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Investigation of ARNT isoform-specific regulation of AHR signaling

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Investigation of ARNT-isoform specific regulation of AHR signaling

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The aryl hydrocarbon receptor nuclear translocator (ARNT) is alternatively spliced into two distinct isoforms, isoform 1 and 3. Although ARNT is found to be critical in immunity, xenobiotic, and hypoxic response, ARNT isoform-specific function has yet to be investigated. We previously demonstrated that primary lymphocytes express both of these isoforms, however malignant T cells overexpress ARNT isoform 1 to promote cell viability. In this study, we find that the ARNT isoforms have opposing roles in aryl hydrocarbon receptor (AHR) signaling, as ARNT isoform 1 suppresses AHR activity, whereas ARNT isoform 3 is needed for AHR target-gene transcription. Furthermore, to explore this suppressive role, we investigated a unique modification specific only to ARNT isoform 1 – phosphorylation of serine 77 (S77). We determined that phosphorylation at S77 is initiated following AHR activation and is critical for the augmentation of AHR-target gene transcription. These results further highlight the importance of investigating ARNT isoform-specific function and reveal an essential role of ARNT isoform 1 phosphorylation in AHR signaling. Collectively, these findings increase our understanding of a complex regulatory mechanism by which ARNT regulates AHR signaling, further aiding in the comprehension of their roles in immunity and supporting the potential of targeting ARNT alternative splicing as a means of therapeutic intervention in hematological diseases and malignancies.

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List of Abbreviations

UTMB	University of Texas Medical Branch
GSBS	Graduate School of Biomedical Science
TDC	Thesis and Dissertation Coordinator
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
AHR	Aryl Hydrocarbon Receptor
CK2	Casein Kinase 2
RA	Rheumatoid Arthritis
SLE	Systemic Lupus Erythematosus
Treg	Regulator T cell
Th17	T-helper 17
IL-17	Interleukin-17
TNF-α	Tumor Necrosis Factor-α
DCs	Dendritic cells
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
ROS	Reactive Oxygen Species
NF-κB	Nuclear factor-kappa B
ER	Estrogen Receptor
Aire	Autoimmune Regulator
FoxP3	Forkhead Box P3
RORγt	RAR-related orphan nuclear receptor gamma t
IDO1	Indoleamine 2.3-dioxygenase 1

DCs	Dendritic cells
HIF-1β	Hypoxia-inducible factor-1β
bHLH-PAS	Basic helix-loop-helix-PER/ARNT/SIM
TAD	Transactivation Domain
XRE	Xenobiotic response element
E-box	Enhancer box
KO	Knockout
VEGFR2	Vascular endothelial cell growth factor receptor 2
AHRR	Aryl hydrocarbon receptor repressor
IFN-γ	Interferon-γ
HSCs	Hematopoietic stem cells
SNPs	Single nucleotide polymorphisms
MDR1	Multidrug resistance 1
COX-2	Cyclooxygenase-2
HCC	Hepatocellular carcinoma
VHL	Von Hippel-Lindau
AHH	Aryl hydrocarbon hydroxylase
HSP90	Heat shock protein 90
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
siRNA	Small interfering RNA
XAP2	Hepatitis B virus X-associated protein 2
ARA9	AHR-associated protein 9
AIP	AHR interacting protein

CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
AIP	AHR-interacting protein
NLS	Nuclear localization signal
PKC	Protein kinase C
KLF6	Kruppel-like factor 6
NC-XRE	Non-consensus XRE
CUL4B	Cullin 4B ubiquitin ligase
HAHs	Halogenated aromatic hydrocarbons
PAHs	Polycyclic aromatic hydrocarbons
BaP	Benzo(a)pyrene
TDO	Tryptophan 2,3-dioxygenase
IDO	Indoleamine 2,3-dioxygenase
KYN	Kynurenine
I3C	Indole-3-carbinol
ICZ	Indolo-[3,2-b]-carbazole
FICZ	6-Formylindolo[3,2-b]carbazole
STAT	Signal transducer and activator of transcription
CA	Cinnabarinic acid
WT	Wild-type
KA	Kynurenic acid
RA	Rheumatoid arthritis
MS	Multiple sclerosis
EAE	Experimental autoimmune encephalitis

PBMCs	Peripheral blood mononuclear cells
AML	Acute myeloid leukemia
CML	Chronic myeloid leukemia
IL4I1	Interleukin-4-induced-1
BNF	β-Naphthoflavone
HDAC	Histone deacetylase
JAK-STAT	Janus kinase-STAT
cDNA	Complementary DNA
CK2	Casein Kinase 2
EMSA	Electrophoretic mobility shift assay
HAT	Histone Acetyltransferase

Chapter 1 Introduction

T CELL DISORDERS

Overview

The immune system is comprised of a multitude of different cells with the role of protecting the host from invading foreign bodies. However, alterations in this protective role can lead to significant detriments that often take one of two forms: immune deficiency syndromes or autoimmune diseases. An immune deficiency syndrome occurs when a specific component of the immune system is absent, leading to an insufficiency in protecting the host upon challenge from a pathogen[1]. Alternatively, autoimmune diseases are characterized by the presence of lymphoid cells that recognize and react to antigens that are specific to the host and thus fail to distinguish between self and non-self[2].

Nobel Laureate Paul Ehrlich first identified and defined autoimmunity in the early twentieth century with the idea of a process referred to as “horror autotoxicus”. Based on his experiments, he developed an understanding of an internal system that prevents self-reactive responses[3,4]. In building off of these findings, the identification of immune tolerance and autoimmune disease formation were discovered in the middle of the twentieth century by the Nobel Prize winning scientists Macfarlane Burnett and Peter Medawar. Following these pioneering discoveries, the research field of autoimmune diseases has exponentially increased and has dramatically enhanced the current understanding and identification of these diseases.

Since these initial findings, more than 80 autoimmune disease-related illnesses have been identified and more than seven percent of the United States population (23.5 million Americans) has been diagnosed with an autoimmune disease, according to the NIH Autoimmune Diseases Coordinating Committee. Unfortunately, studies have found that the prevalence of autoimmune diseases are on the rise as both epigenetic alterations and environmental factors, including nutrition, xenobiotic exposure, ultraviolet light, metals, and pharmaceutical agents, are being attributed to this increase[5–9]. Hence, with the prevalence of autoimmune diseases on the rise, novel therapies have continued to be developed to combat symptomatic inflammatory responses, however more strategies are needed to target the underlying mechanisms of these diseases.

T cell development and immune tolerance

Immune tolerance is a critical function required for cellular homeostasis. For this to occur, leukocytes must first undergo self-nonself discrimination. T lymphocytes originate in the bone marrow as progenitor cells, which then migrate to the thymus to undergo maturation and determine tolerance of self-antigens. The initial stage of immune tolerance begins in the thymus to initialize cellular homeostasis. T cells migrate from the cortex to the medulla of the thymus and are exposed to different microenvironments to direct T cell development and differentiation. While developing in the thymus, lymphocytes undergo positive selection prior to entering circulation. Lymphocytes that demonstrate potential self-reactivity are subjected to negative selection and are eliminated[10–13]. Immature B cells that recognize and respond to cell surface antigens are also destroyed. Upon maturation, both B and T cells undergo a second selection phase to

further ensure no self-reactivity will take place once released into circulation. Unfortunately, when these processes are negatively manipulated or absent, self-reactive lymphocytes are allowed into circulation, which may lead to uncontrolled inflammation and tissue damage.

Upon leaving the thymus, naïve T cells circulate the body and are ready to be stimulated by a specific antigen, thus initiating differentiation into a specific T cell subset. Uniquely, regulatory T cells (Tregs) are differentiated in both the thymus as well as the periphery[14]. Tregs are responsible for maintaining homeostasis and preventing autoimmunity through the release of anti-inflammatory cytokines, modulation of dendritic cell maturation, and suppression of cytolysis[15–18]. In contrast, T helper 17 (Th17) cells, which have been found to be key mediators of autoimmune diseases, are characterized by the release of the inflammatory cytokines interleukin-17 (IL-17) and Tumor Necrosis Factor-α (TNF-α)[19,20]. Th17 cells perform a crucial role in host defense through the release of pro-inflammatory cytokines and recruitment of white blood cells to defend against pathogens, however aberrant Th17 cellular responses provide evidence for a role in inflammatory disorders[21].

Manifestation of T cell disorders

T cell disorders vary widely in how they are established as well as the organs that are affected. The exact reason for the onset of these diseases is uncertain, as symptoms do not surface until well after the disease has progressed, however these disorders are thought to be primarily initiated by two factors: genetic predisposition and environmental factors.

Genetic polymorphisms have continued to be identified for many autoimmune diseases and T cell malignancies with the help of whole genome sequencing studies of affected individuals[22,23]. As expected, many of the polymorphisms identified are found in genes of proteins that have a significant role in immune function and response. Further, mutations in cytokines and cytokine receptors are also shown to be associated with the progression of autoimmune disorders. For example, polymorphisms of the IL-7 receptor are found to enhance the progression of numerous immunological diseases including multiple sclerosis (MS) and ulcerative colitis[24–27].

Environmental factors have also been shown to induce autoimmunity and are found to be responsible for the increased prevalence of these diseases. Although it is extremely difficult to link an environmental exposure to the manifestation of a disease, medicinally induced autoimmune diseases have provided the strongest evidence of connecting xenobiotic exposures to the development of an autoimmune disease[28]. Chemical occupational exposures have also been linked to significant increases in the development and progression of autoimmune disorders including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and systemic sclerosis[29–31].

Mouse models provide further evidence of the link between environmental exposures and autoimmune diseases. Examples of these correlations include exposure to pesticides, mercury, or silica, which results in autoimmune-like conditions such as lupus, neuropathy, and chronic inflammation, whereas other agents, such as the toxicant 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), influences tolerance mechanisms[32–35]. Interestingly, sun exposure has been found to have photoimmunologic effects, which results in the suppression of pro-inflammatory activities and enhances anti-inflammatory

effects in leukocytes including dendritic cells (DCs), monocytes, and B and T cells[36]. However, studies using Vitamin D to reduce inflammatory cytokine production in patients with MS have had conflicting results, suggesting that a different product generated from UV light is responsible for these observed effects[37,38].

The mechanism by which these environmental factors influence an autoimmune response is through a variety of different processes. Previous studies have revealed that exposure to environmental factors can lead to the generation of anti-mitochondrial and anti-nuclear antibodies, aberrant cell death, altered T cell differentiation, and improper cytokine release[39–43]. For example, alveolar macrophages are significantly affected upon the inhalation of silica, as this leads to an increase in the production of reactive oxygen species (ROS) once internalized[41,44]. ROS in turn activates alternative signaling pathways within alveolar macrophages, resulting in the release of cytokines and spontaneous apoptosis[41,44].

Previous reports provide clear evidence that chronic inflammation from autoimmune disorders can subsequently lead to cancer development[45]. While being in a persistent state of T cell activation, cytokine and chemokine release, and free radical distribution, the risk of cancer development and carcinogenesis is significantly augmented. For example, investigations studying autoimmune disorders have reported significant associations between patients with MS or psoriasis and the development of gastrointestinal cancer[46–48]. Other autoimmune diseases including RA and SLE that affect numerous organs, have a significant association with the manifestation of multiple cancers[49,50]. Additionally, supporting data shows that patients with a malignancy may

also develop autoimmune disease-like symptoms, further highlighting a tight link between tumor development and autoimmune disorders[45].

Overall, T-cell specific autoimmune diseases progress due to an improper ratio of effector T cells and Tregs, resulting in the inability to regulate an inflammatory response and potentially develop into cancer. This impaired balance may be attributed to an increased number of dysfunctional Tregs or to a decrease in the total number of Tregs[51,52]. Studies have also revealed that Tregs are able to differentiate into IL-17 producing cells under inflammatory conditions as in autoimmune diseases, further contributing to uncontrolled inflammation[53]. In summary, autoimmune disease progression can be attributed to both genetic and environmental factors, however precise immunological signaling mechanisms are under continual investigation to gain a superior understanding of this incredibly complex system.

Involvement of AHR and ARNT in autoimmune diseases and malignancies

A mutual trademark of autoimmune diseases is an uncontrolled inflammatory response that subsequently leads to tissue damage. Pro-inflammatory induced carcinogenesis and altered cellular differentiation are found to be main contributors to the destruction caused by autoimmune diseases. The aryl hydrocarbon receptor (AHR) is an essential component to a functional immune system and is susceptible to dysregulation in both malignancies and autoimmune diseases. The AHR signaling cascade has important roles in leukocyte differentiation however disruption of signaling through environmental or genetic factors can consequently lead to unequal ratios of immune cells, resulting in the inability to appropriately regulate immune function[54–56]. AHR is also found to have a

role in the expression of pro-inflammatory cytokines, which can be manipulated and severely augmented in autoimmune disorders[57,58]. In having these major implications in the formation of autoimmune diseases, AHR is becoming increasingly identified as a therapeutic target to treat these disorders. Moreover, the aryl hydrocarbon receptor nuclear translocator (ARNT) is a transcription factor with essential roles in AHR signaling. ARNT is demonstrated to also influence the expression of inflammatory cytokines and promote the viability of tumors and malignant B and T cells, however very little information exists about the role of ARNT in immunity[59–65]. Together, these data reveal a gap in knowledge in how ARNT and AHR may work together in the immune system and suggests that further exploration of ARNT within AHR signaling in hematological malignancies will significantly enhance our understanding of these diseases and the function of ARNT within this critical pathway.

ARYL HYDROCARBON RECEPTOR (AHR)

Introduction

AHR was first identified by Alan Poland in 1976 through radiolabeled binding assays with the AHR ligand TCDD[66]. Many previous investigations aided in the identification of this receptor upon observing that aryl hydrocarbon hydroxylase (*AHH*) activity, also known as *CYP1A1*, was increased in particular mice strains more than others, which also mimicked sensitivity to TCDD[66,67]. These observations promoted a full examination of these different mice strains to determine the reason behind the extreme sensitivity to TCDD in certain mice over others. Alan Poland later identified AHR to be the culprit of this sensitivity through capturing a direct interaction between AHR and

TCDD[66]. A full synopsis of the findings leading up to the identification of AHR has been extensively written by Nebert *et al*[67]. Following the identification of AHR, studies began utilizing isolated clones of Hepa-1c1c7 cells, which rapidly enhanced discoveries within the AHR signaling pathway[68]. Nearly half a century later, countless studies continue to provide further insight into the novel roles of AHR in numerous tissues and organ systems.

AHR is a member of the basic helix-loop-helix-PER/ARNT/SIM (bHLH-PAS) superfamily and more specifically, is a class I bHLH-PAS protein, as it is a transcription factor regulated by environmental signals[69]. The *AHR* gene is amplified at the chromosomal region 7p21.1, which encodes 848 amino acids. The protein of AHR contains four functional domains, which include: bHLH, PASA, PASB, and Transactivation Domain (TAD). Previous studies have demonstrated that the bHLH domain of AHR is required for DNA binding following activation[70,71]. AHR and ARNT together interact with xenobiotic response elements (XREs), and AHR specifically recognizes the 5' end of each XRE, minimally consisting of NGC (N stands for any nucleotide)[72]. In addition to DNA binding, the bHLH domain contains a nuclear localization signal (NLS) and is responsible for interacting with heat shock protein 90 (HSP90) as well as ARNT[73,74]. The PASA domain of AHR is essential for dimerization with ARNT, enhancing the stability of the complex and DNA binding strength, as determined by mutagenesis and biochemical studies[71,75,76]. A previous investigation has reported the crystal structure of the mouse AHR PASA domain, identifying numerous hydrophobic residues that are predicted to be crucial for protein and DNA interaction[77]. Interestingly, the crystal structure of the AHR/ARNT heterodimer complex, specifically

at the bHLH and PASA domains bound to DNA, offers an initial glimpse into the sophisticated actions and structural insight of each protein in binding to target DNA sequences[78,79]. These studies offer extensive structural insights and allow for additional modeling to further predict functions of AHR alone and with ARNT.

A previous report also found that the co-chaperone p23 binds in the N-terminal region of AHR within the first 216 amino acids, which includes both the bHLH and PASA domains[80]. Unique to AHR, the PASB domain contains the ligand-binding site. Notably, the crystal structure of the PASB domain is under intense investigation to better understand the ligand binding pocket, which will allow for a more targeted approach in activating AHR[66,81,82]. Furthermore, the PASB domain is found to have an additional HSP90 binding site and is also involved in dimerization with ARNT[73,74]. Interestingly, deletion of the PASB domain results in a constitutively active receptor, thus suggesting that ligand binding is specifically required for nuclear translocation[83]. Studies have also found allele differences that encode an AHR with a low and high binding affinity for AHR ligands[84]. These specific AHR allele differences that alter ligand binding are located in the PASB domain and are predicted to alter the ligand-binding pocket. Experimental analysis demonstrated that mice with a low affinity for TCDD have a valine at amino acid 375 (V375), whereas mice with an alanine at the same location (V375A) resulted in an AHR with a significantly higher affinity for TCDD[84]. Strikingly, additional studies have demonstrated that these differences in agonist activity do not apply to other AHR ligands including indirubin and quercetin, in which AHR V375 displays a higher binding affinity compared to AHR V375A, further highlighting that AHR-agonist binding is much more complex than currently understood[85]. Finally,

through the generation of AHR deletion mutants, the TAD of AHR has been revealed to mediate transactivation activity and enhance occupancy of target genes[86,87]. Additional data provides evidence that the co-chaperone hepatitis B virus X-associated protein 2 (XAP2), otherwise known as the AHR-associated protein 9 (ARA9) and AHR interacting protein (AIP), interacts with AHR at the TAD prior to interaction with ARNT[88].

Molecular pathways of AHR

The unliganded AHR is found in the cytoplasm of the cell as an inactive complex[66]. AHR is restricted to the cytoplasm bound to co-chaperone proteins including HSP90, XAP2 (also known as AHR-interacting protein (AIP)), and P23 prior to activation[89–93]. Interestingly, each of these co-chaperone proteins have a unique function in retaining AHR outside of the nucleus. HSP90 binds to AHR at two separate locations, as mentioned above, one HSP90 molecule interacts with the PASB domain, while the other molecule binds to the bHLH region of AHR. These interactions not only mask the DNA binding ability of AHR, but also maintains AHR in a ligand-free state able to bind to any AHR ligand[74,94–96]. Additionally, studies have demonstrated that XAP2 maintains high levels of AHR in the cytoplasm by protecting it from ubiquitination and aids in the stabilization of AHR through binding with both AHR and HSP90 in the AHR-chaperone complex[97–100]. XAP2 also aids in cytoplasmic localization of the AHR complex by blocking the interaction of AHR to importin-β, thus repressing the transcriptional enhancer activity of AHR[101]. Investigations of p23 have also revealed roles similar to XAP2, as p23 aids in preventing the ubiquitination of AHR and localizing it to the cytoplasm prior to activation with a ligand[80,102]. Collectively, these co-chaperone proteins

sustain abundant levels of AHR in a steady state position that is prepared for AHR ligand-mediated activation.

When an AHR ligand is in close proximity, which can be from an exogenous ligand outside the cell or one generated within the cell, AHR binds to the ligand with its binding pocket in the PASB domain, which then induces a conformation shift, thus exposing the NLS of AHR[103]. AHR is then shuttled from the cytoplasm to the nucleus by importin-β[104]. Following entry into the nucleus, AHR locates and binds to ARNT and together are recruited to XRE sequences to elicit the transcription of AHR target genes. The precise transport mechanism of the chaperone proteins following AHR activation and entry into the nucleus is still relatively unknown due to ligand and cell-type specificities, however previous studies suggest that the entire complex translocates into the nucleus before dissociation[105,106]. Specifically, studies have determined that HSP90 dissociates from AHR upon AHR/ARNT heterodimerization (Illustration 1.1)[107,108].

AHR is also found to modulate the expression of genes that do not contain a consensus XRE sequence through interactions with other transcription factors following activation. The AHR/ARNT heterodimer is able to interact with the estrogen receptor (ER) to regulate ER target genes following AHR ligand exposure without ER activation[109]. Crosstalk between the ER and AHR can also lead to antagonistic effects on either signaling pathway depending on the presence of ER or AHR ligands, causing the utilization of common co-factors[109,110]. Genome-wide expression studies also identified AHR target genes that were responsive to TCDD independent of ARNT, yet were divergent from canonical XRE sequences known as non-consensus XRE (NC-XRE)[111].

Ligand-activated AHR is found to interact with Kruppel-like factor 6 (KLF6) to regulate these unique sequences without ARNT[111].

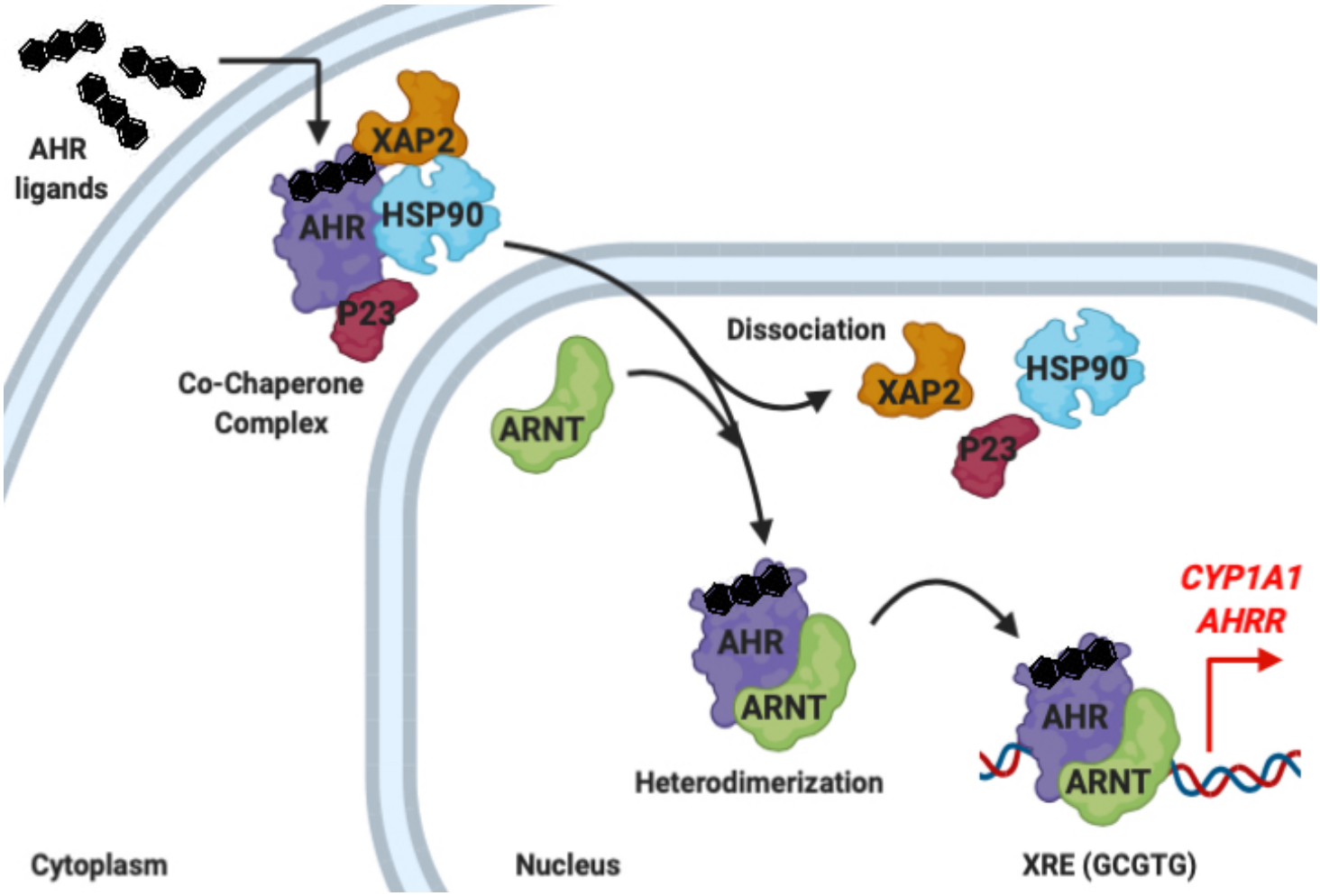

Illustration 1.1: AHR signaling pathway following activation.

AHR, residing in the cytoplasm bound to chaperone proteins, translocates into the nucleus following activation by an AHR ligand. Upon entry into the nucleus, AHR forms a heterodimer complex with ARNT, while also dissociating from the co-chaperone proteins. Together, AHR and ARNT bind to XRE sequences to induce the transcription of target genes.

AHR is also able to control the half-life of other proteins through ubiquitination. Previous reports have provided evidence that AHR acts as an E3 ubiquitin ligase that is responsible for targeted ubiquitination and proteasomal degradation of other transcription

factors[112,113]. Ubiquitin is an 8 kDa protein which is used by either E1, E2, or E3 ligase enzymes to specifically tag proteins to be targeted for proteasome-driven degradation. Briefly, following AHR ligand activation, AHR is able to interact with the cullin 4B ubiquitin ligase (CUL4B) and forms an E3 ubiquitin protein ligase complex to target proteins including the ER, P53, MYC, FOS, OCT, and HIF-1α for ubiquitination and degradation[112,113]. Interestingly, reduction or inhibition of ARNT has also been found to enhance AHR-driven ubiquitin ligase activity[114].

Since AHR is critically involved in many cellular processes, it is also tightly regulated through multiple negative regulatory feedback mechanisms to rapidly halt further signaling. One feedback mechanism is through the aryl hydrocarbon receptor repressor (AHRR), which is thought to inhibit AHR signaling through competition with ARNT[115,116]. A second regulatory mechanism involves AHR target gene expression of cytochrome P450 enzymes. Following translation, these enzymes metabolize the ligand that initiated the activation of AHR, making the ligand hydrophilic and thus easily excreted from the body. However, it is worth mentioning that certain AHR ligands are unable to be readily metabolized and have an extended half life, including TCDD, thus resulting in prolonged AHR activation[117]. A third regulatory mechanism in AHR signaling is through proteasomal degradation[118]. The half-life of AHR is limited through targeted ubiquitination and subsequent degradation by the 26S proteasome following activation[119]. Furthermore, AHR nuclear translocation can also be halted through the phosphorylation of AHR by protein kinase C (PKC). This phosphorylation event interrupts the interaction between AHR and importin-β, restricting AHR to the

cytoplasm[120]. Together, these findings demonstrate numerous regulatory elements that allow for a robust and controlled AHR-mediated response.

AHR agonists

AHR activation can occur by a variety of ligands, both from exogenous and endogenous sources. AHR is able to bind to numerous synthetic and natural ligands due to its promiscuous binding pocket made up of a flexible and extendable loop[121,122]. Early studies of AHR focused on its role in response to xenobiotic ligands, however, a growing number of studies have begun to focus on the role of AHR in response to activation from endogenous ligands. Indeed, there is still controversy over the true applicability of endogenous ligands due to the disputed abundance and concentration of these compounds in healthy individuals and in the tumor microenvironment. In addition, many AHR ligands are continually being identified for the ability to induce AHR activation.

The most well studied exogenous AHR ligands are toxicants including halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs). These molecules are commonly by-products of many chemical processes and also result from the incomplete combustion of organic compounds. TCDD is a well-known and heavily utilized AHR ligand, which belongs to the family of HAHs and has one of the highest known binding affinities to AHR[123]. TCDD also resides in the human body for approximately 7-12 years because of the inability to metabolize and excrete it[124]. Alternatively, the AHR ligand Benzo(a)pyrene (BaP) is a well recognized PAH that is able to be metabolized by CYP1A1.

More recently, physiological roles of AHR have been shown to be regulated through endogenous ligands, most of which are derived from tryptophan metabolites[125–128]. An abundance of AHR ligands are derived from vegetables in the diet, and specifically from the *Brassicaceae* family. These types of vegetables contain glucosinolate glucobrassicin, which is metabolized into the AHR ligand indole-3-carbinol (I3C) and further undergoes an acid-catalyzed condensation reaction in the stomach to form the ligand indolo-[3,2-b]-carbazole (ICZ)[129]. Over 90% of tryptophan from the diet is metabolized by two main enzymes: tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO), both of which are responsible for the generation of the AHR ligand kynurenic acid (KA) and kynurenine (KYN), which can be further metabolized into the ligand cinnabarinic acid (CA)[122,130]. Interestingly, KA has been found to be the most efficacious endogenous ligand for activating AHR and has been identified in the feces of both mice and humans, indicating its abundance in the body[131].

Investigators have also identified indoles as another critical endogenous AHR ligand, which is produced by the bacterial metabolism of tryptophan[132]. Although many exogenous ligands have a high affinity for AHR, the endogenous ligand 6-formylindolo[3,2-b]carbazole (FICZ) is also very efficacious in activating AHR at low concentrations, as AHR has a similar affinity for FICZ as it does for TCDD[133,134]. In addition, FICZ is derived from the metabolism of tryptophan through photolysis with UV light[134]. Moreover, it should also be kept in mind that AHR is abundantly expressed in both human and mouse tissues, and can be found more specifically at barrier sites including the skin, lungs, intestines, liver, and immune system[135]. Fascinatingly, these

sites are also where most molecules are being rapidly metabolized into or presented as AHR ligands to allow for enhanced AHR activation[136].

AHR in the immune system

The role of AHR was originally thought to be specifically for the metabolism of xenobiotics, however research efforts to understand the function of AHR in the immune system have drastically increased in the previous 25 years. Early studies began noticing the involvement of AHR throughout the immune system based on observations of hyperinflammation in the absence of AHR and have more recently begun demonstrating AHR as an essential regulator of immunological function and response. Notably, investigations of AHR in the immune system have been concentrated at the barrier sites of the skin, gut, and lungs, where immune cells work to fight against invading foreign bodies.

Hematopoietic stem cells are critical to the immune system as they give rise to all leukocytes. Studies have found that AHR is critical to the function of hematopoietic stem cells as *Ahr*-null mice demonstrate changes in leukocyte proliferation, which further alters the balance of immune cell populations[54,55]. In looking specifically at naïve T cells, AHR mRNA and protein are not expressed, however T cell activation results in the upregulation of AHR[137]. Throughout T cell activation and differentiation, the abundance of AHR varies. In Tregs, AHR is marginally expressed, however expression in Th17 cells is significantly enhanced[138,139]. Due to the increase of protein expression in Th17 cells, AHR is shown to have a substantial role in autoimmune disorders due to the heightened sensitivity and inflammatory response that occurs following AHR activation[140]. The

influence of AHR on T cell differentiation has also been an area of intense investigation as initial reports determined that AHR activation by specific ligands is able to influence population numbers of particular T cell subsets. Previous investigations have demonstrated that exposure of TCDD or KYN to CD4+ T cells results in the enhanced expansion of Tregs, whereas CD4+ T cells exposed to the AHR ligand FICZ, develop into Th17 cells, thus suggesting that AHR has an immunomodulatory role in T cell differentiation[141–143].

Studies investigating the role of AHR in Th17 cells determined that the expression of *AHR* mRNA is driven by signal transducer and activator of transcription (STAT) 3 and AHR, which is initiated upon T cell stimulation by IL6 and IL-21, in turn promoting Th17 differentiation[144]. This enhancement of AHR expression then drives a positive feedback loop to further increase AHR protein levels in Th17 cells. Together, STAT3 and AHR also regulate the expression of *aiolos*, which represses *IL2* expression and allows for Th17 differentiation[145]. In addition, the utilization and interference of specific STAT proteins is required for Th17 cell development[146,147]. For instance, STAT1 and STAT5 are found to inhibit Th17 cell differentiation, however during Th17 polarizing conditions, AHR interacts with both STAT1 and STAT5 to prevent interference of Th17 cell development[139,148]. AHR is also found to modulate the master transcription factor FOXP3, which is required for functioning Tregs[141,142]. Suppressive activity of Tregs is also partly though AHR, which directly controls the expression of both IL10 and CD39[149,150]. Jointly, these findings support the concept that AHR is not only critical to the differentiation of CD4+ T cells, but is required for both inflammatory and anti-inflammatory effects in Tregs and Th17 cells.

The role of AHR in the skin, gut, and lungs is also quite diverse. The skin is the body's primary line of defense against the external environment and protects against harmful factors including pathogenic infection and toxicants. During cellular expansion of the skin, AHR activation enhances the differentiation of specific human epidermal cells known as keratinocytes[151]. Keratinocytes are cells in the skin that are converted to squames to protect the tissue from the external environment and moisture loss. Interestingly, expression of AHR mRNA fluctuates at different stages of keratinocyte differentiation, similar to CD4+ T cells[152]. The function of AHR in cell cycle progression is still not completely understood, yet in vitro studies have found that suppression of AHR in immortalized keratinocyte cells results in decreased proliferation, whereas overexpression causes epithelial cells to grow more quickly[153,154]. Additionally, upon induction of psoriasis, deletion or antagonism of AHR is shown to exacerbate immune cell infiltration and enhance mRNA expression of pro-inflammatory cytokines[57]. However, AHR activation by the endogenous ligand FICZ decreased skin inflammation[57]. The modulatory role of AHR in keratinocyte differentiation, proliferation, and inflammatory response suggests that AHR is critical to the epidermal barrier and functions as an immunological brake.

Another crucial barrier that protects the host from the external environment is the intestinal tract, which is lined with intestinal epithelial cells to aid in defending against pathogenic agents. Studies also find that AHR expression is essential for gut function. Initial investigations determined that AHR is critical for maintaining appropriate inflammatory responses, intestinal epithelial cell populations, and homeostasis of the gut through observations using AHR-null mice[155,156]. The lining of epithelial cells in the intestinal tract is supported by the stromal layer, consisting of immune cell populations

prepared to defend from invading pathogens. Intestinal epithelial cells void of AHR are unable to control an infection when challenged with *C. rodentium*[157]. The inability to overcome *C. rodentium* infection was found to be a result from impaired intestinal stem cell differentiation and thus a deficiency in the intestinal barrier. Alternatively, AHR activation by the endogenous ligands ICZ and FICZ resulted in intestinal homeostasis and controlled intestinal stem cell growth[157]. Specifically, AHR is also able to regulate an inflammatory response in the gut by inducing the expression of the anti-inflammatory cytokine *IL10,* which is shown to occur following activation with KYN[158]. Collectively, these findings demonstrate that AHR activation is required for intestinal epithelial cell differentiation and maintenance of barrier function. Notably, with the abundance of AHR ligands available in the gut from bacterial and microbiotia-derived metabolites, it is no surprise that activation of AHR signaling is needed to maintain homeostasis[131,159].

The lungs are a unique tissue where a significant amount of AHR activation can also result from exogenous ligands as alveolar cells are constantly exposed to the airborne environment. In studies observing AHR-deficient mice, the development and function of the lungs were normal, however the role of AHR was increasingly obvious when the lungs were challenged with *P. aeruginosa* and displayed exacerbated lung bacterial loads and tissue damage compared to wild-type (WT) mice[160,161]. Furthermore, in alignment with previous Treg findings, WT mice exposed to TCDD prior to infection with influenza resulted in a reduction in the proliferation of CD8+ T cells, significantly reducing a bona fide inflammatory response and thus leading to an increase in mortality[162,163]. In conclusion, the function of AHR in different immunological settings has significant overlap and demonstrates critical roles in maintaining homeostasis, aiding in cellular differentiation, and conducting adequate inflammatory responses.

AHR in malignancy and autoimmunity

Dysregulation of AHR signaling has been well demonstrated to initiate the development of autoimmune diseases and tumors. Upon investigating the role of AHR in tumorigenesis, multiple mechanisms have been identified in which AHR activation induces tumor formation through exogenous and endogenous ligands. However, previous studies have demonstrated complex findings that show both beneficial and detrimental outcomes upon activating AHR in tumors and autoimmune diseases. A mechanism that is tightly associated with AHR activation and tumor formation revolves around the metabolism of particular exogenous ligands that break down into carcinogenic molecules. For example, the metabolism of BaP as well as other carcinogenic AHR ligands results in the production of harmful metabolites that form DNA adducts, which can lead to DNA mutations and give rise to cancer[164]. These results were further validated as WT mice treated with BaP showed enhanced tumor formation in comparison to mice absent of AHR, further highlighting the need of AHR activation for tumorigenesis[165]. Additionally, exposure to TCDD has also been shown to be a potent tumor promoter due to continual AHR activation and the disruption of basal AHR activity[166].

An additional mechanism involving AHR activation in tumorigenesis is tightly connected to immunity and endogenous ligands. Certain tumors are able to evade the immune system by increasing the production of the AHR ligand KYN through an overexpression of IDO. Similarly, within a tumor microenvironment, immune cells, including macrophages and dendritic cells (DCs), are also found to participate in tumor evasion through increased IDO expression and production of KYN[143,167]. The increase of IDO results in the depletion of tryptophan in the surrounding environment of the tumor,

increasing the levels of KYN, and thus augmenting AHR activation[168]. These changes then enhance tumor malignancy as well as suppress anti-tumor immunity[168]. This immunosuppressive mechanism is found to involve IL-6 and STAT3 as enhanced AHR activation from KYN results in the increase of *IL-6* mRNA expression and secretion, whereas knockdown of AHR reduces IL-6 levels[169,170]. Expression of IL-6, leads to STAT3 activation, which in turn drives transcription of IDO, thus completing the autocrine signaling loop[169,170].

The depletion of tryptophan and enhanced activation of AHR in surrounding immune cells are both beneficial to malignancies and is used as a protective mechanism against the immune system of the host. Reduction in available tryptophan reduces population numbers of surrounding immune cells, as it is needed for cell survival. The loss of available tryptophan subsequently leads to cell cycle arrest and the downregulation of T cell receptors in CD8+ T cells, further dampening an immune response[143,171]. The enhanced activation of AHR by KYN also results in an increase in Treg populations and immunosuppression[167,168]. Tragically, this autocrine pathway between AHR, KYN, and IDO also prevents interferon-γ (IFN-γ)-induced cell death and instead activates a T cell dormancy mechanism, therefore allowing for continued growth.

An example of this autocrine signaling loop can be specifically found in glioblastomas, which is a highly malignant cancer that is particularly difficult to treat. High concentrations of KYN are found in the cell culture media from glioblastoma cell lines along with an increase in AHR activation[172]. Furthermore, an investigation in mice using a xenograft model with a glioblastoma cell line demonstrated that loss of AHR resulted in an increase in immune cell infiltration due to the lack of suppressive effects

following AHR activation[172]. Overall, these findings highlight the importance of tryptophan metabolites, specifically KYN, in the suppression of the immune system for malignancies.

Recently, interleukin-4-induced-1 (IL4I1) has been found to be more tightly associated with AHR activity in 32 different malignancies compared to IDO or TDO[173]. IL4I1 mediates the metabolism of tryptophan into indoles as well as KA, which is a tryptophan metabolite and AHR ligand from the KYN pathway[173,174]. The upregulation of IL4I1 in malignancies functions similarly to IDO as AHR activation is enhanced, adaptive immunity is suppressed, and cancer cell motility is promoted[173].

Additional supporting data has shown that constitutive AHR activation enhances tumor formation. Investigations utilizing transgenic mice with a deletion of the PASB domain of AHR, resulting in constitutive activity, were found to form tumors both in the stomach and liver[175–177]. Additional data demonstrates an upregulation in AHR activity through elevated levels of nuclear AHR and increased mRNA expression of *AHR* and AHR target genes in the primary tissue of patients with squamous cell carcinoma[178]. Collectively, studies have found that active AHR signaling has a role in tumor growth, survival, infiltration/migration, and chemoresistance[178,179].

AHR has not only been shown to aid in tumor formation, but has also been found to have antitumorigenic roles as well. These findings were initially discovered through observations of spontaneous tumor formation in *Ahr*-null mice. These mice developed malignancies in a variety of tissues including the colon, intestine, liver, prostate, and blood, suggesting AHR has a tumor suppressive role[180–183]. Together, these investigations suggest that AHR possesses protumorigenic and antitumorigenic properties, however it

should also be kept in mind that genetic alterations to AHR may have severe off-target consequences. Overall, these findings provide evidence that AHR activation promotes tumorigenesis, however the presence of AHR is needed to sustain cellular homeostasis and function in an antitumorigenic role.

As previously described, AHR has many crucial roles in the immune system, however reduced or dysfunctional AHR activity is shown to disrupt homeostatic immune function and lead to the progression of autoimmune diseases. Investigations studying the effects of xenobiotic exposures have found that AHR activation from these compounds is linked to excessive inflammation, epigenetic changes, and oxidative stress, hence contributing to the progression of autoimmune diseases[184].

Individuals who smoke or are exposed to environmental contaminants containing AHR ligands are found to have an increased risk of developing RA[185]. Strikingly, mRNA expression of *AHR* is twice as high in peripheral blood mononuclear cells (PBMCs) of patients with RA compared to healthy individuals[186]. Studies investigating RA also found increased levels of KA in the synovial fluid of patients[174,187]. The increase in KA results in enhanced AHR activation, which is found to induce both *IL-6* expression and inflammation, whereas suppression of AHR reversed these effects[174,187,188]. Alternatively, patients with MS have lower levels of circulating AHR ligands compared to healthy individuals[189]. Studies show that in mouse MS models using experimental autoimmune encephalitis (EAE), AHR activation attenuates EAE-induced inflammation through an increase in Treg differentiation, whereas knockdown of AHR results in the enhancement of disease progression[189,190]. Similar to MS, antagonism or suppression of AHR in psoriasis models results in excessive inflammation, whereas AHR activation reduces

psoriasis and inflammatory symptoms[57]. Synonymously, studies of human acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) show that AHR signaling is repressed, however activation by FICZ results in impaired leukemic growth and lack of self-renewal[191,192]. These findings together suggest that AHR has a central role in the progression of autoimmune disorders and tumors in a disease-specific manner.

ARYL HYDROCARBON RECEPTOR NUCLEAR TRANSLOCATOR (ARNT)

Introduction

The laboratory of Dr. Oliver Hankinson first discovered ARNT in the 1980's by initially observing that AHR was unable to translocate into the nucleus and induce target gene transcription in an isolated mutant Hepa-1c1c7 cell colony and through Hepa-1c1c7 cell somatic hybridization studies. Subsequently, a specific gene, later identified as ARNT, was determined to be mutated within these specific cells compared to wild type (WT) cells. Clones were then synthesized to investigate if ARNT had any role in AHR signaling. Performance of gel retardation assays demonstrated a dioxin-induced retarded band in nuclear extracts from cells containing ARNT and AHR. This initial investigation described ARNT to be required for the translocation of AHR from the cytosol to the nucleus following activation[193]. However, a seminal study performed by Dr. Richard Pollenz determined that these initial findings were a mis-interpretation and that through immunofluorescence microscopy, ARNT was found to be localized to the nucleus both prior to and following AHR activation to aid in DNA recognition with AHR[193–195]. Since this initial discovery, investigations have provided further information and insight into

the role of ARNT both constitutively and upon activation of numerous signaling pathways.

ARNT, also known as hypoxia-inducible factor (HIF)-1β, like AHR, is a transcription factor and member of the bHLH-PAS family. Members of the bHLH-PAS family have a wide range of functions including responding to external stimuli, maintaining homeostasis, immune response, cellular differentiation, circadian rhythmicity, and malignancy[118,196–200]. ARNT is a class II bHLH-PAS member, which indicates that it is continuously expressed and is a dimerization partner of class I bHLH-PAS members[69]. The gene of *ARNT* is located within the region of chromosome 1q21.3 and is made up of 789 amino acids[201]. The overall structure of ARNT is comprised of four specific domains: bHLH, PASA, PASB, and TAD. The bHLH domain of ARNT, located near the amino-terminus, is essential for recognizing and binding to DNA as mutation studies of this domain have demonstrated that it is required for binding specifically to the GTG sequence of XREs. However, the recruitment of co-activators does not require the bHLH region[71,72,202–205]. The PASA domain of ARNT is also needed for DNA binding as well as dimerization with AHR[203,204,206]. Investigations of the PASB domain of ARNT have suggested a role in AHR and HIF-2α heterodimerization as well as co-regulator recruitment[79,203,206,207]. Located near the C-terminus, the TAD of ARNT is required for activating transcription, as deletion of the C-terminal TAD abolishes transcriptional activity[208–211].

Molecular pathways involving ARNT

ARNT has been demonstrated to have critical roles in the signaling pathways of AHR, HIF, and NF-κB[70,212]. When utilized in AHR signaling, the role of ARNT is to aid in initiating transcription in response to AHR activation. Following activation, AHR forms a heterodimer complex with ARNT in the nucleus to induce the expression of target genes containing a conserved enhancer XRE sequence including *CYP1A1* and *AHRR*[89,90,213]. Both AHR and ARNT are required for the transcription of XRE-responsive genes as demonstrated by studies showing that cells lacking either protein fail to alter XRE-driven expression[70,214–216]. Previous investigations have also described a negative feedback mechanism in AHR signaling with ARNT and the AHRR following target gene transcription. *AHRR* expression is regulated by AHR and ARNT as XREs have been identified upstream of the *AHRR* gene[116]. Initial hypotheses suggested that following translation of AHRR, AHRR/ARNT heterodimer complexes are formed to repress AHR activity through competitive binding to ARNT at XREs[115]. However, upon further investigation, the mechanism by which this negative feedback occurs between AHRR and ARNT appears to be more complicated than originally postulated as a previous study has demonstrated that neither the competitive binding to ARNT nor the displacement of AHR from XREs are the only mechanisms by which AHRR functions to repress AHR signaling[217].

In addition to the role of ARNT in AHR signaling, ARNT is also utilized under hypoxic conditions by HIF-1α, which initiates activation of the HIF signaling cascade. During normoxia, HIF-1α is hydroxylated at proline 402 and proline 564, leading to its ubiquitination and degradation by the proteasome and not allowing for the initiation of

the HIF pathway[218]. However, in hypoxic conditions, hydroxylation of the HIF-1α proline residues is unable to occur due to an insufficiency of oxygen. This lack of oxygen allows for the accumulation of HIF-1α and heterodimerization with ARNT following nuclear translocation[219]. HIF-1α/ARNT heterodimers then recognize and bind to hypoxia response elements (HRE) most often in reaction to low oxygen conditions to induce target genes that will elicit an adaptive response[220].

It is increasingly evident that ARNT also has roles in the NF-κB signaling pathway as cross talk between AHR, ARNT and NF-κB has begun to surface. Although the direct involvement of ARNT in NF-κB signaling still appears to be widely unknown, a few studies have revealed intriguing insights. In a previous study, we found that ARNT promotes the DNA binding of NF-κB subunit RelB[221]. Additionally, the promotion of RelB binding to the DNA was found to block the activity of RelA-p50 dimers, however upon suppression of ARNT, RelB DNA binding was abrogated and RelA binding was augmented, thus enhancing canonical NF-κB signaling[221]. Moreover, NF-κB is also found to regulate ARNT levels. Through the use of NF-κB inhibitors and small interfering RNA (siRNA) technology, a previous study demonstrated that RelA regulates the mRNA and protein expression of ARNT through direct interaction with the ARNT promoter following NF-κB activation by TNFα[222]. Together, these findings suggest that the NF-κB subunits not only regulate ARNT, but together also regulate DNA binding to increase NF-κB target-gene transcription.

Constitutive function of ARNT

During basal conditions, ARNT mRNA and protein levels are constitutively expressed and unchanging in the nucleus, however reports have also demonstrated that ARNT protein levels can be further stabilized in the presence of HIF-1α[223–225]. Physiological functions of ARNT have been discovered through observational studies with *Arnt* knockout (KO) mice. Throughout development, ARNT has proven to be essential for survival, as many studies have found that transgenic *Arnt* KO mice have deficient vascularization of the yolk sac and placenta, leading to fetal demise by gestational day 10.5[226,227]. Subsequent studies revealed that ARNT is required for the promotion of angiogenesis by enhancing the expression of vascular endothelial cell growth factor receptor 2 (VEGFR2), thus providing a rationale for the deficiencies in placenta vascularization in Arnt deletion studies[228].

Histone deacetylase proteins (HDACs) are responsible for removing acetyl functional groups from the lysine residues of histones, making the DNA less accessible to transcription factors. Studies find that upon depletion of ARNT in keratinocytes, HDAC activity is significantly increased including that of HDAC1, HDAC2, and HDAC3. Conversely, overexpression of ARNT resulted in the opposite affect[229]. These findings were further supported as suppression of ARNT induced the expression of multiple differentiation markers of keratinocytes[229]. Alternatively, *Arnt*-null trophoblast stem cells display reduced HDAC activity, increased global acetylation, and altered nuclear localization of HDACs[230]. The molecular regulation of HDACs by ARNT is still unclear, however evidence suggests a link between DNA accessibility and ARNT activity.

Pancreatic β-cells are responsible for the synthesis, storage, and release of the hormone insulin in response to the needs of the body[231]. Numerous studies have investigated ARNT in relation to β-cell function as expression levels of ARNT are decreased in humans with type 2 diabetes[232,233]. In looking at the role of ARNT in diabetes, β-cells are found to be protected against diabetes induced by a high-fat diet upon loss of ARNT[234]. Additionally, genes required for β-cell function, glucose tolerance, and insulin secretion are significantly attenuated following the loss of ARNT, thus suggesting that pancreatic β-cells require ARNT to maintain function and homeostasis[232,233,235].

In addition, recombinant protein assays and studies within intact cells have provided evidence that ARNT is able to form homodimer complexes that bind to the palindromic enhancer box (E-box) core sequence of CACGTG[236–238]. Although the role of ARNT homodimers remains unknown, complex formation at E-box elements suggests a transcriptional role independent of other major binding partners. Moreover, a whole genome analysis study has addressed the question of ARNT specific target genes[239]. This analysis was performed by reintroducing ARNT into mutant Hepa1c1c7 cells devoid of ARNT due to a point mutation in the coding region of the ARNT gene[240]. Following DNA microarray analysis, 27 genes were found to be upregulated and no genes were found to be downregulated compared to control cells[239]. Interestingly, the 27 genes found to be upregulated involve various cellular processes including cell growth and maintenance, metabolism, stimulus response, homeostasis, and cell communication[239].

All together, the physiological role of ARNT still remains widely unknown in many tissues. However, it is becoming increasingly clear that ARNT has a significant

impact on the gene expression and function of many physiological processes that are required for development and maintaining homeostasis.

The isoforms of ARNT

Upon its initial discovery, select clones of ARNT were found to include 45 nucleotides that were not identified in others[193]. It was later postulated that these differences stemmed from alternative splicing, thus leading to the generation of multiple ARNT isoforms. Since this initial study, additional investigations have validated that ARNT is alternatively spliced into two isoform, which are referred to as ARNT isoform 1 and ARNT isoform 3[59,241]. Previous investigations have also demonstrated the presence of each isoform in both human and mouse cells[59,202,241]. More specifically, structural analysis of each ARNT isoform demonstrates a difference of a single exon, identified as exon 5, which consists of 15 amino acids located near the amino terminus of only ARNT isoform 1[193,241]. Furthermore, fellow bHLH member ARNT 2, which is encoded on a different gene, is found to be nearly 90% similar to ARNT and is primarily expressed in the kidney and central nervous system[242,243]. However in this investigation, we will not be studying ARNT 2 as our focus is specifically on the isoforms of ARNT within the immune system.

ARNT in the immune system

In knowing that AHR is critical to immune function, it is no surprise that ARNT is also vital in controlling immunity. To examine the role of ARNT in the skin, keratinocyte-specific *Arnt*-deficient mice were generated. Investigations found that loss

of ARNT, similar to suppression studies of AHR, resulted in the inadequate construction of the cellular epidermal layer and improper barrier function, suggesting ARNT is critical for the strength and maintenance of the epidermal barrier through AHR and HIF mechanisms[244–246]. Another report demonstrated that ablation of ARNT specifically in the epidermis also revealed roles in skin vascularization, blood clotting, and vessel strength[247].

ARNT also regulates the gene expression of many cytokines needed for inflammatory responses in myeloid cells. Studies have found that mice containing myeloid cells devoid of ARNT exhibit a reduction in skin inflammation and delays in wound healing following exposure to sodium dodecyl sulfate (SDS)[65]. Moreover, loss of ARNT in myeloid cells also causes a reduction in cytokine mRNA expression in macrophages[65]. Conversely in a colitis model, mice with myeloid cells depleted of ARNT demonstrated elevated immune cell infiltration of the colon along with an increase in both mRNA expression and secretion of pro-inflammatory cytokines[64]. Additionally, these mice were found to develop steatohepatitis, accompanied with an increased expression of inflammatory cytokines while on a high fat diet, thus suggesting that ARNT also has a role in the progression of non-alcohol steatohepatitis[248]. Collectively, these findings demonstrate that ARNT is crucial for the regulation of inflammatory cytokine expression in the immune system.

Previously, a study examining inflammation of the gut uniquely found that ARNT and the inflammatory cytokine IFN-γ have an inverse relationship in intestinal epithelial cells with a murine colitis model[249]. This study demonstrates that ARNT in intestinal epithelial cells is transcriptionally repressed through IFN-γ in a janus kinase-STAT (JAK-

STAT) dependent manner. Furthermore, ARNT also has a role in the immunity of the gut microenvironment as mice with ARNT-deficient CD4+ T cells showed alterations in the development of T cell subsets specific to the basement membrane of the intestinal epithelium[250]. Indeed, further investigations are needed to decipher the mechanisms by which ARNT regulates gut immunity, however these observations collectively suggest a role in preserving homeostatic gut function.

Research of ARNT has also revealed a critical role in hematopoiesis similar to AHR, revealing potential AHR-dependent/ARNT-mediated effects. To investigate the function of ARNT in the formation of blood cells, mice with *Arnt*-deficient hematopoietic stem cells (HSCs) were generated, which displayed an increase in the total number of leukocytes in the peripheral blood. Further, analysis by flow cytometry revealed increases in B cell populations within the spleen and bone marrow, however T cell numbers were significantly reduced in the spleen and thymus[251]. This striking phenotype suggests that ARNT has a central role in the proliferation and differentiation of HSCs. An additional investigation observed that the viability of mice with a T cell specific knockout of ARNT was not affected nor were the T cells phenotypically or histologically altered[252]. However, these mice were resistant to thymic involution following exposure to the AHR ligand and environmental toxicant TCDD[252]. These findings indicate that TCDD-mediated thymic involution requires ARNT in T cells and provides further importance to study the immunologic role and function of ARNT.

ARNT and Malignancies

In addition to the roles mentioned above, ARNT, like many proteins, is also utilized in malignancies. In studying the human population, single nucleotide polymorphisms (SNPs) within the *ARNT* gene are found at a very low frequency, however a few SNPs have been identified[253]. The majority of SNPs identified are found in the PAS and TAD regions of ARNT and a mutation specifically at V304M is found to significantly impact protein function and promote degradation via *in silico* analysis[253]. These findings indicate that mutation of ARNT within the human population may compromise the signaling pathways requiring ARNT, thus potentially enhancing formation of malignancies and promoting tumor survival[254].

ARNT has also been identified to provide tumor cells resistance to anti-cancer agents[59,255]. To note, ARNT is found to induce the expression of multidrug resistance 1 (*MDR1*), which encodes an efflux pump responsible for expelling cancer-fighting therapies from tumor cells. Markedly, suppression of ARNT resulted in a significant decrease in *MDR1* and sensitized malignant cells to cisplatin-induced cell death, whereas overexpression of ARNT reversed the effects of cisplatin and promoted cell survival[255]. Similar to AHR, ARNT has also been reported to have a role in squamous cell carcinoma. *ARNT* mRNA expression is shown to be increased in human tissue samples of squamous cell carcinoma and is found to be responsible for the upregulation of the tumor growth related protein cyclooxygenase-2 (COX-2)[256]. Alternatively, intratumoral ARNT protein levels were found to be directly related to a patient's outcome with hepatocellular carcinoma (HCC), as high levels of ARNT protein within the tumor resulted in longer survival and lower rates of recurrence[257]. Knockdown studies further demonstrated that

suppression of ARNT significantly augmented cell proliferation in HCC cell lines, whereas overexpression of ARNT reduced the rate of proliferation[257]. These findings suggest that manipulation of ARNT may have differing effects in specific malignancies as antagonism of ARNT can be a potential strategy in targeting malignancies relying on MDR1 or COX-2, however should be avoided in patients with HCC.

Other studies have also made observations regarding the role of ARNT in malignancy and tumor growth. ARNT is found to be required during the early stages of hepatic tumor development, but less during later stages as diminishment of ARNT throughout the measurable growth period significantly repressed the progression of the tumor[258]. Furthermore, previous results show that ARNT is required for the development of von Hippel-Lindau (VHL) – associated vascularized tumors[259]. Mutation of the *VHL* tumor suppressor gene can lead to the progression of vascular tumors and constitutive HIF-1α activation, however upon inactivation of Hif-1α, development of vascular tumors was unchanged and expression of growth factors were not suppressed. Markedly, only suppression of Arnt blocked the progression of VHL-associated tumors and expression of growth factors in the liver[259]. All together, these investigations provide evidence for targeting ARNT as a potential therapeutic option to combat malignancy.

Chapter 2 Materials and Methods

CELL CULTURE AND REAGENTS

Karpas 299, Jurkat, and Peer cells were propagated in RPMI-1640 medium (Corning, 15-041-CV) complete with 10% FBS (Atlanta Biologicals, S11550) and 2 mM GlutaMAX (Gibco, 35050-061) at 37 °C, 5% CO_2. Hepa-1c1c7 cells were cultured in MEM Alpha (1X) + 2 mM GlutaMAX (Gibco, 32561-037) with 10% FBS at 37 °C, 5% CO_2. Stable BpRc1 cells, a variant of Hepa-1c1c7 cells, were cultured in DMEM (Corning, 15-018-CV) with 10% FBS, 2 mM GlutaMAX, and 2 μg/mL puromycin (Invivogen, ant-pr) at 37 °C, 5% CO_2. HEK293 cells were cultured in DMEM with 10% FBS, and 2 mM GlutaMAX at 37 °C, 5% CO_2. For CK2 inhibitor experiments, 1×10^7 Karpas 299 or Peer cells were treated with 5 μM CX-4945 (Selleckchem, S2248) for one hour prior to AHR ligand exposure. AHR antagonist experiments were conducted by pretreating 1×10^7 Karpas 299 or Peer cells with 10 μM CH223191 (MilliporeSigma, C8125) for 2 hours. AHR ligands are as follows: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (Cerilliant, ED-901-C), 6-Formylindolocarbazole (FICZ) (MilliporeSigma, SML1489), β-Naphthoflavone (BNF) (MilliporeSigma, N3633), L-Kynurenine (KYN) (MilliporeSigma, K8625), both Indolo[3,2-b]carbazole (ICZ) and Cinnabarinic Acid (CA) were obtained from Dr. Cornelis Elferink (The University of Texas Medical Branch, Galveston, TX).

NUCLEAR TRANSLOCATION ASSAY

1×10^7 Karpas 299, Hepa BpRc1, and Peer cells were treated with vehicle control (DMSO), 10 nM TCDD, or 1 nM FICZ for the designated time. Cells were collected, washed with PBS (Corning, 21-040-CV), and cytoplasmic and nuclear protein fractions were collected with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, 78835) and experimental procedures were performed as outlined in the protocol provided.

ANTIBODIES AND IMMUNOBLOT ANALYSIS

Whole cell lysates were extracted by incubating cells with radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling, 9806S) complete with phosphatase inhibitor cocktail 2 (MilliporeSigma, P5726), phosphatase inhibitor cocktail 3 (MilliporeSigma, P0044), complete mini protease inhibitor tablets (Roche, 11836170001), and 50 mM sodium fluoride (MilliporeSigma, S1504-100G) on ice for twenty minutes. Protein samples were resolved on NuPAGE 4-12% Bis-Tris Gels (Invitrogen, NP0322BOX) and transferred onto nitrocellulose membranes (Bio-Rad, 1620215). Membranes were blocked with 1X TBS 1% Casein Blocker (Bio-Rad, 1610782) supplemented with 0.1% Tween 20. Membranes were incubated with the indicated antibodies, washed 4X/7 minutes each with 1X TBST, incubated with specific horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, NA931 (Mouse) or NA934 (Rabbit)) for one hour, and then washed 4X/7 minutes each again. Lastly, ECL substrate (Bio-Rad, 170-5060) was added to the membrane for five minutes and horseradish peroxidase activity was imaged on the

ChemiDoc MP Imaging System (Bio-Rad). Antibodies used are as follows: ARNT-pS77 (ThermoFischer, custom ab to sequence C-KERFARSDDEQSS-amide) ARNT (BD Transduction Laboratories, 611079), AHR (ENZO, BML-SA210-100), Lamin A/C (Santa Cruz, sc-20681), α-Tubulin (Cell Signaling, 2148S), β-Actin (MilliporeSigma, A5316).

MOUSE TISSUE EXTRACTION

Tissue samples were extracted from C57BL/6 mice and snap frozen with dry ice until stored at -80 °C. Protein was extracted from collected tissues homogenized (Polytron) in complete RIPA lysis buffer.

STABLE CELL LINE GENERATION

Hepa-1c1c7 cells and variant BpRc1 cells were kindly provided by Dr. Cornelis Elferink (The University of Texas Medical Branch, Galveston, TX). Lentiviral packaging in HEK 293 cells was performed as previously described (Galbán *et al,* 2009). Packaged virus contained a WT ARNT isoform 1 plasmid or an ARNT isoform 1 plasmid with S77 mutated to an alanine (S77A). The lentivirus was then incubated with 5×10^4 BpRc1 cells per well in a 6 well plate for four hours at 37 °C, 5% CO_2. Media was then replaced with fresh DMEM and incubated forty-eight hours at 37 °C, 5% CO_2. Stable cells were selected based upon resistance to 2 μg/mL puromycin.

CO-IMMUNOPRECIPITATION

Following protein extraction with complete RIPA lysis buffer, 2 μg of ARNT (Novus, NB100-124) or AHR (ENZO, BML-SA210-100) antibody was added to each sample and rotated at 4 °C overnight. The following morning, 30 μL of rProtein G Agarose beads (Invitrogen, 15920-010), prewashed three times with complete RIPA lysis buffer, was added to each sample and placed back onto the 4 °C rotator for an additional hour. Samples were then washed four times with Triton-X 100 lysis buffer (containing 25 mM Hepes pH 7.9, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton-X 100). Loading buffer containing sodium dodecyl sulfate and dithiothreitol was added to bead-bound protein complexes and subsequently boiled for 5 minutes.

TRANSFECTION

HEK 293 cells were transfected using a standard calcium phosphate transfection protocol as follows: 3.5×10^6 HEK 293 cells were seeded onto 10 cm dishes. In a microcentrifuge tube, plasmid DNA (1-3 μg), 250 mM $CaCl_2$, and H_2O (to bring final volume up to 500 μL) were combined. The DNA-$CaCl_2$ solution was then added dropwise to 500 μL of 2X HEPES-buffered saline (HBS) (containing 280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4 \bullet 2\ H_2O$, 12 mM dextrose, and 50 mM HEPES). The solution was then incubated at room temperature for 15 minutes. While incubating, media was then aspirated and a fresh 9 mL of DMEM was added to HEK 293 cells in 10 cm dishes. Following the incubation, the $CaCl_2$-HBS solution was added dropwise onto the cell and incubated at 37 °C, 5% CO_2 for 7 hours. Media was then replaced with 10 mL of fresh DMEM and cells were left in the incubator at 37 °C, 5% CO_2 overnight.

RNA INTERFERENCE

1×10^7 Karpas 299 and Peer cells were transfected with 4 μM of target siRNA duplexes using a Bio-Rad Gene Pulser Xcell electroporator set on infinite resistance, 300 V for Karpas 299 cells or 250 V for Peer cells, and 950 microfarads. Cells were suspended in RPMI-1640 medium containing 20% FBS and 1% GlutaMAX. Sixteen hours post-transfection, dead cells were removed by centrifugation at 400 x *g* for 20 min with Ficoll-Paque PLUS (GE Healthcare, 17-1440-02). Live-cell layer was then transferred to RPMI-1640 medium complete with 10% FBS and 1% GlutaMAX at 0.5×10^6 cells/mL for 24 hours. The siRNA (MilliporeSigma) target sequences are as follows: siARNT-1 5'-UGC CAG GUC GGA UGA UGA GCA-3′, siARNT-3 5'-CGG UUU GCC AGG GAA AAU C-3', siARNT-1/3 5'-GAC UCG UAC UUC CCA GUU U-3' and 5'-CUU UGC UCC UGA GAC UGG A-3', and AHR (MilliporeSigma, SASI_Hs01_00140202). The target sequence for siControl is a scrambled siARNT-1/3 sequence.

CHROMATIN IMMUNOPRECIPITATION

40 hours after RNA interference, 8.43×10^6 Karpas 299 cells were exposed to DMSO or TCDD (10 nM) for 30 minutes. Protein-DNA complexes in Karpas 299 cells were then cross-linked with 1% formaldehyde (Polysciences Inc., 18814-10) for 10 minutes at room temperature, which was followed by quenching in 125 mM glycine for 5 minutes at room temperature. Cells were collected via centrifugation at 300 x g for 5 minutes at 4 °C, then washed in PBS and spun down again at the same speed at 4 °C. Cells were then lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCL pH 8.1), supplemented with phosphatase inhibitor cocktail 2 (MilliporeSigma, P5726), phosphatase inhibitor cocktail

3 (MilliporeSigma, P0044), complete mini protease inhibitor tablets (Roche, 11836170001), and 50 mM sodium fluoride, at a ratio of 100 μL per 2×10^6 cells. Cells were left to lyse on the ice for 10 minutes. Chromatin was then sheared into ~300bp fragments by sonicating lysates for 8, 10 second bursts with a Branson Sonicator attached with a micro tip. The supernatant was collected by centrifugation by spinning at top speed for 10 minutes at 4 °C and transferred into new tubes with 100 μL each. 900 μL of ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL pH 8.1, and 150 mM NaCl) was added to each aliquot. To pre-clear each supernatant, 30 μL of a 50% slurry of blocked (2 μg sonicated Salmon sperm DNA/ 20 μL of 50% slurry) protein G agarose beads was added to each aliquot and rotated at 4 °C for 2 hours. Beads were then pelleted by centrifugation at 2000 rpm for 2 minutes at 4 °C and the supernatant was transferred to a new tube. 2 μg of the immunoprecipitating antibody was added to each supernatant and then rotated overnight at 4 °C.

The following morning, 30 μL of a 50% slurry of blocked (2 μg sonicated Salmon sperm DNA/ 30 μL of 50% slurry) protein G agarose beads was added to each sample and rotated at 4 °C for one hour. Beads were then washed for 5 minutes on the rotator at 4 °C with 1 mL of the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl), LiCl wash buffer (250 mM LiCl, 1% NP-40, 1 mM EDTA, 10 mM Tris-HCL pH 8.1, and 1% deoxycholate), and twice with 1 mL of TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA). Co-immunoprecipitated complexes were eluted from protein G agarose beads with 100 μL of elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 minutes at room

temperature. Beads were collected by centrifugation at 2000 rpm for 2 minutes at room temperature. The supernatant was transferred to a new tube and the elution process was repeated once more. The eluates were combined and the cross-links of the DNA-protein complexes was reversed with the addition of 8 μL 5 M NaCl and incubating at 65 °C overnight.

The next morning, 1 μL of RNase A (10 mg/mL) was added to each sample and incubated at 37 °C for 30 minutes. Then, 4 μL of 0.5 M EDTA, 8 μL 1M Tris-HCl pH 6.5 and 1 μL proteinase K (New England BioLabs, P8107S) was added to each sample and incubated at 45 °C for 2 hours. DNA was purified using PCR purification columns (Qiagen, 28106) per the manufacturer's protocol. PCR reactions were conducted with 5 μL of purified DNA, 1 μL of 10 μM forward and reverse primers, and 12.5 μL of PCR 2x master mix (Promega, M750C). Primers used for the *CYP1A1* promoter: forward: 5'-TAA GAG CCC CGC CCC GAC TCC T-3' and reverse: 5'-CTC CCG GGG TGG CTA GTG CTT TGA-3'.

T CELL ACTIVATION STUDIES

1×10^7 Peer cells were activated with 20 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma, P1585-1 MG) and 1 μg/mL of ionomycin (IO) (Sigma, I0634-1MG) for the indicated times, then collected.

REVERSE TRANSCRIPTION QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR) ANALYSIS

RNA was extracted using QIAshredder (Qiagen, 79656) and RNeasy Kits (Qiagen, 74104). Then 300 ng of RNA was converted to complementary DNA (cDNA) using iScript cDNA Synthesis Kits (Bio-Rad, 1706691). RT-qPCR was performed on the CFX96 Real Time System (Bio-Rad) using SsoAdvanced Universal Probes Supermix (Bio-Rad, 1725284) for Taqman assays and SsaAdvanced Universal SYBR Green Supermix (Bio-Rad, 1725274) for SYBR Green assays. The following Taqman primers (Bio-Rad) were used against homo sapiens: *CYP1A1* (qHsaCEP0058439) and *AHRR* (qHsaCEP0057513). *GAPDH* (qHsaCEP0041396) was the internal reference gene used to determine fold changes with the ΔC_T method. The following primers for SYBR Green assays were used at 10 μM against homo sapiens: *CSF2* 5'-GGA GCA TGT GAA TGC CAT CCA G-3' and 5'-CTG GAG GTC AAA CAT TTC TGA GAT-3'; *IL3* 5'-AAG CAG CCA CCT TTG CCT TTG C-3' and 5'-ACA GCC CTG TTG AAT GCC TCC A-3'; *IL6* 5'-AGA CAG CCA CTC ACC TCT TCA G-3' and 5'-TTC TGC CAG TGC CTC TTT GCT G-3'. Primers against murine for SYBR Green assays were used at 10 μM and are as follows: *Cyp1a1* 5'-CCT CAT GTA CCT GGT AAC CA-3' and 5'-AAG GAT GAA TGC CGG AAG GT-3'; *Tiparp* 5'-GCC AGA CTG TGT AGT ACA GCC-3' and 5'-GGG TTC CAG TTC CCA ATC TTT T-3'. The internal reference gene for all SYBR Green assays was *18S* (10 μM) 5'-CGC TCC ACC AAC TAA GAA CG-3' and 5'-CTC AAC ACG GGA AAC CTC AC-3'; changes in fold gene expression was calculated by using the ΔC_T method.

STATISTICAL ANALYSIS

All data was graphed as mean ± SEM and performed in triplicate. One representative data set was shown for each RT-qPCR experiment. Statistical differences between two individual groups were assessed by performing a two-tailed unpaired Student's *t* test. Analyses of statistics were performed using SigmaPlot 11 (Systat Software Inc). Results from RT-qPCR were considered significant when a *P* value of less than 0.05 was achieved.

Chapter 3 ARNT isoform-specific regulation of AHR signaling

INTRODUCTION

The aryl hydrocarbon receptor nuclear translocator (ARNT), also known as hypoxia-inducible factor (HIF)-1β, is a member of the basic-helix-loop-helix-PER/ARNT/SIM (bHLH-PAS) superfamily of transcription factors and is essential for development[260,261]. ARNT is a nuclear-localized transcription factor that regulates a number of cellular processes including: inflammation, the response to hypoxia, cell survival, proliferation, and clearance of xenobiotic compounds[118,233,262]. To control these biochemical operations, ARNT dimerizes with other class I bHLH-PAS family members such as the aryl hydrocarbon receptor (AHR), which is restricted to the cytoplasm through chaperone proteins prior to activation[80,91,99,100,263,264]. AHR recognizes a variety of ligands and is activated by certain xenobiotic compounds, metabolic products, and chemicals generated by gut flora[125,130,143,265–267]. Upon activation, AHR translocates to the nucleus where it sheds its chaperone proteins and forms a transcriptional complex with ARNT[108]. The AHR-ARNT complex induces the expression of target genes, such as *CYP1A1* and *AHRR* by binding to a conserved enhancer sequence known as a xenobiotic response element (XRE)[213]. ARNT and AHR are both required for transcription of XRE-responsive genes as demonstrated by the fact that absence of either protein results in the failure to drive expression of XRE target genes[70,214–216].

In its initial identification, complementary DNA (cDNA) clones of ARNT generated from specific mutant Hepa-1c1c7 cell colonies included a unique 45-nucleotide fragment that was not present in others[193]. This difference was suggested to be a consequence of alternatively splicing, which would result in the production of different

ARNT isoforms. Alternative splicing is a process that allows for the generation of multiple proteins from a single gene through the inclusion or exclusion of specific exons and provides organisms the ability to exponentially increase the number of proteins able to be translated from a set number of protein-coding genes[268].

Following these initial observations, ARNT was confirmed to be alternatively spliced into a long and short transcript (now referred to as ARNT isoform 1 and isoform 3, respectively)[59,241]. Structural analysis of these ARNT isoforms indicates a difference of only one alternative exon near the amino-terminus of ARNT isoform 1, encoding 15 amino acids[193,241]. Since this initial finding, the alternatively spliced form of ARNT has been identified in both mouse and human cells and in a previous report, we demonstrate that primary human lymphocytes express equal levels of ARNT isoform 1 and 3[59,193,202].

In exploring the role of the ARNT isoforms in the immune system, we previously investigated the ratio of the ARNT isoforms specifically in malignant B and T cell lines and found that ARNT isoform 1 protein is increased in abundance compared to ARNT isoform 3[59]. Interestingly, suppression assays revealed that loss of ARNT isoform 1 resulted in S phase cell cycle arrest, whereas simultaneous knockdown of both ARNT isoforms had no affect[59]. Further examination of ARNT also revealed that isoform 3 induces the expression of cell cycle inhibitors, specifically *CDKN1A* and *CDKN2B*. Jointly, these findings indicate that ARNT isoform 3 activity is responsible for the suppressive effects on cellular proliferation following loss of ARNT isoform 1 and suggests that ARNT isoform 1 is upregulated in malignant B and T cells to overcome the inhibitory function of ARNT isoform 3.

The ARNT isoforms are also found to mediate protein stability, expression, and activity. Upon examination of ARNT, previous findings suggest that isoform 3 is directly

responsible for regulating the gene expression and protein stability of RelB[59]. Furthermore, the tumor suppressor p53 is also influenced by the ARNT isoforms as suppression of both isoforms results in a reduction of protein level and activation of p53, whereas loss of only ARNT isoform 1 is found to stabilize P53 and enhance its activation, indicating active suppression of the tumor. These results further complement the findings of S phase cell cycle arrest following suppression of ARNT isoform 1 and together with other investigations, demonstrate the importance of studying the individual role of each ARNT isoform. Based on these observations of ARNT isoform-specific function, we are seeking to address the role of each ARNT isoform within AHR signaling and we further hypothesize that ARNT isoform 1 and 3 uniquely regulate AHR activity.

As an initial investigation of the molecular mechanism by which the ARNT isoforms control AHR activity, we examined isoform specific functions in various malignant human T cell lines. These studies indicate that ARNT isoform 1 impedes AHR signaling whereas ARNT isoform 3 significantly enhances AHR target-gene transcription. Collectively, these data increase our understanding of the ARNT isoforms and their unique regulation of AHR signaling. These observations also further aid in understanding the comprehensive roles of the ARNT isoforms in controlling immunity and support the potential of targeting ARNT alternative splicing as a means of therapeutic intervention in hematological diseases.

RESULTS

Functional AHR signaling is retained in Karpas 299 cells

Regulatory T cells (Tregs) function to control autoimmunity and self-tolerance in healthy individuals, and in previous work, we demonstrated that Karpas 299 cells, a

human anaplastic large cell lymphoma cell line that retains Treg properties, express both ARNT isoforms. However, it remains unknown if both ARNT isoforms are present in cells outside the immune system[59,269,270]. To determine this, different tissues were collected from individual mice and analyzed by immunoblot. Interestingly, both ARNT isoforms were identified in the majority of tissues examined; however to our surprise, a few tissue samples only appear to express one ARNT isoform (Fig. 1A). These results further highlight the importance of investigating the function of both ARNT isoforms in different tissue and cell types.

To assess AHR signaling, Karpas 299 cells were treated with the high-affinity AHR ligand 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and assayed for AHR activation. Cytoplasmic and nuclear fractionation of cells revealed nuclear translocation of AHR after TCDD treatment (Fig. 1B). In addition, reverse transcription quantitative PCR (RT-qPCR) showed that TCDD exposure increased expression of the AHR target genes *CYP1A1* and *AHRR* (Fig. 1C). These results demonstrate that AHR signaling is functional in Karpas 299 cells, thus providing us confidence in evaluating ARNT isoform-specific regulation of AHR signaling.

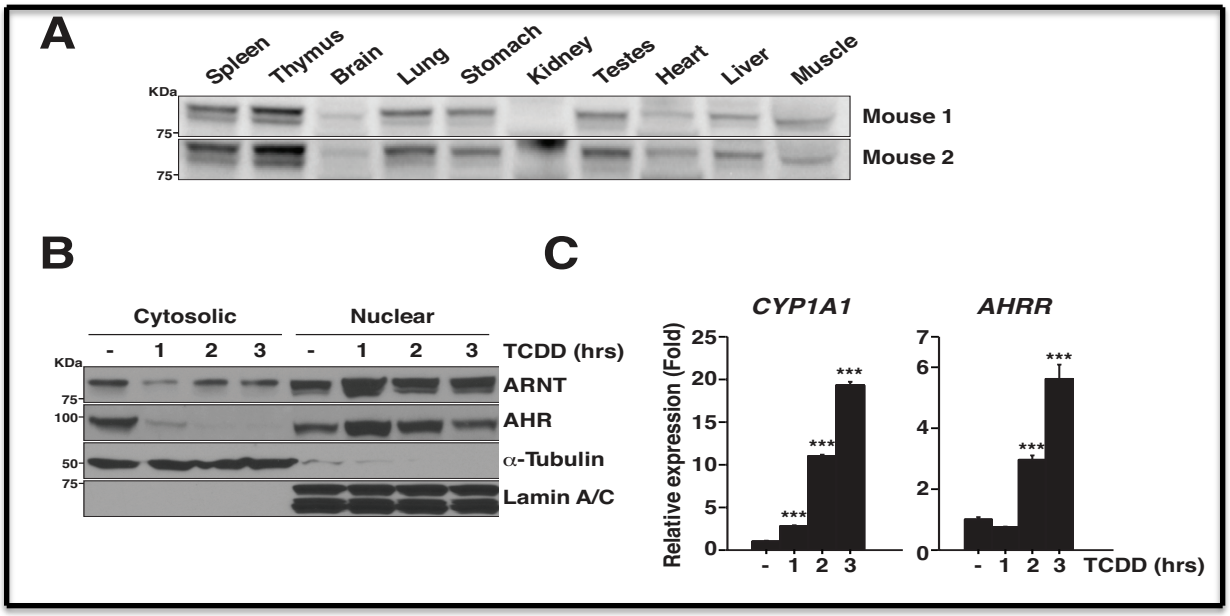

Figure 1. AHR signaling is upheld in Karpas 299 cells. (A) Tissue from the spleen, thymus, brain, lung, stomach, kidney, testes, heart, liver, and muscle were collected from three independent mice (two shown above), lysed in RIPA buffer, and immunoblotted for ARNT. (**B**) Karpas 299 cells were exposed to DMSO or TCDD (10 nM) at the indicated time points; cytoplasmic and nuclear fractions were probed with antibodies specific to ARNT, AHR, α-Tubulin, and Lamin A/C. (**C**) RT-qPCR analysis of *CYP1A1* and *AHRR* gene expression in Karpas 299 cells following exposure to DMSO or TCDD (10 nM) at the indicated time points. Data represent mean ± SEM, ***P<0.001 compared to DMSO control by Student's *t* test.

ARNT isoforms differentially regulate AHR activity in Karpas 299 cells

Upon validating AHR signaling and target gene transcription in Karpas 299 cells, small interfering RNA (siRNA) were utilized to suppress both or either ARNT isoforms (Fig. 2A). Consistent with mice knockout studies, simultaneous suppression of both ARNT isoforms decreased basal AHR activity and prevented TCDD-induced AHR signaling (Fig. 2B)[252]. Strikingly, reduction of ARNT isoform 1 resulted in the augmentation of basal and induced AHR activity while knockdown of ARNT isoform 3 phenocopied the loss of both ARNT isoforms (Fig. 2B). Fractionation experiments were then performed to determine whether removing a specific ARNT isoform affected sub-cellular localization of AHR, thereby affecting activity. These experiments demonstrate that reduction of separate ARNT isoforms has a potential role in AHR localization and further suggests that the ARNT isoforms differentially regulate AHR activity (Fig. 2C). These findings indicate that ARNT isoform 1 is a suppressor of AHR signaling, whereas ARNT isoform 3 is a promoter of AHR activity.

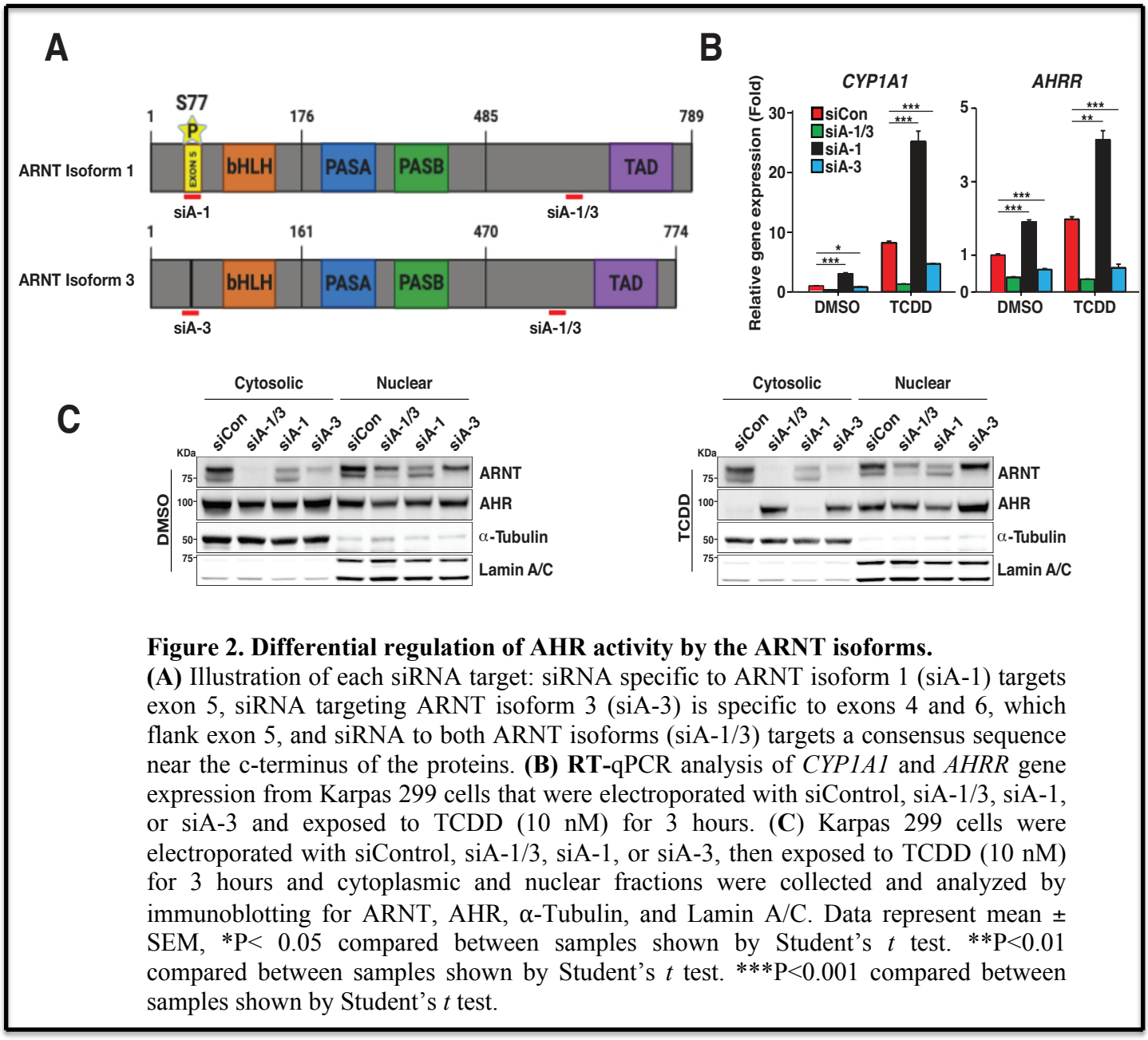

Figure 2. Differential regulation of AHR activity by the ARNT isoforms.
(A) Illustration of each siRNA target: siRNA specific to ARNT isoform 1 (siA-1) targets exon 5, siRNA targeting ARNT isoform 3 (siA-3) is specific to exons 4 and 6, which flank exon 5, and siRNA to both ARNT isoforms (siA-1/3) targets a consensus sequence near the c-terminus of the proteins. **(B) RT-**qPCR analysis of *CYP1A1* and *AHRR* gene expression from Karpas 299 cells that were electroporated with siControl, siA-1/3, siA-1, or siA-3 and exposed to TCDD (10 nM) for 3 hours. (**C**) Karpas 299 cells were electroporated with siControl, siA-1/3, siA-1, or siA-3, then exposed to TCDD (10 nM) for 3 hours and cytoplasmic and nuclear fractions were collected and analyzed by immunoblotting for ARNT, AHR, α-Tubulin, and Lamin A/C. Data represent mean ± SEM, *P< 0.05 compared between samples shown by Student's *t* test. **P<0.01 compared between samples shown by Student's *t* test. ***P<0.001 compared between samples shown by Student's *t* test.

Differential regulation of AHR activity by the ARNT isoforms is not ligand specific

To determine whether regulation of AHR activity by the ARNT isoforms is ligand specific, we assessed AHR signaling by utilizing FICZ as an AHR agonist. Intriguingly, suppression of ARNT isoform 1 protein augmented AHR signaling, while decreasing ARNT isoform 3 protein inhibited AHR activity (Fig. 3A). Nuclear translocation of AHR was again assessed following suppression of the individual ARNT isoforms and exposure

to FICZ. Additionally, fractionation studies found similar differences in AHR sub-cellular localization following the suppression of each ARNT isoform (Fig. 3B). Together, these results identify ARNT isoform 1 as a suppressor of AHR activity and ARNT isoform 3 as a promoter of AHR signaling regardless of the ligand responsible for activating AHR.

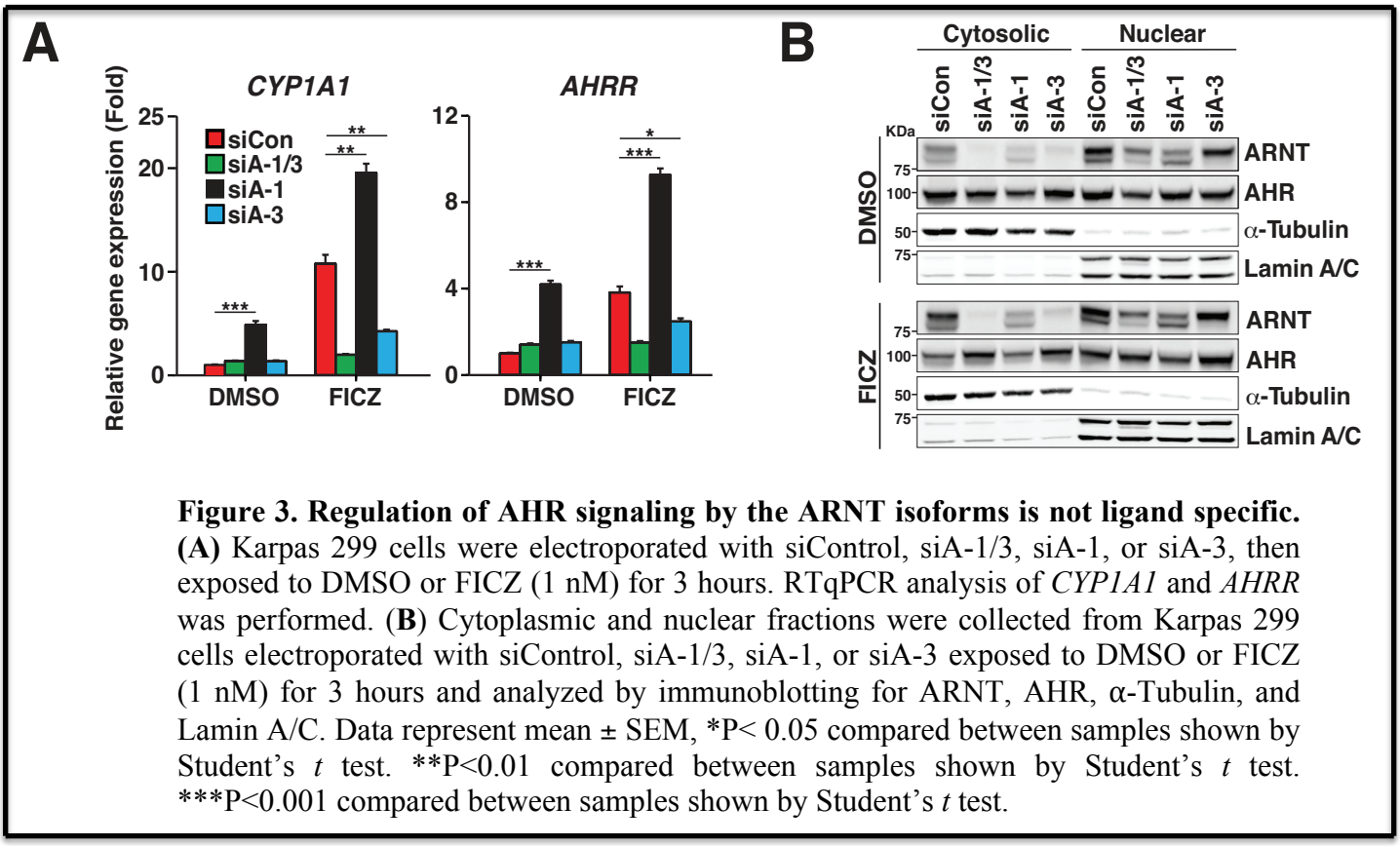

Figure 3. Regulation of AHR signaling by the ARNT isoforms is not ligand specific. **(A)** Karpas 299 cells were electroporated with siControl, siA-1/3, siA-1, or siA-3, then exposed to DMSO or FICZ (1 nM) for 3 hours. RTqPCR analysis of *CYP1A1* and *AHRR* was performed. (**B**) Cytoplasmic and nuclear fractions were collected from Karpas 299 cells electroporated with siControl, siA-1/3, siA-1, or siA-3 exposed to DMSO or FICZ (1 nM) for 3 hours and analyzed by immunoblotting for ARNT, AHR, α-Tubulin, and Lamin A/C. Data represent mean ± SEM, *P< 0.05 compared between samples shown by Student's *t* test. **P<0.01 compared between samples shown by Student's *t* test. ***P<0.001 compared between samples shown by Student's *t* test.

ARNT isoform regulation of AHR signaling is upheld in alternative malignant T cell subtype

Previous investigations have demonstrated a wide variety of roles that AHR possesses in numerous different immune cell subtypes. To further investigate AHR regulation in alternative immune cells, we employed Peer cells, a leukemic cell line that

possesses effector-like properties, to explore ARNT isoform-specific function[271]. To ensure AHR activation and signaling was retained in Peer cells, AHR nuclear translocation and increased expression of the AHR target gene *CYP1A1* was confirmed following exposure to TCDD (Fig. 4A and B). Having established a functional AHR pathway, we next examined ARNT isoform regulation of AHR signaling. Synonymously, ARNT isoform 1 and 3 controlled activity in Peer cells as they did in Karpas 299 cells (Fig. 4C and E). AHR localization further indicated that in Peer cells, the ARNT isoforms have a potential role in regulating sub-cellular localization of AHR (Fig. 4D and F). Given that Peer and Karpas 299 cells are diverse T cell malignancies, these observations indicate that the ARNT isoforms engage a general mechanism to regulate AHR signaling.

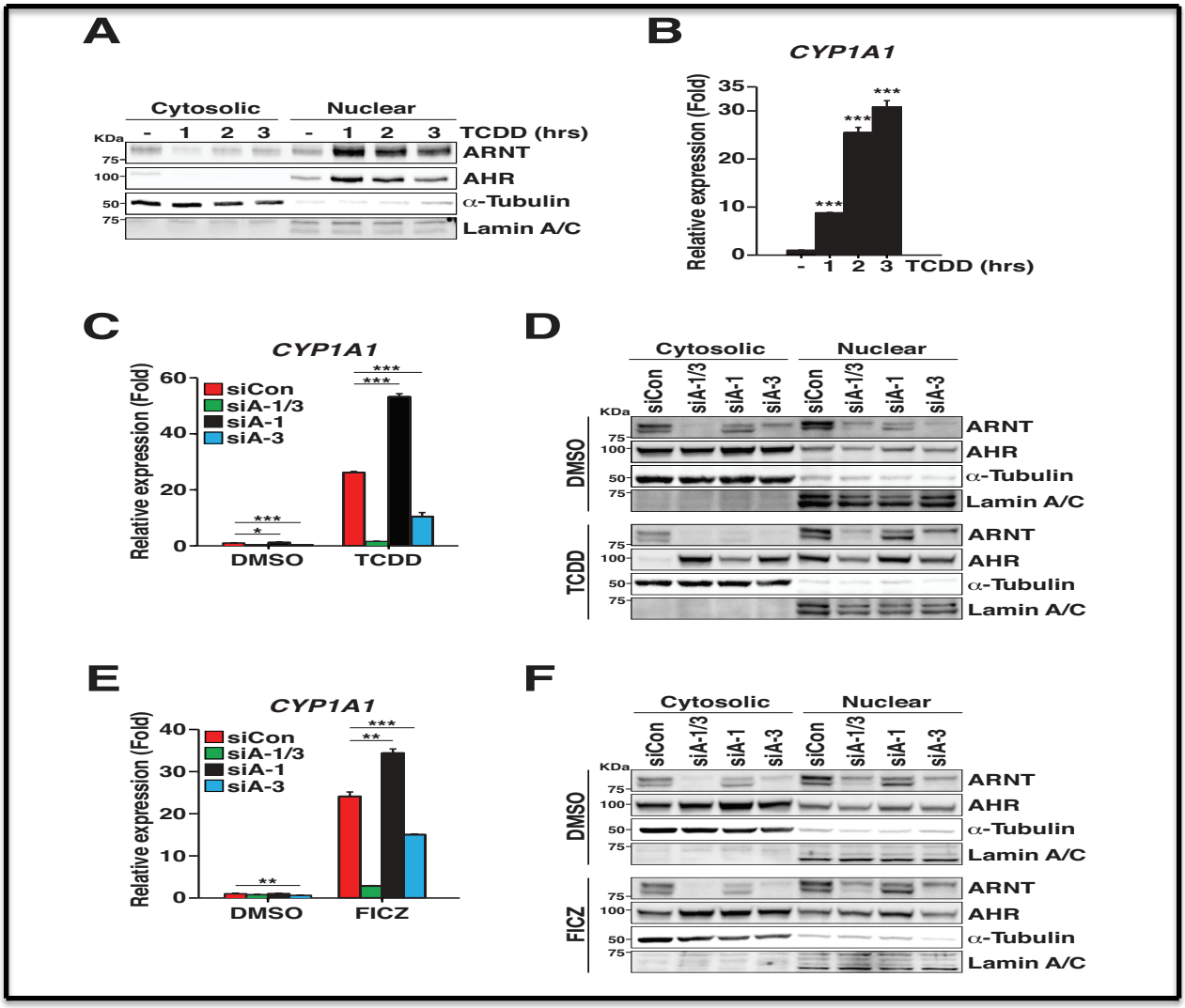

Figure 4. AHR activity in Peer T cells is controlled similarly to Karpas 299 cells. (**A**) Peer cells were treated with DMSO or TCDD (10 nM) at the time points indicated; cytosolic and nuclear fractions were probed with antibodies specific to ARNT, AHR, α-Tubulin and Lamin A/C. (**B**) RT-qPCR analysis of *CYP1A1* gene expression in Peer cells following exposure to DMSO or TCDD (10 nM) at the indicated time points. Gene expression of *CYP1A1* was determined by RT-qPCR analysis in Peer cells following electroporation with siControl, siA-1/3, siA-1, or siA-3 and exposure to TCDD (10 nM) (**C**) or FICZ (1 nM) (**E**) for 4 hours. Peer cells were transfected with siControl, siA-1/3, siA-1, or siA-3 by electroporation and exposed to TCDD (10 nM) (**D**) or FICZ (1 nM) (**F**) for 4 hours; cytoplasmic and nuclear fractions were probed with antibodies specific to ARNT, AHR, α-Tubulin, and Lamin A/C. Data represent mean ± SEM, *P< 0.05 compared between samples shown by Student's *t* test. **P<0.01 compared between samples shown by Student's *t* test. ***P<0.001 compared to DMSO control (B) or between samples shown (C,E) by Student's *t* test.

AHR does not preferentially interact with a specific ARNT isoform

In finding that ARNT isoform 3 promotes AHR signaling and ARNT isoform 1 suppresses AHR activity, we assessed if AHR preferentially bound to ARNT isoform 3 over ARNT isoform 1. To test this, we performed a competition assay by transfecting increased amounts of each ARNT isoform with AHR. Interestingly, immunoblot analysis revealed that AHR bound the ARNT isoforms without preference and interacted with the isoform that was most abundant (Fig. 5A). Given that AHR interacts equally with both ARNT isoforms, we then investigated the recruitment of ARNT isoforms 1 and 3 by AHR following activation. Similarly, AHR activation by the ligands tested resulted in the recruitment of each isoform equal to the ratio of the ARNT isoforms found in the cell (Fig. 5B). These data indicate that AHR ligands do not influence the binding of AHR to a particular ARNT isoform.

Having found no differences in binding between AHR and the individual ARNT isoforms, we next determined if the occupancy of AHR and ARNT at the *CYP1A1* promoter was altered upon suppression of each isoform independently. Analysis by chromatin immunoprecipitation (ChIP) demonstrated an increase in binding of AHR and

ARNT to the *CYP1A1* promoter in cells with ARNT isoform 1 suppressed compared to both control and cells with reduced protein levels of ARNT isoform 3 (Fig. 5C). Collectively, these results demonstrate that AHR does not preferentially interact with a specific ARNT isoform and that interaction of AHR and ARNT with the *CYP1A1* promoter is drastically increased upon suppression of ARNT isoform 1.

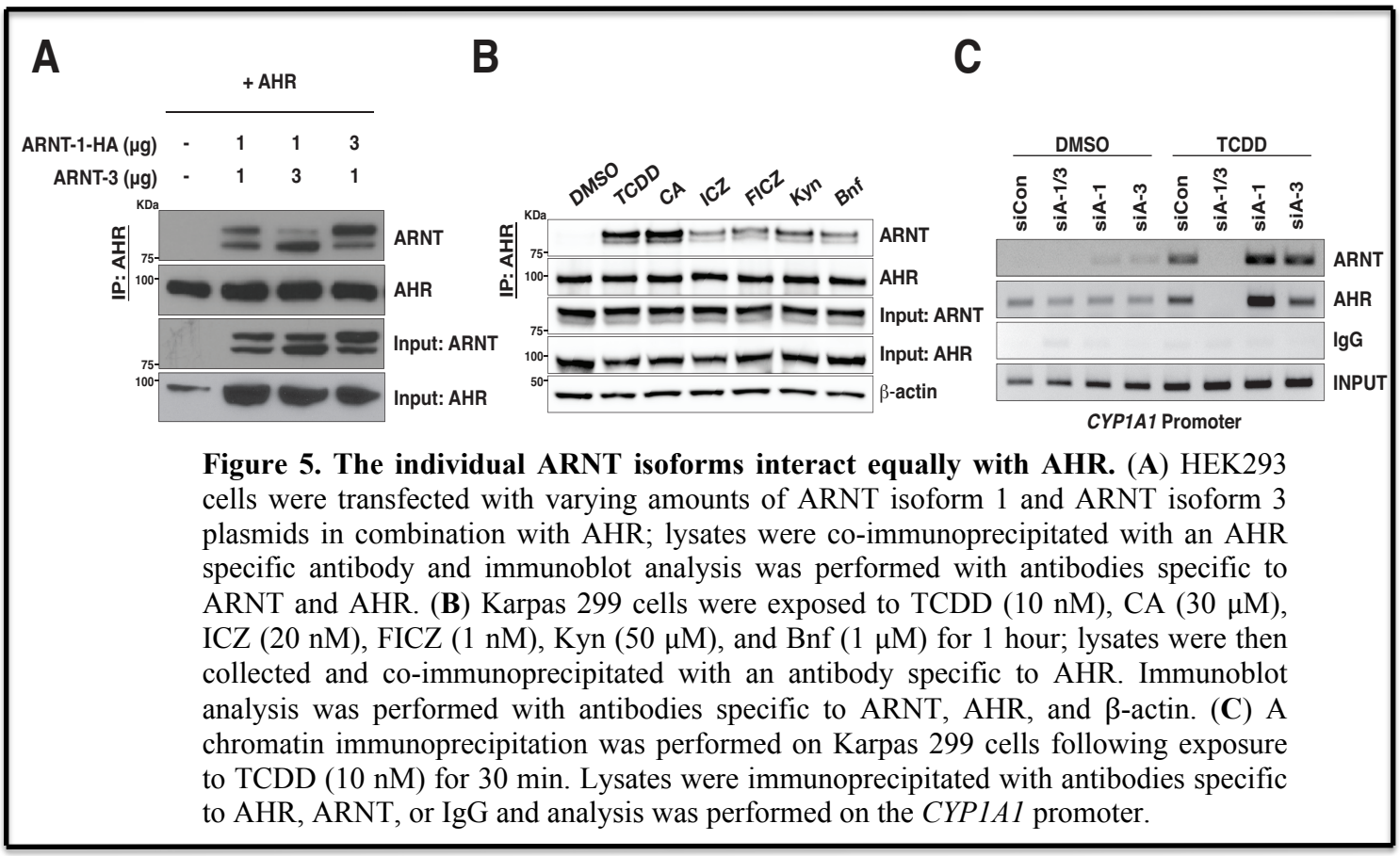

Figure 5. The individual ARNT isoforms interact equally with AHR. (**A**) HEK293 cells were transfected with varying amounts of ARNT isoform 1 and ARNT isoform 3 plasmids in combination with AHR; lysates were co-immunoprecipitated with an AHR specific antibody and immunoblot analysis was performed with antibodies specific to ARNT and AHR. (**B**) Karpas 299 cells were exposed to TCDD (10 nM), CA (30 µM), ICZ (20 nM), FICZ (1 nM), Kyn (50 µM), and Bnf (1 µM) for 1 hour; lysates were then collected and co-immunoprecipitated with an antibody specific to AHR. Immunoblot analysis was performed with antibodies specific to ARNT, AHR, and β-actin. (**C**) A chromatin immunoprecipitation was performed on Karpas 299 cells following exposure to TCDD (10 nM) for 30 min. Lysates were immunoprecipitated with antibodies specific to AHR, ARNT, or IgG and analysis was performed on the *CYP1A1* promoter.

Discussion

AHR and ARNT are essential regulators of XRE-responsive metabolic gene transcription and immune cell function[64,65,141,157,245,248,251,272]. Previously, the role of ARNT and AHR was limited to participating in the detoxification of environmental toxicants, and although greater appreciation of AHR has begun to surface in immunity

and malignancy, the major binding partner of AHR, ARNT, has yet to be comprehensively investigated. Many studies investigating AHR signaling view ARNT as a single protein, however our results support previous reports, that identify two alternatively spliced isoforms of ARNT – isoforms 1 and 3[59,193,241]. In our study, both ARNT isoforms were found in numerous different mouse tissues, indicating potential roles that are unique to each isoform. Alternatively, select tissue samples appear to express only ARNT isoform 1 or ARNT isoform 3, including the liver and the muscle. Notably, ARNT isoforms 1 and 3 were found to have little to no protein expression in both brain and kidney samples, however these are the very same tissues where ARNT 2 is known to be enriched, thus highlighting potentially unique roles of each ARNT protein in various tissues[243]. More specifically in a previous report, we observed differences in the ARNT isoform 1:3 ratio between normal human T cells versus T cell neoplasms, and with the critical nature of AHR signaling in T cell function, we turned our attention to assessing whether the ARNT isoforms might distinctively regulate AHR activity[59]. In accordance with previous reports, we found that abolishment of both ARNT isoforms in T cells results in the abrogation of XRE-target genes[252]. Remarkably, we find that targeted depletion of isoform 1 in Karpas 299 and Peer T cells enhances the expression of the AHR target genes *CYP1A1* and *AHRR*, whereas suppression of isoform 3 abrogates their expression (Illustration 3.1). Other known AHR target genes including *CYP1A2* and *CYP1B1* were also investigated in regards to ARNT-isoform specific affects, however both Karpas 299 and Peer T cells were found to not express these genes following AHR activation. Interestingly, this significant change in AHR target gene expression occurred in the absence and presence of TCDD or FICZ, albeit at a reduced level without the

addition of a ligand, which is most likely a consequence of AHR ligands present in the cells and/or serum. Additionally, the differences in ARNT isoform-specific regulation presented in this study are demonstrated solely in malignant T cells expressing both ARNT isoforms and we postulate that cells expressing only ARNT isoform 1 or 3, including the liver and muscle, function in a cell type specific manner, thus allowing for divergent roles of each ARNT isoform. We predict that in cells with only ARNT isoform 1, such as that of the liver which is capable of significantly augmenting AHR target gene transcription, the suppressive roles of this isoform may not be upheld due to cell type specific differences between hepatocytes and leukocytes. Thus, these findings demonstrate that the ARNT isoforms distinctly regulate AHR signaling in malignant T cells.

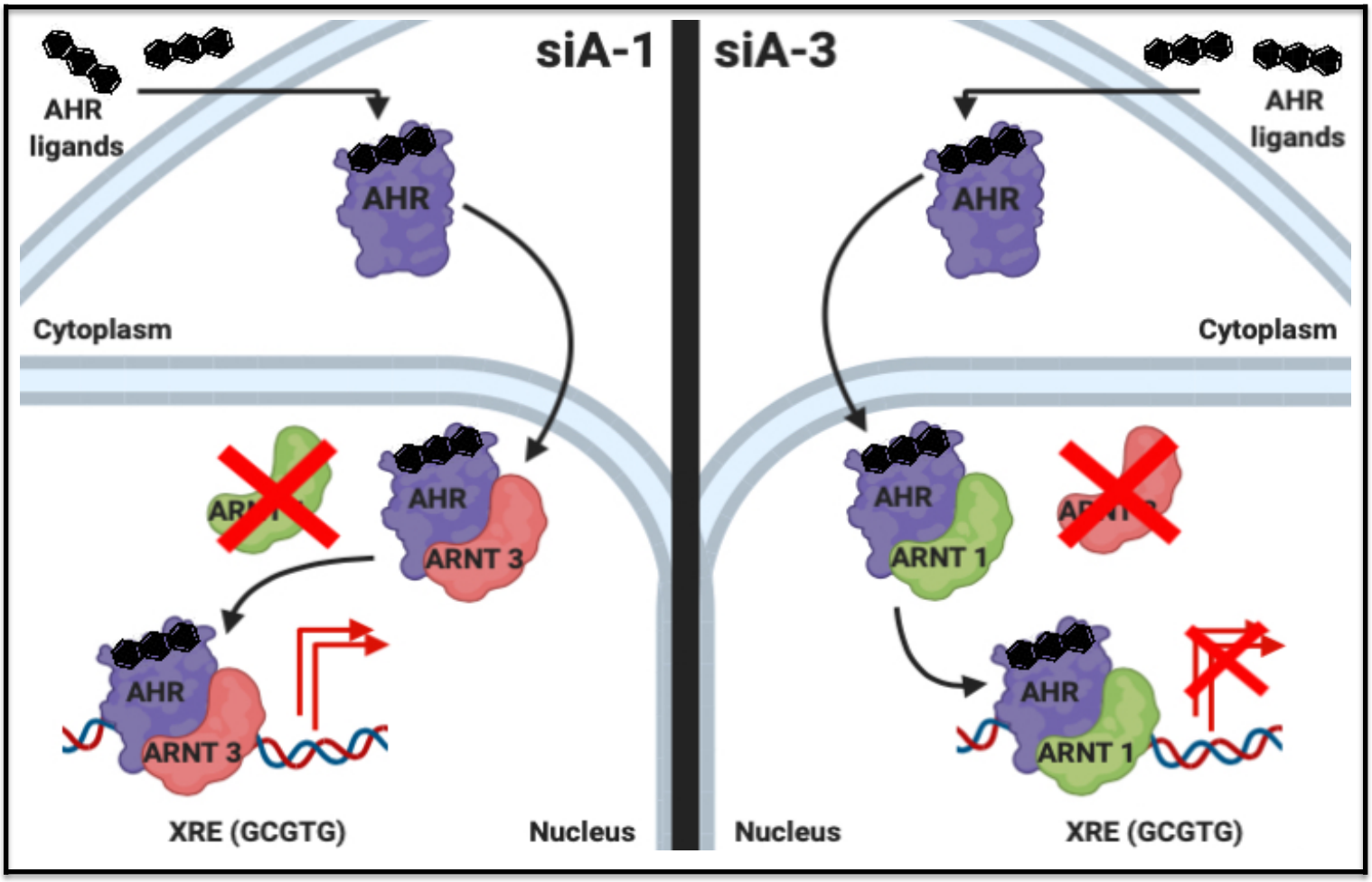

Illustration 3.1: ARNT isoform specific regulation of AHR signaling.

Suppression of ARNT isoform 1 during AHR activation results in a significant increase in expression of the AHR target genes *CYP1A1* and *AHRR*. Alternatively, AHR target gene transcription is significantly reduced following reduction of ARNT isoform 3 protein.

In finding that each ARNT isoform uniquely regulates AHR target gene transcription, we analyzed AHR localization. Intriguingly, cytoplasmic and nuclear levels of AHR were altered following suppression of ARNT isoform 1 or 3. Hence, we postulate that these differences stem from alterations in DNA binding between the individual ARNT isoforms and AHR as an increase in DNA interaction results in nuclear retention, whereas a reduction in DNA binding leads to AHR leaking back into the cytoplasm. These predictions are consistent with the initial investigations of ARNT in that mutation or loss of ARNT resulted in the inability of AHR to be localized in the nucleus after ligand binding[68,70,193]. Furthermore, suppression of ARNT isoform 1 resulted in a drastic increase in the occupation of AHR and ARNT to the *CYP1A1* promoter, whereas AHR and ARNT bound similarly to control after decreasing ARNT isoform 3. These data complement our previous findings as a decrease in ARNT isoform 1 increases target gene expression and the occupation of AHR and ARNT to the *CYP1A1* promoter. Intriguingly, suppression of ARNT isoform 3 results in a similar occupation of the *CYP1A1* promoter as control, however RT-qPCR analysis shows a significant decrease in AHR target gene transcription, which may suggest that the inhibitory role of ARNT isoform 1 involves DNA interaction. Furthermore, future studies will be conducted utilizing an electrophoretic mobility shift assay (EMSA) to further examine AHR-ARNT isoform 1 complex binding to the DNA. By performing EMSA's we will be able to better investigate areas with a potentially low level of DNA binding and will also

be able to mutate binding sites to further validate our findings. However, in conducting these assays, we do understand that these experiments will not be performed within intact cells, will be difficult to quantitate, and that supershifts must be performed to each protein of interest to validate their presence in the DNA binding complex. It is also reasonable to postulate that the ARNT isoforms may interact with different co-regulators as ARNT isoform 1 may employ histone deacetylases (HDACs) to repress transcription, whereas ARNT isoform 3 might recruit histone acetyltransferases (HATs) to promote gene expression[205,273–279]. Additionally, the increased levels of ARNT isoform 1 protein could be involved in sequestering AHR, thus limiting AHR-ARNT isoform 3 complex formation, and preventing the augmentation of AHR target gene transcription. While beyond the scope of the current study we are intrigued with these possibilities and more work is required for delineation of the molecular mechanism used by the ARNT isoforms to regulate AHR target gene expression. Nevertheless, our results demonstrate for the first time that the ARNT isoforms have specific, and in many instances opposite, influences on AHR activity that together ultimately shape the outcome of AHR signaling.

Previous investigations have reported unique roles in T cell differentiation and gene transcription through AHR following activation by particular ligands, however it is unknown if these specific functions of AHR are from differences in the utilization of ARNT isoform 1 and 3[141,172,280,281]. Intriguingly, our data revealed that AHR activation by a variety of ligands results in interactions with each ARNT isoform equal to the ratio of the isoforms already found in the cell. These findings suggest that differences in the response of AHR from diverse ligands may not be through preferential interactions with a particular ARNT isoform.

In conclusion, many reports have demonstrated the role of ARNT in AHR signaling and its function in the immune system, however these previous investigations have overlooked the fact that ARNT is alternatively spliced into two unique isoforms[65,70,213–216,248,282]. In this study, our novel findings significantly enhance our understanding of AHR signaling by revealing an additional layer of AHR regulation by the ARNT isoforms that needs to be considered when investigating AHR signaling, especially in immune cells where ARNT isoforms 1 and 3 are robustly expressed. Unequivocally, our data demonstrate that following suppression of each ARNT isoform individually, we identified ARNT isoform 1 as a suppressor of AHR activity and ARNT isoform 3 as an enhancer of AHR signaling. AHR signaling and activation in malignancies and autoimmune diseases has been identified to either promote or impede disease progression depending on the specific disorder[57,167,168,173,180,183,189]. Thus, targeting the ARNT isoforms for the enhancement or repression of AHR signaling to prevent disease development is an attractive therapeutic option. Together, these significant findings suggest a complex mechanism of AHR regulation and reveal a necessity for considering both ARNT isoforms when investigating immune cell function or AHR signaling.

Chapter 4 The role of ARNT isoform 1 phosphorylation in AHR signaling

INTRODUCTION

Environmental factors are frequently found to have unfavorable effects on the immune system, many of which have been linked to the aryl hydrocarbon receptor (AHR). AHR is a member of the basic-helix-loop-helix-PER/ARNT/SIM (bHLH-PAS) family of transcription factors and is found to have significant roles in xenobiotic response, immune function, and cellular development[161,283,284]. During basal conditions, AHR is bound in the cytoplasm to co-chaperone proteins, however upon activation by endogenous or exogenous ligands, AHR translocates into the nucleus and forms a heterodimer complex with fellow bHLH-PAS member, the aryl hydrocarbon receptor nuclear translocator (ARNT)[121,122,198]. ARNT is often described in AHR signaling as being constitutively expressed, non-regulated, and a protein that solely aids in DNA binding to xenobiotic response elements (XREs)[213]. However, ARNT is alternatively spliced into two isoforms identified as isoforms 1 and 3, which differ by 15 amino acids that encode exon 5 of ARNT isoform 1[59,193,241]. Although these two isoforms have similar genomic sequences, we have previously found that the protein expression of ARNT is altered in malignant B and T cells[59,193,241]. Malignant leukocytes are found to upregulate ARNT isoform 1 protein expression, which was determined to support cellular survival and provide a proliferation advantage[59].

Investigators have revealed significant roles of AHR in immunity as early studies in mice found that the environmental toxicant 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) triggers immunosuppression[141,142,280,285]. AHR binds TCDD with high affinity

and examination of mice exposed to TCDD were found to have a suppressed immunity resulting from an induced differentiation of T cells toward a regulatory phenotype[141,142,280,285]. Since TCDD is a potent inducer of AHR activity, this result suggests AHR signaling is anti-inflammatory. However, research using 6-formylindolo [3, 2-b] carbazole (FICZ), an AHR ligand derived from tryptophan, shows that FICZ-induced AHR activity leads to an excess of interleukin-17-producing T cells, resulting in enhanced inflammation[141]. While research is ongoing to better understand the opposing immunological effects of these AHR agonists, a recent study proposes that these differences stem from the dose of AHR ligand and the duration of AHR activation[286].

As a critical regulator of AHR activity, it is not surprising that ARNT is important for controlling immunity[64,65,245,248–252]. It has been observed that mice containing myeloid cells devoid of ARNT express decreased quantities of pro-inflammatory cytokines and exhibit reduced skin inflammation in response to sodium dodecyl sulfate (SDS) exposure[65]. In contrast, alternative studies found that myeloid cells without ARNT have elevated immune cell infiltration in the colon and the development of steatohepatitis in animals with a high fat diet along with an increase in the mRNA expression and secretion of pro-inflammatory cytokines[64,248]. In addition, mice with ARNT-deficient CD4+ T cells show alterations in the development of certain T cell subsets[250]. Notably, while loss of AHR signaling leads to excessive inflammation, deleting ARNT causes significant tissue-specific changes in immunity. Given that ARNT and AHR form a transcriptional complex, these observations are unexpected and suggest a gap in our knowledge of how ARNT controls AHR signaling.

Activation of cellular receptors elicits the initiation of signaling cascades through numerous post-translational modifications. In many circumstances, proteins undergo the modification of phosphorylation, which influences the activity of a protein through changes in structural conformation and the recruitment of neighboring proteins. This reversible modification is mediated by protein kinases, which add a phosphate group from ATP to the side chains of specific amino acids such as serine, threonine, or tyrosine residues[287]. Previous studies investigating alternatively spliced bHLH proteins determined through electrophoretic mobility shift assays (EMSAs) that phosphorylation influences the DNA binding ability of the transcription factors Max and E47[288,289]. More specifically, exon 5 of ARNT isoform 1 is found to contain a consensus casein kinase 2 (CK2) recognition sequence (S/TXXD/E), and through recombinant protein kinase assays, was further validated to be phosphorylated at serine 77 (S77) by CK2[241,290]. Interestingly, phosphorylation of S77 by CK2 was found to control the promoter affinity of ARNT isoform 1 to enhancer box (E-box) elements, however binding to XREs was unaffected. Collectively, these data show that phosphorylation of S77 is a unique modification that regulates the function specifically of ARNT isoform 1 and warrants further exploration.

Upon demonstrating that ARNT isoform 1 and 3 differentially regulate AHR activity within malignant T lymphocytes in chapter 3, we sought to determine if phosphorylation of S77 had a role in the inhibitory function of ARNT isoform 1 in AHR signaling, as this is a key distinguishing feature between both isoforms. Therefore, we characterized CK2-mediated phosphorylation of ARNT isoform 1 and demonstrated that phosphorylation at S77 is critical for the transcription of AHR target genes. Additional

observations also suggest that unphosphorylated ARNT isoform 1 may have a role in the expression of T cell activation genes. This study provides novel findings that exhibit a complex regulatory mechanism by ARNT isoform 1 for optimal AHR target-gene expression following AHR activation.

RESULTS

ARNT isoform 1 is phosphorylated during AHR activation

To understand the function of ARNT isoform 1 phosphorylation at S77, we sought to investigate the role of this modification in AHR signaling, as a previous study observed by mass spectrometry that S77 undergoes phosphorylation following exposure to TCDD[214,215,291]. To determine if ARNT isoform 1 phosphorylation is involved in the regulation of AHR signaling, we assayed for phosphorylation of S77 following AHR activation in two malignant T cell lines, Karpas 299 and Peer cells. Strikingly, immunoblot analysis revealed a drastic increase in ARNT isoform 1 phosphorylation 30 minutes after TCDD exposure (Fig. 6A and C). To determine if ARNT isoform 1 phosphorylation is specific to AHR activation by TCDD, we investigated whether FICZ treatment also leads to phosphorylation of S77. Intriguingly, induction of AHR signaling by FICZ triggered ARNT isoform 1 phosphorylation similar to TCDD, thus suggesting that AHR activation is required for ARNT isoform 1 phosphorylation (Fig. 6B and D). Having found that ARNT isoform 1 is phosphorylated after AHR activation by TCDD and FICZ, we sought to determine whether other AHR ligands elicit a similar response. Markedly, AHR activation by each tested ligand promoted the phosphorylation of ARNT isoform 1 (Fig. 6E). The ability of these different ligands to induce AHR signaling was

verified by reverse transcription quantitative PCR (RT-qPCR) analysis, which showed increased expression of *CYP1A1* and *AHRR* (Fig. 6F).

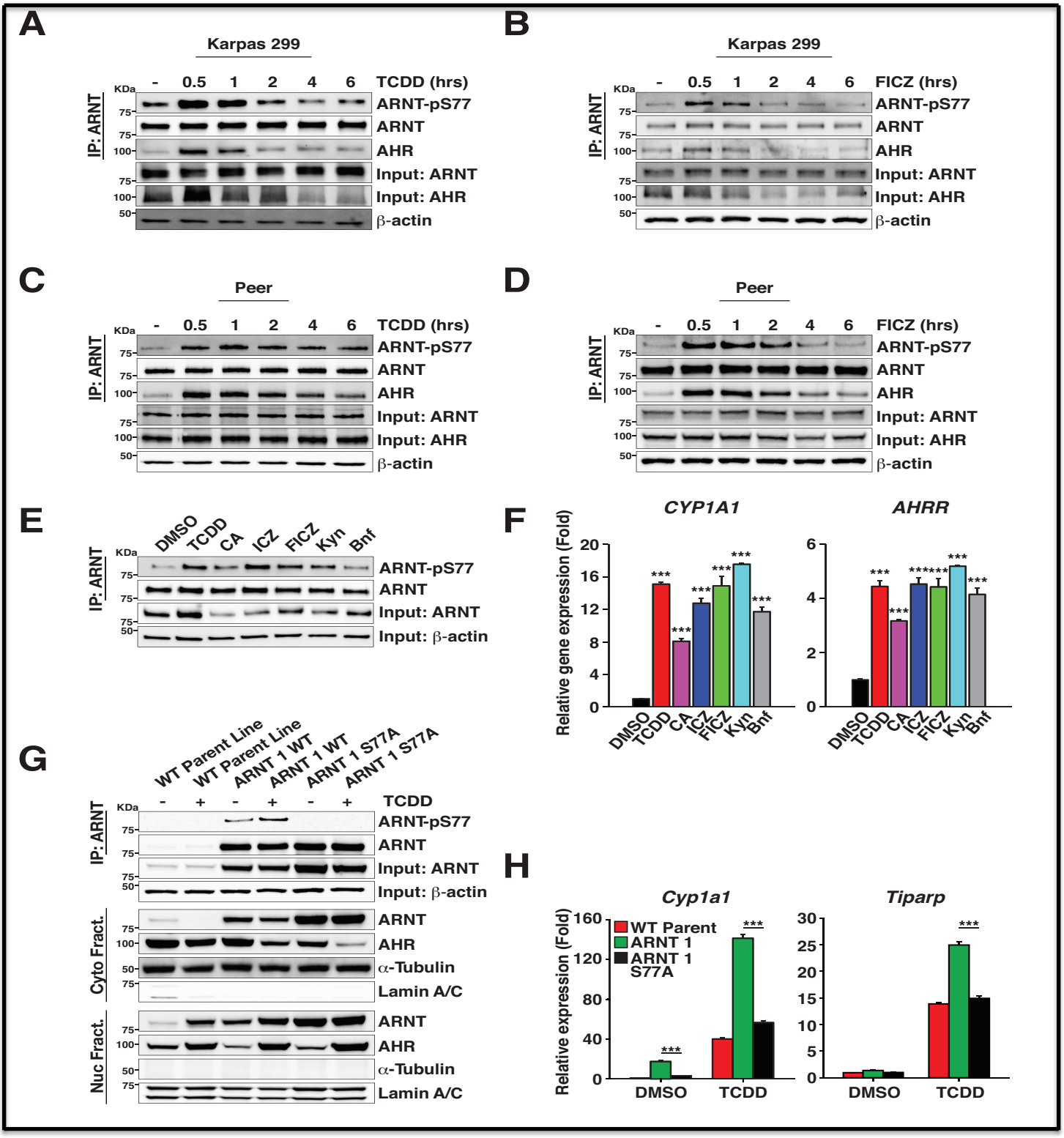

Figure 6. ARNT isoform 1 is phosphorylated following AHR activation. Karpas 299 (**A**) and Peer (**C**) cells were exposed to TCDD (10 nM) at the indicated time points and then lysed in RIPA buffer. Lysates were immunoprecipitated with an ARNT specific antibody and immunoblot analysis was performed with antibodies specific to phosphorylated ARNT isoform 1 (ARNT-pS77), ARNT, AHR, and β-actin. Karpas 299 (**B**) and Peer (**D**) cells were treated with FICZ (1 nM) at the indicated time points, protein lysates were collected and immunoprecipitated with an ARNT specific antibody; immunoblot analysis was performed with antibodies to ARNT-pS77, ARNT, AHR, and β-actin. (**E**) Karpas 299 cells were subjected to AHR ligands including TCDD (10 nM), CA (30 μM), ICZ (20 nM), FICZ (1 nM), Kyn (50 μM), and Bnf (1 μM) for 30 minutes. Cells were then lysed and lysates were probed with antibodies specific to ARNT-pS77, ARNT, and β-actin. (**F**) Karpas 299 cells, treated with the same concentrations of ligand as in (**E**), were exposed for 2 hours and RT-qPCR analysis of *CYP1A1* and *AHRR* gene expression was performed. (**G**) Hepa-1c1c7 (WT Parent Line), ARNT 1 WT stably expressing cell line, and ARNT 1 S77A stably expressing cell line were exposed to TCDD (10 nM) for 30 minutes, whole, cytoplasmic, and nuclear cell lysates were extracted. The whole cell lysate was immunoprecipitated with an antibody to ARNT. Protein levels were analyzed by immunoblotting for ARNT-pS77, ARNT, AHR, α-Tubulin, Lamin A/C and β-actin. (**H**) RT-qPCR analysis of *Cyp1a1* and *Tiparp* gene expression in WT Parent line, ARNT 1 WT stably expressing cell line, and ARNT 1 S77A stably expressing cell line following exposure to TCDD (10 nM) for 2 hours. Data represent mean ± SEM, ***P<0.001 compared to DMSO control (F) or between samples shown (H) by Student's *t* test.

In establishing that ARNT isoform 1 is phosphorylated following AHR activation, we then attempted to mutate the phosphorylation site of ARNT isoform 1 to investigate the effects on AHR-target gene transcription. Initially, we mutated S77 of ARNT isoform 1 in Karpas 299 cells with CRISPR-Cas9 technology. However, through validation studies, we determined that the alternative splicing of each ARNT isoform was impacted, thus resulting in an equal ratio of ARNT isoform 1 and 3. We predict that this shift in splicing was from mutating an amino acid that is close in proximity to where ARNT is alternatively spliced, thus potentially impacting the binding of the spliceosome to the mRNA of ARNT. With this result, we changed approaches and instead transduced Hepa-BpRc1 cells, an *Arnt*-null cell line, with a lentivirus for the stable expression of wild-type

(WT) ARNT isoform 1 or ARNT isoform 1 with a mutation at S77 that renders an alanine (S77A). Validation studies revealed that phosphorylation at S77 could only occur in the WT ARNT isoform 1 cell line (Fig. 6G). Interestingly, RT-qPCR analysis revealed a significant decrease in the AHR target genes *Cyp1a1* and *Tiparp* upon mutation of S77 compared to ARNT isoform 1 WT cells (Fig. 6H). Together, these data demonstrate that ARNT isoform 1 is rapidly phosphorylated following AHR activation and is a necessary modification for optimal AHR target-gene transcription.

ARNT isoform 1 phosphorylation is necessary for optimal AHR target-gene expression

Upon discovering that mutation of ARNT isoform 1 at S77 is critical for AHR-target gene transcription, we sought to explore the role of ARNT isoform 1 phosphorylation in Karpas 299 and Peer T cells. Although CK2 has been shown to phosphorylate ARNT isoform 1 in recombinant protein kinase assays, we sought to validate CK2 as the kinase responsible for ARNT isoform 1 phosphorylation within intact cells at S77. To test this, we assayed for ARNT isoform 1 phosphorylation upon AHR activation with TCDD after first inhibiting CK2. Dramatically, immunoblot analysis showed that ARNT isoform 1 phosphorylation was completely abolished following inhibition of CK2 (Fig. 7A and F). Strikingly, RT-qPCR analysis demonstrated that elimination of ARNT isoform 1 phosphorylation by CK2 inhibition resulted in a significant decrease of the AHR target-genes *CYP1A1* and *AHRR* (Fig. 7B and G). Importantly, AHR translocation and interaction with the ARNT isoforms was unaffected by CK2 inhibition, further suggesting no off-target effects on AHR signaling from the inhibition of CK2 (Fig. 7E).

In finding that ARNT isoform 1 is phosphorylated following AHR activation by TCDD and FICZ, we determined if CK2 inhibition also abolished ARNT isoform 1 phosphorylation following exposure to FICZ. Notably, inhibition of CK2 completely abolished ARNT isoform 1 phosphorylation in cells exposed to FICZ (Fig. 7C and H). Affects on AHR-target gene transcription were determined by RT-qPCR analysis, which revealed a significant decrease in the gene expression of *CYP1A1* and *AHRR* in FICZ exposed samples pretreated with the CK2 inhibitor, synonymous to TCDD treated samples (Fig. 7D and I). To further determine if interactions between ARNT and AHR are influenced by ARNT isoform 1 phosphorylation, we overexpressed WT ARNT isoform 1, ARNT isoform 1 S77A, ARNT isoform 1 with mutations at all three serines in exon 5 that render an alanine (3SA) for complete abolishment of ARNT isoform 1 phosphorylation from overexpression, and ARNT isoform 3 with and without AHR. Markedly, the phosphorylation state of ARNT isoform 1 did not affect interactions with AHR (Fig. 7J). Together, these results suggest that ARNT isoform 1 phosphorylation relieves repression of AHR target gene expression mediated by unphosphorylated isoform 1.

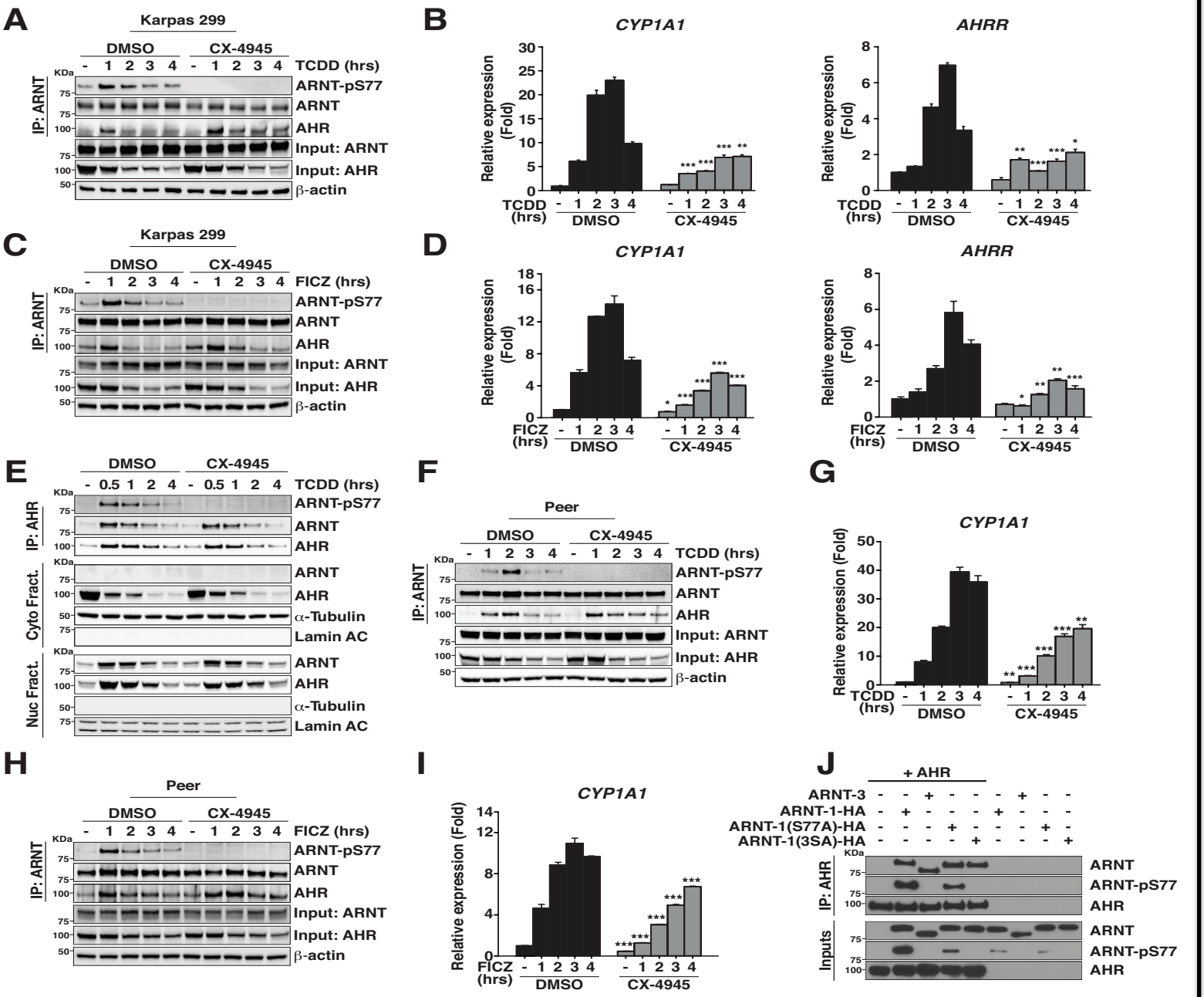

Figure 7. AHR signaling requires ARNT isoform 1 phosphorylation for optimal gene transcription. Karpas 299 cells were treated with DMSO or the CK2 inhibitor CX-4945 (5 μM) for 1 hour. Cells were then exposed to TCDD (10 nM) (**A**) or FICZ (1 nM) (**C**) for the indicated times and whole cell lysates were analyzed via immunoblot with antibodies directed to ARNT-pS77, ARNT, AHR, and β-actin. Total RNA was isolated from Karpas 299 cells following pretreatment of DMSO or CX-4945 (5 μM) for 1 hour and exposure to TCDD (10 nM) (**B**) or FICZ (1 nM) (**D**) for the indicated times. The gene expression of *CYP1A1* and *AHRR* was then examined by RT-qPCR. (**E**) Karpas 299 cells were pretreated with DMSO or CX-4945 (5 μM) for 1 hour, then exposed to DMSO

or TCDD (10 nM) for the indicated times. Cytosolic and nuclear fractions were collected and AHR was immunoprecipitated in the nuclear fraction. Protein fractions were analyzed by immunoblot with antibodies to ARNT-pS77, ARNT, AHR, α-Tubulin, and Lamin A/C. Peer cells were pretreated with DMSO or CX-4945 (5 μM) for 1 hour followed by exposure to TCDD (10 nM) (**F**) or FICZ (1 nM) (**H**) for the indicated times and lysed in RIPA lysis buffer. Lysates were immunoprecipitated with an ARNT specific antibody and immunoblot analysis was performed with antibodies to ARNT-pS77, ARNT, AHR, and β-actin. The gene expression of *CYP1A1* was analyzed by RT-qPCR from Peer cells pretreated with DMSO or CX-4945 (5 μM) for 1 hour and exposed to TCDD (10 nM) (**G**) or FICZ (1 nM) (**I**) for the indicated times. (**J**) HEK 293 cells were transfected with WT ARNT isoform 1, ARNT isoform 1 S77A, ARNT isoform 3SA, or ARNT isoform 3 plasmids in combination with and without AHR; lysates were co-immunoprecipitated with an AHR specific antibody and immunoblot analysis was performed with antibodies specific to ARNT-pS77, ARNT, and AHR. Data represent mean ± SEM. *P< 0.05, **P<0.01, ***P<0.001 compared to the DMSO treated sample of the same TCDD or FICZ exposure by Student's *t* test.

AHR is required for ARNT isoform 1 phosphorylation

Next, we sought to investigate whether AHR has a role in mediating ARNT isoform 1 phosphorylation. To this end, we examined whether the suppression of AHR influenced the phosphorylation of ARNT isoform 1. Strikingly, immunoblot analysis demonstrated that suppression of AHR in cells exposed to TCDD or FICZ lacked ARNT isoform 1 phosphorylation (Fig. 8A and B). To further support these findings that AHR is required for ARNT isoform 1 phosphorylation, we utilized Jurkat cells, which is an acute T cell leukemia cell line devoid of AHR[292]. As expected, TCDD exposure in Jurkat cells did not result in ARNT isoform 1 phosphorylation (Fig. 8C). A lack of AHR signaling in Jurkat cells was validated by RT-qPCR analysis, which showed a significance decrease in *CYP1A1* gene expression (Fig. 8D).

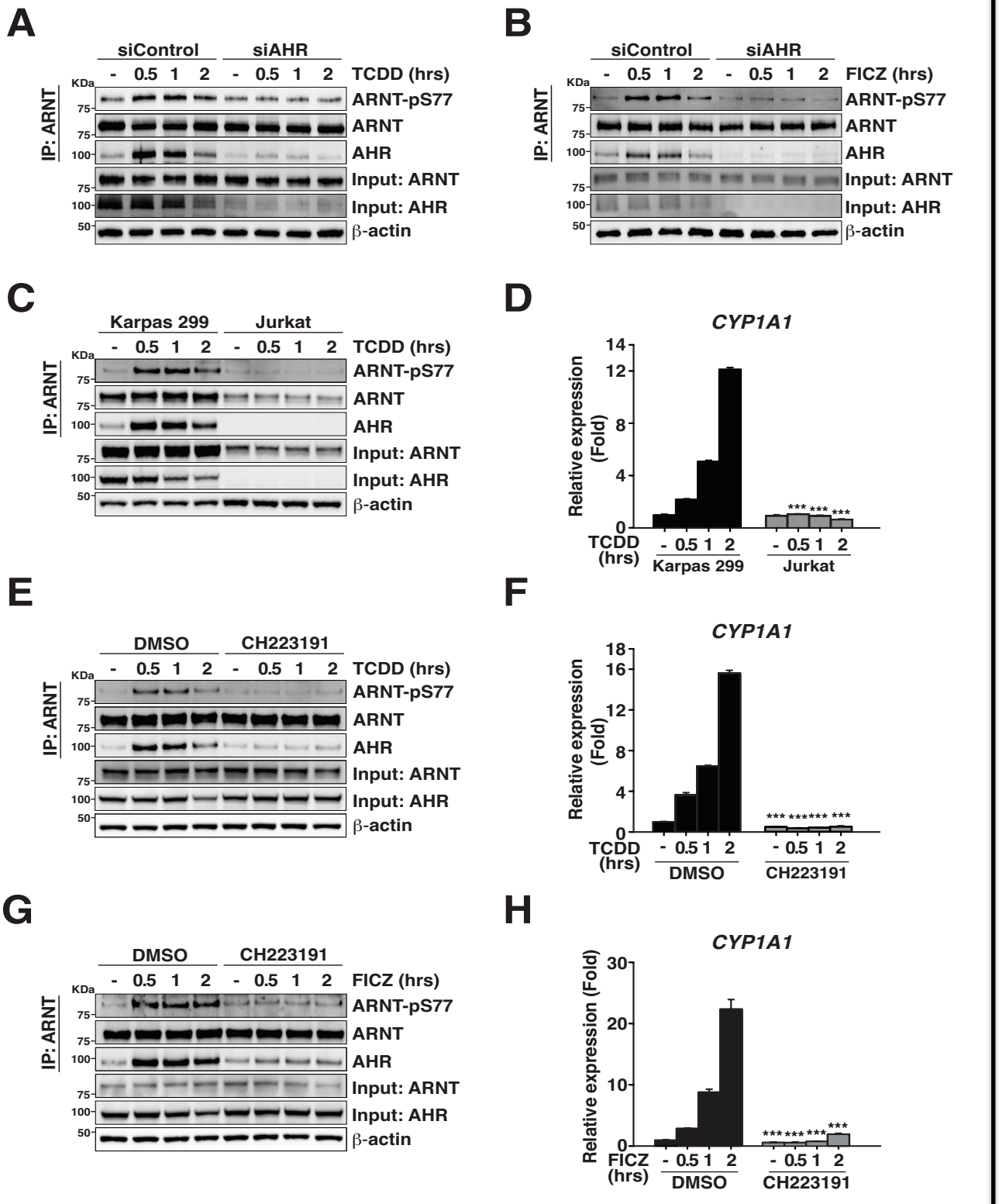

Figure 8. ARNT isoform 1 phosphorylation requires AHR. Karpas 299 cells were transfected via electroporation with scramble or AHR siRNA. 48 hours post-transfection,

cells were exposed to TCDD (10 nM) (**A**) or FICZ (1 nM) (**B**) for the indicated time points and lysed in RIPA buffer. Lysates were immunoprecipitated with an antibody specific to ARNT and analyzed by immunoblotting with antibodies detecting ARNT-pS77, ARNT, AHR, and β-actin. (**C**) Karpas 299 and Jurkat cells were exposed to TCDD (10 nM) for the indicated times and lysed in RIPA buffer; cell lysate was immunoprecipitated with an ARNT specific antibody and immunoblots were probed with antibodies specific to ARNT-pS77, ARNT, AHR, and β-actin. (**D**) Total RNA was isolated from Karpas 299 and Jurkat cells following exposure to TCDD (10 nM) for the indicated times. Gene expression of *CYP1A1* was examined by RT-qPCR analysis. Peer cells were treated for 2 hours with DMSO or the AHR antagonist CH223191 (10 μM) prior to TCDD (10 nM) (**E**) or FICZ (1 nM) (**G**) exposures for the indicated times. Whole cell lysates were analyzed by immunoblotting with antibodies directed to ARNT-pS77, ARNT, AHR, and β-actin. RT-qPCR analysis of *CYP1A1* gene expression following a 2 hour pretreatment of DMSO or CH223191 (10 μM) and exposure to TCDD (10 nM) (**F**) or FICZ (1 nM) (**H**) at the indicated time points. Data represent mean ± SEM, ***P<0.001 compared to Karpas 299 cells of the same TCDD exposure (D) or compared to the DMSO control sample of the same TCDD (F) or FICZ (H) exposure by Student's *t* test.

In light of our observations that ARNT isoform 1 phosphorylation is prevented upon loss of AHR, we utilized the AHR antagonist CH223191 to determine if AHR activation is required for the phosphorylation of ARNT isoform 1. This antagonist competes with other AHR ligands for binding to the ligand-binding pocket of AHR and is found to specifically antagonize AHR activation from halogenated aromatic hydrocarbons and prevent AHR nuclear translocation[293]. Immunoblot analysis revealed that ARNT isoform 1 phosphorylation at S77 was reduced in cells with antagonized AHR following exposure to TCDD or FICZ (Fig. 8E and G). Antagonism of AHR was validated by RT-qPCR analysis of *CYP1A1* (Fig. 8F and H). Moreover, these results indicate that AHR is essential for ARNT isoform 1 phosphorylation at S77.

Unphosphorylated ARNT isoform 1 promotes T cell stimulation-induced gene expression

The essential requirement for AHR in T cell specific cytokine production and differentiation after activation is well established[294]. To assess whether ARNT isoform 1 phosphorylation is critical during T cell activation, we stimulated Peer cells with phorbol 12-myristate 13-acetate and ionomycin (P/I). Notably, phosphorylation of ARNT isoform 1 does not increase after T cell stimulation and instead appears to decrease further from an already low basal level, thus hinting at a possible role of unphosphorylated ARNT isoform 1 in T cell activation-induced gene expression (Fig. 9A). Peer T cell stimulation with P/I was validated by RT-qPCR analysis of *CSF2*, *IL3*, and *IL6* expression (Fig. 9B). To further investigate the role of unphosphorylated ARNT isoform 1 during T cell activation, we simultaneously treated Peer cells with TCDD or FICZ in the absence or presence of P/I. Immunoblot analysis shows that T cell stimulation in the presence of TCDD or FICZ hampers the robustness and longevity of ARNT isoform 1 phosphorylation concomitantly with a reduction in AHR protein levels and in the interaction of AHR with ARNT (Fig. 9C and 9E). Intriguingly, this apparent P/I-mediated obstruction of ARNT isoform 1 phosphorylation correlates with optimal expression of *CSF2*, *IL3*, and *IL6* as levels of transcription are significantly abrogated in the presence of TCDD or FICZ (Fig. 9D and 9F). Given the inverse relationship between ARNT isoform 1 phosphorylation and the expression of *CSF2*, *IL3*, and *IL6*, we surmised that inhibition of ARNT isoform 1 phosphorylation might enhance T cell stimulation-induced cytokine expression. To further explore this hypothesis, we exposed Peer T cells to TCDD, FICZ, or CX-4945 in the presence of P/I. As expected, we observe a decrease in TCDD or FICZ induced ARNT isoform 1 phosphorylation at four hours post-P/I

treatment as compared to the DMSO control, which also corresponds to lower levels of AHR and ARNT interaction (Fig. 9G). Remarkably, abolishment of ARNT isoform 1 phosphorylation by CK2 inhibition enhances the T cell activation response by significantly augmenting the gene expression of *CSF2*, *IL3*, and *IL6*, while simultaneously blocking T cell activation-induced expression of *CYP1A1* (Fig. 9H). Taken together, these data suggest that unphosphorylated ARNT isoform 1 is necessary for robust T cell activation and that induction of AHR, and subsequent ARNT isoform 1 phosphorylation, competes with T cell stimulation-induced gene expression possibly as a means for modulating T cell responsiveness.

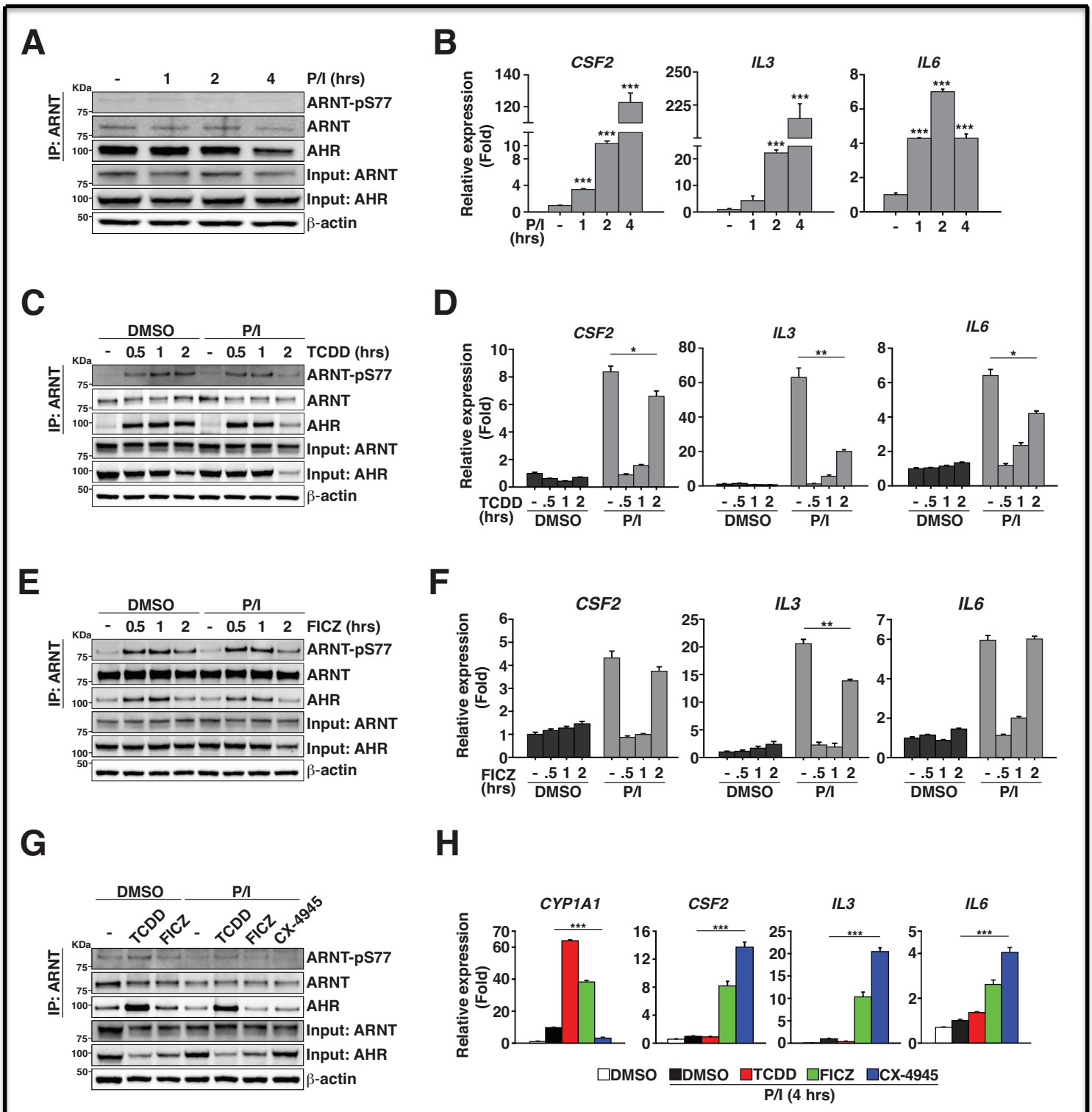

Figure 9. Transcription of T cell activation markers is promoted by unphosphorylated ARNT isoform 1. (**A**) Peer cells were activated with phorbol 12-myristate 13-acetate (20 ng/mL) and ionomycin (1 μg/mL) (PMA + IO) for the indicated times and then lysed in RIPA buffer. Lysates were immunoprecipitated with an ARNT

specific antibody and immunoblot analysis was performed with antibodies detecting ARNT-pS77, ARNT, AHR, and β-actin. (**B**) RT-qPCR analysis of *CSF2, IL3,* and *IL6* gene expression following activation with PMA + IO for the indicated times. Peer cells were activated with DMSO or PMA + IO and exposed to TCDD (10 nM) (**C**) or FICZ (1 nM) (**E**) for the indicated times; whole cell lysates were immunoprecipitated with an antibody to ARNT. Immunoblot analysis was performed with antibodies specific to ARNT-pS77, ARNT, AHR, and β-actin. RT-qPCR analysis of *CSF2, IL3,* and *IL6* was performed using total RNA isolated from Peer cells activated with DMSO or PMA + IO and exposed to TCDD (10 nM) (**D**) or FICZ (1 nM) (**F**) for the indicated times, the 0 hour time point was also activated for 2 hours with PMA + IO. Peer cells were activated with DMSO or PMA + IO and exposed to TCDD (10 nM), FICZ (1 nM), or the CK2 inhibitor CX-4945 (5 μM) for 4 hours; (**G**) Cells were lysed in RIPA buffer and lysates were immunoprecipitated with an antibody to ARNT; immunoblot analysis was performed with antibodies detecting ARNT-pS77, ARNT, AHR, and β-actin; (**H**) Total RNA was isolated and gene expression of *CYP1A1*, *CSF2, IL3,* and *IL6* was determined by RT-qPCR analysis. Data represent mean ± SEM, *P<0.05 compared between samples shown by Student's *t* test. **P<0.01 compared between samples shown by Student's *t* test. ***P<0.001 compared to DMSO control (B) or between samples shown (D,F,H) by Student's *t* test.

DISCUSSION

ARNT is a transcription factor that is vital to the expression of AHR target genes and has been identified in having roles in pro-inflammatory cytokine expression and leukocyte development[64,65,248,251]. Although these studies have offered a closer look at the comprehensive role of ARNT, analysis of the individual ARNT isoforms has been overlooked. Here, we further explored the dampening mechanism of ARNT isoform 1 on AHR signaling, as determined in chapter 3, by investigating its unique phosphorylation site at S77, as this is the most apparent feature that might dictate functional variances between the ARNT isoforms in respect to modulating AHR responses.

A previous study noted through mass spectrometry analysis that ARNT isoform 1 is phosphorylated at S77 following cellular exposure to TCDD[291]. Our data are consistent with these findings as exposure to TCDD resulted in a robust increase in ARNT isoform

1 phosphorylation within 30 minutes. AHR activation by particular ligands has been shown to elicit unique AHR signaling responses, yet it remains to be investigated whether ARNT isoform 1 phosphorylation is responsible for these noted differences in signaling[141,172,281]. Therefore, the majority of studies requiring AHR activation included TCDD and FICZ and also included a variety of ligands in an additional investigation, however all AHR ligands tested promoted ARNT isoform 1 phosphorylation in varying degrees.

Moreover, our results demonstrate that ARNT isoform 1 phosphorylation is necessary for optimal AHR-target gene transcription as revealed in isoform 1 S77A reconstituted Hepa-BpRc1 cells, which exhibit a two-to-three-fold lower expression of *Cyp1a1* and *Tiparp* as compared to control WT ARNT isoform 1 reconstituted cells. Our reconstitution results are even more striking when considering that cells reconstituted with the S77A mutant harbor higher levels of ARNT and nuclear AHR versus cells reconstituted with WT ARNT, indicating that unphosphorylated isoform 1 is a negative regulator of AHR signaling. In concordance with the consequences stemming from mutation of S77, inhibition of ARNT isoform 1 phosphorylation via the CK2 inhibitor CX4945 also significantly reduces the expression of *CYP1A1* and *AHRR* in Karpas 299 and Peer T cells after exposure to TCDD or FICZ, underscoring the importance of ARNT isoform 1 phosphorylation in driving AHR target gene expression (Illustration 4.1).

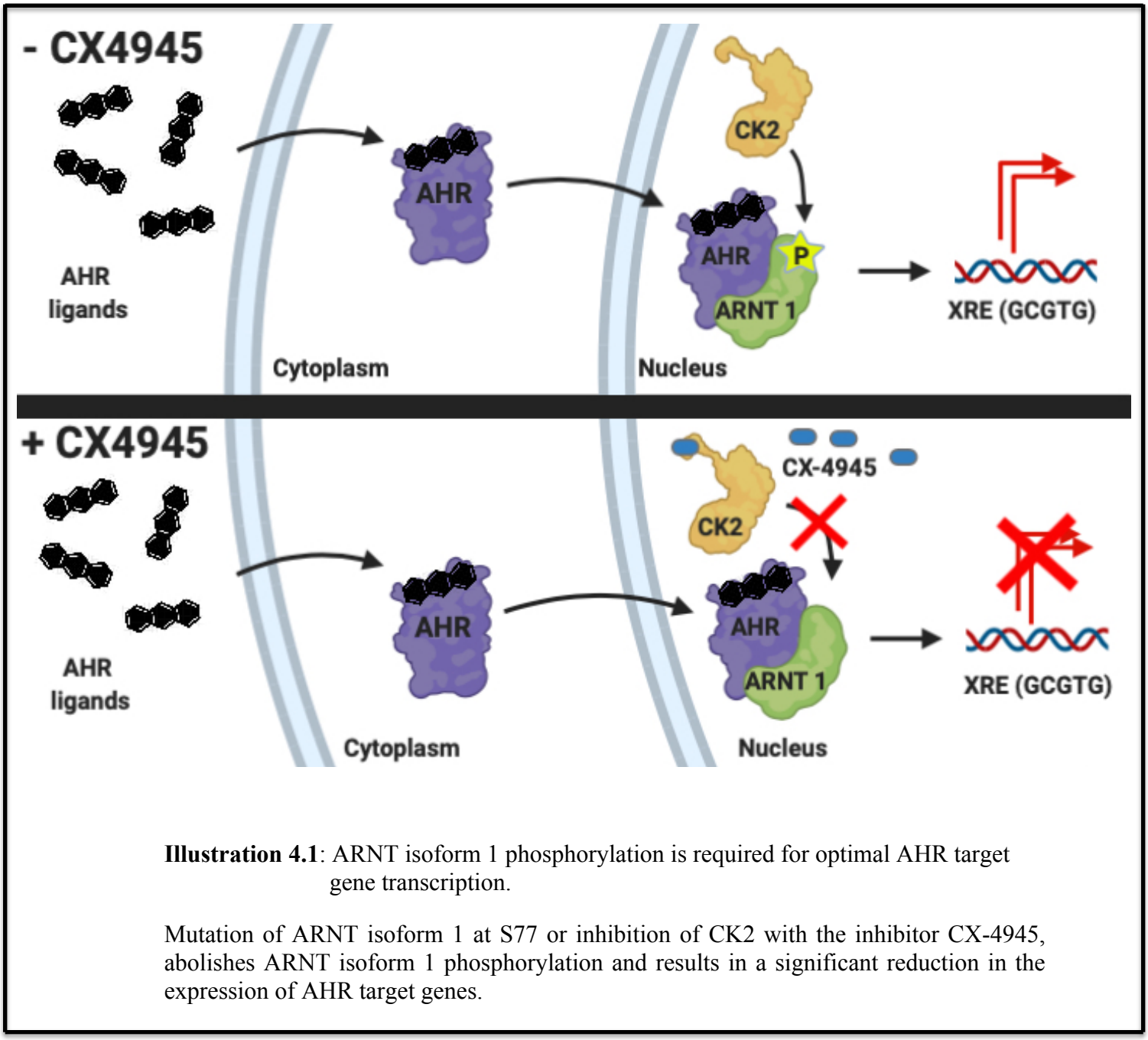

Illustration 4.1: ARNT isoform 1 phosphorylation is required for optimal AHR target gene transcription.

Mutation of ARNT isoform 1 at S77 or inhibition of CK2 with the inhibitor CX-4945, abolishes ARNT isoform 1 phosphorylation and results in a significant reduction in the expression of AHR target genes.

In finding a correlation between AHR activation and ARNT isoform 1 phosphorylation, we surmised that AHR may have a role in S77 phosphorylation[291]. Interestingly, loss of AHR abrogated phosphorylation of S77, which further indicates that AHR has a contributing role in the mechanism of ARNT isoform 1 phosphorylation. In addition, we predict ARNT isoform 1 may need to undergo a conformational change

following AHR activation and binding to allow for ARNT isoform 1 to undergo phosphorylation at S77. With these data, future studies will be employed to address the mechanism of ARNT isoform 1 phosphorylation in AHR signaling and will potentially expand our knowledge of the role of CK2.

Given that AHR activation by particular ligands has been shown to elicit unique AHR-mediated T cell responses, and that NF-κB members, which are known binding partners of ARNT, have been shown to control the expression of T cell activation markers, we also examined whether stimulation of Peer T cells with P/I had any effects on ARNT isoform 1 phosphorylation[294,295]. To this end, we found that T cell stimulation reduces the ability of TCDD and FICZ to induce ARNT isoform 1 phosphorylation. Moreover, TCDD exposure in the presence of P/I promotes a more rapid loss of AHR protein levels compared to TCDD alone, or to FICZ in the presence of P/I, most likely a consequence of the labile nature of FICZ whereas TCDD is not metabolized. The differences in AHR stability between TCDD and FICZ also reveal an interesting correlation with the P/I stimulation-induced expression of cytokines, where TCDD significantly abrogates expression but FICZ does not. These observations suggest that increases in cytokine expression after T cell activation are somewhat reliant on retaining sufficient levels of AHR with a concomitant absence of ARNT isoform 1 phosphorylation. In other words, these data indicate that AHR and/or unphosphorylated ARNT isoform 1 is/are needed to enhance gene transcription following T cell stimulation.. AHR could dimerize with other transcription factors, as has been reported for ROR-γt, STAT3, or RelA, after T cell activation to regulate expression of genes, and different ligands could affect these interactions by influencing AHR stability.

Additionally, incubation of stimulated Peer T cells with the CK2 inhibitor results in the lowest levels of ARNT phosphorylation observed between the various exposure combinations examined, low basal levels of ARNT and AHR interaction, and high levels of total AHR comparable to the DMSO unstimulated control, which corresponds to near complete ablation of *CYP1A1* expression but augmented cytokine expression over that observed for FICZ, further demonstrating that unphosphorylated ARNT isoform 1 is needed to enhance T cell activation-induced gene expression. Furthermore, additional investigations are warranted to determine if AHR is recruited to the specific promoters of *CSF2, IL3, and IL6* following T cell activation and if this recruitment is impacted following the abolishment of ARNT isoform 1 phosphorylation. These findings will provide more insight into the role of AHR and/or ARNT 1 phosphorylation in T cell activation marker gene expression.

In light of these findings, it is tempting to speculate that the strength of ARNT isoform 1 phosphorylation plays a role in the divergent T cell differentiation pathways mediated by AHR ligands TCDD versus FICZ. Thus, these findings suggest that ARNT isoform 1 has different modulatory roles depending on the phosphorylation of S77 and appears to act as a switch for ARNT isoform 1 to function in AHR signaling or in the expression of T cell activation markers. Our data indicate that phosphorylated ARNT isoform 1 is necessary for AHR signaling, whereas unphosphorylated ARNT isoform 1 promotes T-cell stimulation induced gene-expression.

In this investigation, we identified a unique regulatory modification of ARNT isoform 1 by CK2 that is needed for optimal AHR target gene transcription. Our data demonstrates that ARNT isoform 1 phosphorylation at S77 is required to achieve optimal

transcription of XRE target genes. Together, these observations indicate that ARNT isoform 1 phosphorylation is a promising therapeutic target for treating malignancies in a contextual manner. Studies have demonstrated contradictory mechanisms for the role of AHR in tumor cell survival as select malignancies exhibit a dependency on AHR activation, whereas AHR expression has shown to limit proliferative effects in others[296–299]. Targeting ARNT isoform 1 phosphorylation offers a potential therapeutic option to inhibit AHR activity in malignancies dependent on AHR activation. To conclude, these novel studies present findings that demonstrate the importance of studying the ARNT isoforms and highlight a regulatory element of ARNT isoform 1 that further enhances our understanding of AHR signaling in the immune system.

Chapter 5 Conclusion

DISSERTATION SUMMARY

The progression and development of hematological diseases and tumors result most often from disruptions in cellular processes caused by genetic predispositions or environmental factors such as single nucleotide polymorphisms, epigenetic changes, alterations in the gut flora composition, or from environmental toxicant exposures. Genetic variations have been linked to the onset of numerous immune-related disorders, however knowledge on the impact of environmental factors contributing to the progression of autoimmune diseases is still limited. Previous studies have identified several environmental factors that impact immunity including altered compositions of gut flora, infection, and diet[300–303]. Additionally, environmental toxicants have also been demonstrated to be significantly linked to tumorigenesis and autoimmunity[164,166,197]. Although it is becoming increasingly evident that the environment can have drastic impacts on the immune system, many of the specific mechanisms behind these affects still remain unknown.

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that is found to have crucial roles in the immune system. AHR contributes to a wide range of processes in immune function including haematopoiesis, T cell differentiation, homeostasis of the gut, along with a host of functions in monocytes[54,141,148,304,305]. With roles in many facets of immunity, it is no surprise that AHR signaling has been identified as a major pathway involved in the progression of hematological diseases and malignancies[141,149,168,178,184]. The AHR signaling pathway can be initiated by a variety of different ligands ranging from environmental toxicants, compounds from our diet, and

products metabolized from the gut and enzymatic proteins. Activation of AHR then results in nuclear translocation and heterodimerization with the aryl hydrocarbon receptor nuclear translocator (ARNT) to induce target gene transcription.

Although AHR signaling has emerged as a major pathway responsible for the progression of multiple immunological diseases, the major binding partner, ARNT, still remains to be fully investigated. Previous studies have demonstrated a few immunological functions of ARNT including roles in hematopoietic stem cell function and expression of cytokines in myeloid cells, however the vast majority of studies are unaware that ARNT is alternatively spliced into two isoforms, known as ARNT isoform 1 and 3[64,193,251,255]. To address the gap in knowledge of the role of each ARNT isoform in AHR signaling and immunity, we sought to explore the function of ARNT isoforms 1 and 3 following AHR activation in lymphoid neoplasms.

Previously, we found that these two isoforms are present within both B and T cells in equal abundance, but the protein level of ARNT isoform 1 is increased in malignant leukocytes to provide a survival advantage[59]. Having found that each ARNT isoform is expressed in T cells, we initially sought to investigate the abundance of each isoform in different tissues[59]. Hence, we collected 10 different tissue samples from individual mice and observed that both ARNT isoforms were present in each tissue, except for the brain, kidney, and muscle. This finding provides valuable evidence of the presence of each ARNT isoform in numerous tissues, highlighting the importance of studying ARNT not as a single protein, but of two distinct isoforms.

To gain further insight into the role of ARNT in the immune system, we studied the function of each isoform independently upon AHR activation and also examined a

unique phosphorylation event specific to ARNT isoform 1 at serine 77 (S77) for an enhanced understanding of this distinguishing modification in two distinct malignant T cell lines[270,271]. To this end, we suppressed the expression of each isoform individually using siRNA and exposed cells to the environmental contaminant and high affinity AHR ligand 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD). Strikingly, we observed a significant increase in the expression of the AHR target genes *CYP1A1* and *AHRR* in cells with ARNT isoform 1 reduced. Conversely, suppression of ARNT isoform 3 resulted in a significant reduction of both *CYP1A1* and *AHRR* compared to control cells. Collectively, these results demonstrate that ARNT isoform 1 has an inhibitory role in AHR signaling, whereas ARNT isoform 3 is needed to promote AHR activity.

As each ARNT isoform was found to significantly alter AHR target gene transcription, we investigated possible differences in protein and DNA interactions. We found that AHR interacts with each ARNT isoform without preference in the cell, regardless of the activating ligand. Furthermore, upon suppression of ARNT isoform 1, binding of AHR and ARNT to the *CYP1A1* promoter was increased compared to control and cells with reduced levels of ARNT isoform 3. These findings further complement previous data in that suppression of ARNT isoform 1 results in both an increase in AHR target gene expression and *CYP1A1* promoter occupation by AHR and ARNT. Intriguingly, suppression of ARNT isoform 3 is also found to result in the occupation of the *CYP1A1* promoter by ARNT and AHR prior to and after TCDD exposure, hinting that the suppressive role of ARNT isoform 1 may involve DNA interaction.

In finding ARNT isoform-specific differences in the regulation of AHR signaling, we sought to characterize ARNT isoform 1 phosphorylation at S77, as this modification

is the main differentiating feature between each ARNT isoform and may provide further insight into the inhibitory role of ARNT isoform 1 in AHR signaling. Following AHR activation by TCDD, we found that ARNT isoform 1 underwent phosphorylation at S77 within 30 minutes of ligand exposure and was also able to be elicited by numerous other ligands. Since ARNT isoform 1 phosphorylation occurred rapidly after AHR activation, we investigated the role of AHR in the phosphorylation of S77. Through the utilization of cells void of AHR, along with antagonist and knockdown studies, we determined that ARNT isoform 1 phosphorylation at S77 requires AHR activation.

In finding a tight correlation between AHR activation and ARNT isoform 1 phosphorylation, we proceeded to investigate the role of this modification in AHR target gene transcription. To do this, we transduced Hepa-BpRc1 cells, an *Arnt*-null cell line, with a lentivirus to allow for the stable expression of wild-type (WT) ARNT isoform 1 or ARNT isoform 1 with a mutation at S77 to an alanine (S77A) to abolish phosphorylation. Interestingly, activation of AHR in cells with WT ARNT isoform 1 resulted in a significant increase in AHR target gene transcription compared to cells with a mutation at S77. Furthermore, the augmentation of AHR target gene transcription by WT ARNT isoform 1 was not surprising as Hepa-1c1c7 cells express only ARNT isoform 1 protein. Thus, the suppressive function previously shown of WT ARNT isoform 1 may again be specific to cell types expressing both isoforms or may exclusive to leukocytes regarding ARNT isoform specific regulation. In addition, activation of AHR following the inhibition of casein kinase 2 (CK2), which is previously found to phosphorylate ARNT isoform 1 at S77, resulted in the abolishment of ARNT isoform 1 phosphorylation and a significant reduction in AHR target gene transcription. Although not investigated in this

study, a potential increase in the abundance of CK2 may also be attributed to the increase in AHR target gene transcription in Hepa-BpRc1 cells with WT ARNT isoform 1, thus allowing for enhanced phosphorylation of ARNT isoform 1 and an augmentation of target gene transcription. These results show that ARNT isoform 1 phosphorylation at S77 is needed for optimal gene expression driven by AHR in malignant leukocytes.

Collectively, based on these findings, we postulate that ARNT isoform 1 phosphorylation functions as a switch to allow for the promotion of AHR signaling. Our current data supports a model that during basal activities, unphosphorylated ARNT isoform 1 potentially prevents DNA accessibility or hinders ARNT isoform 3 from augmenting AHR signaling, however upon AHR activation and ARNT isoform 1 phosphorylation, the repressive function of ARNT isoform 1 is abrogated, thus allowing AHR and ARNT isoform 3 to promote AHR target gene transcription (Illustration 5.1).

Additionally, with the increased levels of ARNT isoform 1 in hematological malignancies, as demonstrated in a previous publication, one could also postulate that low basal levels of AHR in the nucleus are primarily interacting with unphosphorylated ARNT isoform 1, as AHR is shown to predominantly interact with the most abundant isoform, thus hampering significant increases in AHR target gene transcription[59]. However, as the cell is exposed to higher concentrations of an AHR ligand, increased amounts of AHR translocates into the nucleus, providing more interactions between AHR and the low levels of ARNT isoform 3 while simultaneously inducing ARNT isoform 1 phosphorylation, together allowing for active AHR signaling and an increase in target gene transcription.

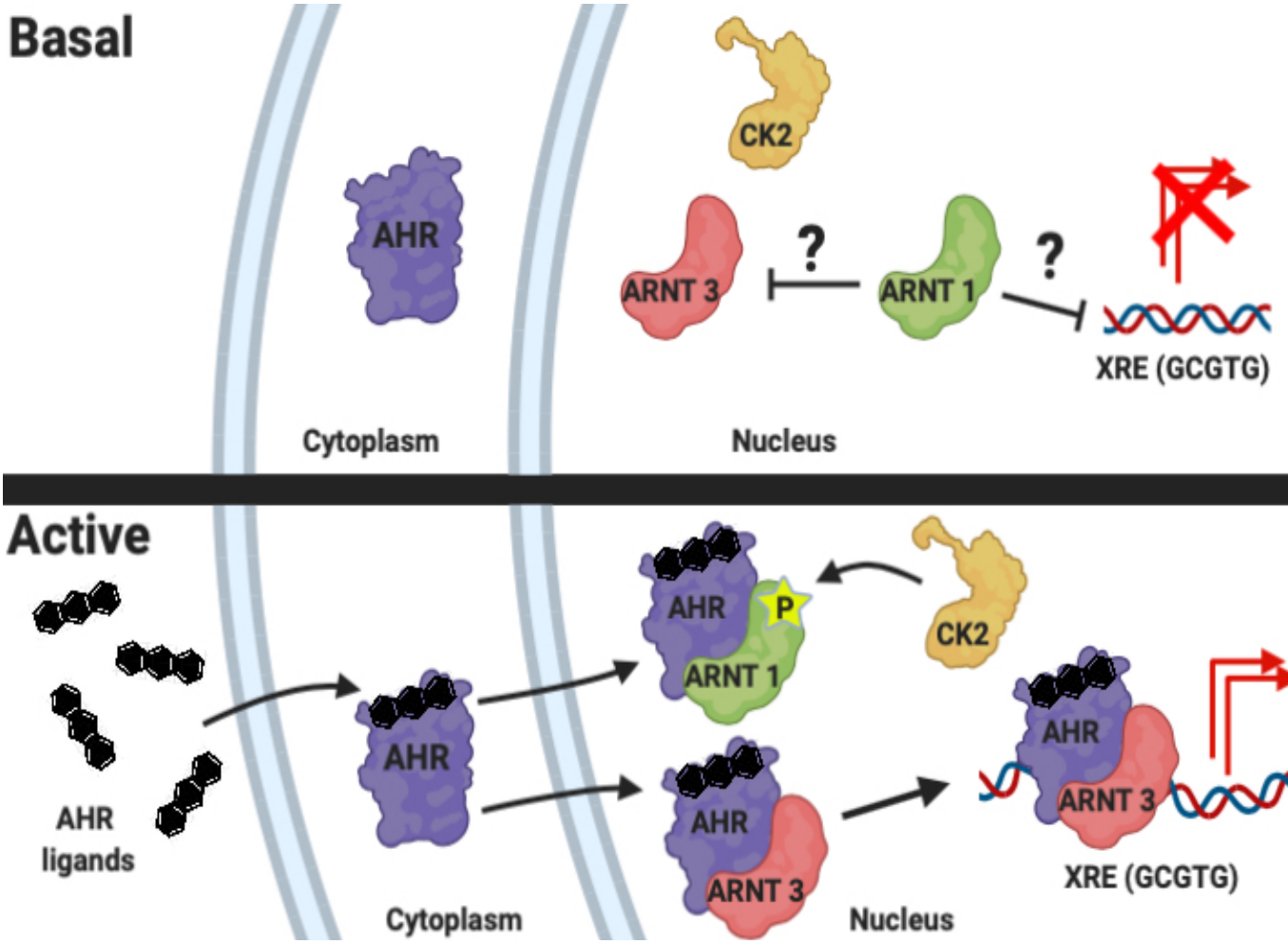

Illustration 5.1: Proposed model of ARNT isoform specific regulation.

During basal activities, we predict unphosphorylated ARNT isoform 1 suppresses AHR target gene transcription by preventing DNA accessibility or suppressing ARNT isoform 3 from increasing gene expression. However, upon AHR activation, ARNT isoform 1 is phosphorylated and its repressive function is repealed, allowing for AHR and ARNT isoform 3 to promote gene transcription at XREs.

Since AHR is found to be involved in T cell activation, we investigated the potential role of ARNT isoform 1 phosphorylation following the activation of malignant T cells[294]. We found that upon T cell activation, ARNT phosphorylation at S77 appears to

decrease from a low basal level and is also reduced when AHR signaling is induced during T cell activation. Further, we demonstrate that T cell stimulation-induced gene expression is significantly reduced in samples with phosphorylated ARNT isoform 1 from activated AHR signaling. These findings suggest that unphosphorylated ARNT isoform 1 aids in the expression of T cell activation markers, which we further demonstrated, as abolishment of ARNT isoform 1 phosphorylation from CK2 inhibition resulted in a significant increase in the expression of genes driven by T cell activation. Collectively, these data indicate a potential role of unphosphorylated ARNT isoform 1 in T cell activation and suggests that ARNT isoform 1 has different roles depending on the status of its phosphorylation at S77.

Previous studies have also found that AHR ligands are able to uniquely modulate AHR activity and influence T cell differentiation. AHR activation by TCDD and kynurenine have both been shown to enhance T cell differentiation into a regulatory T cell phenotype, whereas exposure to 6-formylindolo [3, 2-b] carbazole (FICZ) is found to increase the number of T helper 17 (Th17) populations[141,142,168,280]. Therefore, throughout these studies, we investigated the function of the independent ARNT isoforms following exposure to TCDD and FICZ to determine possible ligand-induced specific differences. To this end, we found that each ligand generated similar responses in regards to ARNT isoform-specific modulation of AHR signaling and ARNT isoform 1 phosphorylation. Although these ligands induce different responses throughout the immune system driven by AHR, we found no such differences when examining ARNT-isoform regulation. The novel findings presented here support and enhance our current understanding of how the ARNT isoforms regulate AHR signaling in the immune system and provide further

evidence of the importance in studying the ARNT isoforms independently. Together, our results indicate that manipulation of the ARNT isoform ratio, or targeting ARNT isoform 1 phosphorylation, offers potential therapeutic options to inhibit or enhance AHR activity for treating hematological malignancies and other immune disorders.

SIGNIFICANCE

These studies address a crucial knowledge gap that exists in understanding the role of the individual ARNT isoforms and the function of ARNT isoform 1 phosphorylation in AHR signaling within T cell malignancies. The identification of both ARNT isoforms in multiple different tissues along with the discovery of isoform-specific functions in AHR signaling emphasizes the necessity and significance of examining each isoform when investigating ARNT. Furthermore, our characterization of ARNT isoform 1 phosphorylation has also provided notable findings in AHR signaling and T cell activation. We have determined that phosphorylation of ARNT isoform 1 at S77 is essential for optimal AHR target gene expression, whereas unphosphorylated ARNT isoform 1 is correlated with the enhancement of T cell stimulation-induced gene expression, thus uncovering a fascinating regulatory role of ARNT isoform 1.

In addition, previous investigations have shown that activating AHR in specific malignancies can result in beneficial or detrimental outcomes[296–299]. Knowledge of ARNT isoform-specific functions in AHR signaling offers an exciting opportunity to uniquely treat these disorders through modulation of the ARNT isoforms. For example, reducing AHR activity by suppressing ARNT isoform 3 in glioblastomas, which rely on AHR signaling to evade the immune system, could provide for an efficacious therapy[172].

Additionally, repressing AHR signaling by targeting ARNT isoform 1 phosphorylation through direct inhibition of CK2 could offer an additional option to target malignancies dependent on AHR activity. With clinical trials being performed utilizing the same CK2 inhibitor used in our studies, CX-4945, the investigation of this notion is exciting[306,307].

With little previously understood about the ARNT isoforms or the role of S77 phosphorylation, investigating these functions and events following AHR activation has not only contributed to the vast knowledge and understanding of AHR signaling, but also to how the ARNT isoforms may contribute to immunological function alone and through AHR. These significant findings provide valuable information as a first step toward our long-term efforts of therapeutically targeting ARNT in hematological diseases and malignancies.

FUTURE DIRECTIONS

Although these studies provide novel and significant findings of the ARNT isoforms and ARNT isoform 1 phosphorylation in AHR signaling, there are many avenues of investigation that would complement these results. To further validate our initial investigation of examining the abundance of the ARNT isoforms in various mouse tissues, we plan to study the presence of the ARNT isoforms in human cell lines from different tissues. To do this, we will collect human cell lines that are from similar tissues investigated in Figure 1 and will extract whole cell lysates to investigate the abundance and prevalence of each ARNT isoform. Furthermore, we aim to study possible sex-specific differences in the abundance of the ARNT isoforms by also investigating tissues from male and female mice.

Despite advances in knowledge of co-regulator recruitment to the AHR/ARNT complex, a disconnect exists in known ARNT isoform-specific co-regulator recruitment[211,273–277,308–310]. In finding stark differences in AHR target gene transcription from each independent ARNT isoform, we aspire to study co-regulator interactions with each isoform to potentially determine unique binding partners. Specifically, we will initially study interactions between histone deacetylases (HDACs) or histone acetyltransferases (HATs) and each ARNT isoform. To investigate this, we will perform co-immunoprecipitations with ARNT in Karpas 299 and Peer T cells following the suppression of each ARNT isoform individually and will then determine binding to HDACs and HATs prior to and following AHR activation through immunoblot analysis. We will also reciprocally co-immunoprecipitate our protein of interest to investigate its binding to each ARNT isoform. With the limitation of needing suitable antibodies to investigate these interactions, we could also perform overexpression studies with our proteins of interest that are tagged with either an HA or FLAG, thus potentially allowing us to overcome the challenge of unspecific antibodies. To further investigate a relationship between the ARNT isoforms with HATs and HDACs, we also seek to perform EMSAs at XREs. Although this technique is not quantitative, we will be able to detect protein-DNA binding complexes and will perform supershift assays to determine the proteins that comprise the complex. Additionally, we will analyze if each ARNT isoform impacts the expression of HATs and HDACs through RT-qPCR. In demonstrating that ARNT isoform 1 functions as a suppressor of AHR activity, we predict this may be through the recruitment of HDACs, whereas the promotion of AHR signaling by ARNT isoform 3 may involve interactions with HATs[211,273–278,309].

Similarly, upon determining that ARNT isoform 1 phosphorylation is needed for optimal AHR target gene transcription, we plan to explore if co-regulator interactions with ARNT isoform 1 are altered depending on the phosphorylation of S77. Initially, we plan to determine if ARNT isoform 1 phosphorylation affects interactions with the aryl hydrocarbon receptor repressor (AHRR) through co-immunoprecipitation studies both with and without CK2 inhibition followed by AHR activattion. AHRR is a well-documented co-factor that is found to interact with ARNT following AHR activation and is suggested to act as a negative feedback in AHR signaling by interacting with ARNT[115,116,217]. Again, if we are unable to capture an interact between ARNT isoform 1 and AHRR, we will turn to performing overexpression experiments in HEK 293 cells utilizing ARNT isoform 1 mutated at S77 in combination with both proteins tagged. In finding ARNT isoform 1 phosphorylation is tightly correlated to AHR activation and that abolishment of S77 phosphorylation results in a significant reduction of AHR target gene transcription, we postulate that AHRR interacts solely with unphosphorylated ARNT isoform 1. The results from this experiment could provide an explanation for the reduction in AHR target gene expression without ARNT isoform 1 phosphorylation and would further complement findings of the correlation between S77 phosphorylation and increases in *CYP1A1* and *AHRR*.

In seeking to gain insight into the mechanism of ARNT isoform 1 phosphorylation, we investigated the role of AHR. Our study revealed that phosphorylation of ARNT isoform 1 requires AHR as well as its activation for phosphorylation to occur. For future experiments, we are aiming to further understand the order of events regarding ARNT isoform 1 phosphorylation by CK2. We will initially

investigate a mechanism involving heat shock protein 90 (HSP90), a co-chaperone of AHR, as previous studies have demonstrated interactions between HSP90 and CK2 as well as between HSP90 and ARNT, thus acting as a potential link for CK2-mediated phosphorylation of ARNT isoform 1 at S77[107,311,312]. To do this, we will attempt to detect a complex consisting of AHR, ARNT, HSP90, and CK2 by performing a co-immunoprecipitation following AHR activation and the use of molybdate to capture this transformative complex[107]. Molybdate has previously been utilized to capture the tertiary complex of AHR, ARNT, and HSP90, thus potentially allowing for the capture of an additional protein such as CK2[107]. If we are unable to capture this fleeting interaction, additional strategies can also be employed to investigate each step of this mechanism. To further study the potential interaction between HSP90 and CK2 and ARNT, we will initially activate CK2 with the agonist spermine, which has been shown to be the most efficacious polyamine to stimulate CK2 activity; we will then perform a co-immunoprecipitation to capture the potential interaction[313]. Activating CK2 directly will allow for an increased opportunity to capture its interaction with ARNT and HSP90. If exposure to spermine is found to also result in AHR activation, we will simultaneously treat cells with an AHR antagonist along with spermine for a more focused activation of CK2. Additionally, we predict that a change in the conformation of ARNT isoform 1 following AHR activation and binding may also be required for phosphorylation at S77 by CK2. The simultaneous use of spermine to activate CK2 and antagonism of AHR, as discussed above, would provide further insight into this thought as we would be able to determine if CK2 is able to phosphorylate ARNT isoform 1 without it undergoing a potential conformational change following AHR activation and binding. These

experiments can also be performed by overexpressing each protein with unique tags to overcome potential complications with antibody specificity. We speculate that following AHR activation, HSP90 may recruit CK2 and upon AHR-ARNT dimerization, CK2 is brought into close proximity of ARNT isoform 1 to phosphorylate S77. Nevertheless, we plan to investigate the complete mechanism of how ARNT isoform 1 is phosphorylated at S77 by CK2.

To further explore the role of ARNT in T cell activation, we will study ARNT isoform-specific function in the activation of T cells. To do this, we will suppress each ARNT isoform independently and study changes in cellular signaling as well as in the gene expression, through RE-qPCR, and promoter occupation of T cell activation markers, through ChIP and EMSAs, following the activation of Peer T cells with phorbol 12-myristate 13-acetate and ionomycin (P/I). To conclude, conducting these future studies elucidating mechanistic signaling and protein interactions with ARNT will build upon this current investigation and enhance our understanding of the role and function of the ARNT isoforms.

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Vita

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