short oligo-ADP-ribose polymers that display no visual slowing of migration by SDS-PAGE. Because catalytic activity assays are non-quantitative, small differences in the level of activity may not be visually observed. For instance,
the TiPARP-I631A and –I631E mutants are catalytically active; however, it is not clear if the level of catalytic activity of either mutant differs from that of wildtype. Our GST-purified TiPARP and mutant protein preparations contain breakdown products making quantification of full-length enzyme difficult. To compare the activities of wildtype TiPARP and these isoleucine mutants requires highly pure protein to ensure equal quantities of wildtype and mutants are used for each assay. We are currently optimizing our protein purification protocol for TiPARP to reduce breakdown products and increase purity.

Our catalytic assays with TiPARP also did not identify specific sites or residues on TiPARP that were auto-ADP-ribosylated or sites of core histones that were hetero-ADP-ribosylated by TiPARP. Determining the sites or residues of modification will demonstrate whether TiPARP has a preference for a particular amino acid. We are currently using mass spectrometry to determine sites of auto-ADP-ribosylation on TiPARP, by using mass spectrometry we are able to identify specific residues of TiPARP that are auto-ADP-ribosylated and confirm that these sites are in fact mono-ADP-ribosylated. Once we purify large enough quantities of AHR protein we will determine whether it is hetero-ADP-ribosylated by TiPARP and use mass spectrometry to identify specific sites of AHR that are mono-ADP-ribosylated.

In vitro catalytic assays may be artificial and may not accurately reflect modification in a cellular context or in vivo. Direct evidence of intracellular protein mono-ADP-ribosylation is quite limited due to the lack of antibody to detect mono-ADP-ribosylated proteins (Feijs, et al., 2013). The challenge of providing direct evidence for intracellular mono-ADP-ribosylation is compounded by the lack of specific mono-ARTD inhibitors (Feijs, et al., 2013). Although TiPARP displays auto- and hetero-ADP-ribosylation in vitro our understanding of whether this occurs in cell or in vivo is limited.

16 Overall significance of research

The objective of this research was to understand TiPARP and its function in AHR transactivation. We provided evidence that TiPARP is a nuclear MART that mono-ADP-ribosylates itself and core histones. We identified TiPARP as a novel AHR ligand-induced negative regulator of AHR transactivation in vitro and in vivo. We also identified its NLS sequence providing further evidence for its nuclear localization. TiPARP specifically targets AHR and promotes its proteolytic degradation and represents a candidate for the elusive ADPF.
The catalytic and zinc finger functions are essential for TiPARP to repress AHR transactivation. Comparison with the AHRR reveals some notable similarities and differences suggesting TiPARP and the AHRR use partially overlapping but also distinct mechanisms to repress AHR activity. Overall we have established a new mechanism of negative feedback regulation of AHR transactivation and identified a nuclear mono-ADP-ribosyltransferase.


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