IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF THE ARYL HYDROCARBON RECEPTOR PROTEIN INTERACTION NETWORK

By

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A Dissertation

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Biochemistry and Molecular Biology-Environmental Toxicology

2010

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Poly aromatic hydrocarbon (PAH) compounds are pervasive environmental contaminates. PAHs are by-products of industrial processes such as, paper bleaching, pesticide production, fossil fuel combustion, and waste incineration. These compounds are highly stable and lipophilic, leading to their bio-accumulation in soil and the food chain. Exposure to PAHs elicits toxic responses in mammals. Some of these responses include chloracne, liver hyperplasia, and immune system suppression. Moreover, increased risk of diabetes, cancer, infertility, and birth defects correlate with PAH exposure. There are several families of compounds classified as PAHs. Two of the more infamous PAH groups are polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins (PCDDs). 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) is the hallmark dioxin congener and one of the most toxic PAHs known. Toxic responses to PAHs are mediated through the aryl hydrocarbon receptor (AHR). The AHR is a ligand activated member of the PER-ARNT-SIM (PAS) superfamily of transcription factors. PAS protein family members act as environmental sensors. PAHs bind the AHR, instigating its translocation to the nucleus, heterodimerization, and activation of its ability to regulate the transcription of genes. Though AHR gene regulation is well characterized, a complete understanding of the receptor mediated mechanisms underlying dioxin toxicity is lacking. This project focused on AHR protein interactions and potential roles these interactions have in toxic responses to TCDD exposures. The first aim was to establish the AHR protein interaction network (AHR-

PIN). Using tandem affinity purification (TAP) and mass spectrometry we have established AHR-PINs in the presence and absence of TCDD. Changes in the PIN were observed between the dosed and vehicle treated samples. In addition, flux in the network dependent on length of exposure was observed. There were a number of protein hits identified in the PINs that are of interest. These proteins include Smarcad (a DNA helicase), Cfcl and Alcam (immune response proteins), and Arf-GAP. Finally, an interaction between the AHR and ATP5α1, a subunit of the ATP synthase complex which controls cellular energy homeostasis, is of particular interest. This interaction is the first to link the AHR to mitochondrial energy production. The second aim of this project was to establish functional relevance associated with the identified protein interactions. To that end, further investigation revealed a TCDD induced AHR dependent hyperpolarization of the mitochondrial inner membrane. This novel function may provide insight into the underlying mechanisms of TCDD induced toxicity. Mitochondrial dysregulation could play a key role in wasting syndrome and metabolic diseases. The findings establish the dynamic nature of the AHR-PIN and identify a potentially novel function of the AHR in cellular energy homeostasis.

DEDICATION

For my first father Roy J. Bauer. He has not been here to see the accomplishments of my life, but he never doubted me or my abilities. He ensured I learned all the lessons needed to traverse the wilds of life.

Thanks for everything, Dad.

ACKNOWLEDGEMENTS

First, I give my heartfelt thanks and gratitude to my boss, Dr. John LaPres, who is not only an outstanding teacher, but a man of infinite patience. I regret having tried that patience as much as I did. I have learned more from him than I can itemize here, but I value each and every lesson he gave. He provided me the room I needed to experiment and grow as a scientist. At the same time he was always accessible and receptive to me. He's encouraged and facilitated my participation in the greater scientific community, provided valuable counsel, and always treated me as an equal. He understood the extenuating circumstances of my life and allowed me enough freedom to meet all my commitments. Finally, he refused to allow me to give up when that was the thing I wanted to do.

I would like to extend special thanks to Dr. Bill Henry. He provided valuable scientific insights, moments of great humor, and, most importantly, believed in and supported me on numerous occasions. I have learned much from you and am more grateful than I can say for everything. Without John & you I would not be here.

Many thanks to the other members of my graduate committee, Dr. Gavin Reed, Dr. Kathy Gallo, and Dr. Norbert Kaminski for their support. They have provided valuable insights into my research and future directions. Moreover, they have always been willing to offer council on scientific matters and career directions during the course of my graduate work.

Next, thanks to Dr. John Wang and Dr. Tom Sharkey for support at a crucial time, Dr. Kaillathe (Pappan) Padmanabhan for always solving every computer problem with a smile and never making me feel like the pain I knew I was being, Dr. James

Geiger for hiring me as an undergrad, overseeing my initial research experience, and being a great Boss, Teresa Vollmer, Jessica Lawrence, Katie Gallagher, and Amy Swagart for taking care of every administration detail, Ron Norris for keeping the film developer going long after its life span, and finally to Laura Thurlow and Larissa Nemoianu for starting me down the road that has brought me here.

LaPres lab members, Dr. KangAe Lee, Yogesh Saini, Dr. Ajith Vengellur, Krista Greenwood, HyeJin Hwang, and Steve Proper you have been great lab mates. Thank you for all your input and insights during the last five years.

To my “science” friends Adam Cornish, Kate Higginbotham, Stacy Hovde, Gauri Jawdekar, Ania Kopec, Marcin Nowicki, Sandhya Payankaulam, Joyce Robinson, Dean Shooltz, Priya Telang, and Annette Thelen, you have all provided so many things to me. You’ve been sounding boards for ideas, technical support crew members, invaluable resources of materials and methods, emotional anchors, and supremely forgiving of my temperament and shortcomings. I am most fortunate, being able to count you all close and dear friends. To my “non-science” friends Deb Bradley, Danielle England, Margaret Lee, Ethel Walton, and Jim Williams, thank you for the years of friendship and laughter we’ve shared. You helped me keep my life balanced and steady. Even when this was a crazy dream, you did not treat it as such. You all have been instrumental in my achieving this goal.

My parents Roger and Betsy Alvarado, Kate and David Baca, Roy and Dolly Bauer, and Tom and Linda Tappenden, and grandmothers Barbara Alvarado and Ann Tappenden Vercellino, all helped shape the person I am. You have supported and

encouraged me, unconditionally, through this entire process. I would not be here or anywhere, for that matter, without you. Thank you for everything.

My sister Katherine has been with me every step of the way. Though we did not grow up together, in the conventional sense, we have shared every aspect of our lives from children's births to loved ones' deaths. I would be less complete without having you in my life. Thank you for being my sister.

Finally, to my children, Frostie, Kelsey, Audrey, and Gillian Tappenden, I owe the greatest thanks. My pursuit of this degree has impacted your lives tremendously and not always positively. My sacrifices were yours as well; but I chose to make them. You did not have that luxury you were just stuck with them. There was a huge amount of time spent studying that was not spent with you guys. There were missed school functions, soccer games and concerts. There was never enough money for Christmas, birthdays or extra activities. And, there were too many things that took momentary importance over you. I do not know if the example I've set is a fair trade off for all the draw backs. You will be the ones to decide that. But, the four of you have always had my back. You have made me a better person than I ever could have been alone. You will always be the very best things in my life. Thank you.

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ABBREVIATIONS

12(R)-HETE	12(R)-hydroxy-5(Z),8(Z),10(E),14(Z)-eicosatetraenoic acid
ACTB	β-actin
AHH	aryl-hydrocarbon hydroxylase
AHR	aryl hydrocarbon receptor
AHR-PIN	aryl hydrocarbon receptor protein interaction network
AIP	aryl-hydrocarbon receptor interacting protein
Alcam	activated leukocyte cell adhesion molecule CD166
AR	androgen receptor
ARA9	aryl-hydrocarbon receptor interacting protein 9
ARNT	aryl-hydrocarbon nuclear translocating protein
Asap2	ArfGAP with SH3 domain, ankyrin repeat and PH domain 2
ATP5α1	ATP synthase F1 complex, alpha subunit
Bag3	Bcl2-associated athanogene 3
Bcl6	B-cell lymphoma 6 protein
Bhlh	basic helix-loop-helix
CaCl2	calcium chloride
CBB	calmodulin binding buffer
Cbr3	carbonyl reductase 3
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CCND1	cyclin D1
Cd36	CD36 antigen

CDK4	cyclin dependent kinase 4
ChIP-on-chip	microarray-based chromatin immunoprecipitations
CID	collision induced dissociation
Clcf1	cardiotrophin-like cytokine factor 1
Co-IP	co-immunoprecipitation
COX IV	cytochrome c oxidase IV
Creb3l3	cAMP responsive element binding protein 3-like 3
cyto	cytosolic
DBD	DNA binding domain
Ddef1	development and differentiation enhancing factor 1
DMSO	dimethyl sulfoxide
DOK1	downstream of tyrosine kinase 1
DRE	dioxin response elements
DTT	Dithiothreitol
DV	ductus venosus
EDTA	Ethylenediaminetetraacetic acid
Eef1a1	eukaryotic translation elongation factor 1 alpha 1
EPA	Environmental Protection Agency
ER	estrogen receptor
Fabp4	fatty acid binding protein 4
Fabp5	fatty acid binding protein 5
FCM	flow cytometry
Gas1	growth arrest specific

GFP	green fluorescent protein
Gsta2	glutathione -S-transferase A2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hes6	hairy and enhancer of split 6
HGNB	high glucose normal buffer
Hpcal1	hippocalcin-like 1
hrs	hours
Htatsf1	HIV TAT specific factor 1
Ig	immunoglobulin
Il18	interferon gamma inducing factor
ITE	2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester
JNK	Jun NH2-terminal kinase
KCl	potassium chloride
kDa	kilo dalton
LD50	lethal dose
LDH	lactate dehydrogenase
Lp1	lipoprotein lipase
MEF	mouse embryonic fibroblasts
MF1	crude mitochondrial fraction
MF2	pure mitochondrial fraction
MFI	mean fluorescence intensity
MgCl2	magnesium chloride
MgOAc	magnesium acetate

mins	minutes
µM	micromolar
mM	millimolar
MMP	matrix metalloproteinases
Mrpl40	mitochondrial ribosomal protein L40
MS	mass spectrometry
MTG	MitoTracker Green
N	normal
Na3VO4	sodium orthovanadate
NaCl	sodium chloride
NaF	sodium fluoride
NaOH	sodium hydroxide
NLS	nuclear localization signal
nM	nanomolar
NP40	nonyl phenoxypolyethoxylethanol
NQO1	NAD(P)H: quinone oxidoreductase 1
Nrf2	nuclear factor erythroid 2- related factor
PAH	poly aromatic halogenated
P-ampho	phoenix-ampho
PAS	PER-ARNT-SIM
Pax5	Paired box protein
PBB	polybrominated biphenyls
PBS	phosphate buffered saline

PCB	polychlorinated biphenyls
PCDD	polychlorinated dibenzo-p-dioxins
PCDF	polychlorinated dibenzofurans
P-eco	phoenix-eco
pRb	retinoblastoma tumor suppressor protein
Prdm1	PR domain zinc finger protein 1
qRT-PCR	quantitative real-time PCR
RET	tyrosine kinase receptor
SDS	sodium dodecyl sulfate
sec	seconds
Slc27a2	solute carrier family 27, member 2
Smarcad1	enhancer-trap-locus-1
Socs2	suppressor of cytokine signaling 2
Stat1	Signal Transducers and Activators of Transcription 1
Stat3	Signal Transducers and Activators of Transcription 3
TAD	transactivation domain
TAP	tandem affinity purification
TCDD	2,3,7,8 tetrachloro-dibenzo-p-dioxin
TEV	Tobacco Etch Virus
Tfrc	transferrin receptor
Th17	IL17 producing T helper cell
TMRM	tetramethylrhodamine methyl ester
Tnfaip2	tumor necrosis factor, alpha induced protein 2

TOM20	translocase of the outer membrane of mitochondria 20
Tregs	Fox3 regulatory T cell
TPR	tetratricopeptide repeat
TTLB	TAP-tag lysis buffer
Tuba1b	alpha-tubulin isotype M-alpha-2
Tubb5	tubulin
Ubap2l	ubiquitin associated protein 2-like
UGT	UDP-glucuronosyltransferase
VCFS	Velo–cardio–facial syndrome
WCL	whole cell lysate
WHO	World Health Organization
WT	wild type
XAP2	X-associated protein 2

INTRODUCTION

1.1 Background

A poly aromatic halogenated (PAH) xenobiotic compound is a general classification for a broad range of environmental contaminates. PAHs include benzo-pyrenes, polychlorinated dibenzofurans (PCDFs), polybrominated biphenyls (PBBs), and polychlorinated biphenyls (PCBs) (Fig 1.1A-D); all of which elicit toxic responses in mammals [1, 2]. Polychlorinated dibenzo-ρ-dioxins (PCDDs) (Fig1.1E) are a family of more than 100 different chemicals each classified as PAHs and commonly referred to as 'dioxins' [3]. PAHs are primarily produced as by-products of industrial processes such as paper bleaching, waste incineration, and pesticide production over the past two centuries [2, 4]. The aromatic carbon backbone structure of these compounds makes them highly stable. In addition, the hydrophobic nature of PAHs readily allows diffusion across cell membranes [5]. Once in the intracellular space, PAHs can exert a host of responses. For example, some PAHs, including PCDD, induce the expression of xenobiotic enzymes that lead to the clearance of these compounds [2].

Today, the Environmental Protection Agency (EPA) has classified dioxin and dioxin-like compounds as human carcinogens. The World Health Organization (WHO) considers them a highly toxic family of compounds that elicit adverse effects on the immune system, reproduction, development, and hormone signaling [6, 7]. Though dioxins were originally identified as manmade compounds recent research has also shown the natural production of dioxins in forest fires [8]. The dioxin family of compounds are highly stable and pervasive [9]. 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) (Fig 1.1F) is one of the most potent compounds in the dioxin family and the

canonical dioxin used in research [10] (Fig1.1F). In early studies, compounds in this family were shown to produce chloracne in workers exposed to them, as well as to induce aryl-hydrocarbon hydroxylase (AHH) enzymatic activatity in laboratory rodent livers [5, 11]. During the interceding years of investigation, these environmental pollutants have been shown to produce a wide variety of toxic responses in mammals. These effects are both tissue and species specific [12, 13].

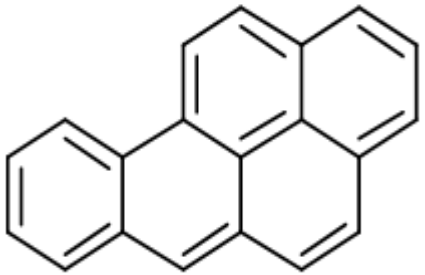
A battery of toxic physiological endpoints has been reported in dioxin exposures. These include immune system suppression, thymic involution, wasting syndrome, chloracne, tissue hyperplasia and hypertrophy, and endocrine disruption [2, 14-16]. Moreover, increased risk of heart disease, diabetes, reproductive and birth defects, as well as tumor promotion, cell cycle deregulation influencing proliferation and differentiation have been linked to dioxin exposure [2, 14-16]. It is noteworthy that dioxin induced toxicity differs between species. For example, chloracne is a hallmark of the toxic response following dioxin exposure and provides a clear example of variation in species response. In the 1950s, Kimmig and Schultz reported chloracne in humans exposed to PAHs in the workplace, and similar epidermal responses have been observed in rabbits and monkeys [5]. Laboratory mice, however, do not develop chloracne following dioxin exposure [17]. These types of species-specific toxic responses in mammals are only part of the complex nature of dioxin toxicity.

Sensitivity to these compounds varies widely among mammals as well. There is a 5000-fold difference in acute exposure LD50 across tested species, with the guinea

Figure 1.1 Poly aromatic Halogenated (PAH) Compounds

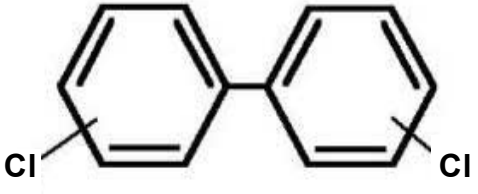
Generic structures of various classes of PAHs. These figures include dioxins (**A**), benzopyrene (**B**), polychlorinated biphenyls (**C**), polybrominated biphenyls (**D**), polychlorinated benzofuran (**E**), and the canonical dioxin compound, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, **F**)

A



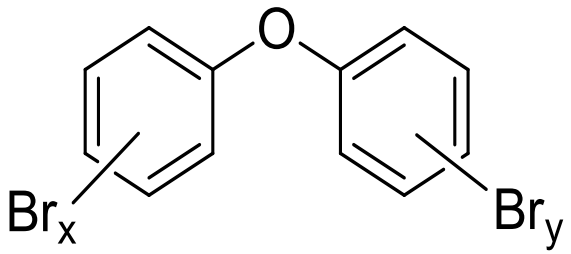
Benzo pyrene

B



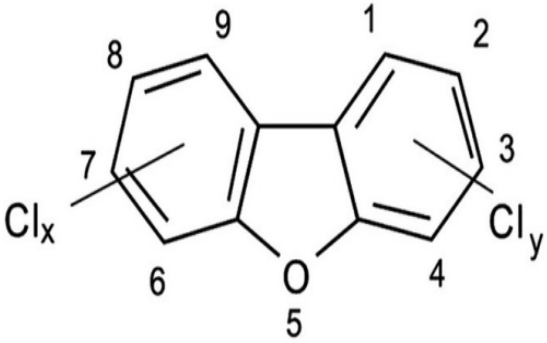
Polychlorinated biphenyl

C



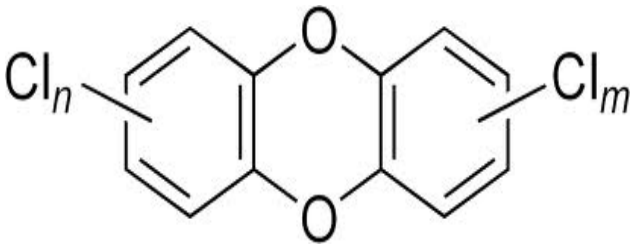
Polybrominated biphenyl

D



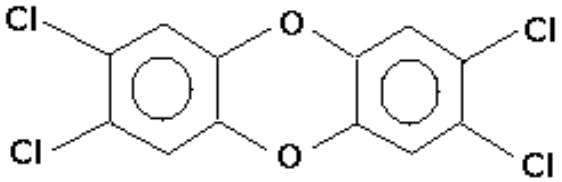
Polychlorinated dibenzofuran

E



Dioxin

F



2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD)

pig being the most sensitive to TCDD (LD50 = 1 μg/kg) and the hamster being the most resident species tested (LD50 = 5000 μg/kg) [2]. Most TCDD induced toxicity experimentation has been performed in species that are relatively sensitive, namely mice (LD50 = 114 μg/kg) and rat (LD50 = 22 μg/kg, males and 45μg/kg, females) strains [5, 18-20]. Assessing the parameters of harmful dioxin exposure to humans, however, has remained problematic. Several studies have attempted to extrapolate a human LD50 based on data from other species. Deriving an accurate measurement is complex given the variables of age, gender, weight, and total fat body burden in a human population [21, 22]. To further confound matters the mixture of dioxin compounds in a given exposure, as well as the duration of exposure are factors for consideration [21, 22]. Acute human exposures to dioxin compounds have not resulted in any documented human LD50 [23-25].

Dioxin exposure in humans has been examined in numerous cohort studies with varying results. One population of interest has been Vietnam veterans, exposed to dioxin via Agent Orange pesticide handling during Operation Ranch Hand. This group was followed for 25 years post exposure. A 2008 report indicates increased risk of diabetes and cancer in the Agent Orange exposed veteran group over the control group after calendar days, spraying time, and time spent in the region are factored into the analysis [26]. This data contradicts two earlier reports showing no link between dioxin exposure and prostate cancer risk [27, 28]. Roberts *et al* reported that paternal exposure to Agent Orange increased the occurrence of spina bifida in offspring. However, their analysis of cohort data did not demonstrate an increased risk for disease in the actual exposure group [29]. A study of trichlorophenol plant workers in New

Zealand did not indicate an increased morbidity rate in the exposure group versus the control group [30]. Reports on the long term effects of the Seveso, Italy dioxin contamination site indicated an increased incident of infertility and circulatory disease associated deaths in the first years after exposure [16, 31]. An increased incidence of diabetes was reported in females of the Seveso group as well as in females from the long term study of the Yucheng China exposure group [31, 32]. An increase risk for toxicity, carcinogenicity, and cardiovascular disease, and mortality rates after dioxin exposure has been interpreted from cohort study data [15, 33]. Finally, cohort studies of PBB and PCB food contamination in Michigan demonstrated differences in health effects among PAHs. These studies found exposure to PBBs or PCBs did not affect the on-set time of menopause, lactation or breast feeding habits [34, 35]. Endometriosis and diabetes occurrences did not increase in the women exposed to PBBs. However, PCB exposure did increase diabetes and endometriosis in women [36, 37]. These studies indicated chlorinated PAH contaminates present greater health risks than brominated compounds. Taken together, these cohort studies link dioxin and other PAHs to increase risk of cancer, metabolic disorders, reproductive and developmental defects, and chronic circulatory and respiratory disease (Table 1.1). The adverse consequences of many of the PAHs are attributed to a single genetic locus, the aryl hydrocarbon receptor.

1.2 Aryl Hydrocarbon Receptor: Identification, Structure, and Function

The aryl hydrocarbon receptor (AHR) has been shown to mediate virtually all adverse biological responses to dioxins [38-41]. An early observation detected a difference in TCDD induced toxicity among inbred laboratory mouse strains. Certain

Table 1.1 Summary of Cohort Studies

Five cohort studies demonstrate a correlation between dioxin exposure and disease states. Table details the location and nature of the accidental exposure, as well as, major adverse health effects.

Cohort Studies

Location	Exposure	Findings
Seveso Italy	Chemical plant explosion: Air, soil, food contamination *Dioxins*	Increased incidents of cancer, diabetes, chronic circulatory and respiratory disease, infertility
Vietnam: Operation Ranch Hand	Pesticide handling *Dioxins*	Increased incidents of diabetes*, cancer*, and spina bifida in off spring
New Zealand	Occupational exposure: chemical plant employees *Dioxins*	No significant increase in morbidity or cancer
Yucheng, China	Food contamination *Dioxins*	Increased incidence of diabetes in females
Michigan, USA	Food contamination *PBBs and PCBs*	Increased incidence of diabetes and endometriosis in females**

* Most recent findings with calendar days , spraying time, and duration in region factored into results
** Findings correlate to PCB exposure.

Table 1.1 Summary of Cohort Studies

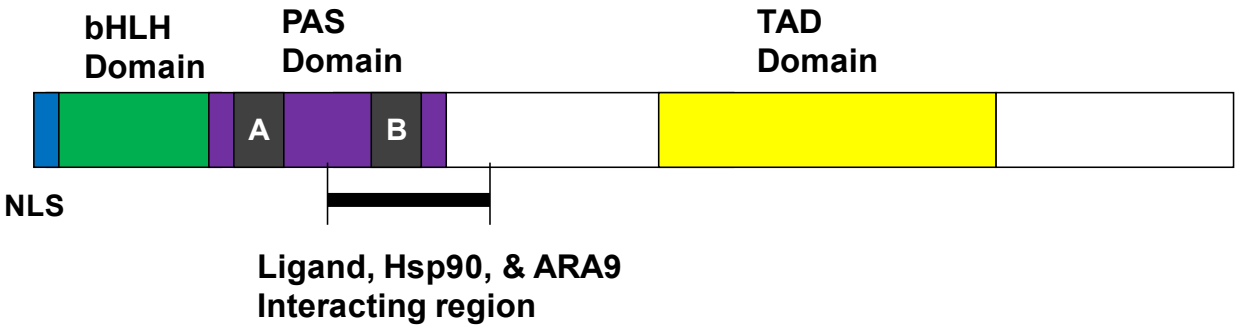
laboratory mice demonstrated a higher level of AHH activity than others, upon exposure to dioxins [42]. Further investigation led to the identification and characterization ofgenetic polymorphisms in the *ahr* locus, which codes for the AHR, and resulted in the differing levels of AHH induction. It was reported that mice carrying the *ahr*b allele have a receptor with high affinity ligand binding properties thus resulting in higher AHH activation levels and subsequent higher sensitivity to TCDD exposure. While mice carrying the *ahr*d allele were shown to have a receptor with low ligand binding affinity and thus a decrease in TCDD sensitivity [43]. The receptor itself was first detected with radiolabeled TCDD and confirmed the existence of high affinity binding in specific tissue types [38].

The AHR is characterized as a ligand activated transcription factor and a member of the PER-ARNT-SIM (PAS) superfamily of environmental sensors. This family of transcriptional regulators are widely conserved and found in prokaryotes, plants, and animals [44, 45]. They are characterized by the common PAS domain, a protein region containing A and B box repeats. Most, but not all PAS family members also contain a basic helix-loop-helix (bHLH) DNA binding domain [44, 45]. This transcription factor superfamily has been shown to play a role in development and to alter gene expression in response to environmental signals such as hypoxia, xenobiotic exposure, and circadian rhythms [46, 47]. Recently, the wide range of PAS family members has created a movement to establish clearer nomenclature for members of this superfamliy [48]. It was the AHR's ability to regulate xenobiotic metabolizing enzymes that lead to it being one of the earliest characterized PAS proteins [45].

Figure 1.2 Structure of the AHR Protein

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

The AHR consists of several domains, at the N terminus is the nuclear localization signal (**blue**) followed by the basic helix-loop-helix domain (**bHLH, green**) involved in DNA binding. The Per-ARNT-Sim (**PAS**) domain (**purple**) is central and contains A and B box motifs. This region is responsible for receptor heterodimerization with ARNT. The binding of Hsp90, ARA9, and ligand spans the C terminus of the PAS domain and the adjacent region of the protein. The transactivation domain (**TAD, yellow**) is in the C terminus of the receptor.

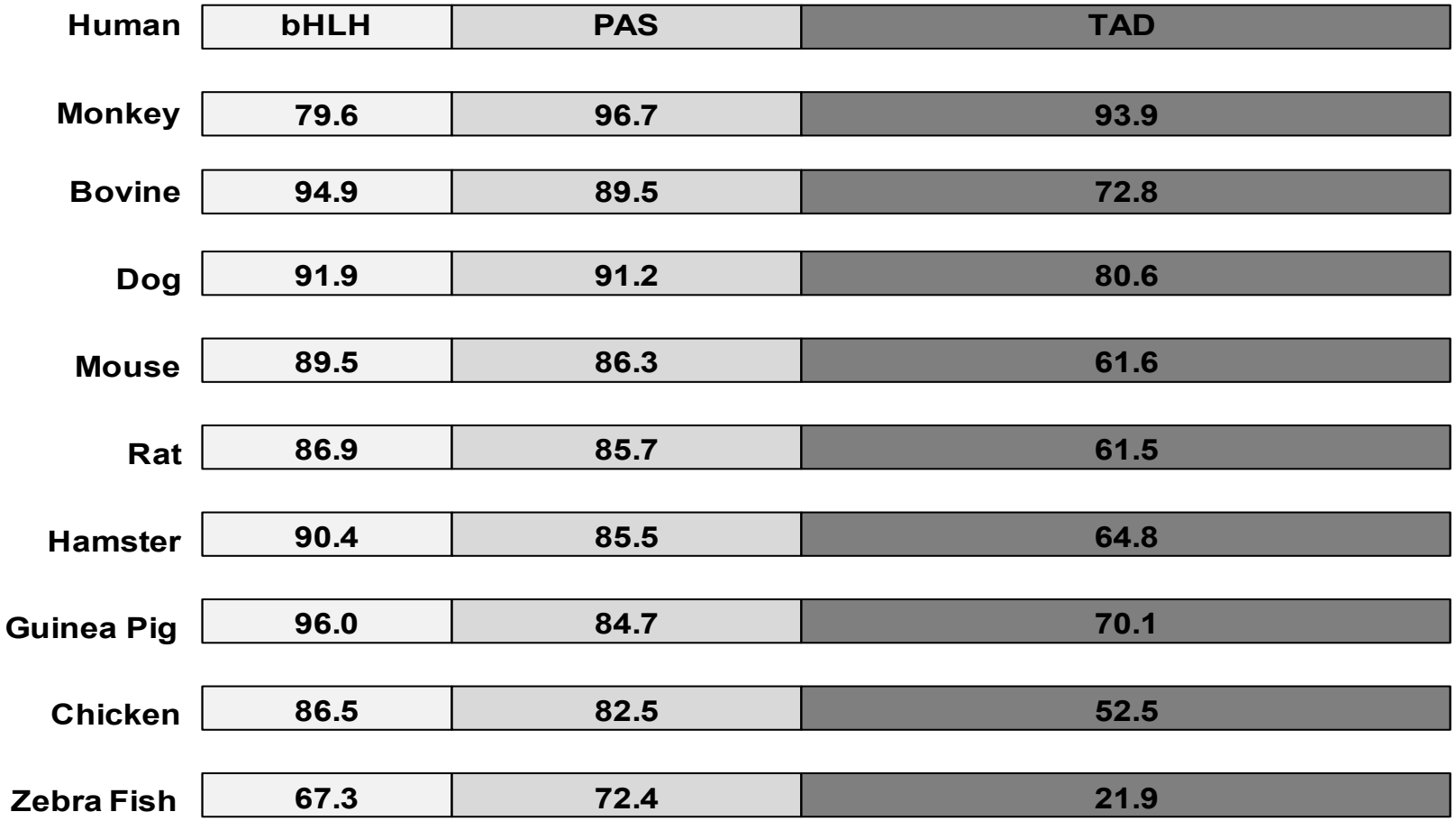

bHLH
Domain
PAS
Domain
TAD
Domain
A
B
NLS
Ligand, Hsp90, & ARA9
Interacting region

The AHR is comprised of several protein domains. At the N terminus is a nuclear localization signal (NLS) followed by a classical DNA binding bHLH domain. This domain is thought to act as a primary dimerization surface. In the central part of theprotein is the PAS domain, which contain A and B box repeats [45]. Finally, in the C terminus of the protein is a transactivation domain (TAD) responsible for the recruitment of transcriptional machinery [49] (Fig 1.2).

Investigation of the murine receptor using genetic back crossing and photoaffinity labeling resulted in the characterization of three different high affinity binding *ahr*b alleles. Each high affinity loci produce receptors of various molecular weights [50, 51]. The three different *ahr*b alleles are: *ahr*b-1, which is found in C57bl mice and codes a 95 kDa AHR, the *ahr*b-2 allele was found in C3H/He and BALB/c laboratory mice strains and code a 104 kDa receptor, and the *ahr*b-3 allele has been identified in non-laboratory mice such as *Mus caroli, spretus and molossinus* and codes a 105 kDa receptor [52, 53]. Only one low affinity *ahr*d allele has been reported, the receptor produced from this allelic variant is 104 kDa [53, 54]. Continued research identified the AHR, at various molecular weights in different mammalian systems. Some examples of this are, the hamster (124 kDa), monkey (113 kDa), human (106 kDa), rat (106 kDa), guinea pig (103 kDa), chicken (95 kDa), dog (95 kDa), cow (94 kDa), and zebra fish (89 kDa). The AHR size differs by 40 kDa amongst these species [13]. This difference in receptor size has been attributed to wide variation in the C terminus of the protein. It is this characteristic which has a central role in the receptor's ability to induce a toxic response to dioxin exposure [44, 55-57]. In fact the bHLH and PAS domains are highly

Figure 1.3 Comparison of homology among domains of the AHR from various species

The AHR from various species was compared to the human receptor. The basic helix loop helix (**bHLH**), PER-ARNT-SIM (**PAS**) and transactivation domain (**TAD**) regions of the AHR were used for the comparison. The percent similarity to the human receptor is reported for each region.


Human
bHLH
PAS
TAD
Monkey
79.6
96.7
93.9
Bovine
94.9
89.5
72.8
Dog
91.9
91.2
80.6
Mouse
89.5
86.3
61.6
Rat
86.9
85.7
61.5
Hamster
90.4
85.5
64.8
Guinea Pig
96.0
84.7
70.1
Chicken
86.5
82.5
52.5
Zebra Fish
67.3
72.4
21.9

conserved between the species mentioned above. In the TAD domains larger sequence variations are observed (Figure 1.3)

In the absence of ligand the AHR is part of a cytosolic complex. The primary members of this complex are the receptor, a dimer of a 90 kDa chaperone heat shock protein (Hsp90) and the aryl-hydrocarbon receptor interacting protein 9 (ARA9). The ARA9 is also known as the aryl-hydrocarbon receptor interacting protein (AIP) and X-associated protein 2 (XAP2). The Hsp90 dimer and ARA9 are considered the core scaffolding proteins of the cytosolic complex. They function to ensure the receptor's proper protein folding and maintain it in a high affinity ligand binding conformational state [58-61]. The co-chaperone protein, p23, has also been shown to be a transient member of this cytosolic complex, but is not required for ligand binding or activation of the receptor [62, 63]. Upon ligand activation, the AHR disassociates from its cytosolic complex and translocates to the nucleus. Once in the nucleus, the AHR heterodimerizes with its partner, the aryl-hydrocarbon nuclear translocating protein (ARNT), becoming an active transcription factor [44, 64]. The AHR/ARNT dimer localizes to genomic DNA at specific sequences known as dioxin response elements (DREs) and modulates gene expression [65] (Fig 1.4). In order to regulate its transcriptional activity, the ligand bound receptor is also ubiquitinated, marking it for proteosomal degradation.

The AHR is also classified as an orphan receptor [66, 67]. Though an endogenous ligand for the receptor has been difficult to identify, the AHR does have endogenous functions. For example, the receptor has been reported to have an

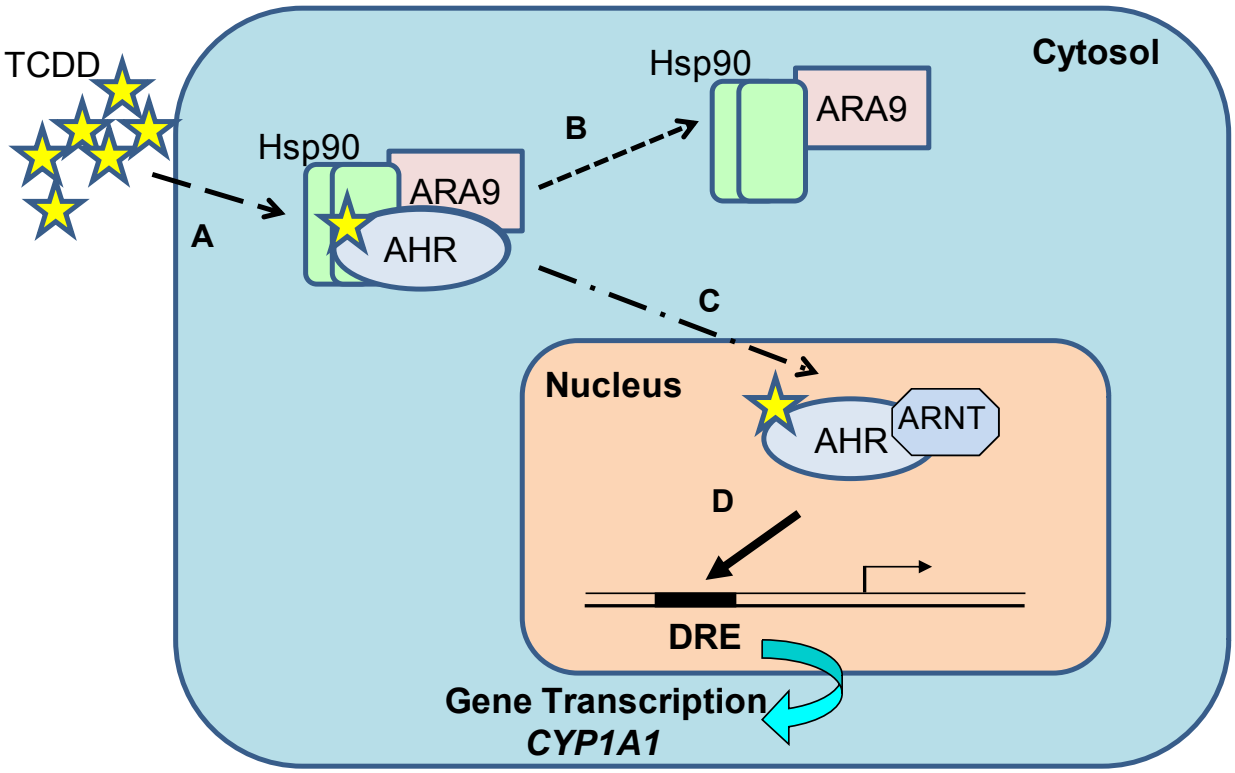
endogenous role in liver and vascular development [41]. Recent finding demonstrated the AHR influences the immune system through cell differentiation [68]. Nguyen and Chowdhury have characterized two D-tryptophan metabolites which may serve as endogenous AHR ligands [69, 70]. However, the physiological relevance of these potential AHR biological ligands is not yet understood.

In fact numerous potential AHR ligands have been reported over the years. There are three different classes of AHR endobiotics; indole derivatives, eicosanoids, and heme metabolites [71] with numerous candidates in each group. For example, Quintana et al demonstrate 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), an indole derivative, activated the AHR in Treg and dendritic cells in mice [72]. Heme metabolites bilirubin and lipoxinA4 are AHR ligand candidates reported to mediated immune responses in mice [73]. Moreover, indirubin-3-monoxime, another indole derivative, influenced gene expression in human monocyte model cell line U937 [74]. Another candidate, indirubin-3-monoxime, may act as an AHR ligand or indirectly regulate the AHR [74]. Finally, an arachidonic acid metabolite, 12(R)-hydroxy-5(Z),8(Z),10(E),14(Z)-eicosatetraenoic acid (12(R)-HETE), does not bind the AHR, but activates the receptor indirectly [75]. It is hypothesized that an unidentified metabolite of 12(R)-HETE is the AHR ligand [75]. The diversity of this group reveals the complex nature of endogenous AHR ligands rivals that of the receptor's exogenous ligands.

AHR gene regulation is most noted for its induction of phase I and phase II drug metabolizing enzymes. The phase I enzymes include, monooxygenases of the cytochrome P450 family, *CYP1A1*, *1A2* and *1B1*. CYP1A1 is considered the hallmark

Figure 1.4 AHR signaling pathway

TCDD is a lipophilic molecule allowing it to cross the plasma membrane and bind the AHR (**A**), Upon ligand binding, the AHR undergoes a conformational change and disassociates from its cytosolic partners (**B**). The ligand bound receptor translocates to the nucleus and heterodimerizes with ARNT (**C**). The AHR/ARNT dimer is an active transcription factor that recognizes dioxin response elements (DREs)within the genome and regulate gene transcription. CYP1A1 is the canonical gene regulated by AHR/ARNT (**D**)


TCDD
Cytosol
Hsp90
ARA9
B
Hsp90
ARA9
AHR
A
C
Nucleus
AHR
ARNT
D
DRE
Gene Transcription
CYP1A1

biomarker of AHR activation [76, 77]. Some of the phase II enzymes regulated are NAD(P)H: quinone oxidoreductase 1 (NQO1), glutathione-S-transferase A2 (Gsta2), carbonyl reductase 3 (Cbr3), and members of the UDP-glucuronosyltransferase (UGT) family, (e.g. UGT1A1) [77, 78]. Comparison of AHR gene regulation between the mouse and human AHR gene battery demonstrates species variability. This data revealed only an 18% overlap for induced gene expression and a 48% overlap in gene repression. Overlapping genes include those involved in cell proliferation and metabolism. Interestingly, the overlapping genes displayed a temporal difference in their expression patterns [79]. While the regulation of xenobiotic metabolizing enzymes is focused on frequently in AHR gene regulation, the AHR gene battery is quite complex and includes genes involved in development, differentiation, fatty acid transport, metabolism, cell growth and proliferation, apoptosis, and tumor promotion [78, 80].

The AHR has also been demonstrated to play a role in mammalian development and reproduction [81]. In AHR null mice, vascular biology is impacted. Observations in these mice included prolonged extramedullary hematopoiesis, transient microvesicular steatosis in perinatal hepatocytes, incomplete development of the ductus venosus (DV) a fetal vascular structure, and reduced liver size throughout their life span [41]. These null mice also have reduced total numbers of T and B-cell counts when compared to their wild type counter parts, indicating immune system suppression. Adult null female mice present with fibrosis and significant calcification in the uterus [82].

Gene regulation has been proven to be a major component of AHR mediated toxicity. For example, AHR mediated dysregulation of developmental genes, tumor necrosis factor alpha induced protein 2 (Tnfaip2), hairy and enhancer of split 6 (Hes6), and growth arrest specific 1 (Gas1) may influence cell differentiation in a TCDD dependent manner [78]. TCDD induced fat vacuolization in liver cells is linked to AHR mediated gene regulation involved in fatty acid uptake and metabolism [78]. The AHR has been shown to induce fatty acid binding protein 4 and 5 (Fabp4 and 5), lipoprotein lipase (Lp1), solute carrier family 27, member 2 (Slc27a2), and CD36 antigen (Cd36) [78]. It is possible the upregulation of these genes plays a central role in the fatty liver phenotype observed in TCDD exposure. The most convincing evidence for the central role AHR mediated gene regulation has in TCDD induced toxicity comes from mouse models. Bunger *et* al have reported on transgenic mice expressing an AHR with a deleted NLS or with the receptor's DNA binding ability ablated. In both models, the mice did not exhibit liver or thymic toxicity upon TCDD dosing. Furthermore, TCDD exposure to pregnant dames did not result in progeny with cleft pallet another hallmark toxic response [40, 41].

There is a substantial body of work investigating gene expression cooperatively regulated by the AHR and other factors or indirectly mediated by the AHR. Included in this is the receptor's crosstalk with other transcription factors. For example NFκB pathway and nuclear factor erythroid 2- related factor (Nrf2) regulated genes. NFκB is a pleiotropic transcription factor which governs many of the biological processes impacted by dioxin exposure, including the immune system, thymic involution, and carcinogenesis

[83]. Dioxin-induced AHR activity appears to have a synergistic effect on NFκB gene regulation in a tissue specific manner. This effect has been demonstrated in the CH12.LX B-cell line and human breast cancer cells [83, 84]. In contrast, AHR activity represses NFκB function in COS cells [85]. The complex relationship between AHR and NFκB is poorly defined to date. The relationship between the AHR and NFkB and the influence exogenous activation of the AHR has on it may play a critical role in dioxin toxicity. Two potential mechanisms for the cooperative gene regulation have been proposed. Protein/protein interactions between the AHR and the RelA (p65) subunit of NFκB have been reported in Hepa1C1C7 wild type cells using co-immunoprecipitations [83]. The first mechanism based on this evidence depicts activated AHR and RelA influencing gene expression as members of each other's functional transcriptional complex. The second proposed mechanism is a cooperative DNA binding model between AHR/ARNT and NFκB leading to a synergistic gene regulation effect. Sulentic *et al* demonstrated an overlap of DREs and NFκB binding sites upstream of immunoglobulin heavy chain enhancer gene [84]. It is also noteworthy that dioxin exposure has been shown to induce NFκB DNA binding in the AHR deficient BCL1 B-cell line, indicating an AHR independent mechanism also exists [84]. A sub-set of the AHR gene battery has recently been shown to require Nrf2 for their expression. Thus, indicating another cooperative mechanism involved in AHR gene regulation [86]. This group includes Nqo1, a hallmark gene of Nrf2 regulation, and members of the UGT family. Though these findings demonstrate the central role AHR mediated gene

regulation plays in dioxin-induced toxicity they do not account for all aspects of AHR biology.

The AHR has been implicated in estrogen and androgen receptor mediated hormone signaling [87-94]. Both the AHR and estrogen receptor (ER) can be activated by environmental pollutants. Cross talk between these receptors has been extensively investigated. Reports indicate activation of one receptor inhibits the other receptor's activity. This potentially involves the RIP140 transcriptional co-activator or via direct protein/protein interactions between the AHR/ARNT complex and the ER [87-89]. Recent studies show estrogen inhibits AHR activity in an ER-independent mechanism in human breast cancer [90]. Moreover, in endometrial cells, activation of the AHR induced a cell stress response and metabolism in an ER-independent manner [91]. Similarly, cross talk has been observed between the AHR and the androgen receptor (AR). The AHR and AR share a common co-activator NcoA4, similar to the AHR/ER relationship, the amount of available co-activator can be a limiting factor when both receptors are activated [92]. Furthermore, activated AHR has been shown to enhance, as well as inhibit AR signaling in a cell specific way. In kidney cells, activated AHR enhances AR function whereas the AR is inhibited by AHR activity in prostate cells [93, 94]. Given these and numerous other reports, there is strong evidence for AHR-mediated influence in hormone signaling that is not the result of direct receptor-mediated gene regulation.

1.3 The AHR's Role in Cellular Processes

Research into AHR biology has demonstrated the receptor's influence on numerous cellular processes. Kinase signaling cascades, cell cycle regulation and proliferation, cell motility, intercellular communications and tumor promotion have all been shown to be influenced by the AHR and are impacted in TCDD exposure [66, 95-99]. The AHR also has a demonstrated role in modulating the immune system following dioxin exposure. Though AHR-mediated gene regulation might play a direct role in some of these cellular processes, the scope of the reported AHR gene battery and the reported evidence suggests other modes of action.

The influence AHR activity has on kinase signaling pathways, cell cycle regulation, and proliferation is an area of extensive study. This is especially important, given the links between the AHR and these cellular processes, tumor formation, and cancer progression. An early finding of the AHR's involvement in cell cycle processing was a prolonged doubling time in an AHR deficient Hepa1c17 cell line when compared to wild type cells. This difference was attributed to an extended G1 phase in the AHR inactive cell line [100]. Subsequently, multiple reports have linked the AHR to the retinoblastoma tumor suppressor protein (pRb), a master regulator of cell cycle progression [101-105]. The hypophosphorylated active form of pRb has been shown to directly interact with the AHR and it has been proposed that this interaction acts in both a co-activator and co-repressor relationship influencing expression of cell cycle proteins [101, 102]. Activation of the AHR with TCDD leads to the phosphorylation of pRb and to cell cycle arrest in these cells [105]. More recent findings demonstrate AHR interaction

with cyclin-dependent kinase 4 (CDK4) and cyclin D1 (CCND1) in the absence of exogenous ligand in breast cancer cell lines, thus allowing for cell cycle progression [98, 105]. Moreover, it has been shown that pretreatment with TCDD inhibits liver regeneration in partial hepatoectomy [106]. Taken together, these finding indicate a role for the AHR in normal cell cycle progression and deleterious effects of dioxins on that role, leading to cell cycle arrest. In contrast, other evidence suggests that TCDD exposure promotes hyperplasia in various species and tissue types [13, 53]. Ray and Swanson demonstrated TCCD- induced activation of the AHR inhibits cell senescence in skin cells; the loss of senescence has been linked to tumor promotion [99]. Mitchell *et al* elucidated a link between prolonged AHR activity and mitogen activated cell proliferation in cases of TCDD-induced hyperplasia in mouse whole livers [106]. Moreover, in rat oval cells WB-F334, a liver stem cell line, under confluent conditions, TCDD exposure leads to escape from G1 arrest and cell cycle progression [107]. These observations provide further evidence for an association between the AHR, kinase signaling pathways, and cell cycle dysregulation. More importantly, these examples demonstrate the complex nature of the AHR's role in different species' and tissue type's cell cycle and the implications that role has in tumor promotion.

Cell motility and intercellular communication are other cellular processes with active roles in cancer progression that have been shown to be influenced by activated AHR. Prolonged activation of the AHR by dioxins has been correlated to an upregulation of the Jun NH2-terminal kinase (JNK) and the extracellular signal related kinase pathways that mediates intracellular communication [103]. Observed

morphological changes in MCF7 breast cancer cells resulted in a loss of cell-cell interaction and remodeling of the cytoskeleton which increased contact with the extracellular matrix upon dioxin exposure [108]. These changes are consistent with increased tissue plasticity a hallmark of metastasis and cancer progression. In the human liver cell line, HepG2, activated AHR induced the same effects on the cytoskeleton and activation of JUN. In addition, repression of E-cadherin and up regulation of the metastasis marker NEDD9 were exhibited [109]. From a clinical study of patients with upper urinary tract urothelial cancer, poor prognosis correlated to the level of AHR expression. Further investigation using the urothelial carcinoma T24 cell line revealed AHR-mediated expression of matrix metalloproteinases (MMP) 1, 2, and 9 proteins involved with cell invasion. However, in human breast cancer cells TCDD-induced AHR activation plays an inhibitory role in cell mobility and invasion in an ER-independent manner [110]. The mechanism by which the AHR influences these cellular events is not clearly defined. Evidence indicates the receptor does not impact these cellular functions through traditional AHR/ARNT gene regulation [103, 107]. There is significant evidence that AHR biology encompasses several cellular processes that are central to carcinogenesis.

Immune suppression is a hallmark response of dioxin-induced toxicity. As mentioned previously, the AHR and NFκB act in concert influencing immunoglobulin (Ig) heavy chain production [111]. In addition, cytokine levels, as well as T and B-cell differentiation are regulated, in part, through AHR activity. TCDD treatment induced expression of suppressor of cytokine signaling 2 (Socs2) in murine B-cells in an AHR-

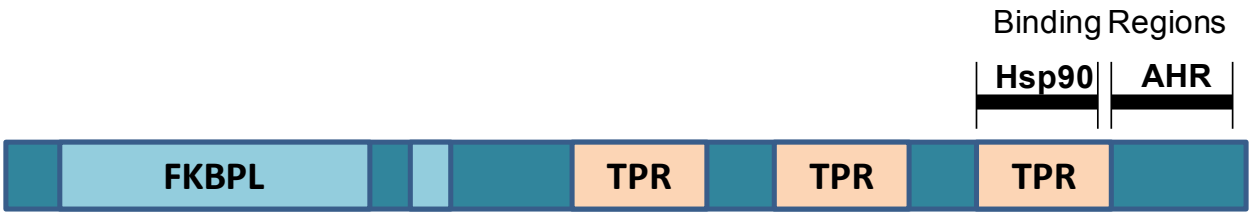
dependent manner [112]. Repression of cytokines leads to reduced immune system response. De Abrew *et al* recently reported a comprehensive genomic analysis of the AHR's role in B-cell differentiation, implicating dioxin-induced AHR activity in both *cis* and *trans* regulation of this process. Using microarray-based chromatin immunoprecipitations (ChIP-on-chip) methodology, the study elucidated potential AHR *cis* regulation at the three transcription factors responsible for B-cell differentiation (Prdm1, Bcl6, and Pax5). Of the reported 420 genes which demonstrated altered expression profiles under dioxin treatment, 78 genes also showed AHR localization to their transcriptional start sites, indicating a *cis* acting regulation. The remaining genes' expression may be altered by the AHR via a *trans* regulator mechanism, cooperative regulation, or other signaling events [113]. T-cell differentiation has also been shown to be modulated by the AHR. IL17 producing T helper cells (Th17) derive from parental T helper cells in an AHR-dependent process [114]. In the absence of exogenous ligand, the AHR regulates signal transducer and activator of transcription 1 and 3 (Stat1 and Stat3) activity allowing for T helper cell differentiation. In addition to the receptor's role in T helper cell formation, two independent investigations have demonstrated a role for the AHR in Fox3+ regulatory T cells (Tregs) production [115, 116]. Mezrich et al demonstrated AHR activation by kynurenine, a proposed endogenous ligand for the receptor, resulted in Treg production [116]. Gandhi et al reported TCDD-induced AHR-mediated upregulation of the transcription factor *c-MAF* which is responsible for IL10 expression in mouse T-cells, leading to Treg differentiation [115]. Taken together these

data depict a complex role for the AHR in cytokine production and several immune cell differentiation events which are impacted by exogenous ligand exposure.

Clearly, AHR-mediated gene regulation has a pivotal role in dioxin toxicity. AHR-mediated transcriptional regulation, however, may not be the sole mechanism by which toxic responses are induced via the receptor. There is potential for contribution from protein/protein interactions, pathway cross talk, translocation events, and metabolic function in AHR-mediated toxicity. One question that arises is, does the disassociation of the cytosolic complex upon ligand activation of the receptor play a part in TCDD induced secondary toxic responses? Extensive research on Hsp90 demonstrated its role as a chaperone protein, responsible for protein homeostasis [117]. Moreover, evidence suggests Hsp90 participates in protein transport to the mitochondria, neurodegenerative diseases, cancer progression, and immune function [118-120]. ARA9 is the other member of the cytosolic complex. ARA9 is comprised of two domains, in the N terminus is an FK506 binding protein (FKBP)-like domain and at the C terminus is a region of tetratricopeptide repeat (TPRs) (Fig 1.5). Like Hsp90, ARA9 has important cellular functions. It has been shown to interact with the tyrosine kinase receptor (RET) *in vivo,* effecting survivin levels, revealing a potential role for ARA9 in RET-mediated apoptosis [95]. Knockout of the ARA9 is embryonic lethal in mice resulting from disruption in cardiac development [121]. In humans, predisposition to pituitary adenomas is linked to mutations in the ARA9 loci [122]. The overlap the three members of the AHR cytosolic complex proteins have in cellular processes is

Figure 1.5 Structure of the ARA9 Protein

The amino terminus of ARA9 contains an FK506 binding protein (FKBP)-like (FKBPL) domain, however, the protein is unable to bind macrolidic drugs, such as FK506 and rapamycin. The carboxy-termuinus of ARA9 contains three tetratricopeptide repeat (TPR) domains that are responsible for mediating interactions with Hsp90 and the AHR.

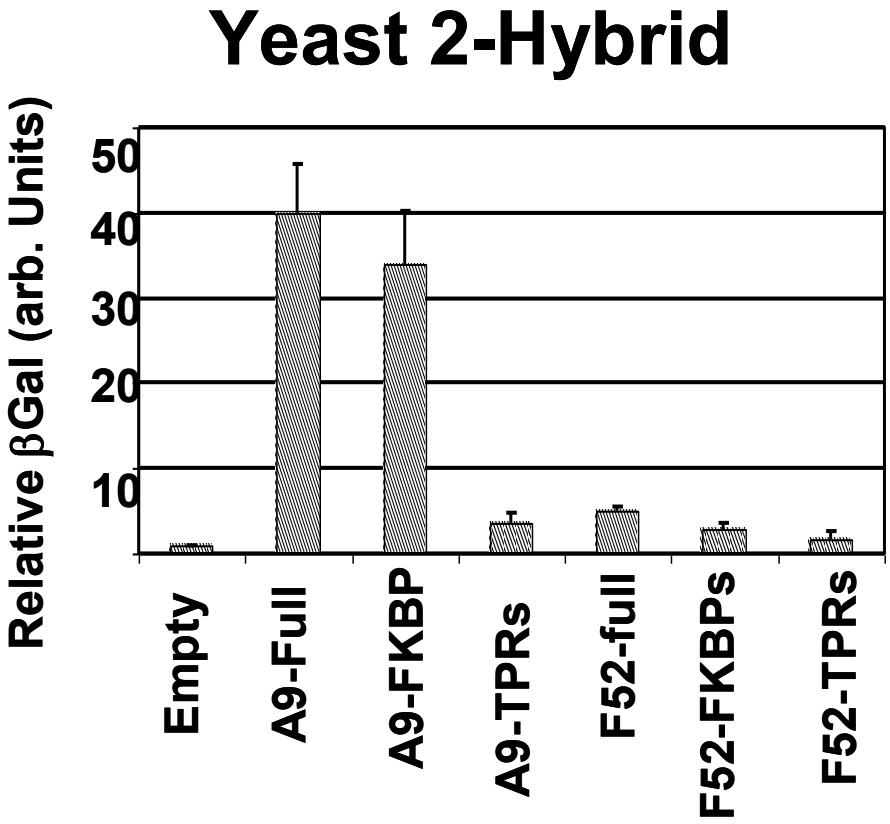

Binding Regions
Hsp90
AHR
FKBPL
TPR
TPR
TPR

noteworthy. Moreover, the effect of dioxin on the dynamics of this complex is of interest.

Preliminary investigations using the ARA9 as bait in a yeast-two hybrid screen of a murine B-cell library resulted in the identification of a protein interaction with the downstream of tyrosine kinase 1 (DOK1) protein [123, 124]. The family of DOK proteins has been characterized as docking proteins that facilitate phosphorylation events in kinase activities, specifically in ras-GAP associated signaling cascades [123, 124]. Notably, they are involved in the negative regulation of signaling events in B-cells [125]. Subsequent investigation determined the N terminal FKBPL domain of ARA9 was necessary and sufficient for the interaction with DOK1 (Fig 1.6). The ARA9 interacts with the AHR and Hsp90 through the TPRs of its C terminus. Given this concurrent association with the AHR complex and DOK1 is possible. To explore ARA9-mediated influence of DOK1 on AHR-mediated gene expression, transient transfection and a luciferase reporter assay were used (Fig 1.7).

Figure 1.6 ARA9/DOK1 Interaction

The FK506 binding protein like domain (**A9-FKBP**) of ARA9 was used as “bait” in a yeast two-hybrid screen of a human B-cell library. DOK1 was identified in multiple clones and further investigation confirmed the interaction between full length ARA9 and the N terminus truncated construct (**A9-FKBP**) but not the C-termanl tetratricopeptide repeat domain (**A9-TPR**). This interaction was specific to ARA9, as similar constructs from the closely related FKBP52 (**F52**) protein, wild type (**F52-FulI**) or n-terminal (**F52-FKBPs**) or C-terminal (**F52-TPRs**) constructs, were unable to interact with DOK1.

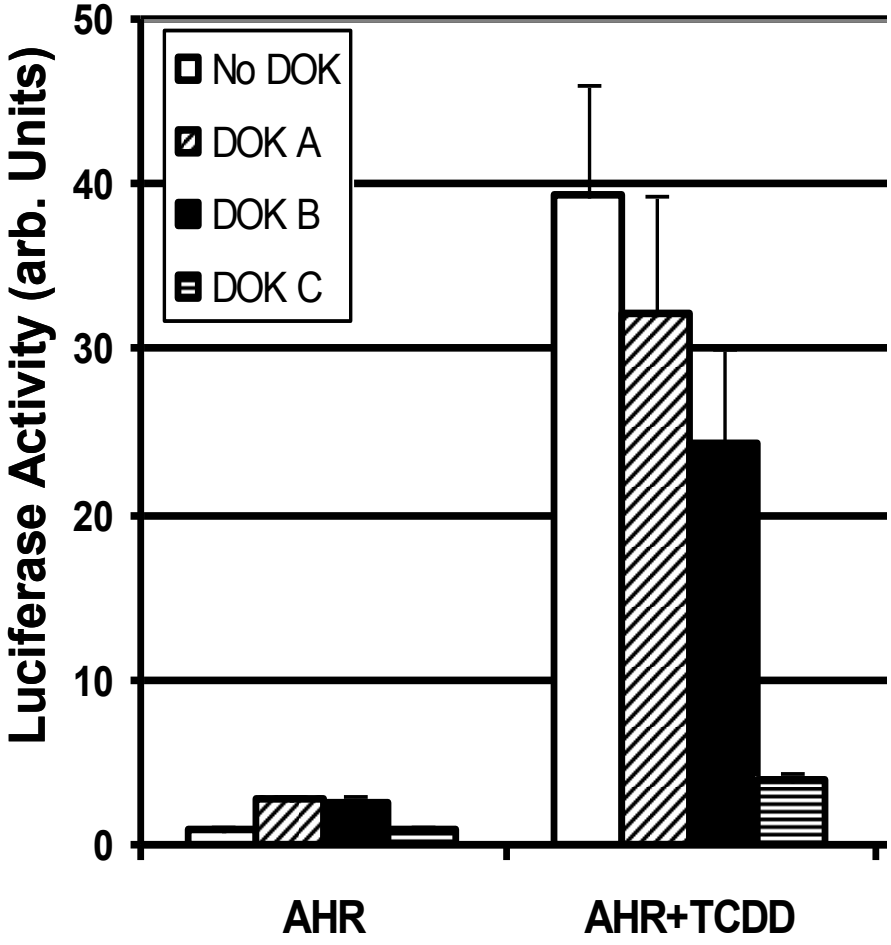

Yeast 2-Hybrid
Relative βGal (arb. Units)
50
40
30
20
10
Empty
A9-Full
A9-FKBP
A9-TPRs
F52-full
F52-FKBPs
F52-TPRs

(J. LaPres)

Figure 1.7 DOK Influence on AHR-mediated Transcription

Hepa1C1C7 cells were transiently transfected with the AHR in the absence (**white bars**) and presence (**hatched, black, and striped bars**) of increasing amounts of DOK1 and dosed with DMSO (**AHR**, vehicle, 0.01%) or TCDD (**AHR+TCDD**, 10 nM). A DRE driven luciferase reporter plasmid was co-transfected and used to measure DOK1 influence on AHR mediated transcription.

Transient Transfection



DOKA =1µg, DOKB=3µg, DOKC=5µg

(J. LaPres)

The luciferase reporter assay demonstrated the ability of DOK1 to attenuate AHR-driven transcription. The results suggest that secondary signaling is capable of influencing the AHR's function. It was these initial findings that led to the AHR-PIN project's hypothesis that **secondary signaling plays an important role in TCDD-induced toxicity mediated by the AHR through direct influence on its cytosolic complex and perturbations of downstream signaling cascades.**

The following specific aims have been set for this investigation.

Specific Aim 1:

Verification of DOK1/AHR interaction and functional relevance.

Specific Aim 2:

Identify AHR interacting proteins in liver and spleen cell types from mouse and human systems.

Specific Aim 3:

Elucidate the functional relevance of AHR associating proteins isolated in tandem affinity purification (TAP) tagging.

Specific Aim 4:

Assemble AHR-PIN database using data compiled in Aims 2-3

(Collaborative effort with Superfund grant members).

MATERIALS AND METHODS

2.1 Materials

Oligonucleotide synthesis was performed at the Macromolecule Synthesis Facility at Michigan State University. pTarget and pGEM-T Easy vectors were obtained from Promega (Madison, WI) and pZome1C vector was obtained from Cellzome (Cambridge, UK). The Phoenix-eco cell line was a generous gift from Dr. Garry Nolan (Stanford University, Palo Alto, CA). The AHR -/- mouse embryonic fibroblast cell line and rabbit polyclonal anti-AHR BEAR3 were generous gifts from Dr. Christopher Bradfield (University of Wisconsin-Madison). The other antibodies used were obtained from the following sources, mouse monoclonal anti-ATP5α1 (cat #ab14748) and rabbit polyclonal anti-Hsp90 (cat #ab19021) were obtained from Abcam (Cambridge, MA), mouse monoclonal anti-COXIV (cat #A21349) was obtained from Invitrogen, rabbit anti-LDH was a generous gift from John Wang (Michigan State University). Goat polyclonal anti-SODII (cat #sc18503), donkey anti goat (cat #sc2033), and goat anti rabbit (cat # sc2004) and mouse (cat #sc2005) and normal mouse IgG (cat #sc2025) and protein G resin were purchased from Santa Cruz (Santa Cruz, CA). Protease inhibitor tablets (cat # 11836170001) were purchased from Roche (Indianapolis, IN). All other chemicals were reagent grade and purchased from Sigma Aldrich (St. Louis, MO).

2.2 Plasmids

The cDNAs for the murine AHR, human AHR and GFP were amplified using the following primers:

mAHR: forward - 5’-ggatccccaccatgagcagcggcgccaacatcacc-3’

reverse - 5'-ggatcctgcactctgcaccttgcttagg-3',

hAHR forward - 5'- gggcccccaatgaacagcagcagcgccaacatc-3'

reverse - 5'- tcttctcttttccatggatcctgggcccataggcgcgccggccggcgcctag-3'

GFP: forward - 5'-gggggatccaccatggtgagcaagggcgac-3'

reverse - 5'-gtggatccccgggcccgcggtaccgtcgactgc-3'.

The mAHR and hAHR amplicons were cloned into p-Target vector and the GFP amplicon was cloned into the pGEM-T Easy vector. Each cDNA was subcloned into the pZome1C vector. Use of the pZome1C vector places the TAP-tag at the C terminal of the mAHR, hAHR and GFP genes. The hAHR was subcloned into the target gene vector pLH-Z12I-PL of the ARGENT inducible expression system (Ariad, Cambridge, MA). Clones were isolated with ampicillin selection and sequence verified

2.3 Inducible Expression System

The ARGENT regulated transcription retrovirus kit is a dual vector system. The target gene vector mentioned in the section above is regulated by a transcription factor cassette which is housed on the pL2-N2-RHS3h/ZF3 vector. This transcription factor is constitutively expressed, but only becomes activated in the presence of the rapamycin or analogs such as AP21967. The system is fully described at http://www.ariad.com/pdf/Reg_Tx-Retrovirus.pdf

2.4 Transfection/Stable Cell Line Infection

The retroviral vectors, mAHR-TAP-P-ZOME, hAHR-TAP-P-ZOME, GFP-TAP-P-ZOME, hAHR-TAP- pLH-Z12I-PL and transcription factor pL2-N2-RHS3h/ZF3, were transfected into Phoenix-eco (P-eco) cells, for non-human mammalian cell infections, or

Phoenix-ampho (P-ampho) cells, for human cell infections, for packaging using Lipofectamine (Invitrogen) via manufacturer's instructions. After the initial incubation (5 to 8 hrs, 37°C) in the presence of DNA, the media was removed and replaced with Phoenix cell growth media containing chloroquine (25 μM). Cells were incubated for 24 hrs (37°C) and media was replaced with fresh Phoenix cell growth media and incubated for an additional 24 hrs (32°C). The media was collected and cellular debris was removed by centrifugation (3 mins, 67 x g, in RT7, Sorvall, Rockford, IL), and purified by filtration through a 0.45 μm membrane filter (Millipore). Hepa1c1c7 wild type, AHR-/- HepaC12, MEF AHR -/-, and Hep3B target cell lines were exposed to virus containing media for 3 hrs at 32°C and 5% CO2. After this incubation, media containing 15 μg/mL polybrene was added and target cells were incubated for 24 hrs (32°C). Media was replaced and target cells were incubated (37°C) until plates reached 80% confluence. Cells were then passaged and selected using media containing selection antibiotics.

2.5 Cell Culture and Dosing Regimen

The Phoenix-eco and wild type mouse hepatocytes, Hepa1c1c7, were maintained in DMEM w/L-glutamate and supplemented with 10% cosmic calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5% sodium pyruvate. Tissue culture media and supplements were purchased from Invitrogen Co (Carlsbad, CA) and cosmic calf serum was purchased from HyClone (Waltman, MA). mAHR-TAP, hAHR-TAP and GFP-TAP Hepa1c17 cell lines were grown in the above media, supplemented with puromycin (2 μg/mL) (US Biological, Swampscott, MA). Hepa1c1c7 cells expressing ARGENT transcription factor were maintained in the above media and

supplemented with G418 125 µg/mL (Sigma). Hepa1c1c7 cells expressing ARGENT transcription factor and hAHR were maintained in the above media and supplemented with G418 (125 µg/mL) and hygromycin (250 µg/mL) (Sigma). The AHR -/- mouse embryonic fibroblast cell line was maintained in DMEM w/L-glutamate and supplemented with 10% FCS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 5% sodium pyruvate, and 5% non-essential amino acids. MEF AHR-/- with mAHR-TAP or GFP-TAP were grown in the above media and supplements with 3 µg/mL puromycin. The AHR-/- hepatocyte cell line HepaC12 was maintained in DMEM w/L-glutamate and supplemented with 10% FCS, and 5% sodium pyruvate. HepaC12 with hAHR-TAP or GFP-TAP underwent antibiotic selection in the above media with 1.5 µg/mL puromycin. Human hepatocytes, Hep3G, were maintained in MEM w/L-glutamate and supplemented with 10% cosmic calf serum, 5% sodium pyruvate, and 5% non-essential amino acids. Hep3G cells with hAHR-TAP or GFP-TAP were maintained in the above media supplemented with 0.5 µg/mL puromycin.

2.6 Western Blot Analysis

Total protein samples were prepared and concentrations determined as previously described [126, 127]. Proteins were separated on 4-12% Nu-Page Bis-Tris gels, transferred to nitrocellulose membranes and probed with various antibodies as previously described [128]. Westerns were visualized using ECL Western blotting substrate (Pierce, Rockford, IL).

2.7 Quantitative Real-time PCR Analysis

mAHR-TAP and GFP-TAP MEF AHR-/- cell lines were used for functional analysis of the TAP-AHR. CYP1A1 gene expression was examined after exposure to DMSO vehicle (0.01%) or TCDD (10 nM) for 6hrs. Total RNA was extracted from the cells using TRIzol (Invitrogen) via manufacturer's protocol. RNA concentrations were determined by UV spectrophotometry (Nanodrop, Wilminton, DE). One microgram of total RNA was reverse-transcribed using anchored oligo (dT)18 primers and M-MLV reverse transcriptase (Invitrogen). Reactions were carried out according to manufacturer's protocol. CYP1A1 gene expression was measured by quantitative real-time PCR (qRT-PCR) using the SYBR-Green system and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) [129]. The primers used were: mCYP1A1, 5'-AAGTGCAGATGCGGTCTTCT-3' and 5'-AAAGTAGGAGGCAGGCACAA -3'; mHPRT 5'-AAGCCTAAGATGAGCGCAAG-3' and 3'-TTACTAGGCAGATGGCCACA-5'.

2.8 Tandem Affinity Purification

Approximately 2.5 x 10^9 Hepa1c1c7 mAHR-TAP, and GFP-TAP cells were dosed with vehicle (DMSO, 0.01%) or TCDD (10 nM) for 30, 120, or 240 mins. Pilot experiments were performed on naïve TAP-tagged cells. Media was removed and cells were washed three times with phosphate buffered saline and harvested in TAP-tag lysis buffer (TTLB) (5% glycerol, 50 mM Tris, pH 7.5, 50 mM MgCl2, 100 mM NaCl, 0.1% NP40, 1 mM DTT, 1 mM Na3VO4, 25 mM NaF, protease inhibitor tablets). Cells were disrupted by two cycles of freeze/thaw, and insoluble material was removed by

centrifugation (7000 x g, 20 mins). Samples were then incubated with 200 µL of IgG Sepharose beads for 4 hrs, at 4°C, and rotation. Beads were collected by centrifugation (45 x g, 30 sec) and transferred to Poly-Prep Chromatography columns (BioRad, Hercules, CA). Columns were extensively washed with TAP-tag lysis buffer, followed by washing with tobacco etch virus (TEV) cleavage buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT). Beads were incubated (18 hrs, 4°C) in 1.5 mL TEV cleavage buffer containing AcTEV protease (450 units). Elutes were collected by gravity flow. IgG sepharose resin (GE Healthcare, Waukesha, WI) was washed with 1.5 mL of TEV buffer and 9 mL of calmodulin binding buffer (CBB) (10 mM β-mercaptoethanol, 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 0.1% NP40, 2 mM DTT). Washes were combined with elute. Samples were then incubated with 200 µL Calmodulin affinity resin (Stratagene, La Jolla, CA) (4 hrs, 4°C). Calmodulin resin was transferred to new Ploy-Prep Chromatography columns, supernatant was removed and resin was washed extensively with CBB. Warm 3x SDS buffer (4.8% SDS, 100mM Tris pH 6.8, 16% glycerol, 8% β-mercaptoethanol, 0.4% bromophenol blue) was added to resin to remove protein complexes (Fig 2.1).

2.9 Gel Electrophoresis and Mass Spectrometry

Samples were separated on a 4-12% Bis-Tris gradient gel (NuPage, Invitrogen) by electrophoresis. Gels were silver stained using SilverSNAP staining kit (Pierce, Rockford, IL) according to the manufacturer's protocol and the gels were photographed. Specific bands were excised from the matrix and destained with SilverSNAP destaining kit and subjected to in-gel tryptic digestion as previously described [130]. The extracted

Figure 2.1 TAP-tag Methodology

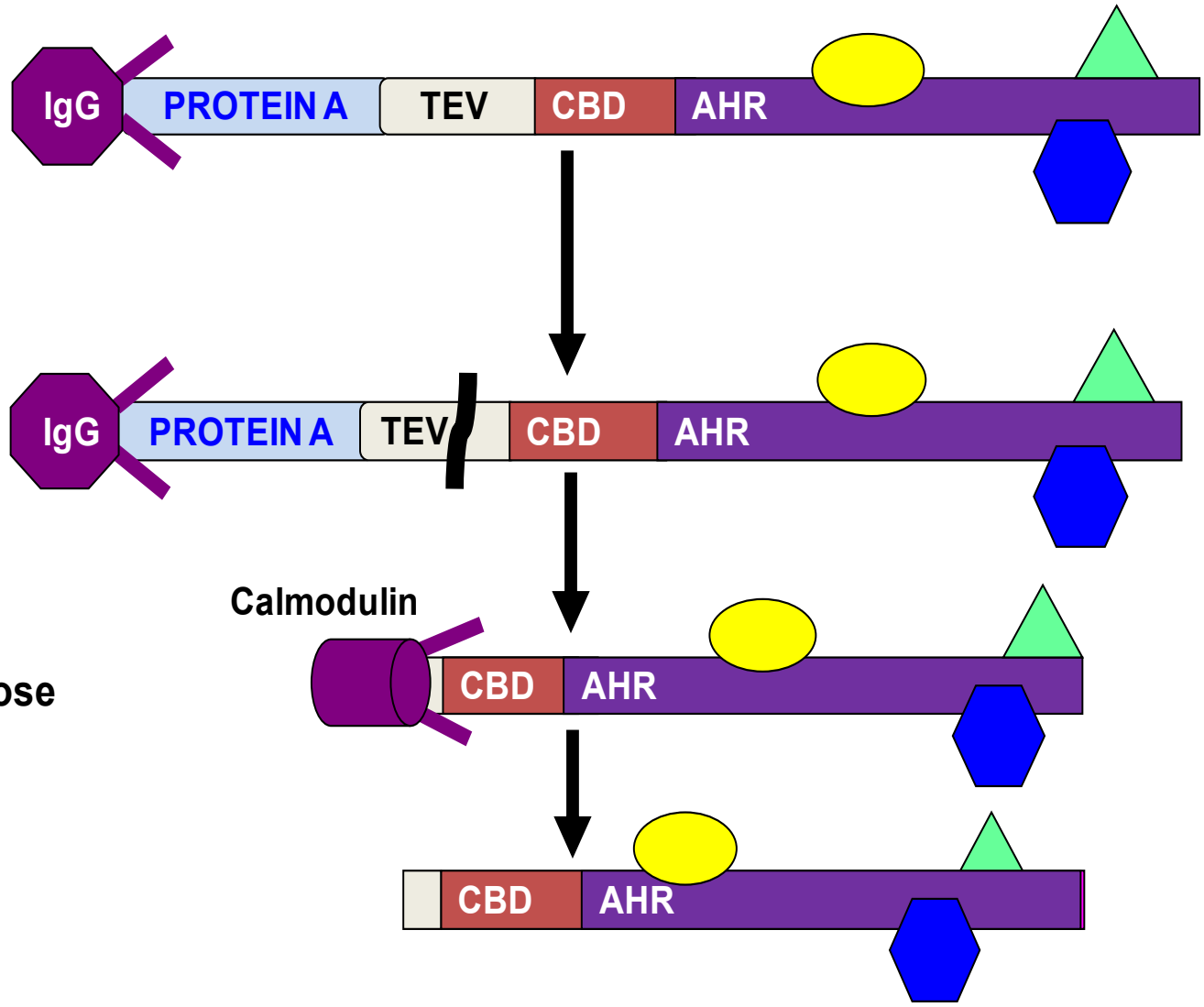
The Tandem Affinity Purification tag (TAP-tag) consists of protein A, a tobacco etch virus cleavage site (TEV), and calmodulin binding domain (CBD). The TAP-tag was fused to the AHR. Cell lysates were prepared from TAP-tagged AHR expressing cells as described in "materials and methods" section and incubated with IgG sepharose beads. The protein A portion of the TAP tag binds to the IgG and facilitates this first interaction. Cell lysate is removed and IgG beads are washed. The TAP-tagged proteins bound to the IgG beads are incubated with AcTEV protease to cleave tagged proteins thus freeing the receptor and interacting proteins from IgG beads. The samples are eluted after cleavage and incubated with calmodulin beads. After the second round of purification, beads are washed. Final eluates are removed from calmodulin beads with warmed SDS buffer. A similar strategy was carried out for the TAP-tagged green fluorescent protein used as a control.

IgG Sepharose Purification

TEV Cleavage

Calmodulin Sepharose Purification

AHR Interacting Proteins



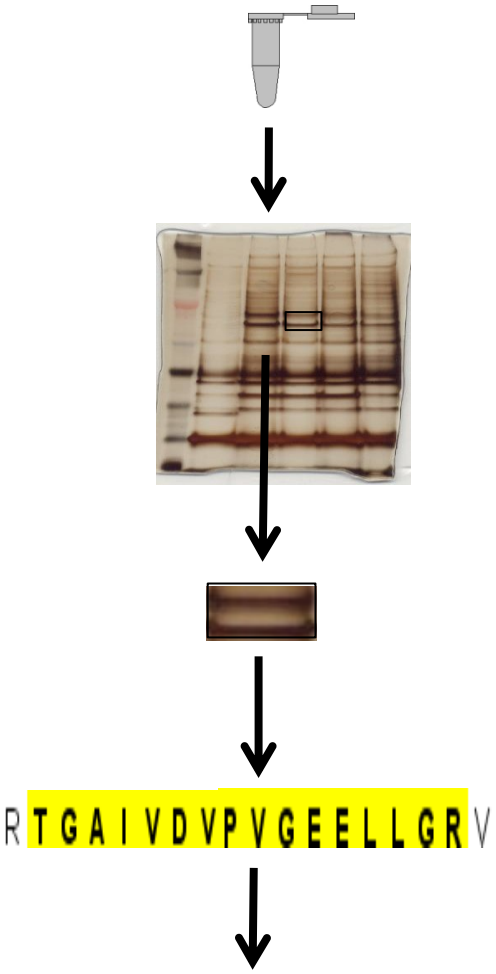
peptides were then automatically injected by a Waters nanoAcquity Sample Manager (Milford, MA) loaded for 5 minutes onto a Waters Symmetry C18 peptide trap (5 µm, 180 µm x 20 mm) at 4 µL/min in 5% acetonitrile /0.1% formic acid. The bound peptides were then eluted onto a Waters BEH C18 nanoAcquity column (1.7 µm, 100 µm x 100 mm) over 35 minutes with a gradient of 2% B to 35% B in 21 min, 90% B from 21-24 min and back to constant 5% B at 24.1 min using a Waters nanoAcquity UPLC (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 99.9% Acetonitrile/0.1% Formic Acid) with an initial flow rate of 600 nL/min, ramping to 700 nL/min at 80 min and back to 600 nL/min at 86min. Eluted peptides were sprayed into a ThermoFisher LTQ Linear Ion trap mass spectrometer outfitted with a MICHROM Bioresources ADVANCE nano-spray source. The top five ions in each survey scan are then subjected to data-dependent zoom scans followed by low energy collision induced dissociation (CID) and the resulting MS/MS spectra are converted to peak lists using BioWorks Browser v 3.3.1 (ThermoFisher, Rockford, IL) using the default LTQ instrument parameters. Peak lists were searched against all mouse sequences available in the NCBI nr database, downloaded on 11-16-2008 from NCBI, using the Mascot searching algorithm, v2.2 (www.matrixscience.com). The Mascot output was then analyzed using Scaffold (www.proteomesoftware.com) to probabilistically validate protein identifications using the ProteinProphet computer algorithm. Assignments validated above the Scaffold 95% confidence filter are considered true (Fig 2.2).

2.10 Co-immunoprecipitation

Hepa1c1c7 and CH12.LX wild type cells were grown to confluence, washed with cold PBS and harvested in TTLB. Equal amounts of the Hepa1c17 and CH12.LX

Figure 2.2 Mass spectrometry (MS) Sample Processing and Analysis

Protein samples from TAP-tag are separated on 4-12% gradient Bis-Tris gel. Protein bands are visualized by silver staining. Gel sections of each sample are excised and destained. These sub-samples then undergo in-gel trypsin digestion and resulting peptide fragments are removed from the gel matrix. The freed peptide undergo LTQ-MS analysis. The molecular weight of the peptide is used to determine the amino acid sequence. The peptide sequence is then compared against the Mascot database and assigned to a protein identification. This data is then subjected to another layer of analysis using the Scoffold software to score the accuracy of both the peptide read and the protein assignment.



Sample Separation:
Gel electrophoresis

Visualization:
Silver stain

Sub-sample Selection:
Protein band excision
Destaining

Peptide Fragmentation:
In-gel trypsin digestion

Peptide Sequencing:
LTQ MS analysis

Protein Identification:
MS peptide data analysis using Mascot and Scaffold software

cellular supernatant were incubated with 2 µg/uL of normal mouse IgG or ATP5α1 antibody for 90 minutes and then Protein G beads (30 µL) were added to the samples and incubated for an additional 90 minutes. The supernatant was removed and the beads were washed in TTLB. Samples were eluted from the beads using 3X SDS buffer and separated by Nu-PAGE Bis-Tris 4-12% gradient gel matrix, transferred to a nitrocellulose membrane, and probed with primary AHR antibody.

2.11 Cellular Fractionation

Hepa1c1c7 wild type cells were grown to near confluence and then dosed with vehicle DMSO (0.01%) or TCDD (10 nM) for 120 mins. Cells were harvested in fractionation buffer (25 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, pH7.4) and dounce homogenized (100 stokes). CH12.LX cells (8 x 10^6) were harvested in fractionation buffer and dounce homogenized, as above. Liver tissue was homogenized with Tissue-Tearor (Biospec, Bartlesville, OK) in fractionation buffer and then dounce homogenized, as above. Insoluble materials were removed by centrifugation (400 x g, 10 mins) and the supernatant was transferred to new tube. The supernatant was cleared by centrifugation (4,500 x g, 10 mins). The supernatant was removed and organelle pellet was resuspended in 1 mL of fractionation buffer and dounce homogenized (14-20 strokes). Large debris was removed by centrifugation (400 x g, 10 mins) and supernatant was transferred to new tube and purified mitochondria were collected by centrifugation (4,500 x g, 10 mins). The isolated mitochondrial pellet was resuspended in 250 µL fractionation buffer.

2.12 Mitochondrial Inner Membrane Potential Determination

CH12.LX and BCL-1 cells were grown in cell type appropriate media to a concentration of 1 x106 cells/ml. Cells were then transferred into a 96 well culture plate at 1 x 10^5 cells per well. The culture plates were immediately centrifuged at 300 x g for 5 min (24 °C), media was decanted and culture plates were gently agitated to disperse the cell pellet. Replicate wells (8 replicates per treatment) were then exposed to control media, vehicle (0.01%, DMSO), carbonyl cyanide m-chlorophenylhydrazone (CCCP, 12.5 μM), or TCDD (3, 10, or 30 nM) for 6 hrs in the presence and absence of transcriptional inhibitor mitomycin C. Culture plates were centrifuged as above, treatment media was decanted and 4 wells per treatment were exposed to 100 μl of high glucose normal buffer (HGNB) (130 mM NaCl, 5.5 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, 25 mM glucose, 20 mM HEPES, pH 7.4) with either 500 nM tetramethylrhodamine methyl ester (TMRM) or 250 nM MitoTracker Green (MTG) for 30 min. Average TMRM per well was divided by average MTG per well per treatment to generate TMRM/MTG fluorescence. Culture plates were then centrifuged, decanted, agitated, and cells were resuspended in 150 μl of HGNB. This procedure was repeated two more times and then the culture plates were analyzed by flow cytometry (FCM). 35,000 events were recorded per well on a BD FACSCanto II (BD Biosciences, San Jose, CA). Singlet gating was applied and the mean fluorescence intensity (MFI) for both TMRM and MTG fluorescence per cell was calculated for each well and then the average MTG across replicates was determined within a treatment group.

2.13 ATP Determination

CH12.LX cells were plated 8 x 10^5/well of a 6 well plate and grown to ~80% confluence. The cells were then treated with vehicle DMSO (0.01%) or TCDD (10 nM) for 6 hrs. An aliquot of each sample was taken for cell counts for normalization. Samples were then precipitated with 1 N perchloric acid, centrifuged (13,000 x g, 15 mins) to pellet cellular debris, aqueous sample was then neutralized with equal volume of 1 N NaOH. ATP levels were measured using ATP determination kit (Invitrogen) according to manufacturer's protocol.

EXPERIMENTAL RESULTS

AHR-mediated gene regulation has been demonstrated to be an important component of dioxin-induced toxicity. A complete understanding, however, of the mechanisms underlying AHR-mediate dioxin toxicity is lacking. Identification of AHR protein interactors and the functional roles these interactions might play in receptor signaling will impact our understanding of dioxin-induced cytotoxicity. Preliminary finding demonstrated DOK1's ability to influence AHR-mediated gene induction. This data raised interest in identifying other AHR interacting proteins and how these interactions vary between tissue type and species. This project's focus was the role protein interactions and signal transduction have on AHR biology and receptor-mediated TCDD toxicity. To identify novel interacting proteins for the AHR, a high through-put proteomics approach was employed. Tandem affinity purification was employed to isolate the AHR and associated proteins. Protein samples were then analyzed by mass spectrometry. Protein interactions of interest were then independently verified and characterized for functional relevance.

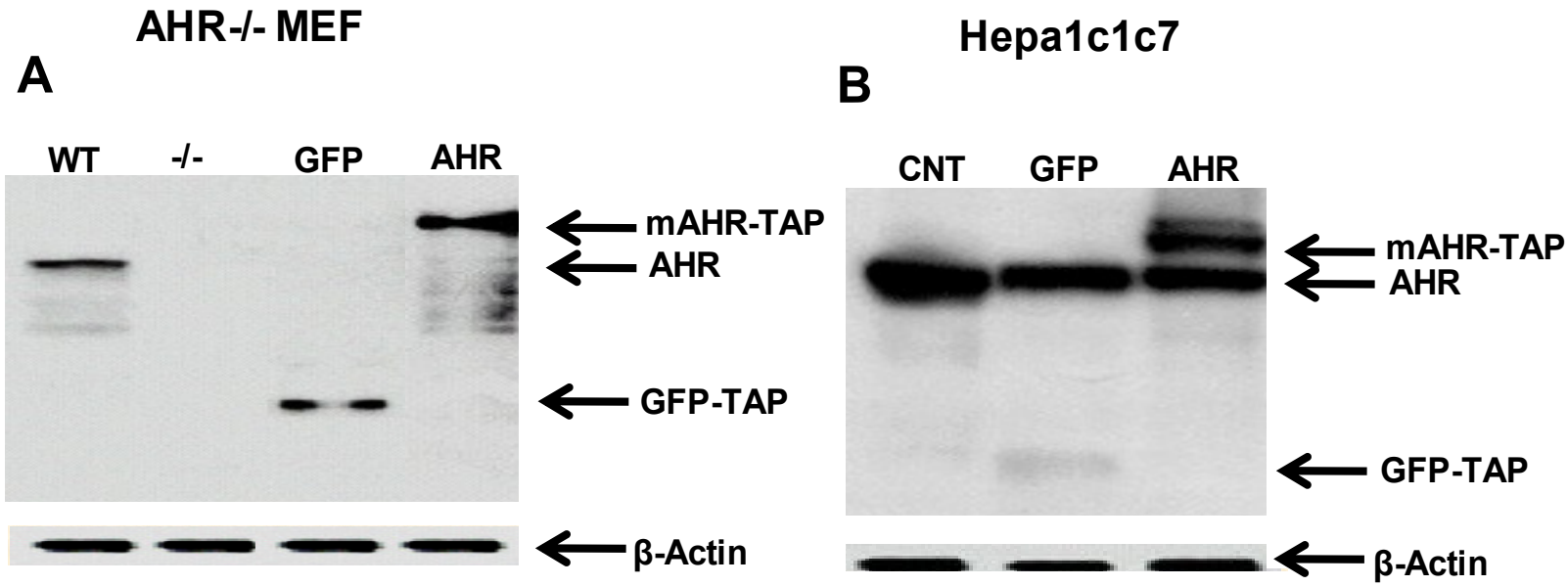
3.1 The Production of Stable cell line expressing TAP-tagged proteins

A. mAHR-TAP and GFP-TAP tagged cell line expression

In order to use the TAP-tag methodology, AHR and GFP protein constructs containing the TAP-tag had to be produced. These constructs were then used to create stable cell lines expressing the tagged receptor and the GFP control. Initial production of mAHR-TAP and GFP-TAP cells was conducted in an AHR-/- mouse embryonic fibroblast (MEFs). The expression levels of the mAHR-TAP and GFP-TAP proteins

Figure 3.1 mAHR-TAP and GFP-TAP Expression

Western blot of wild type (**WT**), AHR null (**-/-**), mAHR-TAP (**AHR**), and GFP-TAP (**GFP**) expression in and AHR-/- MEF cell lines using an AHR specific antibody. The antibody recognizes the protein A region of the TAP tag thus allowing for visualization of the GFP-TAP **(A)**. Western blot analysis of Hepa1c1c7 parental cells (**CNT),** GFP-TAP (**GFP**) and mAHR-TAP (**AHR**) expressing hepa1c1c7 cells using an AHR-specific antibody **(B)**.


AHR-/- MEF
A
WT
-/-
GFP
AHR
mAHR-TAP
AHR
GFP-TAP
β-Actin
Hepa1c1c7
B
CNT
GFP
AHR
mAHR-TAP
AHR
GFP-TAP
β-Actin

were visualized by Western blotting (Fig 3.1A). Over time the expression of the mAHR-TAP protein in AHR-/- MEF cell line diminished. Next, the mAHR-TAP and GFP-TAP were transduced into wild type Hepa1c1c7 cells. Western blotting was again used to visualize expression levels (Fig 3.1B). All further TAP-tag and MS experiments were conducted in these Hepa1c1c7 cell strains expressing mAHR-TAP and GFP-TAP proteins.

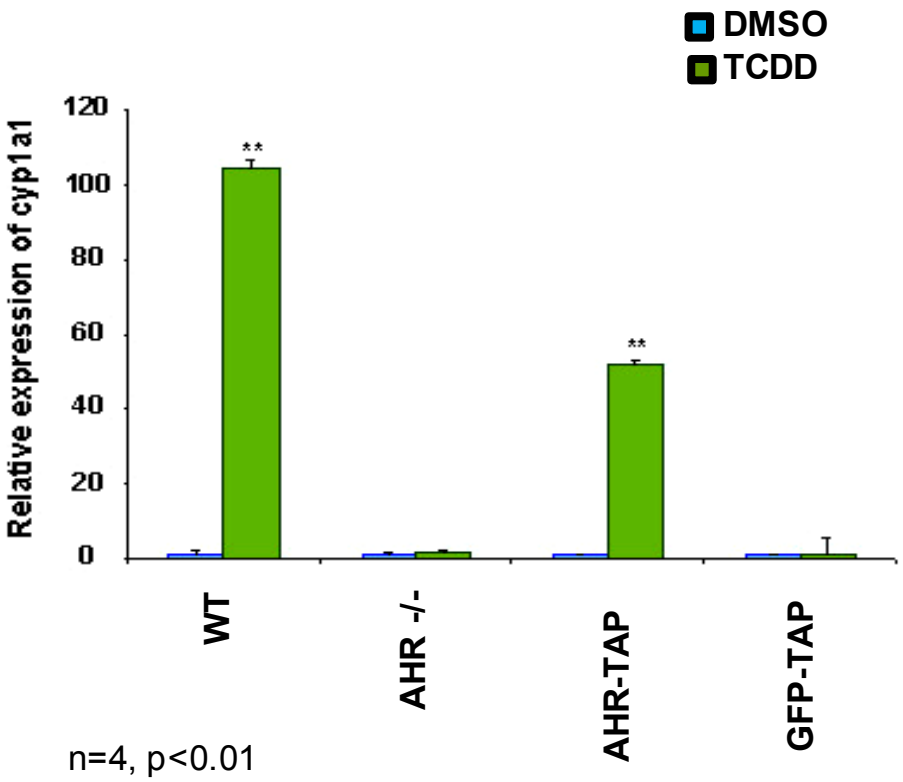
It is noteworthy that mAHR-TAP Hepa cells have lost expression of the TAP-tagged receptor over time. While the cell line remains puromycin resistant; the amount of mAHR-TAP expressed markedly decreased. This has been observed in cells that have undergone several rounds of passaging, as well as, in cells cryo frozen, revived, and then tested for mAHR-TAP expression. It is unclear if this phenomenon is a result of the mAHR-TAP being excised from the DNA by recombination or if there is silencing of the receptor. Moreover, this expression loss is exclusive to the mAHR-TAP cell line. It has not been observed in the GFP-TAP cell line or the other two TAP-tagged cell lines used in our lab (data not shown). Expression of the receptor may be under tight regulation and transcriptional machinery may act in silencing the tagged construct.

B. Functional characterization of mAHR-TAP

CYP1A1 expression is under the control of the AHR. Due to its robust induction upon AHR activation it is the canonical gene used to measure AHR induction. The function of mAHR-TAP was tested in the AHR-/- MEF cell line using Cyp1A1 expression as an endpoint of receptor activation. Cells were treated with vehicle or TCDD followed by measurement of CYP1A1 mRNA levels using qRT-PCR (Fig 3.2). The mAHR-TAP was able to induce CPY1A1 at 60% of the wild type AHR MEF cell line. Neither the

Fig 3.2 mAHR-TAP Functional Transcription Factor

Wild type (WT), AHR null (AHR -/-), mAHR-TAP (AHR-TAP), and GFP-Tap expressing null MEF cells were treated with DMSO (blue bars) or TCDD (green bars). AHR activity was determined by its ability to upregulate Cyp1a1 expression. Cyp1A1 was normalized to the expression of HPRT expression and Cyp1A1 in TCDD-treated WT Hepa1c1c7 cells was set to 100%


DMSO
TCDD
Relative expression of cyp1a1
120
100
80
60
40
20
0
**
**
WT
AHR -/-
AHR-TAP
GFP-TAP

n=4, p<0.01

Figure 3.3 Visualization of Sample Eluates from Complete Tandem Affinity Purification

Protein samples were prepared from mAHR-TAP and GFP-TAP expressing Hepa1C1C7 cells dosed with vehicle (DMSO). Following TAP, samples were visualized by silver staining. Bands were excised and MS data identified AHR, Hsp90, ARA9, ATP5α1, and GFP in the regions of the gel denoted by the black boxes

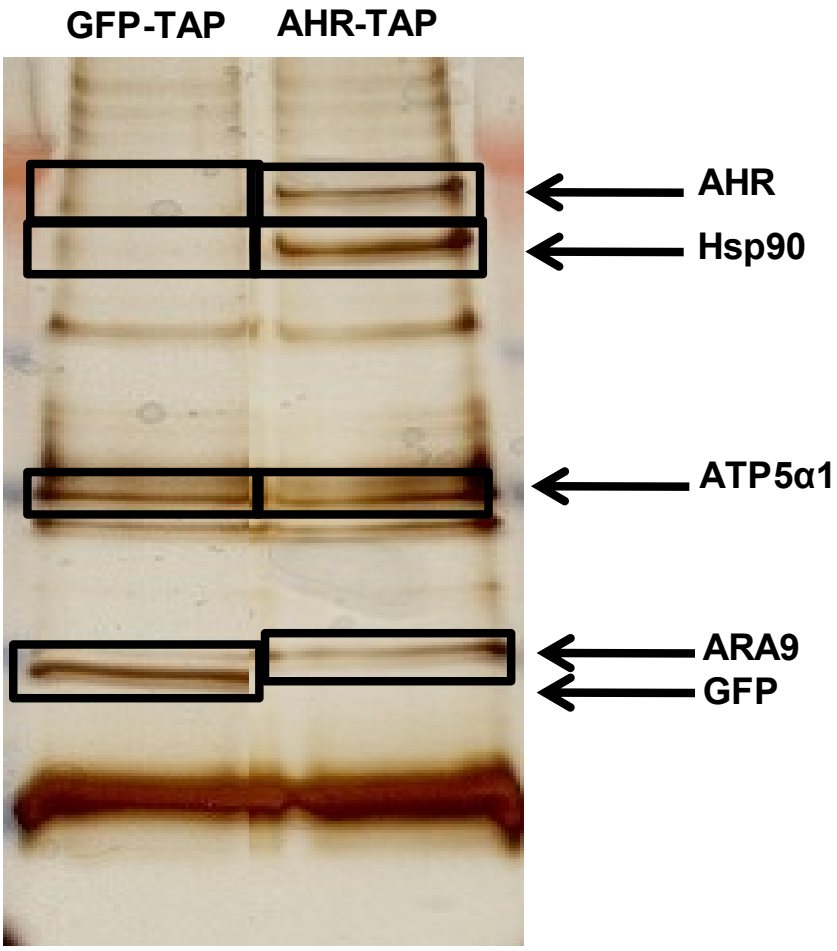
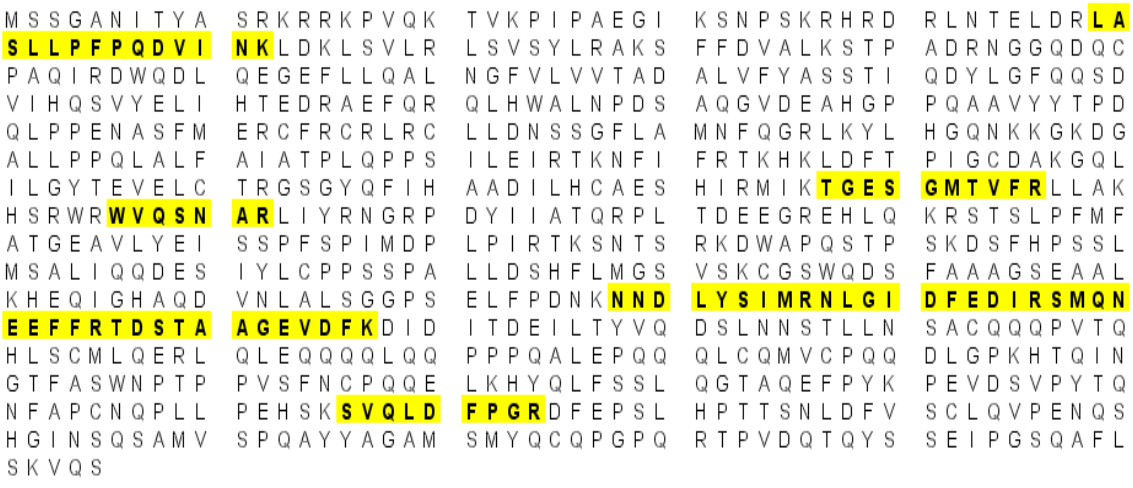

GFP-TAP
AHR-TAP
AHR
Hsp90
ATP5α1
ARA9
GFP

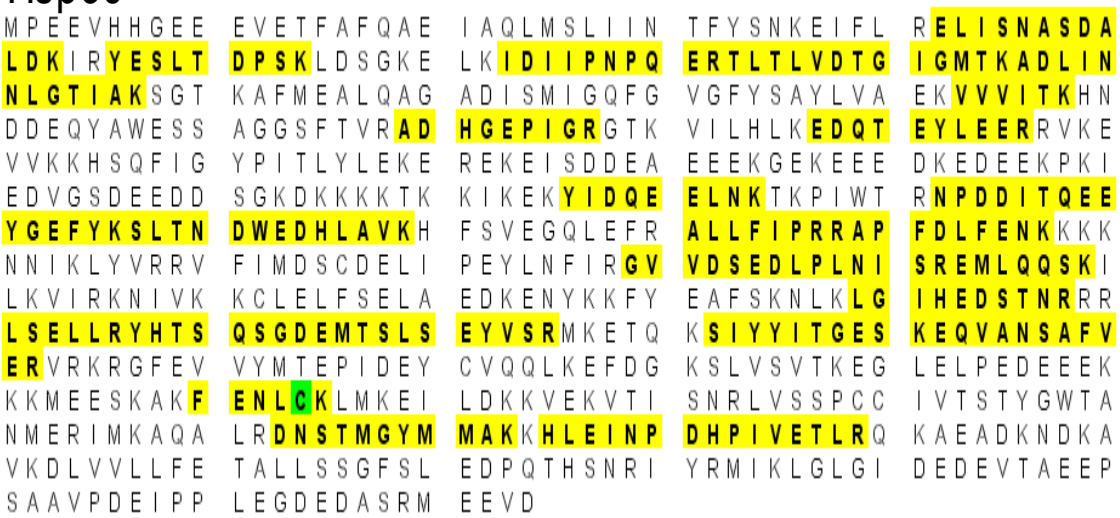
Figure 3.4 MS Data: Sequence coverage of Protein Hits

MS identification of the AHR and cytosolic complex proteins, Hsp90 and ARA9. Highlighted regions show unique identified peptide sequences. AHR, Hsp90, and ARA9 were identified at a >95% confidence using the ProteinProfit algorithm of Scaffold Software.

AHR



Hsp90



ARA9

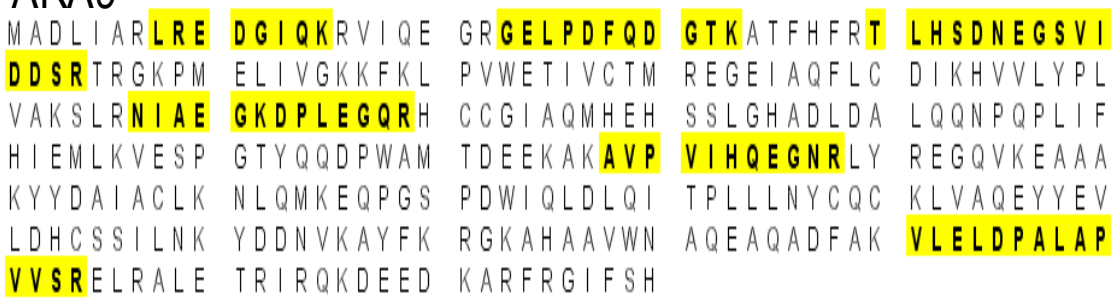
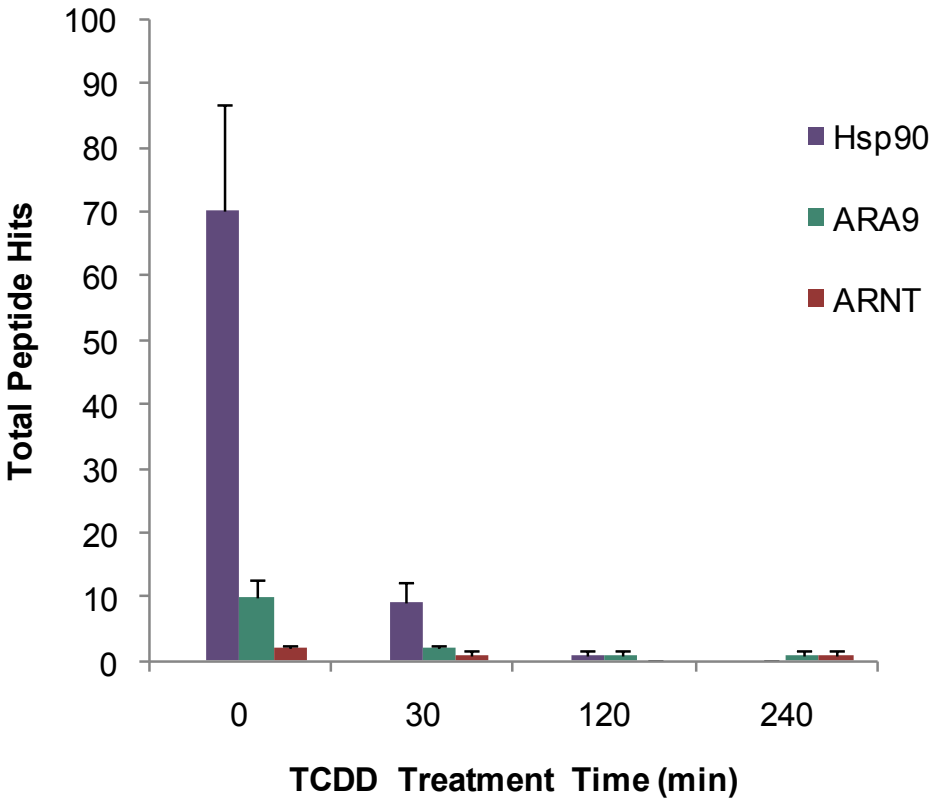


Figure 3.5 Total Number of Identified Peptides of Known AHR Interactors

Graph depicts total numbers of peptides identified by mass spectrometry of Hsp90 (**purple bars**), ARA9 (**green bars**) and ARNT (**red bars**) after TAP of the receptor from mAHR-TAP expressing Hepa!c1c7. Total peptides from each protein were determined in the absence (DMSO, vehicle 0 time point) or presence of TCDD (10 nM, for 30, 60, and 120 minutes).


100
90
80
70
60
50
40
30
20
10
0
Total Peptide Hits
0
30
120
240
TCDD Treatment Time (min)
Hsp90
ARA9
ARNT

AHR-/- nor the GFP-TAP expressing AHR-/- MEF cell line showed induction of CYP1A1.

3.2 Identification of the AHR-PIN

The AHR-PIN was constructed to investigate the role of protein interactions and secondary signaling plays in AHR biology. The influence of TCDD treatment and length of exposure on the AHR-PIN was investigated. Ultimately, four AHR-PINs have been constructed reflecting similarities and differences in PINs between vehicle treatment and three different TCDD exposure time points of 30, 120, 240 mins.

A. Identification of AHR complex proteins

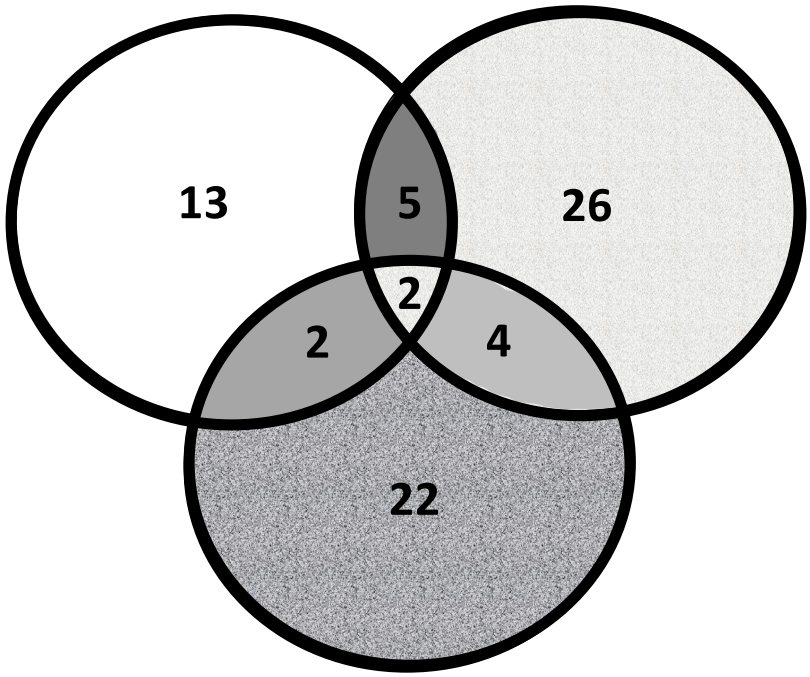
Initially, the AHR-TAP and GFP-TAP expressing Hepa1c1c7 cell strains were used to verify the specificity of known AHR-interacting proteins, Hsp90 and ARA9. TAP of the AHR, followed by MS, identified the AHR, Hsp90, and ARA9 at the correct molecular weights only in AHR-TAP expressing cells (Fig. 3.3). These known interactors were indentified with greater than 95% confidence using MASCOT and Scaffold data analysis software (Fig. 3.4). In time course experimental data sets the number of Hsp90 and ARA9 peptides decreased indicating these interactions were lost. This is in accordance with disassociation of the AHR from its cytosolic complex in the presence of ligand (Fig. 3.5).

B. Identification of Novel AHR Interactors

Novel AHR interactors were identified as follows. Replicated TAP-tag and MS experiments were performed on AHR-TAP Hepa1c1c7 cells dosed with TCDD (10nM) or DMSO (0.01%), as well as on GFP-TAP Hepa1c1c7 cells treated with DMSO (0.1%). Data sets from three independent experiments were then compared and proteins

Fig 3.6 Protein identified in Vehicle treated mAHR-TAP expressing Hepa1c1c7 cells

Venn diagram of proteins identified by mass spectrometry from 3 independent experiments from vehicle (DMSO,0.01%) treated mAHR-TAP expressing Hepa1c1c7 cells.



DMSO Dose Control

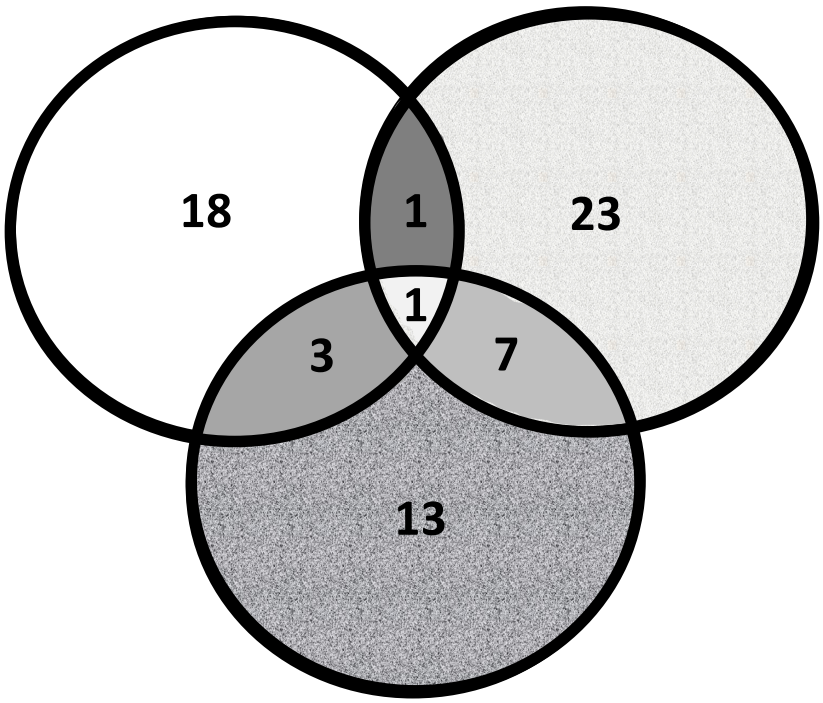
Table 3.1 AHR No Dose Sample Common Proteins

Proteins identified in two out of three data sets. Bold type with * denotes a protein represented in all three data sets. Italicized type denotes nonspecific proteins identified in both AHR-TAP and GFP-TAP samples

Protein	Official Symbol	Entrez Number
Aryl-hydrocarbon receptor-interacting protein *	**Aip**	**11632**
Heat shock protein 1, alpha *	**Hsp90aa1**	**15519**
Heat shock protein 1, beta	Hsp90ab1	15516
ATP synthase F1 complex, alpha subunit	Atp5a1	11946
Mitochondrial ribosomal protein L40	Mrpl40	18100
Cardiotrophin-like cytokine factor 1	Clcf1	56708
Tesp4 protein	1810049H19Rik	435889
HIV TAT specific factor 1	Htatsf1	72459
Beta actin	*ACTB*	*60*
Hippocalcin-like 1	*Hpcal1*	*53602*
Transferrin receptor	*Tfrc*	*22042*
Tubulin	*Tubb5*	*22154*
Interferon gamma inducing factor	*Il18*	*16173*
Unnamed protein product	*A430108E01Rik*	*384382*

Fig 3.7 Proteins identified following tandem affinity purification of the AHR following TCDD exposure – 30 minutes

Venn diagram of proteins identified by mass spectrometry in 3 independent experiments or purification of proteins associated with AHR-TAP following 30 min exposure to TCDD (10 nM).



30min Dose

Table 3.2 Protein identified in Vehicle treated mAHR-TAP expressing Hepa1c1c7 cells

Proteins identified in two out of three data sets. Bold type with * denotes a protein represented in all three data sets. Italicized type denotes nonspecific proteins identified in both AHR-TAP and GFP-TAP samples

Protein	Official Symbol	Entrez Number
Aryl-hydrocarbon receptor-interacting protein *	**Aip**	**11632**
heat shock protein 1, beta	Hsp90ab1	15516
cAMP responsive element binding *protein* 3-like 3	Creb3l3	208677
Enhancer-trap-locus-1	Smarcad1	13990
mitochondrial ribosomal protein L40	Mrpl40	18100
cardiotrophin-like cytokine factor 1	Clcf1	56708
HIV TAT specific factor 1	Htatsf1	72459
Eukaryotic translation elongation factor 1 alpha 1	Eef1a1	13627
hippocalcin-like 1	*Hpcal1*	*53602*
unnamed protein product	*A430108E01Rik*	*384382*
interferon gamma inducing factor	*Il18*	*16173*
Tubulin	*Tubb5*	*22154*

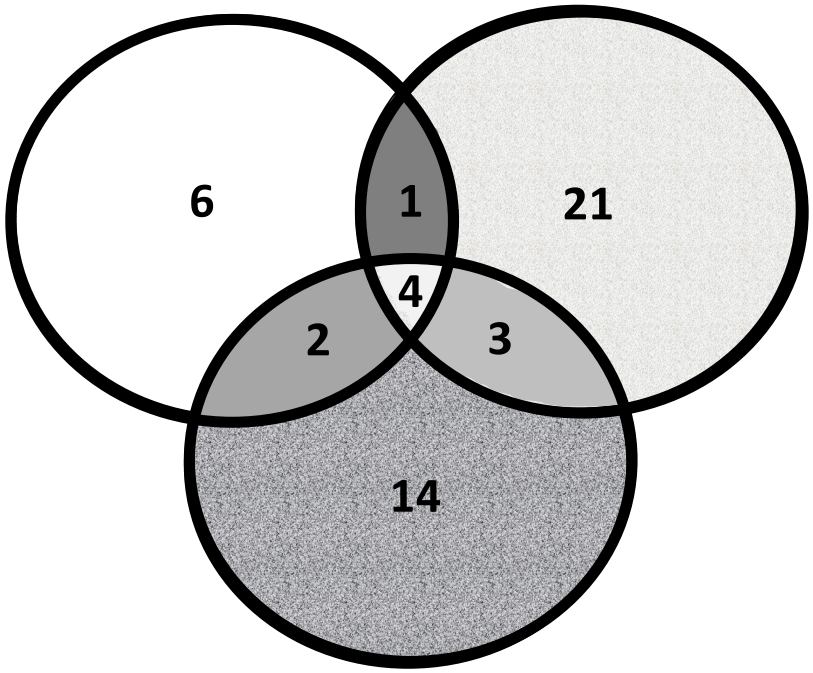
common in two out the three data sets were included in the AHR-PIN reported here. The confidence level of MS protein identification was set at 80%. This cutoff was arrived at to include proteins which were identified at greater 95% confidence levels in one replicate sample, but at a lower level in another replicate sample.

TCDD or vehicle treated AHR-TAP Hepa1c1c7 samples were analyzed for AHR protein interactors. In the DMSO-treated AHR-TAP samples, thirteen proteins were identified in at least two out of three experiments at a greater than 80% confidence level (Fig 3.6). Three of these proteins are the above mentioned Hsp90α, Hsp90β and ARA9. Five of these, ATP synthase F1 complex, alpha subunit (ATP5α1), mitochondrial ribosomal protein L40 (Mrpl40), cardiotrophin-like cytokine factor 1 (Clcf1), Tesp4 protein, and HIV TAT specific factor 1 (Htatsf1), were previously unreported AHR interactors (Table 3.1). The remaining six proteins, β-actin (ACTB), hippocalcin-like 1 (Hpcal1), transferring receptor (Tfrc), tubulin (Tubb5), interferon gamma inducing factor (Il18), and A430108E01Rik were common to both the AHR-TAP and GFP-TAP data set and considered non-specific interactors.

The 30 min TCDD treated AHR-TAP samples contained twelve proteins indentified in at least two out of three experiments at a greater than 80% confidence level (Fig 3.7) Two of these proteins were ARA9 and Hsp90β. Six proteins were previously unreported AHR interactors; three were also found in the vehicle treated data set, Mrpl40, Clcf1, and Htatsf1. There were three unique proteins in this TCDD treatment group, cAMP responsive element binding *protein* 3-like 3 (Creb3l3), enhancer-trap-locus-1(Smarcad1), eukaryotic translation elongation factor 1 alpha 1 (Eef1a1). The remaining four proteins, Hpcal1, Il18, unnamed protein product and

Fig 3.8 Proteins identified following tandem affinity purification of the AHR following TCDD exposure – 120 minutes

Venn diagram of proteins identified by mass spectrometry in 3 independent experiments or purification of proteins associated with AHR-TAP following 120 min exposure to TCDD (10 nM)



120min Dose

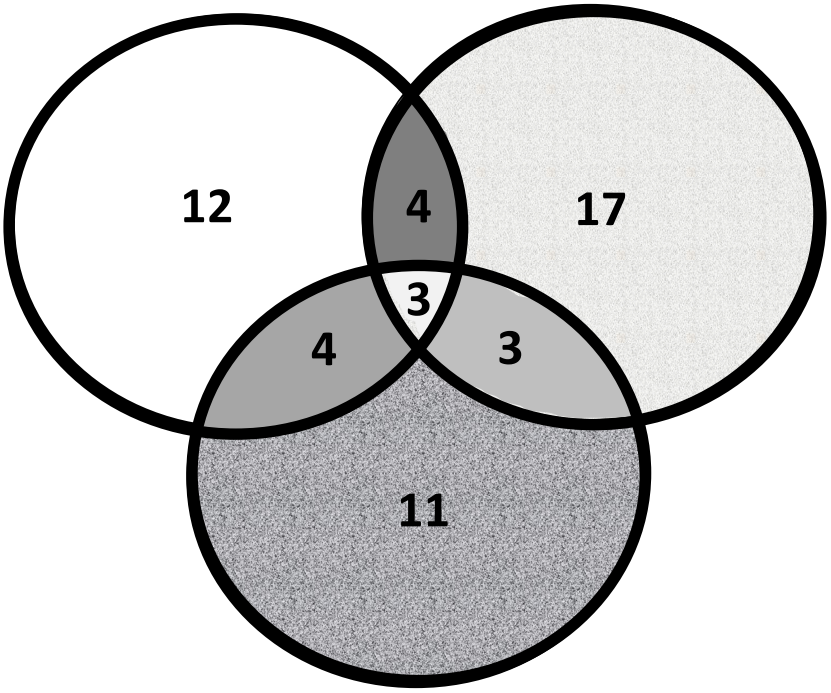
Table 3.3 Proteins identified following tandem affinity purification of the AHR following TCDD exposure – 120 minutes

Proteins identified in two out of three data sets. Bold type with * denotes a protein represented in all three data sets. Italicized type denotes nonspecific proteins identified in both AHR-TAP and GFP-TAP samples

Protein	Official Symbol	Entrez Number
Activated leukocyte cell adhesion molecule CD166 *	**Alcam**	**11658**
Cardiotrophin-like cytokine factor 1 *	**Clcf1**	**56708**
Smarcad1 SWI/SNF-related	Smarcad1	13990
Heat shock protein 1, beta	Hsp90ab1	15516
Eef1a1 eukaryotic translation elongation factor 1 alpha 1	Eef1a1	13627
HIV TAT specific factor 1	Htatsf1	72459
Hypothetical protein LOC56279		56279
Interferon gamma inducing factor	*Il18*	*16173*
Unnamed protein *	***A430108E01Rik***	***384382***
Hippocalcin-like 1 *	***Hpcal1***	***53602***

Fig 3.9 Proteins identified following tandem affinity purification of the AHR following TCDD exposure – 240 minutes

Venn diagram of proteins identified by mass spectrometry in 3 independent experiments or purification of proteins associated with AHR-TAP following 240 min exposure to TCDD (10 nM)



240min Dose

Table 3.4 Proteins identified following tandem affinity purification of the AHR following TCDD exposure – 240 minutes

Proteins identified in two out of three data sets. Bold type with * denotes a protein represented in all three data sets. Italicized type denotes nonspecific proteins identified in both AHR-TAP and GFP-TAP samples

Protein	Official Symbol	Entrez Number
Activated leukocyte cell adhesion molecule CD166 *	**Alcam**	**11658**
Mitochondrial ribosomal protein L40	Mrpl40	18100
Bcl2-associated athanogene 3	Bag3	29810
Cardiotrophin-like cytokine factor 1	Clcf1	56708
Smarcad1 SWI/SNF-related	Smarcad1	13990
Development and differentiation enhancing factor 2	Ddef2	211914
Ubiquitin associated protein 2-like	Ubap2l	74383
HIV TAT specific factor 1	Htatsf1	72459
Eef1a1 eukaryotic translation elongation factor 1 alpha 1	Eef1a1	13627
Hypothetical protein LOC56279		56279
Alpha-tubulin isotype M-alpha-2	*Tuba1b*	*22143*
Unnamed protein *	***A430108E01Rik***	***384382***
Hippocalcin-like 1 *	***Hpcal1***	***53602***
interferon gamma inducing factor	*Il18*	*16173*

Tubb5 were non-specific interactors identified in both AHR-TAP and GFP-TAP data sets (Table 3.2).

The 120 min TCDD treated AHR-TAP samples contained ten proteins indentified in at least two out of three experiments at a greater than 80% confidence level (Fig 3.8). Only one AHR cytosolic complex protein was found in these data, Hsp90β. Six of these proteins were exclusively in the AHR-TAP data sets: activated leukocyte cell adhesion molecule CD166 (Alcam), Clcf1, Smarcad1, Eef1a1, Htatsf1, and hypothetical protein LOC56279. Of this set only one, Clcf1, was also found in the vehicle treated data sets. The three remaining proteins, Hpcal1, Il18, and A430108E01Rik were, again, non-specific interactors (Table 3.3).

Following exposure to TCDD for 240 minutes, AHR-TAP was shown to interact with fourteen proteins in at least two out of three experiments at a greater than 80% confidence level (Fig 3.9). Ten of the identified proteins in this group were novel AHR interactors. Two were common to the vehicle control data set, Mrpl40 and Clcf 1. Of the eight novel AHR interacting proteins identified five, Alcam, Smarcad1, Htatsf1, Eef1a1, hypothetical protein LOC56279, appear in the 30 and/or 120min time point mentioned above. Three proteins, Bcl2-associated athanogene 3 (Bag3), ArfGAP with SH3 domain, ankyrin repeat and PH domain 2 (Asap2), and ubiquitin associated protein 2-like (Ubap2l) are unique to this 240 min TCDD exposure sample. The non-specific interactors of this group are alpha-tubulin isotype M-alpha-2 (Tuba1b), Hpcal1, A430108E01Rik, and Il18 (Table 3.4).

C. Co-immunoprecipitation verification of identified interactors

Independent verification of interactions between the AHR and select proteins identified by mass spectrometry was performed by co-immunoprecipitation in wild type

Figure 3.10 Independent Protein Interaction Verification using Co-immunoprecipitation

Endogenous AHR was isolated from wild type Hepa1c1c7 (**Hepa WT**) and CH12.LX cells via co-immunoprecipitation (Co-IP). Precipitate from an AHR specific antibody or normal mouse IgG (negative control) as precipitation agents was subsequently probed with specific antibodies against MRPL40 (**A**), -ASAP2 (**B**), -p53 (**C**)., or AHR (positive control, **D**)a

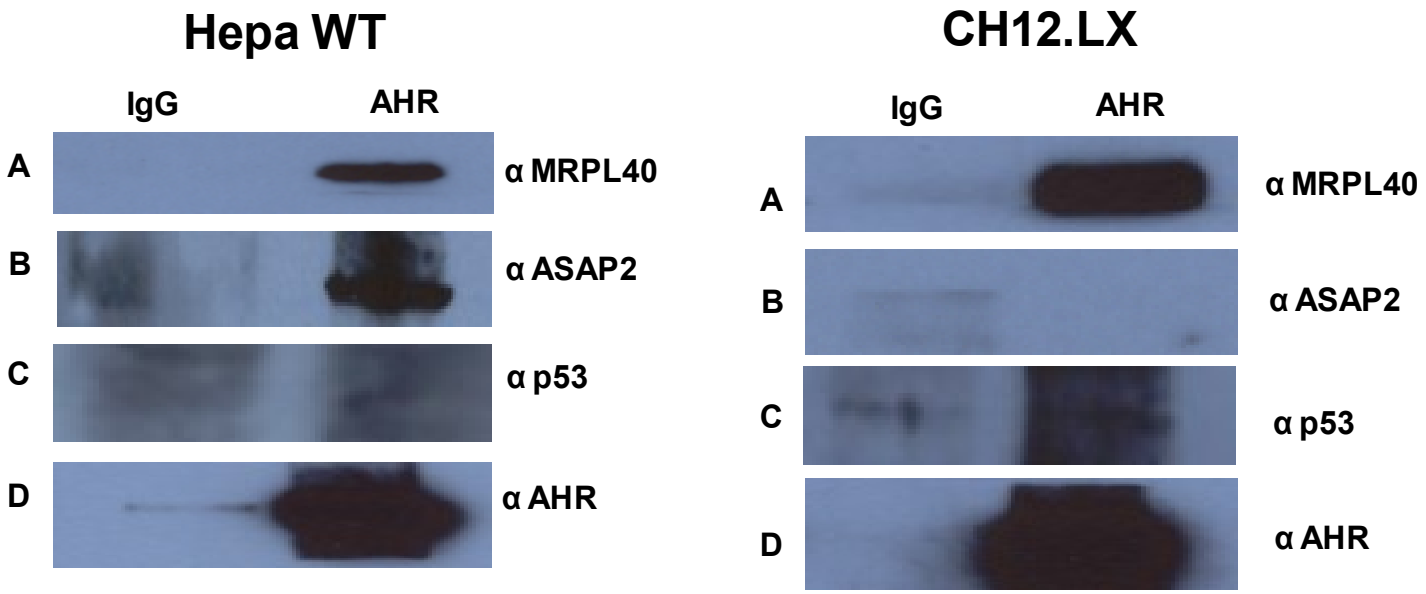

Hepa WT
IgG
AHR
A
α MRPL40
B
α ASAP2
C
α p53
D
α AHR
CH12.LX
IgG
AHR
A
α MRPL40
B
α ASAP2
C
α p53
D
α AHR

Figure 3.11 Identification of ATP5α1 in vehicle treated AHR-TAP expressing Hepa1c1c7 cells.

Following TAP, proteins were separated via SDS-PAGE and Silver stained. One gel fragment (box, A) was subjected to tryptic digestion and MS identification. One protein identified in the fragment was ATP5a1.

A

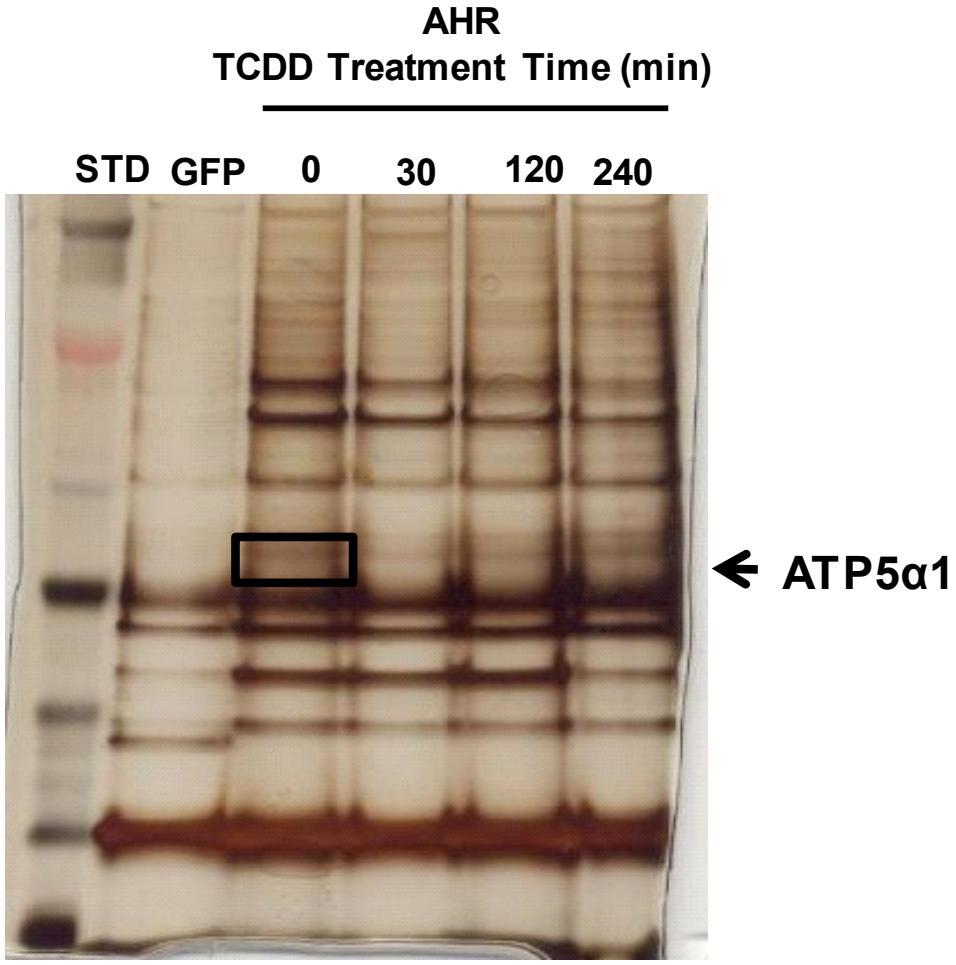
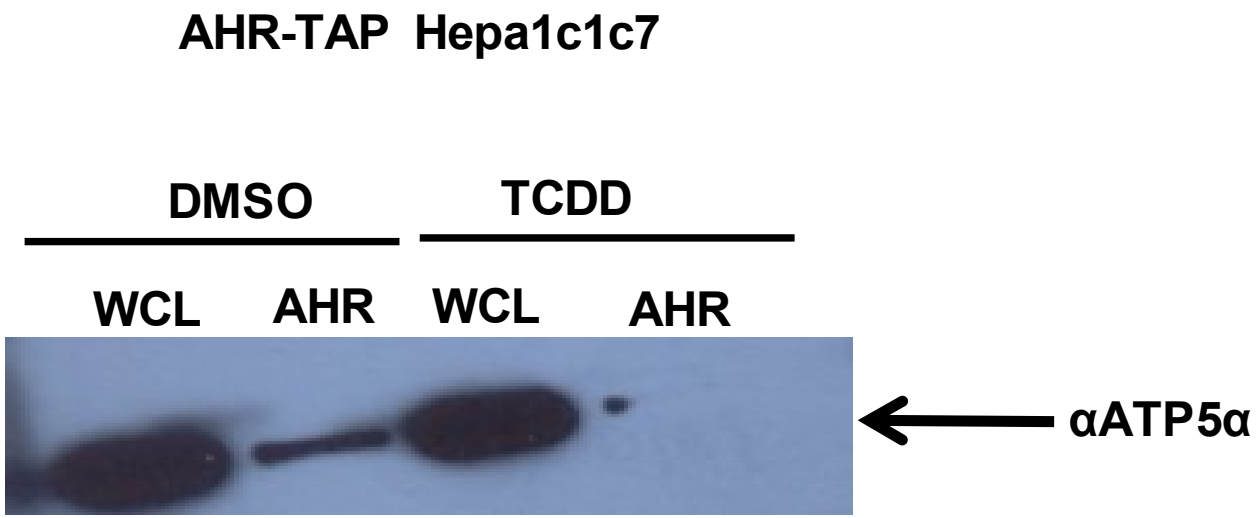


Figure 3.12 The Effect of TCDD Exposure on the Interaction between ATP5a1 and the AHR

Extracts from DMSO or TCDD treated AHR-TAP expressing Hepa1c1c7 cells were immunoprecipitated with an AHR specific antibody. The resulting precipitate was probed with an ATP5α1 specific antibody. WCL= whole cell lysate, AHR= anti-AHR antibody pulldown.


AHR-TAP Hepa1c1c7
DMSO
TCDD
WCL
AHR
WCL
AHR
αATP5α

Hepa1c1c7 and CH12.LX B-cells. Similar to previously reported verification, this method was used to investigate the interaction between the AHR and Asap2, Mrpl40, and p53 (Fig 3.10). Interestingly, a protein interaction between the AHR and p53 was identified by mass spectrometry, in one TCDD treatment data set. Given the previously reported evidence of a role for the AHR in cell cycle regulation and apoptosis, p53 was included in the independent verification [104, 105, 131, 132]. These interactions were reproducible in two of the three trials performed. In both cell lines, Mrpl40 has significant enrichment in the AHR pull down sample over the control. Asap2, however, showed marked enrichment in the AHR Co-IP sample over the normal mouse IgG control in the Hepa cell line, but showed no association in the CH12.LX cells. The AHR/p53 result demonstrates only a minimal enrichment over the IgG control sample in both cell lines. The variability in these results may indicate a weak or transient interaction with the AHR. The functional consequence of these interactions is currently under investigation in our lab.

3.3 AHR Influence on Mitochondrial Function

A. Identification of ATP5α1 protein interaction with the AHR

A novel interaction between the AHR and ATP5α1 was identified using the TAP/MS method. ATP5α1 is a catalytic subunit of ATP synthase and was identified at a confidence level of greater than 95% using the MASCOT and Scaffold analysis software (Fig. 3.11). This protein interaction suggests a link between the AHR and mitochondrial function. A functional link between the two could mechanistically explain AHR mediated toxic responses to dioxin exposure, such as wasting disease and metabolic diseases such as diabetes. Western blot analysis of the purified AHR complex following TAP

confirmed the identity of ATP5α1 (Fig. 3.12). The interaction between the AHR and ATP5α1 was lost upon TCDD treatment, suggesting that activation of the AHR leads to its dissociation from the ATP synthase complex (Fig. 3.12). Given that this interaction was identified using an over-expression system, co-immunoprecipitations from wild type Hepa1c1c7 cells were performed. The interaction was confirmed at endogenous protein levels (Fig. 3.13A). To verify that the interaction was not specific to Hepa1c1c7 cells, co-immunoprecipitations were performed using the murine B-cell lymphoma CH12.LX line. Again, the AHR/ATP5α1 interaction was confirmed in the mouse B-cell line (Fig. 3.13B). These findings indicated that the AHR:ATP5α1 interaction occurs in more than one tissue type. Given the ATP5 α1 central role in mitochondrial function this interaction was of great interest.

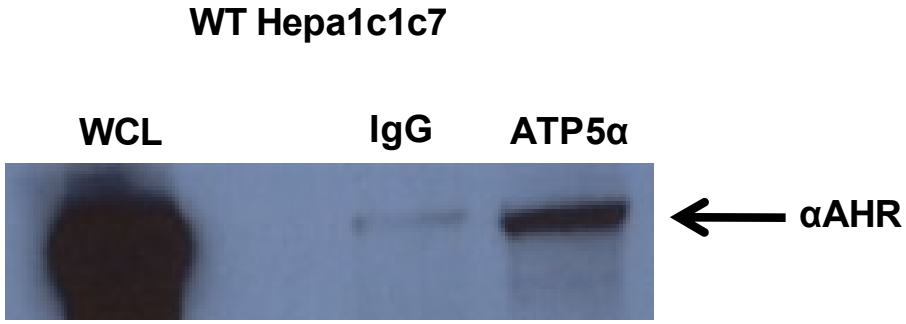
B. AHR mitochondrial localization

As mentioned above, ATP5α1 is part of the multisubunit ATP synthase complex, part of the electron transport chain, and raised the possibility that a portion of the cellular pool of AHR translocated to the mitochondria. To test this hypothesis, cellular fractionation studies were performed. The AHR was co-purified with the mitochondrial specific protein cytochrome c oxidase IV (COX IV), suggesting that some fraction of the total cellular AHR pool does translocate to the mitochondria. ATP5α1 was also observed in the mitochondria, as well as, the cytoplasm, raising the possibility that the AHR:ATP5α1 interaction is not mitochondria-specific. Interestingly, upon TCDD treatment, the amount of AHR localized to the mitochondrial compartment was reduced (Fig 3.14).

Figure 3.13 Co-immunoprecipitation Verification of the AHR/ATP5α1 Interaction

Extracts from WT Hepa1c17 (A) or CH12.LX (B) cells were incubated with an anti-ATP5α1antibody or mouse normal IgG (negative control) for 90 mins. Protein A beads were added to the samples and incubated for an additional 90 mins. Beads were washed and samples eluted from beads with warm SDS. Western blot analysis was performed on isolated protein samples with an anti-AHR antibody. WCL= whole cell lysate.

A



B

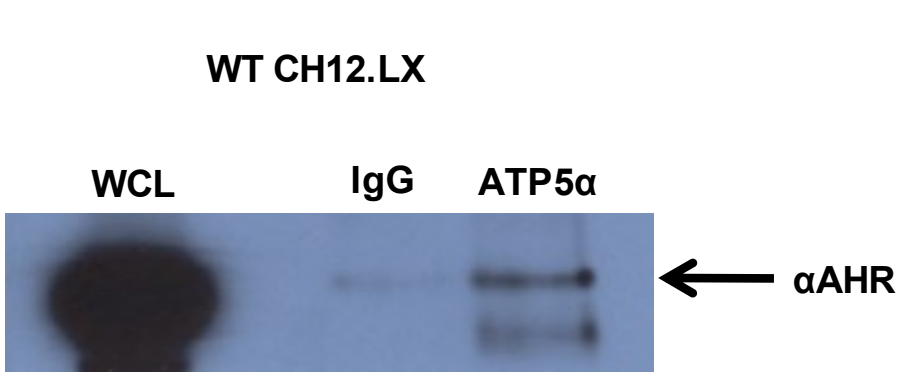
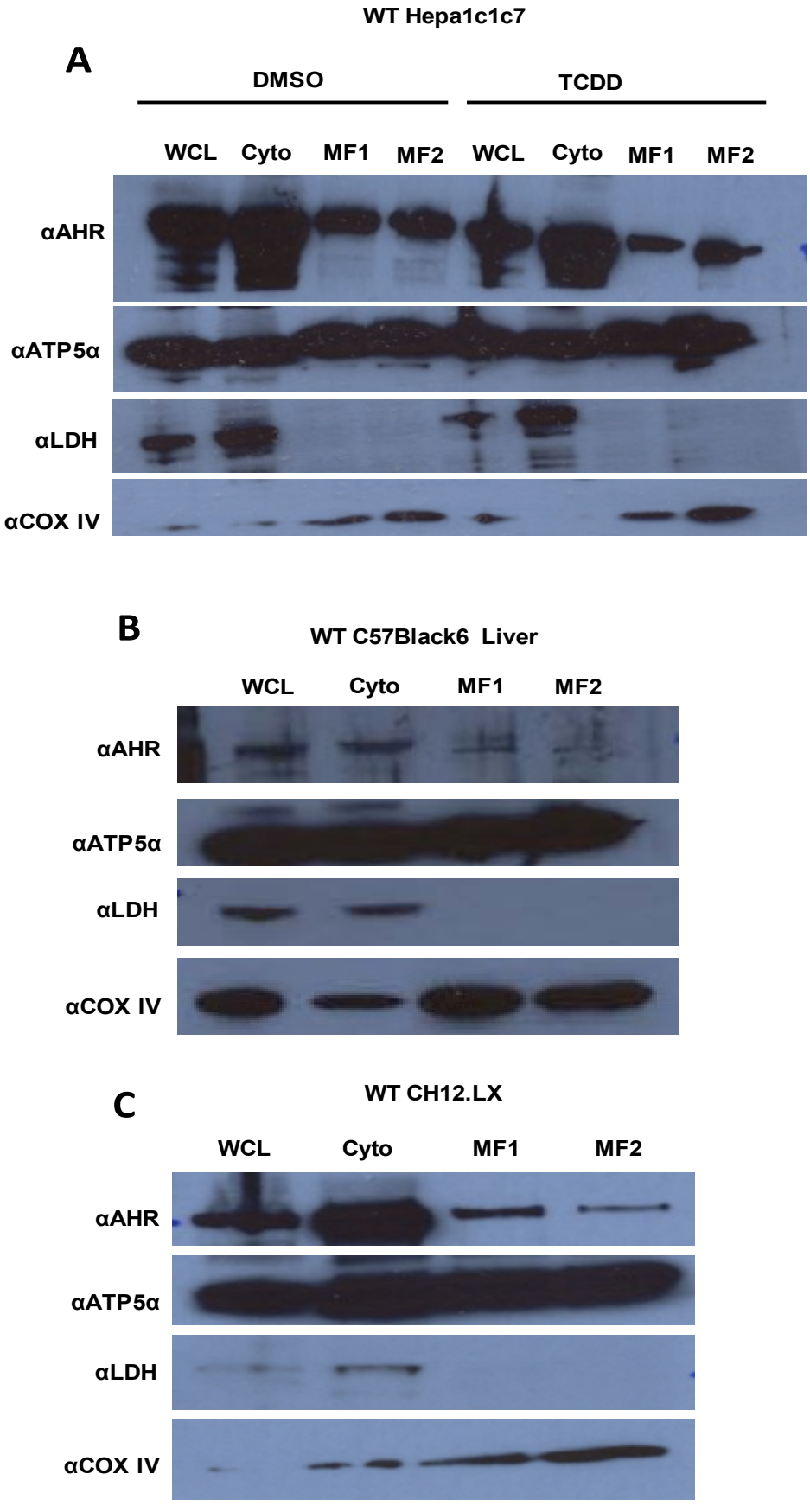


Fig. 3.14 AHR Mitochondrial Co-Localization

Cell fractionation was performed on WT Hepa1c1c7 following DMSO or TCDD (**A**), liver samples from a C57Bl/6 liver (**B**), and CH12.LX (**C**)were fractionated by differential centrifugation. Whole cell lysates (**WCL**), cytosolic (**Cyto**), crude mitochondrial (**MF1**), and pure mitochondrial (**MF2**) fractions were obtained and analyzed by Western blot using an AHR specific antibody (**top panel**), anti-ATP5α1 (**second panel from top**), anti-lactate dehydrogenase (**LDH, third panel from top cytosolic specific**), and anti-Cytochrome C oxidase subunit IV (**COXIV, bottom panel, mitochondrial specific**).


WT Hepa1c1c7
A
DMSO
TCDD
WCL
Cyto
MF1
MF2
WCL
Cyto
MF1
MF2
αAHR
αATP5α
αLDH
αCOX IV
B
WT C57Black6 Liver
WCL
Cyto
MF1
MF2
αAHR
αATP5α
αLDH
αCOX IV
C
WT CH12.LX
WCL
Cyto
MF1
MF2
αAHR
αATP5α
αLDH
αCOX IV

C. AHR influence on mitochondrial function

To investigate the functional relevance of AHR: ATP5α1 protein interactions two murine B-cell lymphoma lines, CH12.LX (AHR+/+) and BCL1 (AHR-/-) cells were employed. These two cell lines were chosen to observe the effect TCDD had on mitochondrial function in the presence and absences of the AHR. The mitochondrial inner membrane potential of these cell lines was indirectly measured using tetramethylrhodamine methyl ester (TMRM) and flow cytometry (Fig 3.15). The level of red fluorescences emitted by the TMRM was used as an indirect measure of membranepotential and normalized to total number of mitochondria using mito-tracker green (MTG). In CH12.LX cells, a concentration-dependent hyperpolarization of the mitochondrial inner membrane was observed with TCDD treatment, when compared to the vehicle control. Interestingly, BCL1 cells demonstrates a basal inner membrane polarization that was 1.5-fold higher in magnitude compared to the basal level observed in CH12.LX cells (Fig 3.16). These results indicate the AHR has a role in maintaining a lower inner membrane potential. The AHR may act to facilitate mitochondrial function and homeostasis of cellular energy levels through its association with the ATP synthase complex. Moreover, results indicate TCDD was capable of disrupting this role.

To elucidate what influence transcription may have in the AHR-dependent TCDD-induced hyperpolarization of the mitochondrial inner membrane, the transcriptional inhibitor mitomycin c was used. Using CH12.LX cells the TMRM experiments were repeated after pretreatment with mitomycin c. Hyperpolarization of the inner membrane was again observed at the same elevated 3.5-4 fold level in the 30 nM TCDD treated cells when compared to the polarization levels in the vehicle control

cells. These results further suggest that the AHR:ATP5α1 protein interaction may have a role in maintaining mitochondrial function in the absence of AHR-mediated transcription (Fig 3.17).

To evaluate the effect of the inner membrane hyperpolarization on cellular energetics, ATP levels were examined. In the CH12.LX cells, only a moderate decrease in ATP levels was detected upon TCDD (30 nM, 6hrs) treatment (Fig 3.18). The same decrease was also observed in the vehicle treated cells. Given that the inner membrane had a higher potential and also a slight decrease in ATP, these data suggest that the TCDD-induced membrane hyperpolarization serves to maintain cellular energy levels. Moreover, the hyperpolarization event corresponds to a decrease in the mitochondrial pool of the AHR, suggesting that AHR plays a role in maintaining the efficiency of mitochondrial energetics.

Figure 3.15 The Effect of TCDD Exposure on Mitochondrial Inner-membrane Hyperpolarization

Flow cytometric analysis of mitochondrial membrane potential was performed on CH12.LX (AHR+/+) cells exposed to DMSO (0.01% vehicle control, shaded area) or TCDD (3, 10, and 30 nM) for 6h. CCCP, a mitochondria uncoupler, was included as a positive control. Increased TMRM fluorescence correlates to a hyperpolarization of the mitochondrial inner membrane potential.

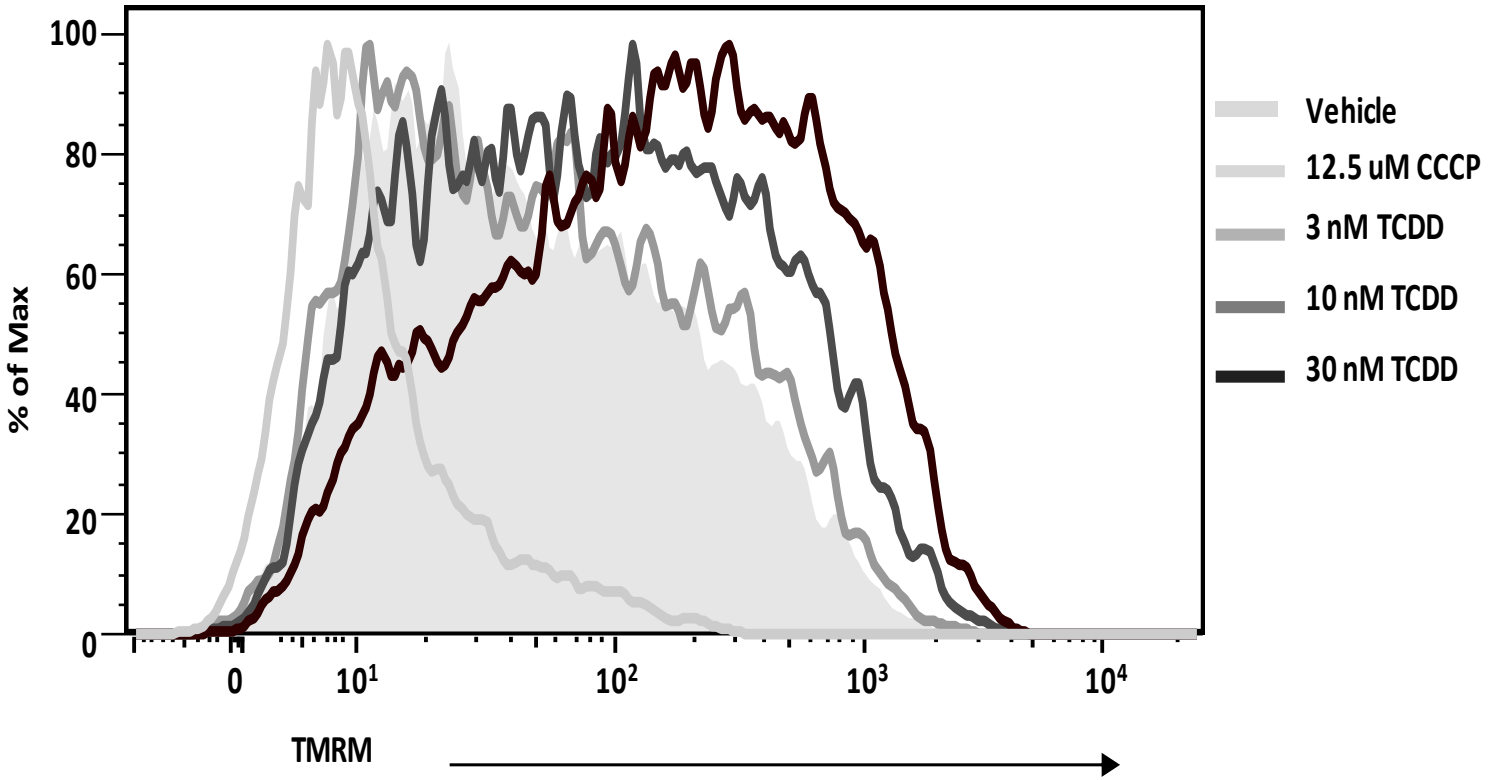

100
80
60
40
20
0
% of Max
0
10^1
10^2
10^3
10^4
TMRM
Vehicle
12.5 uM CCCP
3 nM TCDD
10 nM TCDD
30 nM TCDD

Figure 3.16 TCDD Influence on Membrane Polarization is Mediated by the AHR

CH12.LX (AHR +/+) and BCL1 (AHR-/-) B-cell lines were treated with vehicle (DMSO, white bars), CCCP (negative control, black bars), or 3 (gray bars), 10 (medium gray bars), or 30 (dark gray bars) nM TCDD for 6 hrs. Membrane polarization was assessed using TMRM and the signal was normalized to mitotraker green (MTG). Samples were analyzed by flow cytometry. The value of DMSO treated CH12.LX cells was set to 1. Values are the average of four independent experiments and SEM are represented.

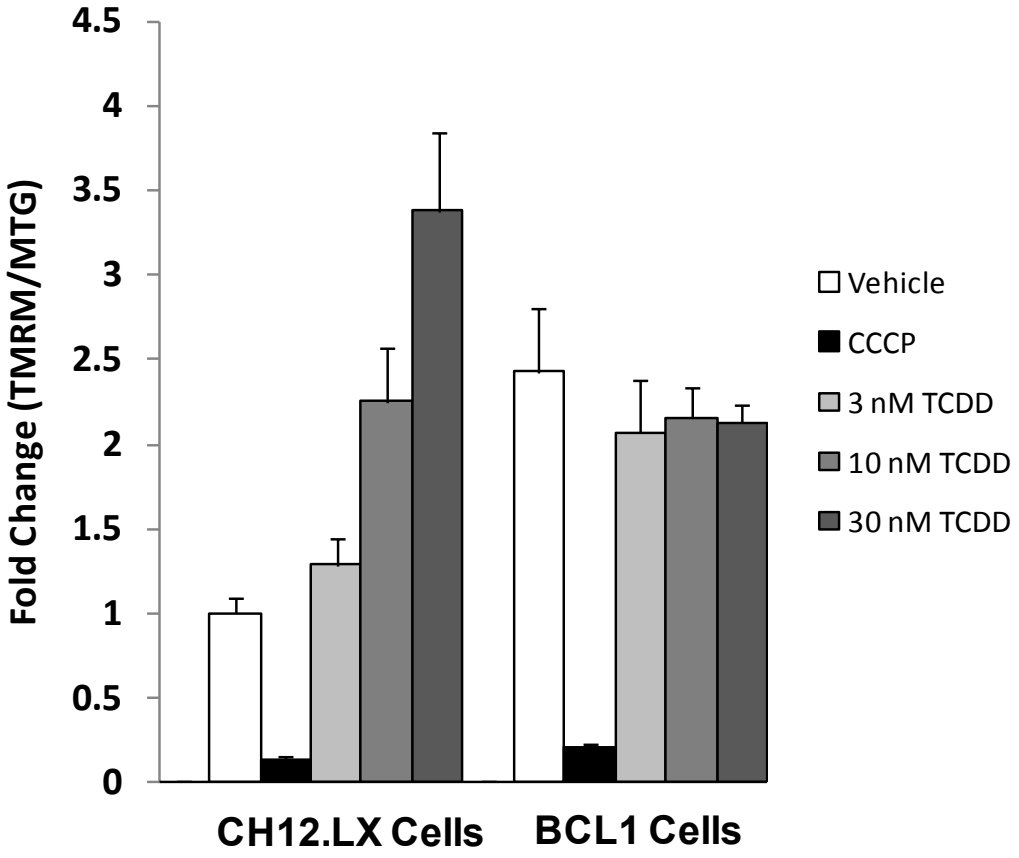

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Fold Change (TMRM/MTG)
CH12.LX Cells
BCL1 Cells
Vehicle
CCCP
3 nM TCDD
10 nM TCDD
30 nM TCDD

Figure 3.17 TCDD Influence on Membrane Polarization is Mediated by the AHR and Independent of Transcription

CH12.LX (AHR +/+) B-cell line was left untreated (white bars) or were dosed with CCCP (negative control, black bars), vehicle (DMSO, light gray bars), or 30 nM TCDD (medium gray bars) for 6 hrs, in the presence and absence of transcriptional inhibitor mitomycin c. Membrane polarization was assessed using TMRM and the signal was normalized to mitotraker green (MTG). Samples were analyzed by flow cytometry. The value of DMSO treated CH12.LX cells was set to 1. Values are the average of four independent experiments and SEM are represented.

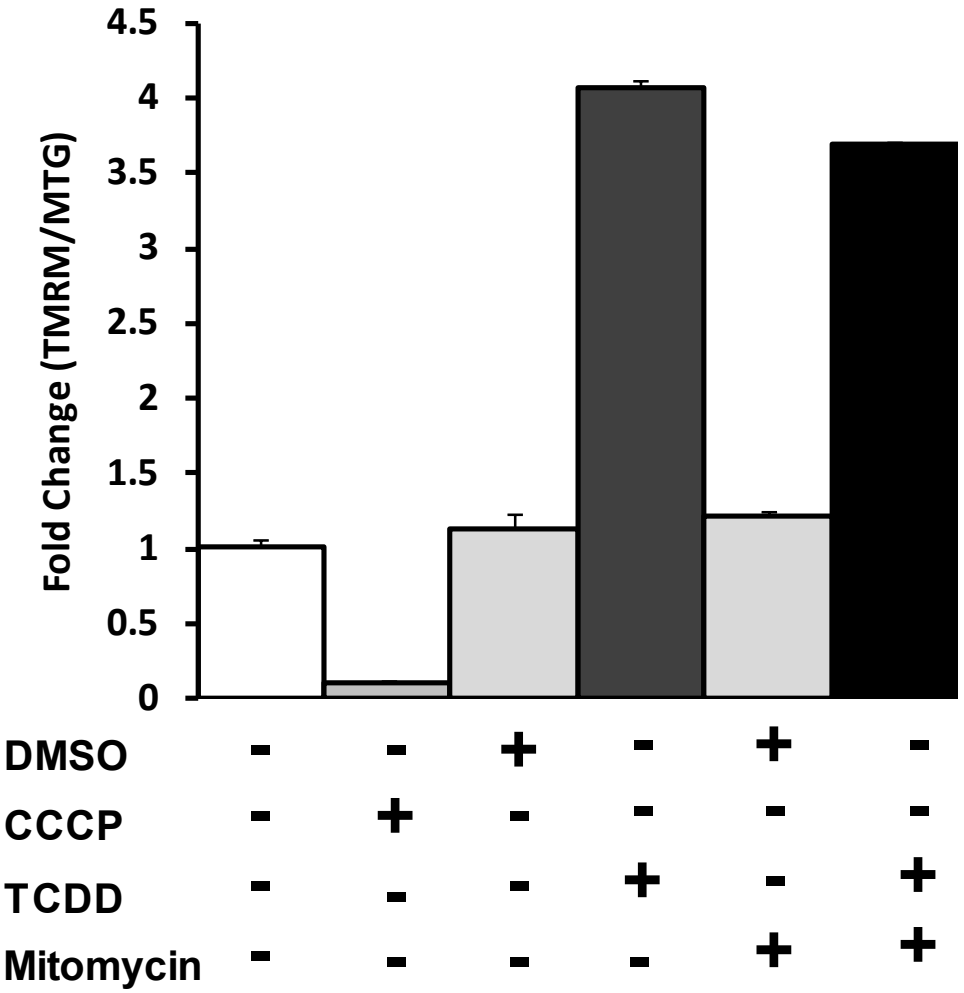
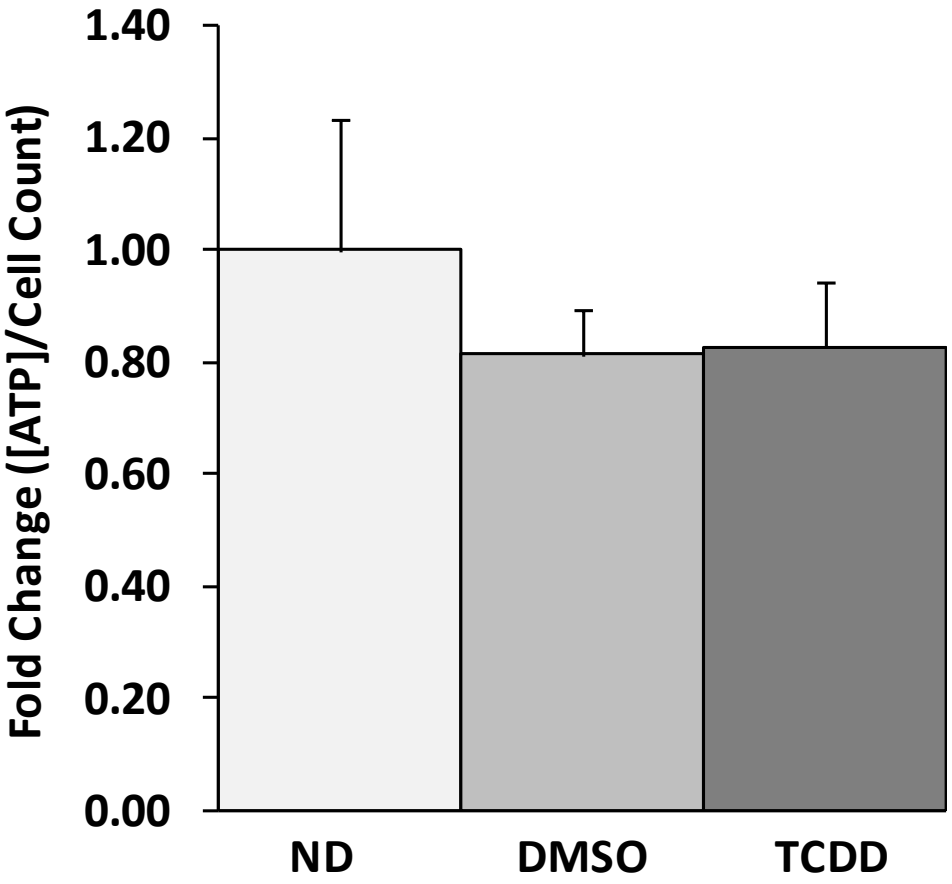

Fold Change (TMRM/MTG)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
DMSO - - + - + -
CCCP - + - - - -
TCDD - - - + - +
Mitomycin - - - - + +

Figure 3.18 TCDD Effect on ATP Levels

ATP levels were measured in three dosing groups of CH12.LX cells, naive (ND), vehicle (DMSO), and TCDD (30 nM). The cells were treated for 6 hrs, harvested, and counted. ATP levels where determined by luciferase assay. The ATP levels were normalized to the cell counts and ND value was set to 1.


Fold Change ([ATP]/Cell Count)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
ND
DMSO
TCDD

DISCUSSION

4.1 Identification of the AHR-PIN

The AHR is important for sensing environmental pollutants and regulating developmental processes. Identifying interacting partners for the AHR, therefore, is important to our understanding the dioxin-induced changes in signaling pathways involved in these processes. The research detailed here represents the first comprehensive analysis of the AHR-PIN in a mammalian system using proteomic technology. The AHR-PIN reveals physical interactions with proteins involved in several cellular processes and disease states with which the receptor has been previously linked. These include; cell cycle, apoptosis, immune response, mitochondrial function, and cancer [103, 105, 133-136]. In addition to AHR gene regulation, this data suggests the receptors potential influence on cellular biology through protein/protein interactions and signaling pathways.

Tandem affinity purification (TAP) was used to identify the AHR:ATP5α1 interaction. This interaction led to the demonstration of an AHR-dependent hyperpolarization of the mitochondrial inner membrane that was independent of transcription. These results indicate a role for the AHR in mitochondrial function and cellular energetics. In addition to the interaction between the AHR and ATP5a1, three other mitochondrial associated proteins have been identified. The first is a mitochondrial large ribosomal protein known as Mrpl40. In yeast, this protein has been shown to play a role in growth rate, mitochondrial protein folding, and mitochondrial function [137, 138]. In humans, the Mrpl40 gene is part of a chromosomal deletion of 22q11 in Velo–cardio–facial syndrome (VCFS) and DiGeorge syndrome [139, 140].

Interestingly, these diseases present with heart defects and immune deficiencies. AHR null mice have been shown to have abnormal heart development and the immune system is highly sensitive to TCDD-induced toxicity [111, 141, 142]. Bag3, the second mitochondrial associated protein identified, is a co-chaperone protein involved in the stress response and disease [143, 144]. Bag3 is considered a pro-oncoprotein, by inhibiting the apoptotic response though its influence on Bcl-2 family members [145, 146]. Moreover, Bag3 has been shown to influence the NfκB pathway through its interaction with p65. The interaction between Bag3 and p65 has been shown to play a role in the human immunodeficiency virus type 1 (HIV-1) LTR response to NfκB signaling [147]. The physical interactions between Bag3 and the AHR provide an interesting connection between the AHR and cell cycle regulation, apoptosis, and the NfκB pathway, which the AHR has been previously linked to [83]. These AHR protein interactions could indicate other roles for the receptor in cellular energy homeostasis and gene regulation not mediated directly by the receptor's transcriptional activity.

There are other proteins in the AHR-PIN that stand out and further connect the receptor to transcriptional cross-talk. First, Creb3l3, also known as CrebH, functions as an endoplasmic reticulum bound transcription factor responsible for hepatic gluconeogenesis [148]. Second, is Htatsf1, a transcriptional co-factor that regulates expression of the HIV-1 LTR [149]. These AHR protein interactions could indicate an association with a larger protein complex involved in transcription. The third and perhaps least surprising, but novel interaction identified in the AHR-PIN, is the Smarcad helicase protein. Smarcad has been linked to genetic instability and interaction with TRIM28, a transcription factor implicated in cancer [150-152]. Since the AHR is first

considered a transcription factor, identifying its interaction with a helicase is not that unexpected. However, the Smarcad helicase is reported to interact with the SWI/SNF complex [150], this being its first reported interaction with a PAS transcription family member.

Other proteins of interest in the AHR-PIN include two immune system proteins and a GAP protein which provide more connections between the receptor and immune response, gene regulation, and disease states. First, is the receptor's interaction with Alcam, a leukocyte adhesion molecule. Alcam has become widely recognized as a cellular marker for several forms of cancer, including melanoma, squamous cell carcinoma, prostate, breast, colorectal, bladder, oesophageal, and ovarian cancers [153-155]. Second, is the AHR:Clcf1 interaction; Clcf1 is a cytokine, highly expressed in tissues of the immune system and has been reported to be an activator of the Jak, Erk, and MAP kinase pathways [156, 157]. Moreover, Clcf1 also activates a select group of the STAT transcription factors via tyrosine phosphorylation signaling cascades and plays a role in neuronal development [156, 158]. Lastly, the AHR's interaction with Asap2 is of note. Asap2, also known as development and differentiation enhancing factor 1 (Ddef1), is an Arf-GTPase activating protein [159, 160]. It has been shown to work cooperatively with adenomatous polyposis coli (APC), a known tumor suppressor, regulating microtubules and focal adhesions and has been implicated as a drug target in breast cancer [160, 161]. The AHR has been independently linked to cell plasticity, E-cadherin repression and cell motility, and all cellular changes observed in metastasis [103, 108-110]. Here again we observe AHR interactions with signaling pathways that

are involved with disease and influence gene regulation not directly mediated by the AHR, but that the receptor has been previously linked.

A 2004 study conducted using *S. cerevisiae* yeast as a model system reported 54 genes which influence AHR biology [162]. Interestingly, we have identified proteins that demonstrate some correlation with this data. In yeast, SNF12 and SWI3 were shown to influence the AHR and here we report a protein interaction between the AHR and SMAD-CAD, a helicase linked to the SWI/SNF complex. In addition, yeast genes encoding kinase and GTPase proteins where among the 54 genes reported and we observed interactions with Asap2, an ARF-GTPase and Clcf1, a cytokine shown to have a role in kinase signaling. While these similarities are not direct correlations they do provide another layer of evidence to the complexity of AHR biology and the possible mechanisms involved in AHR-mediated toxicity and cellular function

There are previously reported AHR protein interactions that were not observed in the proteomic analysis of the AHR-PIN. The cytosolic complex protein p23 was not identified in any of our MS data. Given the transient nature of its interaction with the AHR its absence is not wholly unexpected. We did not identify Rb or RelA in any of the data sets. These interactions are also of a transient nature making identification problematic and we cannot rule out cell type specific interactions [85, 163]. Interestingly, we did identify Cdk4 in some of our MS data, however this result was not consistent across multiple data sets.

The novel interactions reported here demonstrate potential roles for the AHR in signaling pathways which may function in concert with AHR-mediated gene regulation in dioxin-induced toxicity. While the physical interactions reported here provide another

layer of evidence for the AHR's potential roles in several signaling pathways, disease states and gene regulation events, the functional relevance of these interactions remain in question. This AHR-PIN presents new avenues of investigation to better define a comprehensive understanding of AHR biology.

4.2 AHR Influence on Mitochondrial Function

The xenobiotic-induced toxicity mediated through the AHR is complex in its species- and tissue-specific effects. However, the underlying mechanisms remain poorly defined. This could be due to a lack of an integrative global understanding of the role different cellular processes, such as gene regulation, protein interactions, and cellular energetics, have in the AHR-mediated toxic response. AHR research has described numerous aspects of the receptor's function in regards to xenobiotic responses and development. It has been shown, using nuclear localization signal (NLS) and DNA binding domain (DBD) mutant mice, that nuclear translocation is necessary but not sufficient for TCDD-induced injury [40, 41]. Moreover, knockout mice of the canonical xenobiotic metabolizing enzymes regulated by the AHR, *CYP1A1, 1A2*, and *1B1*, exhibit some protection from xenobiotic-induced toxicity [164-166]. For example, CYP1A1, 1A2, and 1B1 triple knockout mice are protected from benzo(A)pyrene-induced toxicity [167]. These results support the classic view that most TCDD-induced toxicities involve AHR nuclear translocation and transcription. However, they do not rule out the possibility that other AHR-interacting proteins play important roles in the process [101, 163, 168, 169]. For example, cellular localization of the DBD mutant AHR is constitutively nuclear, while mutations in the NLS will disrupt normal compartmental trafficking of the receptor. In addition, the triple knockout mouse

displays aberrant bioavailability of the xenobiotics that will influence the AHR's normal cellular signaling. These results, therefore, do not directly address nor rule out the possibility of secondary effects, mediated by AHR protein interaction, in xenobiotic-induced toxicity. This research sought to identify novel AHR interacting proteins and to correlate their function to changes in TCDD-induced toxicity.

The findings reported here demonstrate a novel link between the AHR and the mitochondrial machinery responsible for cellular energy production. The ATP synthase is a multi-subunit complex, as part of the electron transport chain it is localized to the inner mitochondrial membrane. ATP5α1 is a subunit of the soluble catalytic F1 subunit of this complex. Changes in ATP5α1 levels have been linked to various disease processes, including cancer, and these changes have been suggested to play a role in tumor progression and the Warburg effect [170-173]. The characterization of the interaction between the AHR and ATP5α1 and subsequent changes in mitochondrial function, might link these observations with TCDD-induced tumor promotion [99]. More importantly, the AHR:ATP5α1 interaction might explain previous observations linking TCDD exposure with changes in mitochondrial function and gene expression [132, 135, 174-176]. For example, Senft and colleagues observed an AHR-dependent increase in TCDD-induced H_2O_2 and superoxide production that was independent of Cyp1a1 and Cyp1a2 [176]. In contrast to what was observed in CH12.LX cells, several reports have described a TCDD-induced decrease in mitochondrial membrane polarization [132, 135]. This difference might be attributed to cell type-specific and methodological reasons. Finally, it is tempting to speculate that this interaction plays a broader role in TCDD-induced metabolic syndrome [177].

Taken together, the data presented here indicates a direct role for the AHR in mitochondrial function and cellular energy production. Exposure to TCDD not only decreased the amount of AHR found in the mitochondria, but it also created a hyperpolarization of the inner membrane in a transcription-independent manner. The absence of AHR in the BCL1 cell line appeared to create innate hyperpolarization condition for the inner membrane of this cell line. Interestingly, although perhaps only coincidental, CH12.LX cells express remarkably high levels of AHR and exhibit a very rapid doubling rate, approximately every 12-14 hrs. In contrast, the BCL1 cells, which do not express AHR, exhibit a remarkably slow doubling rate of approximately every 24-28 hrs.

We propose the hyperpolarization of the inner membrane occurs in response to decreased efficiency of the ATP synthase complex. Moreover, this decreased efficiency is a direct influence of decreased AHR in the mitochondria and its association with the ATP synthase complex. The discovery of a role for the AHR in cellular energy production establishes a possible mechanism by which dioxin-induced toxic endpoints such as wasting syndrome or immune system suppression may be occurring.

Upon ligand exposure the AHR becomes an activated transcription factor and is localized to the nucleus. This cellular localization event reduces the pool of cytosolic AHR and mitochondrial AHR. It is not yet known how the mitochondrial pool of AHR is decreased. This may occur through mass action, where an equilibrium shift of the activated receptor to the nucleus requires the cytosol and mitochondrial to adjust levels

of the receptor. It may also occur through TCDD entering the organelle, binding the AHR, and activating the receptor to translocate to the nucleus.

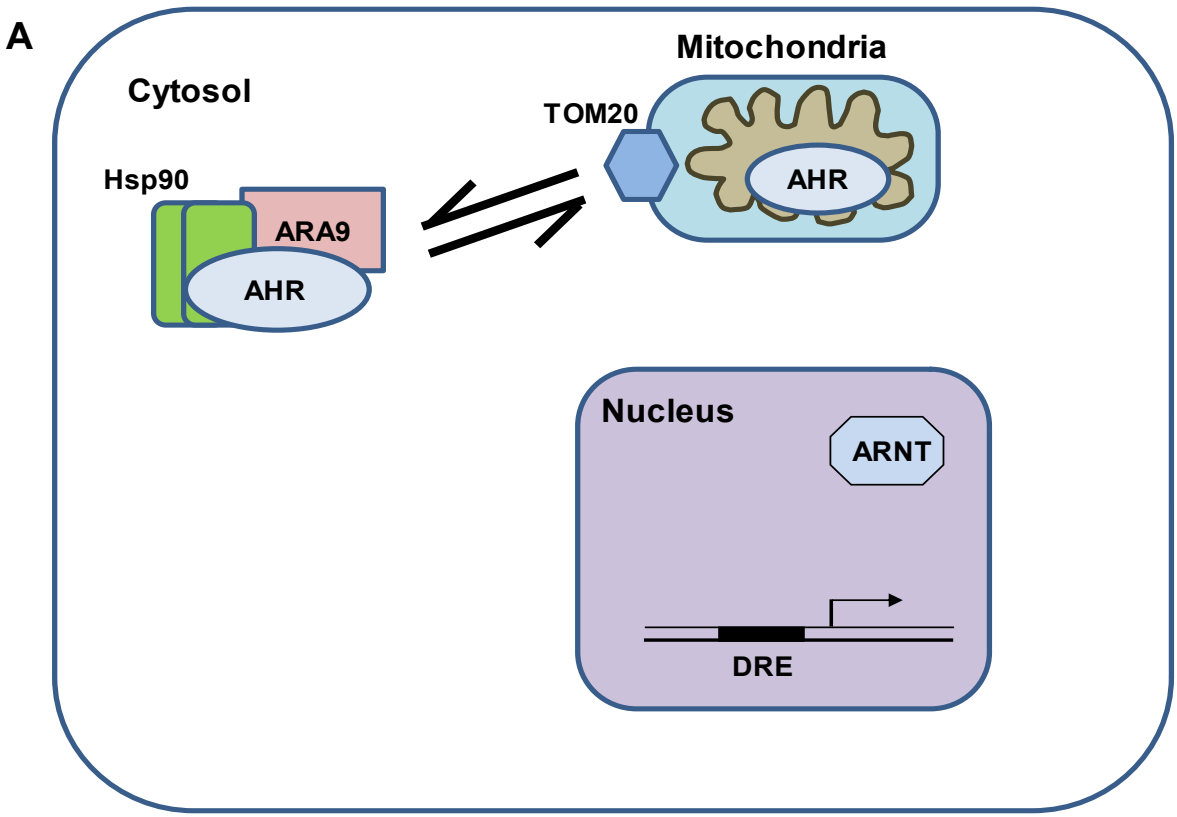
By whichever mechanism the loss of AHR occurs, the efficiency of the ATP synthase complex is affected. Here we observed hyperpolarization of the inner membrane in B-cells in an AHR-mediated response to TCDD exposure. Whether this event occurs to maintain energy production rates or a result of a decrease in the ATP synthase complex efficiency is not yet known. The most interesting questions remain, such as how long can this hyperpolarizaiion of the inner membrane compensate for the decreased in ATP synthase complex energy production? Moreover, how does this decrease in mitochondrial function affect a whole animal model? Thought further investigation is needed; data presented here introduces a new role for the AHR in mitochondrial function and energy homeostasis. These novel findings further elucidate AHR biology and offer possible mechanisms to AHR-mediated toxicity.

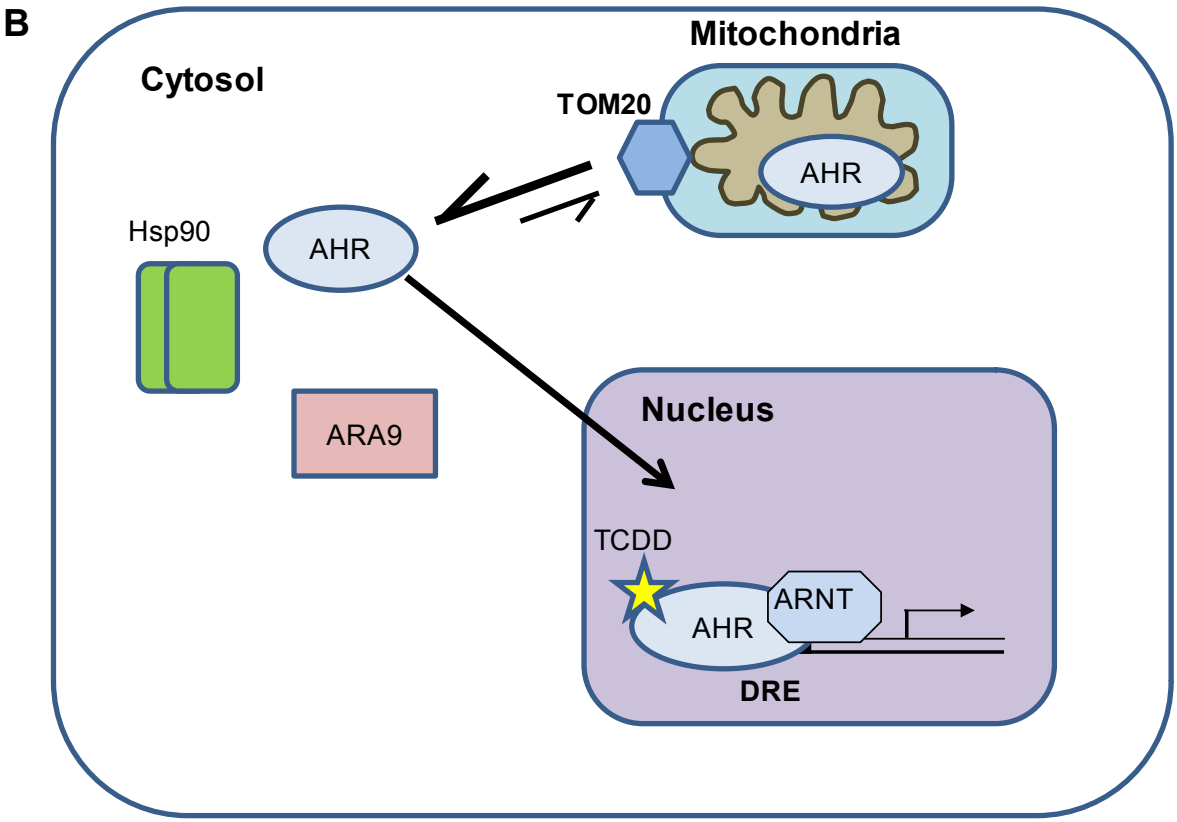
We have constructed a model of AHR cellular localization from this data. In the absence of ligand twos pool of the AHR exist. One pool is in the cytosol where the AHR complexes with its cytosolic partners, Hsp90 and ARA9. The second pool is in the mitochondria, where we have shown, the receptor interacts with the ATP synthase complex (Fig 4.1A). The mechanism by which a pool of the AHR is localized to the mitochondrial is still unknown. It has been reported that the ARA9 interacts with translocase of the outer membrane of mitochondria (Tom) 20 [178]. The TOM pathway has also been shown to interact with Hsp90 to facilitate the translocation of connexin 43 [179]. Though there is no direct evidence that these AHR cytosolic complex proteins act in the translocation of the receptor these reported TOM interactions provide a

possible mode of transport. Equilibrium between these two pools of AHR is maintained regardless of the transportation mechanism employed. Upon exposure to ligand the cytosolic pool of the AHR is shifted to the nucleus after AHR activation. This decrease in cytosolic AHR disrupts the equilibrium of the receptor and the mitochondrial pool of AHR is decreased by an unknown mechanism (Figure 4.1B). This decrease in mitochondrial AHR leads to a loss in ATP synthase efficiency. The hyperpolarization of the inner membrane acts to maintain ATP production levels and cellular energy homeostasis. This model depicts a new cellular distribution of the AHR and a novel role for the receptor in cellular energetics.

Figure 4.1 Model of the AHR Cellular Distribution and TCDD's Disruption of this Equilibrium

In the absence of ligand the AHR is found in its cytosol, complexed with Hsp90 and ARA9, and in the mitochondria, associated with a subunit of the ATP synthase complex. The pool of receptor is at equilibrium between the cellular compartments **(A)**. In the presence of ligand the cytosolic pool of AHR becomes depleted. The AHR is activated upon ligand binding, translocates to the nucleus, heterodimerizes with the ARNT, and becomes an active transcription factor. The decrease in the cytosolic pool of AHR disrupts the cellular equilibrium of the receptor, thus decreasing the mitochondrial pool of AHR. This decrease in mitochondrial AHR leads to a decrease in mitochondrial effiency.


A
Mitochondria
Cytosol
TOM20
Hsp90
ARA9
AHR
AHR
Nucleus
ARNT
DRE


B
Mitochondria
Cytosol
TOM20
AHR
Hsp90
AHR
ARA9
Nucleus
TCDD
ARNT
AHR
DRE

SUMMARY

5.1 TAP-tag Stable Cell Line Production

The GFP-TAP cell line was established and maintained without complication. The mAHR-TAP can be readily produced in Hepa cells. However, stable expression of the mAHR-TAP in these cells is lost over time. The mAHR-TAP Hepa cell line has been reproduced three times to date. Creation of a hAHR-TAP stable cell line has proven the most challenging. Constitutive hAHR-TAP expression has failed in both mouse and human cell lines. An inducible expression system is currently being tested. To date, a stable hAHR-TAP cell line has not been created.

5.2 Identification of the AHR-PIN

This project has focused on a greater understanding of AHR biology by defining its protein interaction network. Aspects of the receptor, such as its role in gene regulation and vascular development are well characterized. Questions remain regarding AHR-mediated mechanisms of dioxin-induced toxicity. As well as the receptor's influence on cellular processes. For example, cell cycle regulation, hormone signaling pathways, non-AHR mediated gene regulation and immune responses are linked to receptor activity. The AHR's modes of action in these processes however, are not defined. The newly expanded AHR-PIN has indentified proteins that fall into three categories. First is cellular signaling, including inter and intra cell communications. Second, are gene regulation events, which are not directly mediate by the AHR. Third, and previously unreported, is mitochondrial function. By investigating AHR protein interactions we have established the AHR-PIN and elucidated a functional role for the receptor in cellular energetics.

Three proteins involved in cellular signaling processes were identified. We observed AHR interactions with two immune response proteins, Clcf1 and Alcam. These interactions were identified in TCDD-treated and untreated samples. Given Clcf1 and Asap2 involvement in kinase signaling pathways it is of interest how AHR activation may influence both the MAP and GAP kinase signaling cascades. These three proteins, Clcf1, Alcam, and Asap2 are involved in cellular signaling pathways closely associated with tumor formation and cancer progression. The indirect evidence of TCDD exposure correlating to an increased risk of some cancers makes these interactions of great interest.

Three proteins involved in gene regulation were identified in our studies. Creb3l3 is an ER bound transcription factor that mediates gluconeogenesis in hepatic tissue. Htatsf1 is a cellular co-factor associated with HIV driven transcriptional events. Lastly, Smarcad, is a DNA helicase that is associated with transcriptional machinery associated with cancer progression. None of these proteins are directly associated with AHR-mediated gene regulation; however, AHR interaction with these proteins establishes connections to metabolism and disease states.

Finally, we identified AHR interactions with three mitochondrial associated proteins; Mrpl40, Bag3, and ATP5α1. Two of these proteins are linked to cancer promotion, heart defects, gene regulation and the immune response. Bag3 is classified a pro-onco protein and is known to influence NFκB signaling. Mrpl40 deletions are associated with VCFS, which results in heart defects, and DiGeorge syndrome, which impacts the immune system. Here again, we observed the AHR physically interacting with proteins reported to have functional roles in biological events the receptor has

previously been associated with. The AHR:ATP5α1 interaction raised the possibility of the receptor's direct involvement in mitochondrial function.

There are AHR interactions that are not addressed in this body of work. Some of the proteins identified in this project are as yet uncharacterized. In these cases we have reported the interaction with no further discussion. Previous reported AHR protein interactions with p23 and RelA were not observed in any of our data sets. However, we did identify an interaction with Cdk4 in some of the data sets. Given the AHR protein interactions reported here and their connection to cellular processes, it is clear the AHR-PIN is a dynamic network that will continue to be refined.

5.3 AHR Influence on Mitochondrial Function

Interaction between the AHR and a subunit of the ATP synthase complex was a novel finding of this research. This protein interaction raised the question of a role for the receptor in mitochondrial energy production. Investigation of this question revealed AHR co-localization to the mitochondria in Hepa and B-cell lines as well as in liver tissue. Moreover, the AHR influences the polarization state of the inner membrane of the mitochondria. Upon TCDD exposure the inner membrane becomes hyperpolarized in a transcriptional-independent manner. In addition, the basal inner membrane polarization level in an AHR null B-cell line is higher than in a WT AHR B-cell line. ATP levels were not significantly affected after TCDD exposure. These findings lead us to propose that the mitochondrial pool of the receptor is decreased after ligand activation of the AHR. This decrease in mitochondrial AHR adversely impacts the ATP synthase complex efficiency. To compensate the inner membrane becomes hyperpolarized in an

effort to maintain cellular energy homeostasis.

FUTURE WORK

6.1 TAP-tagged Stable Cell Line Production

Production of a stable hAHR-TAP cell line will continue. The inducible Ariad system and clonal selection will be used. The establishment of the hAHR-TAP cell line is necessary to compare species differences in the AHR-PIN. Species-specific response to dioxin-induced toxicity is an important factor of AHR biology. Comparison of species AHR-PINs may hold clues to these varied responses.

6.2 Identification of the AHR-PIN

The AHR-PIN demonstrated physical interactions between the receptor and proteins involved in cell processes and diseases the AHR has been linked to. The next step is to investigate the functional relevance of these interactions. Independent verification of AHR interaction with Mrpl40, Asap2 and p53 has been undertaken. Others protein interactions, such as Clcf1, Alcam, Smarcad, and Bag3, still need to be confirmed. These verifications will be prioritized and carried out depending on antibody availability. This will be followed by investigation of the functional relevance of the verified protein interactions.

6.3 AHR Influence on Mitochondrial Function

Further investigation is needed to identify the mechanism by which the AHR influences the hyperpolarization of the inner mitochondrial membrane. It is also of interest if the hyperpolarization occurs in other tissue types. Currently we are investigating polarization of the inner membrane in Hepa cells. Also of interest is the impact of prolonged TCDD exposure on the cell's ability to maintain energy homeostasis. Is there a point where the hyper-polarization of the inner membrane can

no longer compensate for the hypothesized decrease in ATP synthase efficiency? If so, what are the downstream cellular responses to this energy decrease? Does this prolonged mitochondrial dysregulation lead to the metabolic disorder, such as increased risk of diabetes, steatosis, and wasting syndrome, observed following exposure to TCDD. Answers to these questions will lead to a better understanding of AHR biology.

APPENDIX

APPENDIX A: Cell line production

A.1 hAHR-TAP tagged cell line expression

The manufacture of a hAHR-TAP expressing cell line has proven problematic. Three different cell lines have been investigated, human Hep3B liver cells, mouse AHR-/- Hepac12 and wild type Hepa1c1c7 cells, over numerous rounds of infection. Each cell line was incapable of maintaining stable expression of the hAHR-TAP. However, two of the cell lines have been able to maintain GFP-TAP expression. The AHR-/- HepaC12 cells suffered large cell death during infection and subsequent antibiotic selection. This occurred in both the hAHR-TAP and GFP-TAP infected cells. Visualized GFP expression in these cells is markedly lower than in the wild type Hepa and Hep3B-cells (Fig A.1). The cells did recover sufficiently for testing freeze/thaw survival; however none survived the thawing process. Cells harvested prior to cryo freezing did not demonstrate any hAHR-TAP expression by Western blot.

Hep3B-cells survived viral infection and puromycin selection. The selected hAHR-TAP cells were then cyro frozen and revived to test viability. The cells were maintained under puromycin selection upon thawing with moderate cell death observed. Though the cells continued to grow under puromycin selection, expression of the hAHR-TAP could not be verified after Western blot (Fig A.2) or TAP-tag (Fig A.3). Wild type Hepa1c1c7 cells were robust through infection, selection, and cryo screening steps; hAHR expression could not be verified by Western blot (Fig A.2) or after TAP-tag (Fig A.3). The GFP-TAP expression in both these cell lines was verified by Western blot (Fig A.2) and after TAP-tag (Fig A.3).

Figure A.1 Visualization of Infection Efficiency using GFP

UV visualization of GFP was used to evaluate efficiency of infection in Hep3B, Hepa1c1c7, and HepaC12 expressing cell lines. GFP is expressed in approximately 60% of Hep3B-cells, 90% of Hepa1c1c7 cells, and 5% of HepaC12 cells.

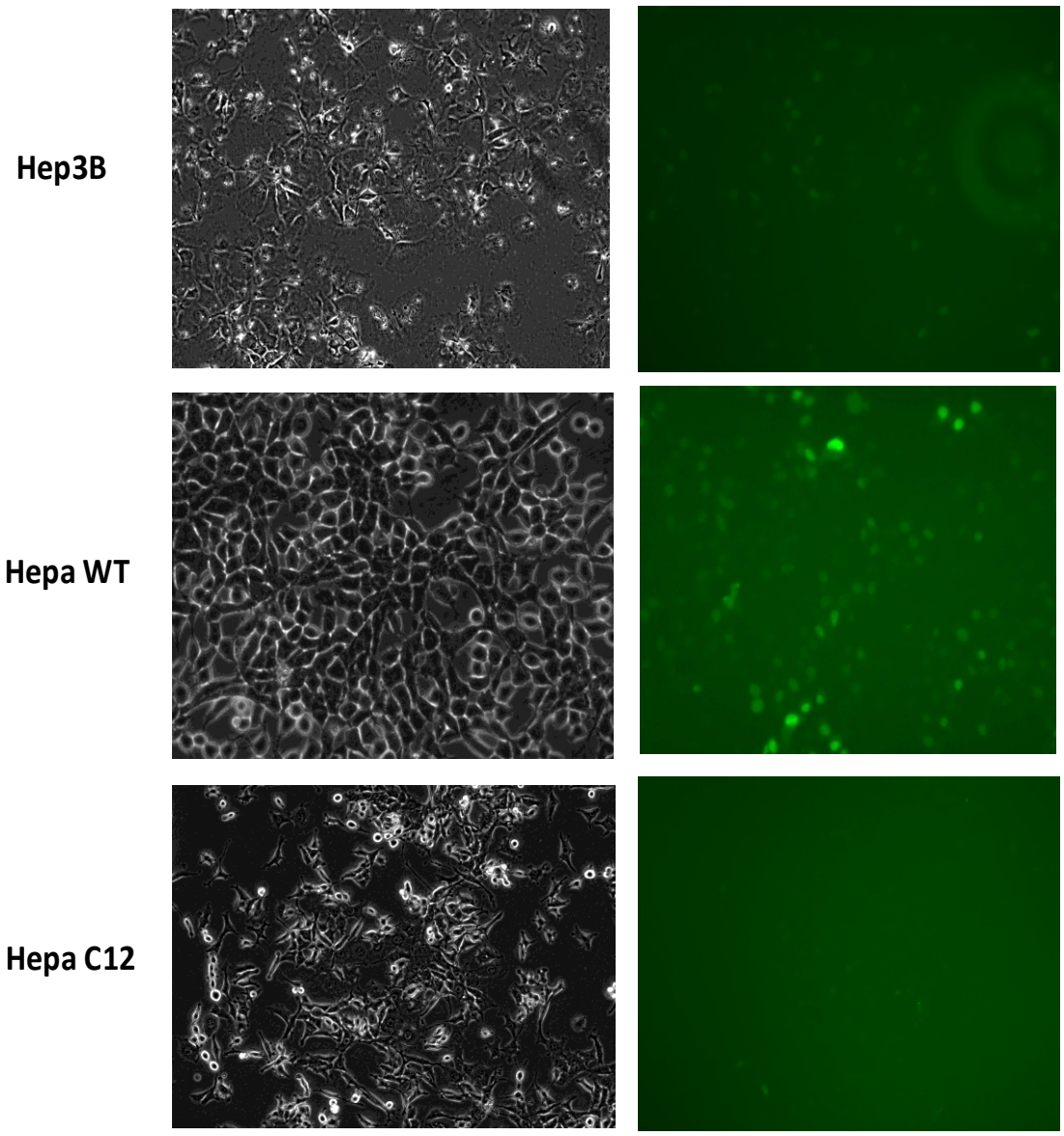

Hep3B
Hepa WT
Hepa C12

Figure A.2 Expression of hAHR Post Infection

Western blotting for hAHR-TAP expression in Hep3B, Hepa1C1C7, and HepaC12 cell lines was performed. Cells from three replicate hAHR-TAP infections in Hep3B, Hepa1C1C7, and HepaC12 cell lines were used (R1, R2, R3).

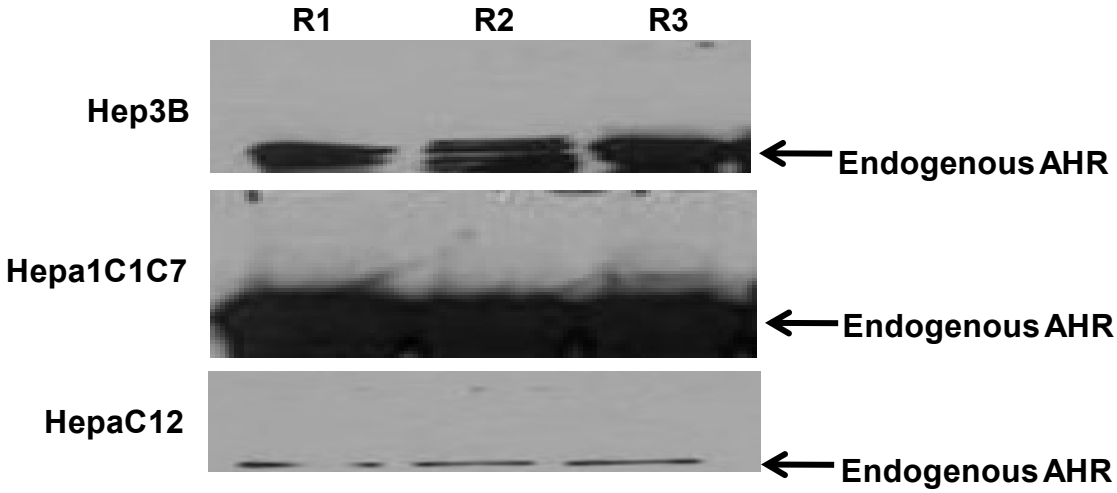
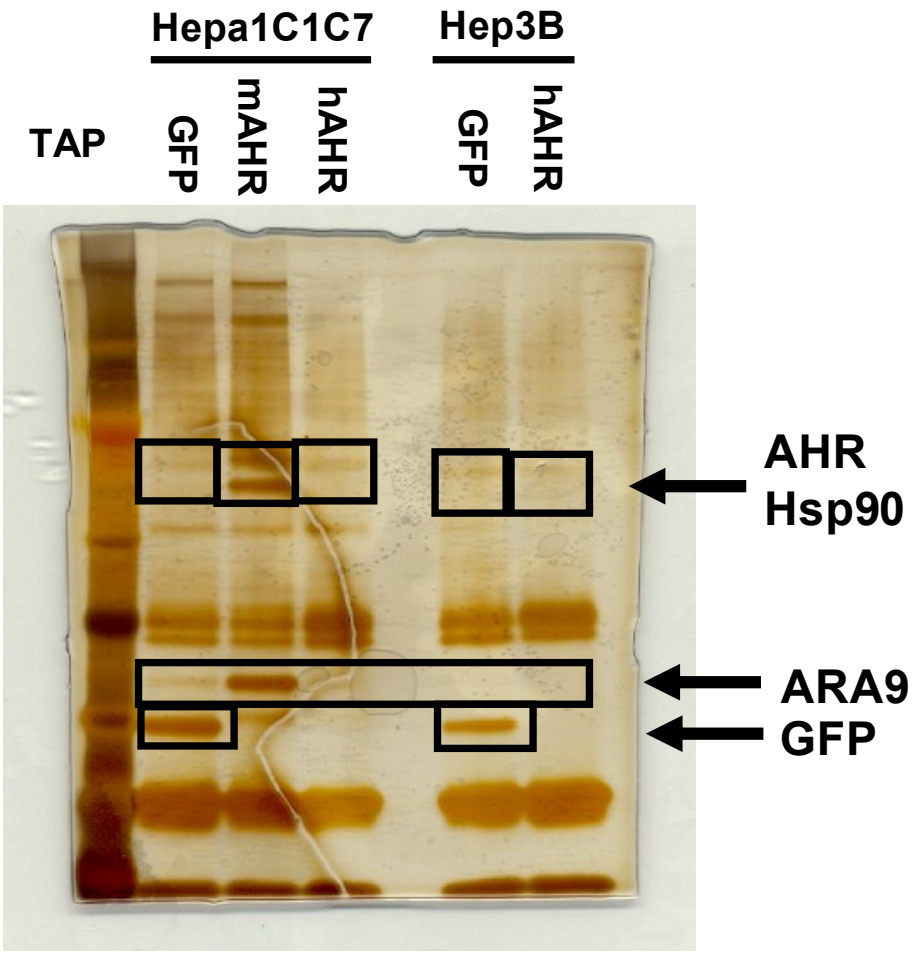

R1
R2
R3
Hep3B
Endogenous AHR
Hepa1C1C7
Endogenous AHR
HepaC12
Endogenous AHR

Figure A.3 Comparison of TAP-tag between mAHR and hAHR Cell Lines

Silver stain gel of final TAP-tag eluates from GFP-TAP, mAHR-TAP and hAHR-TAP expressing Hepa1c1c7 cells and GFP-TAP and hAHR-TAP expressing Hep3B cells. Gel fragments in which AHR, Hsp90, ARA9, and GFP are normally identified are boxed.


Hepa1C1C7
Hep3B
TAP
GFP
mAHR
hAHR
GFP
hAHR
AHR
Hsp90
ARA9
GFP

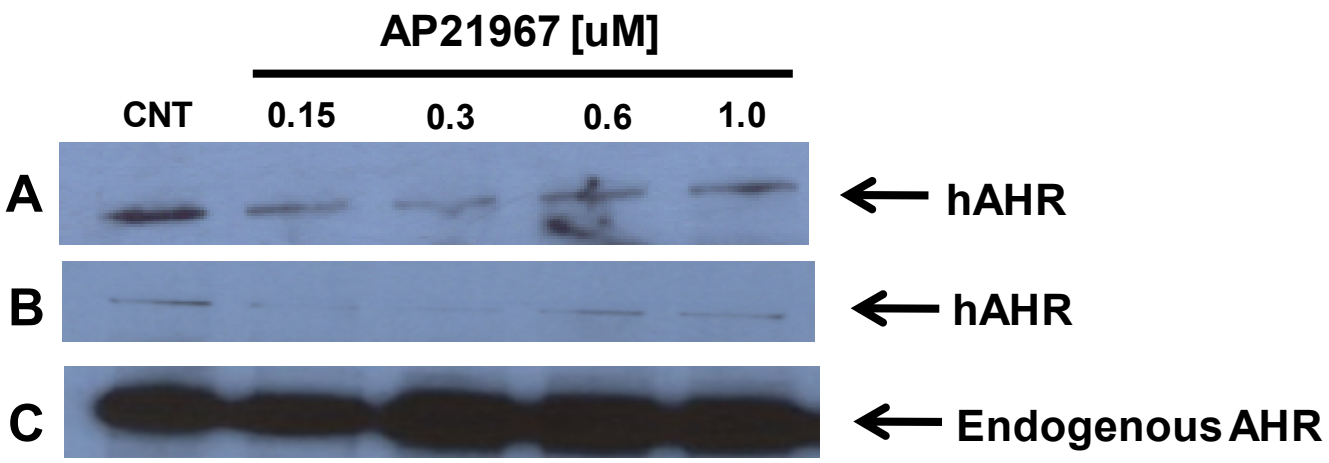
The Ariad inducible expression system is currently being tested on the Hepa1c1c7 cell line. A stable Ariad transcription factor expressing Hepa1c1c7 cell line has been established. In two separate rounds, hAHR infection has been performed with this cell line. The first round demonstrated no induction of hAHR-TAP expression in the presence of the rapamycin analog, but leaky basal expression of the receptor was observed. This expression was silenced after two round of passaging (Fig A.4). For the second round of infection, in the Hepa1C1C7 cell line, clonal selection was performed after infection and hygromycin selection. There were 19 clones picked from this selection, none of which survived clonal expansion. Infection and clonal selection in the Hepa1c1c7 cell line will be repeated.

A.2 TAP-tagged Stable Cell Line Production

The establishment of TAP-tagged AHR and GFP was the first step in investigating the AHR-PIN and the role it has in dioxin induced toxicity. In order to explore species variation of the AHR-PIN we wanted to elucidate the PINs of the mouse and human AHR. The mouse model has been extensively used in laboratory dioxin research and the consequences of dioxin exposure is a human health concern. The comparison of these two PINs could provide differences and similarities between the two species sensitivity to dioxin exposure. The comparisons of these PINs were to be examined following expression of TAP-tagged mAHR and hAHR in both mouse and human liver cell lines. This would allow us to observe how orthologous variation altered the PIN of a given species. Generation and maintenance of stable TAP-tagged cell

Figure A.4 Expression of an Inducible hAHR

Visualization of hAHR expression using the Ariad induction system via Western blot with AHR specific antibody. AHR expression was assessed in non-induced (CNT) and cells exposed to AP2197 at various concentrations. Cells were analyzed immediately upon the completion of selection (A) and following two passages over 7 days (B). Endogenous AHR (C) is included as a control.


AP21967 [uM]
CNT
0.15
0.3
0.6
1.0
A
hAHR
B
hAHR
C
Endogenous AHR

lines have presented unique challenges. We have been able to maintain GFP-TAP cell lines and two other TAP-tagged proteins in various cell lineages. These cell lines survive cryo preservation and have robust growth after thawing. These cell lines also maintained consistent expression levels of the TAP-tagged proteins. However the AHR-TAP constructs have proven more problematic.

Cell lines expressing the mAHR-TAP and GFP-TAP were first established in the AHR-/- MEF cells. These cell lines were used to test the functionality of the mAHR-TAP construct. They were also intended to be used in the analysis of the AHR-PIN. Given the null background the cellular proteins interactions with the AHR would be exclusively with the tagged AHR with no competition from endogenous receptor. However, the mAHR-TAP cell line could not be maintained. Expression of the mAHR-TAP and puromycin resistance was lost over time, indicating loss of the inserted gene cassette or toxicity from receptor overexpression.

Hepa1c1c7 cells were chosen as the next target cell line because of the cell line's historical presence in AHR research. The GFP-TAP again established and maintained in these Hepa cells. The mAHR-TAP Hepa cell line was viable and retained expression of the tagged receptor. However, loss of mAHR-TAP expression was observed over the course of multiple passages of the cell line. Moreover, the cells retained puromycin resistance. Further investigation revealed frozen cell stocks experienced this same loss of mAHR-TAP expression but retained antibiotic resistance. The cause of this loss in expression is unknown, however, it is consistent (the cell lines have been made 3 separate times). Taken together with the MEF experiments, the results suggest that cells have a certain level of AHR that can be tolerated and if this

amount is exceeded, the cells will compensate by removal of exogenous gene or through selection.

A stable hAHR-TAP cell line has yet to be generated. Three cell lines from two different species, human liver cell line Hep3B, and two mouse liver cell lines, wild type Hepa1c1c7 and AHR-/- HepaC12, were employed as target cell lines. After numerous attempts of infection and selection, no viable hAHR-TAP expressing cell line was established. Each cell line had unique issues during the process.

The AHR-/- HepaC12 cells were also tried as target cell line for the benefit of a null background. Uninfected HepaC12 cells grew slowly with thin cell body morphology. Cell death during infection and antibiotic selection was greater than the observed cell death in other infected cell lines. Recovery was slow in both hAHR-TAP and GFP-TAP cells. Both cell lines did expand enough to test freeze/thaw survival, but never survived the thawing process. Though expression of AHR constructs has been challenging this was the first time a GFP-TAP cell line could not be established. The AHR-/- HepaC12 cells appeared resistant to infection.

The Hep3B-cells were used as a target cell line to establish a human AHR-PIN for comparison with the mouse AHR-Pin. This cell line proceeded through the infection and selection process with average cell death. Though the cells had a slow growth rate after undergoing freeze/thaw, they did expand under continued puromycin selection pressure. However, a TAP-tag using these cells did not result in observable isolation of AHR, Hsp90 or ARA9.

Finally, wild type Hepa1c1c7cells were used as the target cell line for hAHR-TAP. The hAHR-TAP cells survived infection, puromycin selection, and freeze/thawing as the

mAHR-TAP counterparts did. Similar to the Hep3B-cells using this cell line for TAP-tagging, no AHR, Hsp90, or ARA9 was detected. A subsequent Western blot detected no hAHR-TAP protein expression in the cell line.

Given the consistent loss of mouse and human AHR over expression in numerous cell lines we next tried an inducible expression system. This system involves the use of ligand-activated transcription factor that is responsive to rapamycin-like drugs. Then ARGENT transcription factor expressing cell line was created in the Hepa1c1c7 cells. Another round of infection inserted the gene hAHR gene cassette into this cell line. In the presence of a rapamycin analog, A21967, the transcription factor becomes active and hAHR is expressed. Western blots for hAHR induction revealed leaky expression of the tagged receptor in the absence of A21967. No induction of hAHR was observed over a concentration gradient of A21967 either. Moreover, the exogenous hAHR expression was lost over time.

In the latest attempts to create this inducible cell line, clonal selection is being attempted. It is possible the basal expression of the hAHR is enough to prevent the inducible over expression of the receptor. The aim is to isolate a colony that has minimal basal expression, but has inducible hAHR expression. The first round of infection/selection yielded minimal colonies for expansion. None of these produced a viable cell line. The unsuccessful attempts at expressing the hAHR have made identifying species-specific differences unattainable. The lack of viability in multiple cell lines and with differing methodology, suggest that over-expression of hAHR is detrimental to the cell. Though this inability to produce a hAHR-TAP cell line hampers

this portion of the specific aim, the creation of the mAHR-PIN in the Hepa1c1c7 cells does offer new insights into AHR biology.

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