Regulation of the aryl hydrocarbon receptor (AHR) activity through intracellular transport processes

Inaugural-Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry, Pharmacy

of Freie Universität Berlin

by

Rashad Haidar

2024

This thesis was carried out at the Federal Institute for Risk Assessment (BfR) in Berlin from December 2018 to December 2022 under the supervision of Prof. Dr. Dr. Andreas Luch.

1st Reviewer: Prof. Dr. Dr. Andreas Luch

2nd Reviewer: Prof. Dr. Gerhard Wolber

Date of defense: 05.12.2024

Declaration of Independence

I hereby declare that I have independently prepared this thesis entitled 'Regulation of the aryl hydrocarbon receptor (AHR) activity through intracellular transport processes' without the use of any other than the approved resources. All citations are marked as such.

The submitted work has not been accepted or found to be unsatisfactory in any previous doctoral proceedings.

Berlin, den 22.05.2024

Rashad Haidar

Table of contents

Abbreviations

All abbreviations occurring in the text are listed in the following table:

ITE	2-(1' *H*-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester
TCDD	2,3,7,8-Tetrachlordibenzo-*p*-dioxin
3-MC	3-Methylcholanthrene
FICZ	6-Formylindolo[3,2-b]carbazole
DMBA	7,12 Dimethylbenzanthracene
AHR	Aryl hydrocarbon receptor
$AHR^{-/-}$	AHR knockout
AHRC	AHR/ARNT complex
ARNT	AHR-nuclear translocator
AKR	Aldo-keto reductase
AHH	Aryl hydrocarbon hydroxylase
bHLH-PAS	Basic helix-loop-helix, Per-ARNT-Sim
B[*a*]P	Benzo[*a*]pyrene
CRE	cAMP response elements
CRM1	Chromosome region maintenance 1
COX	Cyclooxygenase
CYP	Cytochrome P450-dependent monooxygenase
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
FBS	Fetal bovine serum
FDA	Food and drug administration
Q-rich	Glutamine-rich region
GST-α	Glutathione *S*-transferase α
RanGTP	Guanosine triphosphate-bound Ran
HAH	Halogenated aromatic hydrocarbon
HSP90	Heat shock protein 90
XAP2	Hepatitis x-associated protein-2
hAHR	Human AHR
HIV-1	Human immunodeficiency virus-1
IMP	Importin

IPZ	Importzaole
IND	Indirubin
I3C	Indole-3-carbinol
IDO	Indoleamine-2,3-dioxygenase
ICZ	Indolo[3,2-b]carbazole
IL	Interleukin
IVM	Ivermectin
JAK	Janus kinase
Kyn	Kynurenine
LMB	Leptomycin B
LPS	Lipopolysaccharide
NQO1	NADPH quinone oxidoreductase 1
NES	Nuclear export signal
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signal
OM	Omeprazole
Per	Period circadian protein
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzodioxine
PCDF	Polychlorinated dibenzofuran
PAH	Polycyclic aromatic hydrocarbon
PST	Proline/Serine/Threonine
PKA	Protein kinase A
RanGEF	Ran guanine exchange factor
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription
Sim	Single-minded protein
Sp	Specificity protein
TIPARP	TCDD-inducible poly-ADP-ribose polymerase
TAD	Transactivation domain
TF	Transcription factor
TGF	Transforming growth factor
TNPO	Transportin

Trp	Tryptophan
TDO	Tryptophan-2,3-dioxygenase
UGT1A1	UDP-glucuronosyltransferase 1A1
WT	Wild type
XRE	Xenobiotic response element
XME	Xenobiotic-metabolizing enzymes
ANF	α-naphthoflavone
BNF	β-naphthoflavone

1 Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor (TF) with a famous history in the field of toxicology. Originally, the AHR was identified in the 1970s for mediating an enzymatic response to environmental toxicants, especially benzo[*a*]pyrene (B[*a*]P) and 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD) (Poland *et al.*, 1976) (Okey *et al.*, 1979). This cellular response was described initially as "aryl hydrocarbon hydroxylase (AHH)" (Nebert & Gelboin, 1968a) (Nebert & Gelboin, 1968b), but turned out to be the cytochrome P450 -dependent monooxygenase (CYP) (Nebert & Gielen, 1972) (Nebert, 2017), thus presenting early evidence for AHR's role in xenobiotic metabolism. In the following years, the N-terminal domain of the AHR was sequenced (Bradfield *et al.*, 1991) and subsequently AHR knockout ($AHR^{-/-}$) mice were generated in many laboratories (Lahvis & Bradfield, 1998) (Fernandez-Salguero *et al.*, 1995). These knock-out mice exhibited a low sensitivity to xenobiotics underscoring the importance of the AHR in xenobiotic metabolism (Fernandez-Salguero *et al.*, 1996). Furthermore, studies on $AHR^{-/-}$ mice gave first hints for more complicated AHR functions than xenobiotic metabolism (Gonzalez & Fernandez-Salguero, 1998), involving organ development and fertility (Fernandez-Salguero *et al.*, 1995) (Lahvis *et al.*, 2005). Moreover, current research revealed that the AHR participates in many physiological processes, including cell cycle, apoptosis, and immune cell functions (Xue *et al.*, 2018).

Evolutionary, the AHR is highly conserved in vertebrates and invertebrates (Hahn *et al.*, 2017) and its expression is well characterized in human und mice. mRNA of the human AHR (hAHR) has been detected in all tested human tissues, even in retina (Y. Zhou *et al.*, 2018) and different blood cells types (Karlsson *et al.*, 2021). High AHR expression is noticed in physiological barriers, like lung, liver, placenta, and the gastrointestinal system (Fagerberg *et al.*, 2014).

1.1 Regulation of the *AHR* gene

The promoter of *hAHR* contains multiple binding sites belonging to different TFs, like cAMP response elements (CRE). The *AHR* promoter contains close to the transcription initiation sites no TATA box but many GC boxes, which are binding sites for Sp1 (specificity protein 1) (Eguchi *et al.*, 1994). Further research confirmed many similarities between human and murine *AHR*, which were found to be regulated through members of Sp family of TFs (Fitzgerald *et al.*, 1998). In general, induction of the *AHR* is cell type specific. As for example, activation of transforming growth factor-β (TGF-β) can induce the expression of *AHR* in the human hepatoma cell line HepG2 but not in the lung carcinoma cell line A549 (S. Wolff *et al.*, 2001), or in other studied human cancer cells (Döhr & Abel, 1997). Another study reported that treatment of HepG2 cells with interleukin 6 (IL-6) leads to binding of the signal transducer and activator of transcription 3 (STAT3) to a binding site upstream of *AHR*, resulting in

initiation of its transcription (Stobbe-Maicherski *et al.*, 2013). Comparably, IL-4 induces *AHR* expression in human B cells in a STAT6-dependent manner (Tanaka *et al.*, 2005).

Furthermore, many other factors regulate *AHR* gene expression (Shivanna *et al.*, 2022), for example epigenetic factors like histone acetylation (Garrison *et al.*, 2000) or DNA methylation (Mulero-Navarro *et al.*, 2006).

1.2 The AHR signalling pathway

In general, the AHR signaling pathway can be subdivided into the classic (also called canonical or genomic) signaling pathway, referring to AHR's involvement in xenobiotic metabolism, and the non-classic (non-canonical or non-genomic) signaling pathway that rely on AHR's interactions with other signaling pathways.

1.2.1 The classical AHR pathway

According to the classic activation pathway, the AHR remains in the cytoplasm prior to ligand-binding as a part of a multiprotein complex. Binding to ligands triggers rapid import of the AHR into the nucleus whereby it is released from the complex. Next, the AHR builds a dimer with the AHR-nuclear translocator (ARNT) and then binds to the DNA leading to induce the expression of AHR target genes (Beischlag *et al.*, 2008).

Considering more details in the classical AHR pathway, the cytoplasmic multiprotein complex of AHR consists of two molecules of heat shock protein 90 (HSP90) (Perdew, 1988) and single molecules of co-chaperone p23 (Kazlauskas *et al.*, 1999) and hepatitis x-associated protein-2 (XAP2) (B. K. Meyer *et al.*, 1998). More recently, an association between AHR protein complex and c-Src protein kinase has been reported in cell lines from colon cancer (Xie *et al.*, 2012) or prostate cancer (Ghotbaddini *et al.*, 2017). Nevertheless, only limited studies investigate this association.

Members of the AHR multiprotein complex collaborate to ensure high AHR activity (Beischlag *et al.*, 2008), since experimental evidence showed that the monomeric AHR has no capacity for ligand-binding (Pongratz *et al.*, 1992). The AHR interacts directly in the complex with both HSP90 and XAP2 (B. K. Meyer & Perdew, 1999), while P23 interacts only with one HSP90 (Chadli *et al.*, 2000) (Larigot *et al.*, 2022). Moreover, HSP90 is involved in maintaining a proper folding of the newly synthesized AHR (Petrulis & Perdew, 2002), while XAP2 and co-chaperone p23 contribute to protect the AHR from ubiquitination and subsequent proteasome-mediated degradation (Kazlauskas *et al.*, 2000) (Pappas *et al.*, 2018).

Endogenous and exogenous ligands of the AHR are typically hydrophobic and thus they are able to cross the cell membrane through diffusion (Stevens *et al.*, 2009), where they bind the cytoplasmic AHR. This binding leads to a conformational change that exposes a regulatory motive, termed nuclear

localization signal (NLS), to a nuclear transporter, which mediates the nuclear import of the AHR (Esser & Rannug, 2015). In the nucleus, AHR dissociates from the protein complex (Tsuji *et al.*, 2014) and dimerizes with ARNT (Figure 1) (Reyes *et al.*, 1992). The AHR/ARNT complex (AHRC) recruits coactivators with histone acetyltransferase and histone methyltransferase activity (Esser & Rannug, 2015), which gives the AHRC access to the DNA. Subsequently, the AHRC binds a special DNA sequence located in the enhancer region of its target genes, called XRE (xenobiotic responsive element), also known as dioxin responsive element (Figure 1) (Swanson *et al.*, 1995b). XREs have the minimal sequence 5´-TNGCGTG-3´ (N stands for any nucleotide), in which AHR recognizes (TNGC), and ARNT (GTG) (Bacsi & Hankinson, 1996). Finally, binding of AHRC to XRE induces the expression of different xenobiotic metabolizing enzymes (XMEs) (Esser & Rannug, 2015).

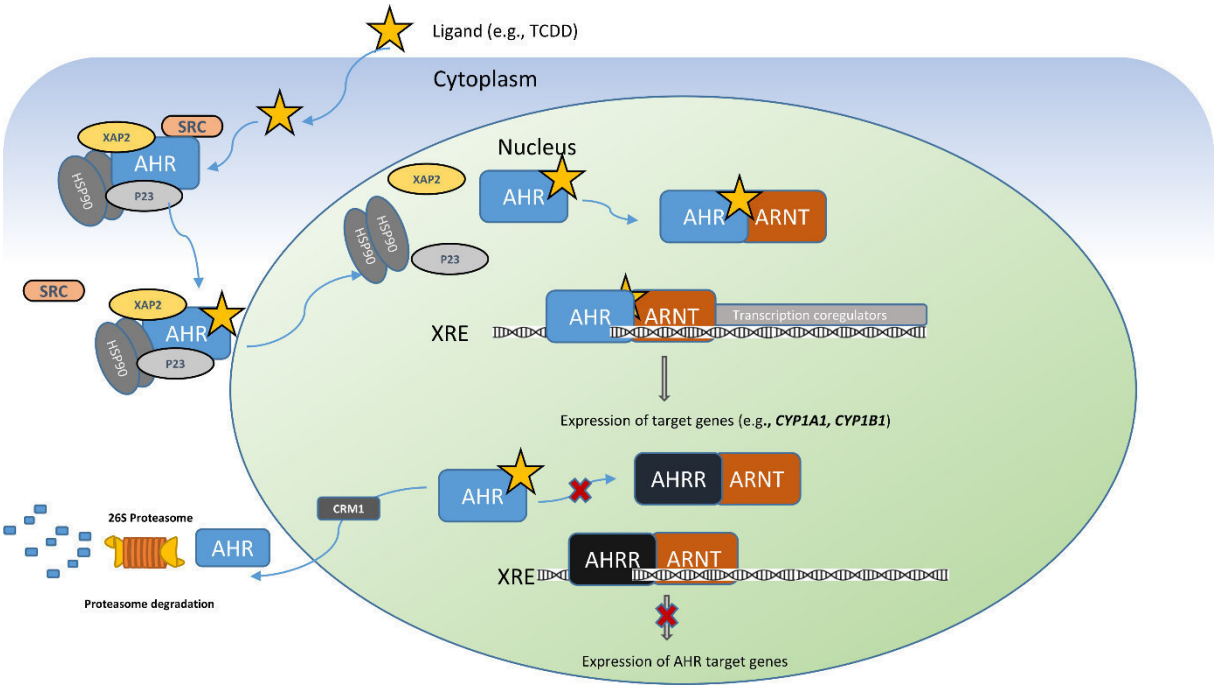


Figure 1: Model of the classical activation pathway of the AHR: Prior to ligand binding, the AHR is located in the cytoplasm as a member of a protein complex that contains HSP90, XAP2, p23, and SRC. Ligand binding triggers release of SRC in the cytoplasm. Subsequently, the AHR is imported into the nucleus, where it is released from its protein complex, builds a dimer with ARNT, and binds to a sequence in the DNA called XRE. Binding to XRE induces the expression of its target genes, e.g., *CYP1A1* or *AHRR*. The expressed AHRR builds a dimer with ARNT and binds to XRE, thereby blocking the activation of the AHR. Finally, the AHR gets exported through CRM1 to the cytoplasm, where it undergoes an ubiquination followed by 26S proteasome-mediated degradation.

1.2.1.1 Role of the AHR in xenobiotic metabolism

The AHR induces the expression of many XMEs. This comprises phase I XMEs like *CYPs*. The most prevalent CYPs in the research on the AHR are *CYP1A1* and *CYP1B1*, which are used as markers for the AHR activation. CYP enzymes catalyze insertion or unmasking of polar functional group e.g., -OH, -SH

and -NH_2. Additionally, the AHR promotes the upregulation of many phase II XMEs, like UDP-glucuronosyltransferase 1A1 (*UGT1A1*), glutathione *S*-transferase α (*GST-α*), and NADPH quinone oxidoreductase 1 (*NQO1*) (Nebert *et al.*, 2000) (Yueh *et al.*, 2003). Reactions of phase II mediate conversion of compounds to even more polar metabolites, by conjugation of subgroups to one of the functional groups -OH, -SH, or -NH2 to form a chemically-inactive metabolite. Typically, the inactive metabolites are excreted through the kidney into urine, via liver into fecal matter, and less often via lung (Guerrina *et al.*, 2018).

However, many AHR-regulated enzymes, for instance CYP1A1, cyclooxygenase (COX), and AKR (Aldo-keto reductase) give also rise to reactive oxygen species (ROS) resulting in oxidative damage to DNA or other macromolecules in the cells (Kennedy *et al.*, 2013) (Grishanova & Perepechaeva, 2022). This implies toxic outcomes for a chronic active AHR, which may result from exposure to environmental toxicants for example. For instance, the B[*a*]P contained in tobacco smoke promotes an overexpression of CYP enzymes and is associated with elevated ROS formation (Vogel *et al.*, 2020).

1.2.1.2 Post activation of the AHR

AHR activation increases the oxidative metabolism and thereby forms oxidative stress. Accordingly, the cellular homeostasis induces a feedback mechanism to limit AHR activity. First, the AHR induces the expression of its own inhibitor, the AHR-repressor (AHRR) (Mimura *et al.*, 1999). In the N-terminal domain, the AHRR and AHR are structurally very similar (Mimura *et al.*, 1999). This allows AHRR to compete with AHR for binding the same target sequence on the DNA, the XRE (Figure 1) (Vogel & Haarmann-Stemmann, 2017). Further investigation revealed that the C-terminal domain of AHRR is also involved in AHR inhibition through recruitment of corepressors (Oshima *et al.*, 2009). In this context, it is interesting to note that the AHRR can also control AHR function. Indeed, ARNT availability is the main factor determining for AHR to serve as ubiquitin ligase or to initiate its work as transcription factor, respectively. More precisely, when ARNT is occupied through dimerization with AHRR, AHR possesses a ubiquitin ligase function (Luecke-Johansson *et al.*, 2017).

Secondly, the AHR is also a target for TCDD-inducible poly-ADP-ribose polymerase (TIPARP), which is known to repress AHR's transcriptional activity through ADP-ribosylation of the nuclear AHR (Gomez *et al.*, 2018). Notably, both systems, meaning TIPARP and ubiquitin, collaborate towards AHR-degradation (Rijo *et al.*, 2021).

At last, AHR is re-exported into the cytoplasm after activation, mediated by CRM1 (chromosome region maintenance 1, also known as exportin). CRM1 recognizes and binds a sequence within the AHR, namely nuclear export signal (NES) (Marlowe & Puga, 2010). This returns the AHR back into the cytoplasm, where it undergoes a ubiquitination followed by 26S proteasome-mediated degradation (Figure 1) (Davarinos & Pollenz, 1999).

1.2.1.3 The dimerization partner ARNT

In the initial phase of AHR research, ARNT was mistakenly described as the nuclear translocator of the AHR. In fact, the *ARNT* gene was discovered accidentally while attempting to clone the *AHR* and was thought to be responsible for the nuclear import of the AHR after ligand-binding (Hoffman *et al.*, 1991). Following this, the AHR/ARNT-XRE was acknowledged as a core complex for AHR's transcriptional activity (Bacsi *et al.*, 1995) (Swanson *et al.*, 1995a) (Bacsi & Hankinson, 1996). Thereafter, a thorough investigation of the subcellular localization of ARNT demonstrated a predominantly nulcear localization of the protein (Sojka *et al.*, 2000) (Eguchi *et al.*, 1997).

1.2.2 The non-classical AHR pathway

The non-classic pathway comprises direct physical interactions between the AHR and members of other signaling pathways, including but not limited to, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) components RelA (DiNatale *et al.*, 2010) and RelB (Vogel *et al.*, 2007b). More interesting, ligand binding to AHR promotes c-SRC release from AHR complex, which itself contributes to many signaling events as mediator of phosphorylation. For instance, an *in vitro* study demonstrated that exposure to B[*a*]P results in activation of the oncogenic protein epidermal growth factor receptor (EGFR) via c-SRC mediated phosphorylation (Vogeley *et al.*, 2022). Another study illustrated that UVB radiation leads to c-SRC translocation to the cell membrane through AHR-activation and consequently initiates EGFR internalization, thus pointing to AHR as mediator in UVB stress response (Fritsche *et al.*, 2007).

The involvement of the AHR in immune regulation mainly relies on non-classic functions. Indeed, AHR participates in Th17 differentiation by inhibiting STAT1 phosphorylation. Notably, this effect is reported in the absence of ligands (Kimura *et al.*, 2008). Consistent with this finding, multiple studies investigated the interaction between AHR and the JAK (janus kinase)/STAT pathway, which is known for its impact on the innate and adaptive immune system (Liu *et al.*, 2021) (Masuda *et al.*, 2011). In macrophages, the AHR builds a complex with STAT1 after lipopolysaccharide (LPS) stimulation. This complex interacts with the NF-κB subunit p50 to block the promoter activity of IL-6 and therefore the proinflammatory responses induced by LPS (Kimura *et al.*, 2009).

Furthermore, the non-classical pathway may result from AHR-binding to non-XRE sequences. For example, the AHR interacts physically with the NF-κB component RelB to form a complex that binds to a responsive element of the IL-8 promoter, thereby inducing its expression (Vogel *et al.*, 2007a).

1.3 AHR ligands

The AHR can be bound and activated by a wide range of ligands. These ligands vary greatly in origin, structure, and binding affinity (Denison & Nagy, 2003). However, AHR ligands can be typically described as hydrophobic, small (12.0 - 14.0 A° in length), and planar (Nguyen & Bradfield, 2008) (Waller & McKinney, 1995).

Moreover, AHR ligands include various phytochemicals, pharmaceuticals and synthetic flavonoids (Stejskalova *et al.*, 2011), which are weak inducers of AHR target genes compared to TCDD (Denison & Heath-Pagliuso, 1998).

1.3.1 Exogenous ligands

1.3.1.1 Halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs)

The first characterized AHR ligands are classified under the group of halogenated aromatic hydrocarbons (HAHs). This comprises dioxin-like compounds: polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and polychlorinated dibenzodioxins (PCDDs) (Busbee *et al.*, 2013), which show high persistency against XMEs (Q. Wang *et al.*, 2016) (Busbee *et al.*, 2013). Therefore, they are metabolically stable and bioaccumulative (Avilla *et al.*, 2020). HAHs can enter the environment as contaminants of consumer products or through waste incineration processes (Nguyen & Bradfield, 2008). TCDD belongs to the group of PCDDs and is the most investigated AHR ligand (Figure 2). Through a high binding affinity to the hAHR and impaired metabolism in humans, the half-life of TCDD can be up to 13 years (Matsumoto *et al.*, 2015) (Wolfe *et al.*, 1994). Exposure to TCDD can clinically be identified through induction of chloracne and results typically in hepatotoxicity, liver cancer, or immunosuppression (Schecter *et al.*, 2006).

In addition to HAHs, many compounds of the group of polycyclic aromatic hydrocarbons (PAHs) are AHR ligands, as for example 3-methylcholanthrene (3-MC) and 7,12-dimethylbenzanthracene (DMBA) (Figure 2) (Avilla *et al.*, 2020). PAHs occur in the nature through incomplete combustion of organic compounds and are contained in cigarette smoke (Guerrina *et al.*, 2018). PAHs are found in soil, air, and water, underlining the importance of PAHs as environmental pollutants (Sampaio *et al.*, 2021). They can also enter the food chain when food is processed with high temperatures, like grilling and frying (Sampaio *et al.*, 2021). Through AHR signaling, some PAHs can be metabolically activated to carcinogens, one prominent example is B[*a*]P (Figure 2) (Luch, 2005). Biotransformation of B[*a*]P leads to the formation of a diol epoxide derivate, which covalently binds to macromolecules and forms genotoxic DNA-adducts (Luch, 2005).

1.3.1.2 Synthetic flavonoid

5,6-Benzoflavone or β-naphthoflavone (BNF) is a non-carcinogenic synthetic flavonoid that belongs to the first compounds tested to induce AHR activity (Figure 2) (Boobis *et al.*, 1977) and is now acknowledged as a strong inducer of AHR target genes (Sinal *et al.*, 1999) (Fujii-Kuriyama & Kawajiri, 2010). Early studies on the anticarcinogenic effect of BNF were carried out even before the discovery of the AHR (Wattenberg & Leong, 1968). Moreover, a study on female rats showed that BNF exhibited antitumoral effects against mammary carcinomas by activating the AHR (Lubet *et al.*, 2011). It was also shown that BNF mediated a cell cycle arrest of estrogen receptor (ER)-positive breast cancer cells *in vitro* (C. Wang *et al.*, 2014).

Additionally, BNF reduces the oxidative stress caused by hydrogen peroxide significantly, indicating a strong potential as an antioxidant (Zhu *et al.*, 2017). The antioxidative activity of BNF was used in *in vivo* studies and it improved the survival rates of mice exposed to radiation therapy (X. Zhou *et al.*, 2020). Nevertheless, the clinical application of BNF is restricted by its low water-solubility. Hence, it was beneficial to improve BNF water solubility, for example through supramolecular complexation (Choi *et al.*, 2016). In contrast to BNF, 7,8-benzoflavone or α-naphthoflavone (ANF) has a poor affinity to AHR and is a very weak inducer of AHR activity (Figure 2) (Sinal *et al.*, 1999).

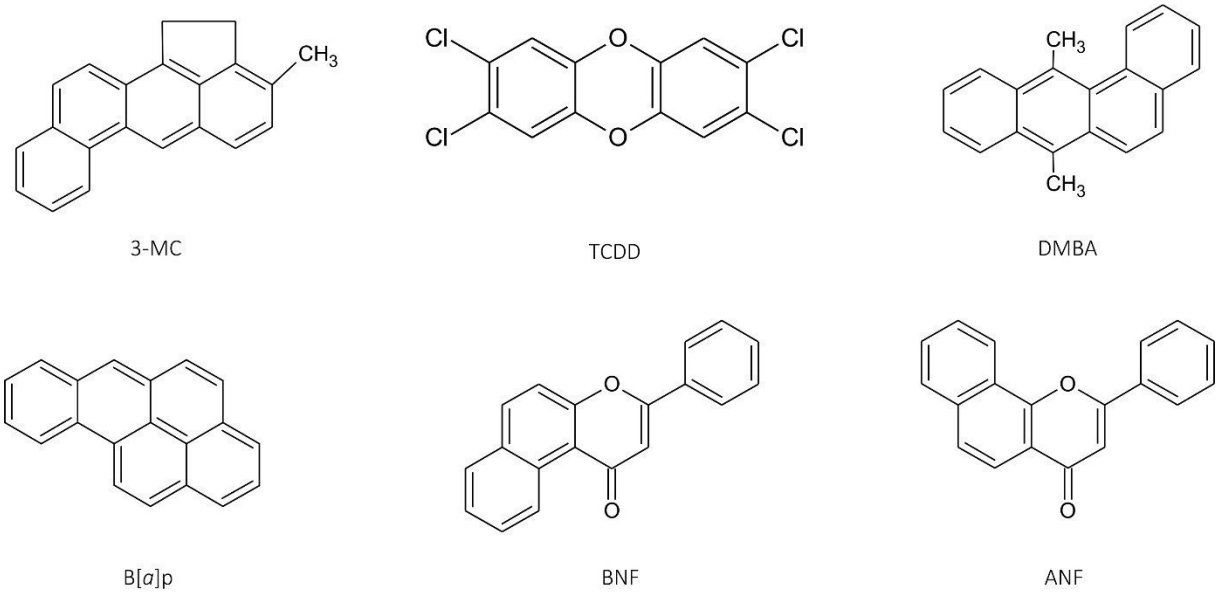


Figure 2: Chemical structures of well known exogenous ligands of the AHR: 3-methylcholanthrene (3-MC), 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD)**,** 7,12-dimethylbenzanthracene (DMBA), benzo[a]pyrene (B[*a*]P), β-naphthoflavone (BNF), α-naphthoflavone (ANF).

1.3.1.3 Naturally occurring compounds

The diet represents a great source for natural compounds that are able to activate the AHR (De Juan & Segura, 2021). These are weak AHR activators and mostly competitive inhibitors of AHR ligands. This means, they compete with other ligands to bind the AHR, thereby triggering a nuclear import but

inducing very low expression of AHR target genes (Busbee *et al.*, 2013). This category contains many famous compounds, like resveratrol (a polyphenol found in red wine) (Ciolino *et al.*, 1998), curcumin (found in *Curcuma longa*) (Garg *et al.*, 2008) and catechins (flavonols found in green tea extract) (Williams *et al.*, 2000). Another well-studied compound is indolo[3,2-b]carbazole (ICZ) (Figure 3), a metabolite of indole-3-carbinol (I3C) found in *Cruciferous* plants, such as broccoli (Bjeldanes *et al.*, 1991).

1.3.1.4 Nonligand activators or nonspecific ligands

Omeprazole (OM) is a proton pump inhibitor that blocks the gastric acid production (Figure 3) (Massoomi *et al.*, 1993). OM is prescribed to treat infection with *helicobacter pylori*, peptic ulcer and many other gastric acid disorders (Langtry & Wilde, 1998). Importantly, OM induces the nuclear import of AHR and subsequently induces the expression of *CYP1A1* (Daujat *et al.*, 1992). However, a physical interaction between AHR and OM is still not proven (Daujat *et al.*, 1992). Many hypotheses aimed to explain OM's effect on the AHR, proposing that a metabolite of OM is actually activating the receptor (Dzeletovic *et al.*, 1997) or that AHR phosphorylation leads to an indirect activation (Kikuchi *et al.*, 1998). Nevertheless, the precise mechanism for the AHR activation through OM remains unknown.

1.3.2 Endogenous ligands

Endogenous ligands are compounds synthesized in higher organisms (Denison & Nagy, 2003) and cover heme and arachidonic acid metabolites, e.g., bilirubin (Sinal & Bend, 1997) and lipoxin 4A (Schaldach *et al.*, 1999), respectively. The search for endogenous ligands revealed agonists with high importance, like indigoids and tryptophan (Trp) metabolites (Nguyen & Bradfield, 2008).

1.3.2.1 Indigoids

Indigo is a dark blue pigment that was used originally to dye jeans, while indirubin (IND) in this context is an undesirable isomer with red color. Historically, indigo was manufactured by fermentation of plants like *Isatis tinctoria* (Gillam *et al.*, 2000). Interestingly, old Chinese medicine used a plant-based powder with a dark blue color to treat leukemia. This powder is rich with indigo, but the major antileukemic effect is caused by indirubin (Figure 3) (Hoessel *et al.*, 1999).

Surprisingly, both indigo and indirubin are detected in the urine of healthy humans with a sufficient amount to activate the AHR (Adachi *et al.*, 2001). It is believed, that dietary Trp is degraded by intestinal bacteria to indole, absorbed, and transported to the liver, where it is oxidized to indoxyl and then excreted in the urine. Hydrolyzed by bacteria in the urine, the free indoxyl can undergo spontaneous oxidation and dimerization to form indigo or IND (Meijer *et al.*, 2006).

IND is more potent as an AHR ligand than indigo (Sugihara *et al.*, 2004) and was used in many studies for its strong potency of AHR activation (Haidar *et al.*, 2021). Notably, IND is a strong inducer of AHR-regulated enzymes like CYP1B1 and CYP1A1 and is at the same time a substrate of it. Hence, IND has only a transient effect on the AHR (Spink *et al.*, 2003).

IND was never used for any clinical application due to its limited water-solubility (Cheng & Merz, 2016). Thus, more effort is now made on its water-soluble derivates, like indirubin-3'-monoxime (Lo & Chang, 2013) (Springs & Rice, 2006) (Rebl *et al.*, 2022).

1.3.2.2 Tryptophan metabolites

Trp is an essential amino acid (Koper *et al.*, 2022). The majority of Trp can be metabolized through kynurenine (Kyn) or serotonin pathways, while small amounts take the indole pathway mediated by gut microbiota (Yang *et al.*, 2023).

In the Kyn pathway, the AHR-regulated enzymes indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO) metabolize Trp to N-formyl-Kyn, which is reduced to Kyn (Figure 3). Kyn can be further catabolized to kynurenic acid and xanthurenic acid (Badawy, 2017) (Murray & Perdew, 2020).

In particular, Trp itself is a weak activator of the AHR, but the metabolic product kynurenic acid exhibits great binding affinity and capacity to induce AHR activity within the measured physiological concentration (Murray & Perdew, 2020). Besides that, Kyn is produced in many cancer types in sufficient amount to activate the AHR (Z. Wang *et al.*, 2020). Hence, constitutive AHR activity can be sustained in malignant cells through IDO, TDO, and Kyn (Z. Wang *et al.*, 2020).

Moreover, another Trp metabolite is 6-formylindolo[3,2-b]carbazole (FICZ) (Figure 3), which is a potent AHR activator and strong inducer of *CYP1A1* (Rannug *et al.*, 1987) (Murray & Perdew, 2020). Primarily, FICZ was identified as a photo-oxidation product of Trp (Rannug *et al.*, 1987). By now, it has been shown that FICZ can be generated in an UV-independent way through enzymatic deamination and oxidative rearrangement of an indole derivate from microbial origin (Smirnova *et al.*, 2016).

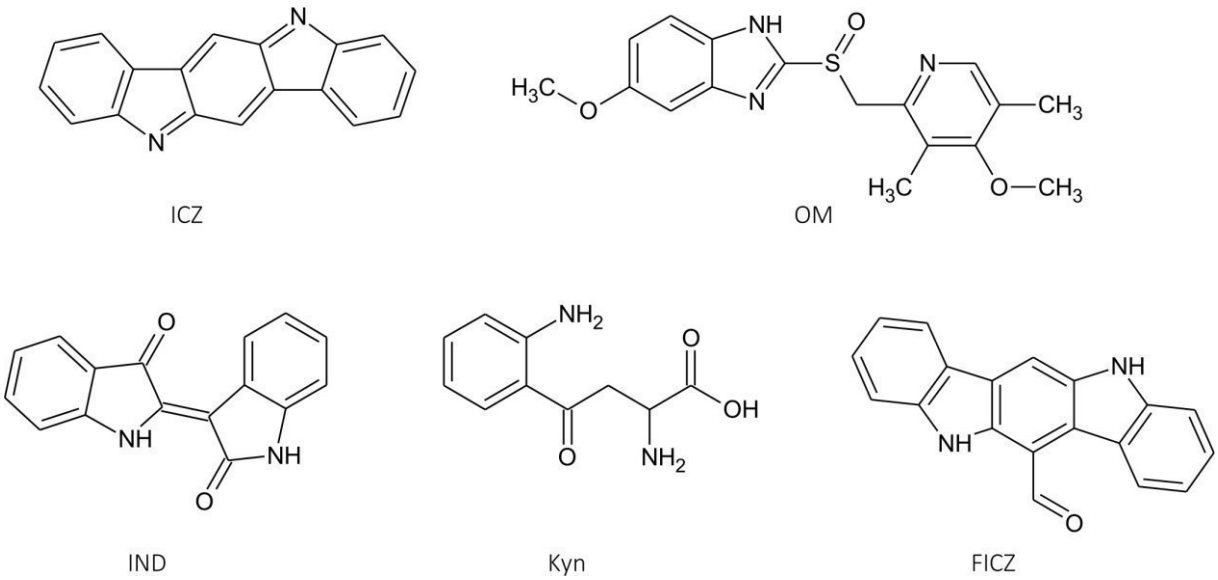


Figure 3: Chemical structures of most famous nonligand activators, naturally occuring compounds, and endogenous ligands of AHR: indolo[3,2-b]carbazole (ICZ)**,** omeprazole (OM), indirubin (IND), kynurenine (Kyn), 6-formylindolo[3,2-b]carbazole (FICZ).

1.4 Structure of the AHR

The AHR and its dimerization partner ARNT belong to the same family of transcriptional regulators: bHLH-PAS (basic helix-loop-helix, Per (period circadian protein)-ARNT-Sim (single-minded protein) (Kolonko & Greb-Markiewicz, 2019). Proteins of this superfamily are classified into two classes: Proteins of class I are activated in a specific manner in response to physiological or environmental stimuli, like AHR, while proteins of class II are expressed continuously and serve as dimerization partners, like ARNT (Kolonko & Greb-Markiewicz, 2019). In humans, the *AHR* gene consists of 11 exons and is located on chromosome 7p15 (Micka *et al.*, 1997). The *hAHR* gene encodes a 848 amino acid protein with a molecular weight of 96 kDa (Dolwick *et al.*, 1993). Notably, it has been concluded from studies on murine AHR that the amino acids (1-10) in hAHR are leader peptide, which are cleaved during translational processing (Burbach *et al.*, 1992).

1.4.1 Domain structure of the AHR

At the well-conserved N-terminus, the AHR contains the bHLH domain followed by the PAS domain (Jones, 2004). The basic (b) region possesses the DNA-binding motive and the helix (HLH) contributes to AHR protein interactions (Schulte *et al.*, 2017). Next to bHLH, two imperfect repeats separated by a low conserved linker, called PAS-A and PAS-B, form the PAS domain (Crews, 1998). Both PAS-A and -B mediate direct interactions with other proteins, but PAS-B holds the specific ligand binding domain (Soshilov & Denison, 2008). The C-terminus of the AHR is less conserved, which explains the difference in AHR length between human and mouse (Ramadoss & Perdew, 2005). At the C-terminus, the transactivation domain (TAD) mediates recruitment of coactivators to facilitate the AHR's

transcriptional activity (Kumar & Perdew, 1999) (Ramadoss & Perdew, 2005). TAD can be subdivided into three domains: starting by an acidic region (due to enrichment with glutamic and aspartic acid), followed by a glutamine-rich region (Q-rich), and a region rich in proline/serine/threonine (PST) (Kumar *et al.*, 2001).

On the other hand, ARNT shares the same domain structure like the AHR. However, the PAS B domain of ARNT is unable to bind ligands (Kolonko & Greb-Markiewicz, 2019).

The crystal structure of the N-terminal region of both AHR and ARNT bound to XRE was first disclosed a few years ago in two different laboratories (Seok *et al.*, 2017) (Schulte *et al.*, 2017). Owing to these results, the crucial amino acids in the AHR for ARNT dimerization and DNA-binding were identified. Recently, advances in cryo-electron microscopy helped to elucidate the AHR structure further. The reported structure describes the first step of AHR activation prior to nuclear import, while the AHR, as a part of the protein complex with HSP90 and XAP2, binds a ligand. The essential amino acids for the interaction with HSP90, XAP2, and ligand were identified in the mentioned study (Gruszczyk *et al.*, 2022).

1.4.2 Regulatory motives for the nucleo-cytoplasmic translocation of the AHR

As mentioned above, the AHR's transcriptional activity demands nuclear import as an essential step towards dimerization with the nuclear ARNT. The nucleo-cytoplasmic translocation of the AHR is regulated through a special sequence, referred as NLS and NES (Ikuta *et al.*, 1998). NES is typically a cluster of hydrophobic residues, mostly leucine (Wen *et al.*, 1995). Classical NLS motives are generally of two types: monopartite, consisting of a single cluster rich in basic residues (lysin and arginine), and bipartite NLS, with two clusters enriched with basic residues separated by a linker (Freitas & Cunha, 2009). The bipartite NLS ($_{13}$RKRR$_{16}$-$_{37}$KRH$_{39}$) of AHR along with the NES ($_{63}$K**L**DK**L**SV**L**R**L**S$_{73}$) are contained in the bHLH domain (Ikuta *et al.*, 1998).

Nevertheless, studies on the C-terminal domain of the human AHR demonstrated a significant effect of this domain on the protein trafficking, especially the residue valine 647 (Tkachenko *et al.*, 2016).

1.5 Physiological significance of the AHR

More than five decades of research left no doubts on the crucial role of the AHR in various physiological processes additionally to the xenobiotic metabolism, like lung health (Guerrina *et al.*, 2018), interactions with skin and gut microbiome (Dong & Perdew, 2020), skin homeostasis (Fernández-Gallego *et al.*, 2021), immune responses (Rothhammer & Quintana, 2019), nervous system (Juricek & Coumoul, 2018), and circadian rhythmicity (Anderson *et al.*, 2013).

1.5.1 Androgenic, estrogenic and anti-estrogenic AHR-mediated effects

As previously mentioned, the AHR serves as a E3 ubiquitin ligase. More specifically, the AHR recognizes specific signals on other proteins and transfer ubiquitin molecules to the specific substrate. Next, proteasomal degradation targets the substrates marked with ubiquitin molecules. Multiple studies reported estrogenic or androgenic effects of AHR ligands. This ligand specific effect has been reported for BNF and TCDD originally (Brauze *et al.*, 1997) (Lin *et al.*, 2002). Later research demonstrated that the AHR is a component of a ubiquitin ligase complex, namely $CUL4B^{AHR}$ (Sondermann *et al.*, 2023). In this complex, the ligand-activated AHR took the role of substrate-specific component targeting sex steroid receptors (e.g., androgen receptor, ER-α, and ER-β) towards their degradation (Ohtake *et al.*, 2007) (Ohtake *et al.*, 2003). This function as E3 ligase occurs in a ligand-dependent manner and is also tissue specific (Sondermann *et al.*, 2023).

1.5.2 Involvement in carcinogenesis

Clearly, AHR activation by some ligands like B[*a*]P can lead to generate mutagenic metabolites (Luch, 2005). Despite that, AHR's role in cancer progression is more complex than to be limited to xenobiotic metabolism.

The first hint on potential associations between the AHR and cancer arose from increased AHR expression in blood cancer, like adult T-cell leukemia (Hayashibara *et al.*, 2003), as well as solid tumors, like breast cancer (Baker *et al.*, 2020) [reviewed in (Paris *et al.*, 2021) and (Z. Wang *et al.*, 2020)]. According to Murray *et al.*, 2014 , the enhanced AHR expression correlates with activation of STAT6 and NF-κB, which is common in tumor microenvironment (Murray *et al.*, 2014). In addition to hyper-expression, a constitutively active AHR is detected in various cancer types, like in liver, prostate, or breast (Xue *et al.*, 2018).

Notably, it is not possible to describe a single and unique role of the AHR on tumor progression, since the relevant effects of AHR depend completely on cancer type, cellular context, and presence of ligands (Paris *et al.*, 2021). Many studies searched for the probable trigger for the constitutive activity of the AHR in cancer cells (Xue *et al.*, 2018). A well-researched example is the IDO/TDO-Kyn-AHR loop. Trp-derived AHR ligands, like Kyn, are produced in malignant cells in high amounts due to high Trp circulation by the enzymes IDO and TDO. This loop ends with the generation of immune-tolerant dendritic cells as well as regulatory T cells that promote a tumor immunological microenvironment. This tumor environment lacks the ability to recognize and suppress tumor cells by immune cells (Cheong & Sun, 2018) (Campesato *et al.*, 2020).

Further research on Kyn exhibited its ability to drive an AHR-dependent breast cancer migration (Novikov *et al.*, 2016). In another study, it promotes epithelial-to-mesenchymal transition (EMT), a key step in metastasis, in lung cancer cells (Duan *et al.*, 2018). In accordance, Kyn stimulates specific AHR-

driven EMT in thyroid carcinoma cells (Moretti *et al.*, 2020). Additionally, the Trp photoproduct, FICZ, advances an inflammation-induced melanoma cell dedifferentiation in a mouse model (Mengoni *et al.*, 2020). Complementary, in a study on oral squamous cell carcinoma in mice, application of AHR inhibitors blocked the rapid migration of cancer cells (Stanford *et al.*, 2016).

All above-mentioned facts classify the AHR rather as oncogene. However, recent studies showed tumor suppressor properties of the AHR as well (Elson & Kolluri, 2023). In fact, it has been shown that AHR enhances the expression of some microRNAs that act as tumor suppressors in many cancer types, like in prostate cancer cells (Yu *et al.*, 2018) or breast cancer cells (Mobini *et al.*, 2019). Moreover, the AHR interacts with the tumor suppressor p53 to promote antiproliferative effects and protect against carcinogenesis (Phillips *et al.*, 2022).

1.5.3 The AHR as pharmaceutical target

Due to the oncogenic and tumor suppressor properties, the AHR has promising potential as a drug target (Kolluri *et al.*, 2017). First of all, antagonism of the AHR activity is a logic strategy to deal with carcinogenesis related to AHR hyperactivity. However, the search for applicable AHR antagonists is still ongoing. A classic and early-described AHR antagonist is ANF (Merchant *et al.*, 1993). However, the weak agonistic effect reported when applied in high concentrations limited its use (Santostefano *et al.*, 1993). The search for AHR antagonists identified many novel compounds. As for example, CH-223191 that blocks the AHR activity driven by TCDD and other HAHs, but fails to antagonize other AHR ligands, like IND and BNF (Zhao *et al.*, 2010). Another compound is CB7993113, which was first identified in an *in silico* study. CB7993113 reduces invasion of breast cancer cells in 3D-cultures, inhibits the AHR-triggered activity by TCDD and PAHs, and blocks tumor cell migration in 2D-cell cultures (Parks *et al.*, 2014). A recent investigation in targeting the AHR was carried out with the inhibitor BAY2416964. The inhibitory effect of BAY2416964 relies on antagonizing the immunosuppressive effects of the ligand-activated AHR (Dumbrava *et al.*, 2023).

Moreover, the AHR hyperactivity observed in various cancer types can be targeted through blocking the IDO/TDO-Kyn-AHR loop, for instance by minimizing the production of the ligand Kyn. Recently, a study examined a selective synthetic antagonist of the AHR, namely Kyn-101, that led to delayed tumor progression through AHR inhibition (Campesato *et al.*, 2020).

Furthermore, targeting the AHR can be achieved through ligand driven activity. Reasonably, most research focuses on the AHR-agonistic activity of molecules already approved by the US Food and Drug Administration (FDA). Of note, many clinically approved drugs are ligands of the AHR, for instance, Raloxifene, Sulindac, and Leflunomide (Baker *et al.*, 2020). The latter is prescribed to treat rheumatoid arthritis (O'Donnell *et al.*, 2010), but an AHR-related therapeutic value of Leflunomide in melanoma treatment was also demonstrated (Hanson *et al.*, 2018). On the other hand, the AHR endogenous

ligands are still interesting for current research due to their high potency and their transient activation e.g., ITE (2-(1' *H*-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester) (Piwarski *et al.*, 2020) and FICZ (Arabnezhad *et al.*, 2020).

Interestingly, in the year 2022 the first and only FDA-approval of an AHR-agonist based drug, namely Tapinarof, was announced (Keam, 2022) (Smith *et al.*, 2017). A cream containing 1% of the active substance Tapinarof can now be employed to treat plaque psoriasis, an immune-mediated skin disease (Keam, 2022).

1.6 AHR subcellular localization and nucleocytoplasmic transport

The AHR, as a member of the bHLH-PAS transcriptional regulators, is identified as a cytoplasmic sensor for intracellular and extracellular signals, while its activation demands a nuclear import (Greb-Markiewicz & Kolonko, 2019).

1.6.1 The precise mechanism of importin-mediated protein transfer through the nuclear envelope

In a general sense, TFs stay in the cytoplasm, before stimuli initiate their activation. Towards effective function, TFs should bind to specific target DNA after entering the nuclear envelope and accumulating in the nucleus (Komeili & O'Shea, 2000). The mentioned nuclear import is regulated via a highly selective nuclear pore complex (NPC). Notably, nucleo-cytoplasmic transport is the main function of NPC, and thereby they contribute to many cellular processes, like cell cycle progression and chromatin organization (Strambio-De-Castillia *et al.*, 2010).

In the NPC, small molecules take a central viscous channel as their exclusive route. Meanwhile large macromolecules take periphery transport pathways (J. Ma *et al.*, 2012). Solutes and small molecules (e.g., ATP) can traverse the NPC through passive diffusion, with the molecular mass being the limiting factor for this process (Keminer & Peters, 1999) (Timney *et al.*, 2016). By contrast, transport receptors and the related cargo complexes use the facilitated diffusion to ensure a rapid diffusion, which is generally, but not necessarily, energy dependent (Quimby & Dasso, 2003) (Çağatay & Chook, 2018). In this context, the molecular mass of proteins contributes to affect their cellular distribution (Wühr *et al.*, 2015). Even though it is agreed in some reviews that only proteins under 45 kDa can undergo passive diffusion (Kosyna & Depping, 2018) (Jans *et al.*, 2019), many *in vitro* studies demonstrated that larger proteins undertake passive diffusion as well (R. Wang & Brattain, 2007) (Popken *et al.*, 2015).

In brief, proteins using the facilitated diffusion should carry an NLS sequence. This sequence can be recognized by nuclear transporter, typically importin β1 (IMPβ1). As some proteins cannot be bound by IMPβ1, an adaptor protein called IMPα mediates the binding between cargo protein and IMPβ1. After cargo recognition, either the complex IMPβ1/cargo or the complex IMPβ1/IMPα/cargo translocates to the nucleus through an interaction between IMPβ1 and the main units of NPC,

nucleoporins (Figure 4). On the nuclear side of NPC, IMPβ1 binds to guanosine triphosphate-bound Ran (RanGTP) releasing the cargo protein and IMPα. Next, the IMPβ1/RanGTP returns to the cytoplasmic side of NPC, where RanGTP is hydrolyzed, with help from the GTPase, RanGAP, to RanGDP, releasing IMPβ1 (Kosyna & Depping, 2018) (van der Watt *et al.*, 2016) (Jans *et al.*, 2019). Very similar, exportins bind to their cargos through the NES sequence and subsequently associate with RanGTP (Figure 5). Following the export, RanGTP is hydrolyzed to RanGDP and the exportin/cargo protein complex dissociates. At the end, RanGDP is imported into the nucleus where the GDP on Ran (RanGDP) is switched to GTP through RanGEF (Ran guanine exchange factor) (Kosyna & Depping, 2018).

Importantly, the human genome encodes 7 isoforms of IMPα, namely IMPα1 to IMPα7, and different isoforms bind to distinguishable NLSs. In addition, more than 20 isoforms of IMPβ are encoded in humans. This includes IMPβ1, transportin 1 (TNPO1), and CRM1 (Kosyna & Depping, 2018).

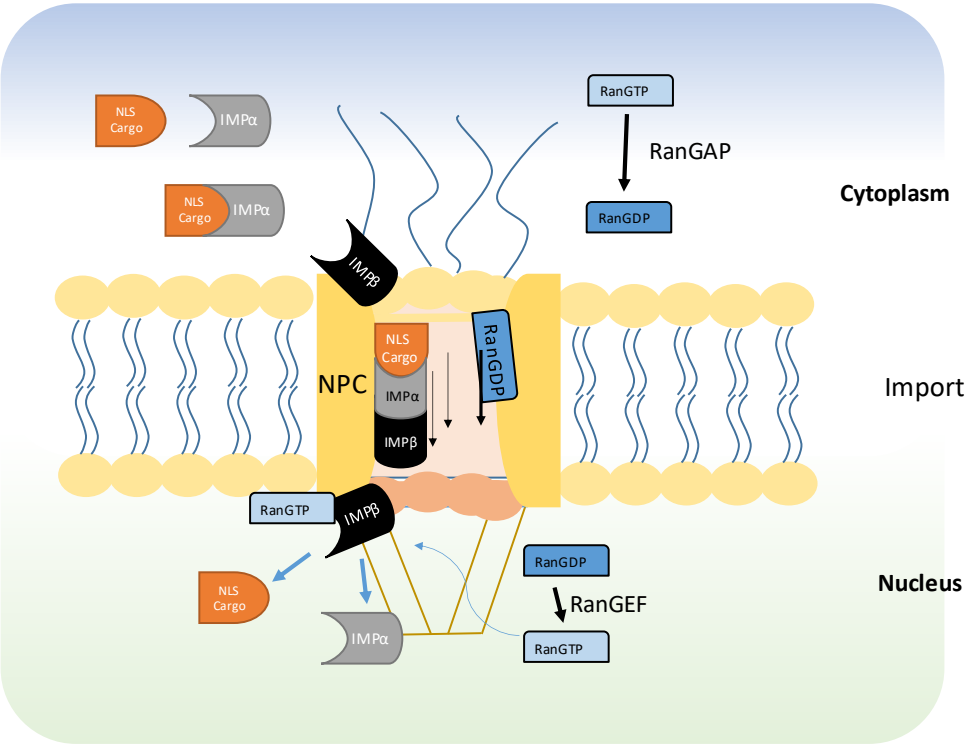


Figure 4: Model of the import pathway of NLS-carrying cargos. NLS is recognized in the cytoplasm by IMPβ1 or its adaptor IMPα. Next, the complex IMPα/IMPβ1/cargo translocates to the nucleus through an interaction between IMPβ1 and NPC. On the nuclear side, IMPβ1 binds to RanGTP, thereby releasing the cargo protein and IMPα. Subsequently, the IMPβ1/RanGTP returns to the cytoplasmic side of NPC, where RanGTP is hydrolyzed through RanGAP to RanGDP releasing IMPβ1. RanGDP returns to the nucleus and gets converted into RanGTP with help from RanGEF.

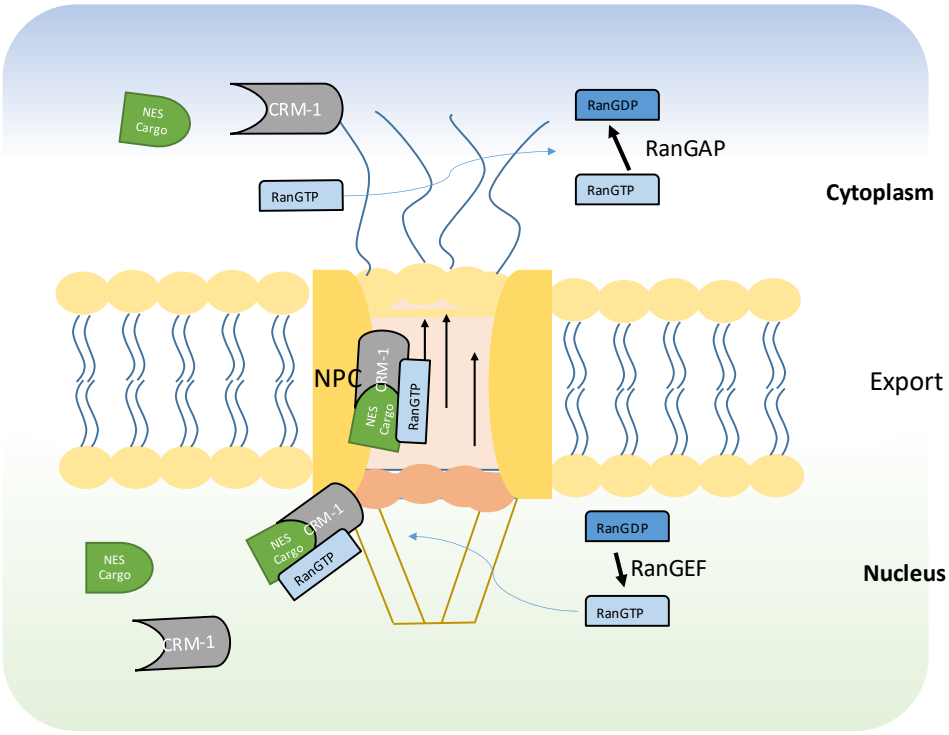


Figure 5: Model of the export pathway of NES-carrying cargos. Exportin or CRM-1 binds to NES sequence and thereby build an association with RanGTP in the nucleus. This complex goes through the NPC to the cytoplasm. In the cytoplasm, RanGTP is hydrolyzed to RanGDP and the exportin/cargo protein dissociates and is released. RanGDP returns to the nucleus and is converted into RanGTP with help from RanGEF.

1.6.2 Inhibitors of nucleo-cytoplasmic transport

Due to the clear association between nucleo-cytoplasmic transport of many TFs and significant cellular processes (e.g., cell differentiation), manipulation of nucleo-cytoplasmic transport is of great research interest. Notably, the existence of endogenous compounds inhibiting nucleo-cytoplasmic transport was also investigated. As for example, the anti-inflammatory and anti-viral prostaglandin has been shown to block the export of CRM1 substrates by binding CRM1 directly (Hilliard *et al.*, 2010).

Furthermore, the search for inhibitors of the cytoplasmic export revealed leptomycin B (LMB), which was originally discovered as an antifungal in the 1980s (Hamamoto *et al.*, 1983). Research on LMB demonstrated that it is a highly efficient and specific inhibitor of CRM1 by targeting only one residue (Kudo *et al.*, 1999). LMB was widely used in *in vitro* studies leading to export block and nuclear accumulation of many proteins, including the AHR (Tkachenko *et al.*, 2016) (Haidar *et al.*, 2021) and viral proteins like HIV-1 (Human Immunodeficiency Virus-1) (B. Wolff *et al.*, 1997).

In contrast, research on import inhibitors reached its highest point in the last decade (Jans *et al.*, 2019). Until today, many import inhibitors were identified and can be applied to study the nuclear import of TFs, like the AHR. Ivermectin (IVM) was described in the 1980s as an antiparasitic agent (Campbell *et al.*, 1983) but later, it turned out to be one of the most effective inhibitors of IMPα/β1 mediated

translocation. In fact, a study on IVM showed that it selectively targets the nuclear import mediated by the adaptor IMPα, while IMPβ1 recognized cargoes are still unaffected (Wagstaff *et al.*, 2011) (Wagstaff *et al.*, 2012). In another study, IVM reduces the nuclear accumulation and the related transcriptional activity of HIF1α (Kosyna *et al.*, 2015), which belongs to the same family of proteins as the AHR. In addition, IVM inhibits the replication of many viruses, like dengue virus (Tay *et al.*, 2013) and influenza (Götz *et al.*, 2016). Due to its antiviral properties against SARS-CoV-2 in cell culture (Caly *et al.*, 2020), IVM was suggested to reduce the severe progression of COVID-19. However, clinical trials showed no beneficial effect of IVM (Popp *et al.*, 2022).

Another promising compound in inhibiting the nuclear import is importazole (IPZ). IPZ interferes with IMPβ1 and RanGTP binding (Soderholm *et al.*, 2011), showing thereby anti-tumor capabilities in malignant breast cancer cells *in vitro* (Kuusisto & Jans, 2015).

1.6.3 Nuclear and cytoplasmic AHR

The AHR is generally identified as a cytoplasmic protein and the nuclear import is only associated with ligand binding. In this theory, ligand binding to the AHR triggers a conformational change, which unmasks the bipartite NLS. Thereafter, nuclear transporters target the exposed NLS and import AHR into the nucleus. In this context, the nucleo-cytoplasmic shuttling of the AHR was primarily described in the year 2000 (Ikuta *et al.*, 2000), two years after characterizing its NLS and NES (Ikuta *et al.*, 1998). Another study from the same group reported a relation between cell density and the intracellular distribution of AHR (Ikuta *et al.*, 2004a). Notably, nucleo-cytoplasmic shuttling is not unique for the AHR and was observed in many TFs, like GATA (Cai *et al.*, 2014) and STAT (T. Meyer & Vinkemeier, 2004).

Although the nuclear import of the AHR was initially linked to ligand binding (Ikuta *et al.*, 2000), an independent study elucidated that the AHR shuttles between the nucleus and the cytosol in the absence of exogenous ligands (Richter *et al.*, 2001). More recently, it was demonstrated that ligand binding increases the nuclear import rates of the AHR, while shuttling itself happens continuously and does not lead to any receptor activation (Tkachenko *et al.*, 2016). In the same study, co-treatment with the export inhibitor LMB and ligand resulted in a stronger AHR nuclear accumulation compared to ligand alone, thus indicating a parallel re-export for ligand-free AHR (Tkachenko *et al.*, 2016).

Notably, the AHR localization is used as a biomarker in advanced cancer research to determine tumor grade and prognosis level for patients (Z. Wang *et al.*, 2020) (Paris *et al.*, 2021). In ovarian cancer, low cytoplasmic AHR was linked to improved survival. In the same context, expression levels of the cytoplasmic or nuclear AHR correlates with tumor size (Deuster *et al.*, 2019). Meanwhile, the AHR nuclear translocation is associated with poor survival in patients with squamous cell carcinoma (Su *et al.*, 2013). In addition, the nucleo-cytoplasmic immunoreactive score of the AHR was measured in

around 300 breast cancer samples. These scores showed an association between nuclear AHR and poor outcome (Jeschke *et al.*, 2019). Remarkably, both nuclear and cytoplasmic AHR were detected in 80% of ERα-positive or ERα-negative breast tumors, which gives a hint to a nucleo-cytoplasmic shuttling to be existing *in vivo* (Vacher *et al.*, 2018).

2 Aim of the thesis

The AHR is a master regulator in the human body gaining a lot of attention from researchers. First of all, several studies considered identifying its endogenous agonists and described their physiological relevance. Beyond this, more advanced research focused on its involvement in different cellular processes along with the pathological relevance. Moreover, the AHR localization is used in cancer studies in the assessment of tumor grade and prognosis among patients. Taken together, the therapeutical potential and the need for targeting AHR in living cells increased dramatically.

Despite all of that, studies trying to address the localization of AHR are very limited and many inquiries on the basal import are yet to be addressed. The proposition that the AHR is a cytoplasmic receptor or remains in the cytoplasm prior to ligand-binding is considered a fact in many publications, even though evidence is mounting that contradicts this simplistic picture.

In this thesis, the localization of the AHR in living cells was thoroughly examined. Hence, a visualization system was established to track overexpressed EYFP-AHR in live modus *in vitro* and examine its intracellular movement. Furthermore, a validation of this system was carried out to confirm the intracellular distribution to be similar to endogenous AHR.

In the first part of the current work, I addressed the factors regulating AHR accumulation in the nucleus. More exactly, ARNT-dimerization and XRE-binding, independent of their significance on the functional output, were analyzed on their impact to stabilize the receptor in the nucleus after ligand-binding or in the basal state. Thereby, I addressed the characteristics of the nucleo-cytoplasmic translocation in absence of the ability to form the AHR/ARNT-XRE complex.

In the second part of the present thesis, I analyzed the nuclear import of the AHR in detail in order to characterize and distinguish between the basal and ligand-induced import. For this reason, various AHR variants with semi or full impaired translocation-functions were generated. This was used to study the nuclear import of the AHR in absence or presence of ligands. Additionally, import inhibitors were applied and tested on the AHR. Through that, I aimed to clarify the involved nuclear transporters in the nuclear import of the AHR. Especially since these import inhibitors target different stages and work on different molecular components of the import pathway.

Taken together, this work aimed to help clarifying the AHR's internal residues that are specifically involved in basal and ligand-induced nuclear import and the cellular components contributing in the nucleo-cytoplasmic translocation as well as the nuclear accumulation of AHR in living cells.

3 Results

3.1 Paper 1

The role of DNA-binding and ARNT dimerization on the nucleo-cytoplasmic translocation of the aryl hydrocarbon receptor

Rashad Haidar, Frank Henkler, Josephine Kugler, Aline Rosin, Doris Genkinger, Peter Laux & Andreas Luch

This chapter was published on 14 September 2021:

Scientific Reports, volume 11, Article number: 18194 (2021)

DOI: 10.1038/s41598-021-97507-w.

Link: https://doi.org/10.1038/s41598-021-97507-w

Author contributions within this chapter:

Project planning (80%)

Experimental work (90%)

Data analysis (100%)

Writing of the manuscript (80%).

Supplementary materials for the following publication are detailed in Annex I.

scientific reports

Check for updates

OPEN

The role of DNA-binding and ARNT dimerization on the nucleo-cytoplasmic translocation of the aryl hydrocarbon receptor

Rashad Haidar[1,2]✉, Frank Henkler[1], Josephine Kugler[1], Aline Rosin[1], Doris Genkinger[1], Peter Laux[1] & Andreas Luch[1,2]

The human aryl hydrocarbon receptor (AHR) is predominantly located in the cytoplasm, while activation depends on its nuclear translocation. Binding to endogenous or xenobiotic ligands terminates the basal nucleo-cytoplasmic shuttling and stabilizes an exclusive nuclear population. The precise mechanisms that facilitate such stable nuclear accumulation remain to be clarified as essential step in the activation cascade. In this study, we have tested whether the sustained nuclear compartmentalization of ligand-bound or basal AHR might further require heterodimerization with the AHR-nuclear translocator (ARNT) and binding to the cognate XRE-motif. Mutagenesis of the DNA-binding motif or of selected individual residues in the ARNT-binding motif did not lead to any variation in AHR's nucleo-cytoplasmic distribution. In response to ligands, all mutants were retained in the nucleus demonstrating that the stable compartmentalization of activated AHR in the nucleus is neither dependent on interactions with DNA, nor ARNT. Knocking down the ARNT gene using small interfering RNA confirmed that ARNT does not play any role in the intracellular trafficking of AHR.

The aryl hydrocarbon receptor (AHR) is a ligand induced transcription factor that activates oxidative phase I metabolism by the induction of cytochrome P450-dependent monooxygenases (*CYP*), especially *CYP1A1* and *1B1*[1]. Early-identified AHR ligands included environmental toxicants, as for example 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD)[2] and benzo[*a*]pyrene (B[*a*]p)[3], as well as β-naphthoflavone (BNF)[4] and other xenobiotic compounds, thereby pointing to the role of the AHR in xenobiotic substance metabolism. However, more current studies demonstrate the AHR to be more than a simple sensor, indicating further functional interactions with other signalling pathways[5,6]. The AHR plays also a major role in organ development[7,8], regulation of adaptive immunity[9] and the maintenance of lung health[10]. Furthermore, several endogenous ligands have been identified consistent with various physiological functions, such as skin differentiation[11] or intestinal homeostasis[12,13]. Endogenous AHR agonists include tryptophan metabolites, such as kynurenine (KYN), and indole derivatives, such as indirubin (IND)[14]. The latter is confirmed to be present in the urine of healthy people with levels sufficient to activate the AHR[15]. Recent research has shown that bacterial products from *Pseudomonas aeruginosa* can bind and activate the AHR, which indicates that the AHR collaborates in antibacterial responses[16].

Activation of the AHR is linked to increased oxidative metabolism and consequently the formation of, e.g., reactive oxygen species. In fact, prolonged activation of the AHR can enhance carcinogenic effects[17]. Transcriptional activation of the AHR is therefore a tightly regulated process that depends on the interactions with several co-factors and the heterodimerization with the AHR-nuclear translocator (ARNT). ARNT is a nuclear protein that acts as dimerization partner for several transcription factors including hypoxia-inducible factors (HIF)[18], single-minded proteins (SIM)[19] or the estrogen receptor (ER)[20]. Studies on the anti-estrogenic effects of AHR-activation confirmed the competition between AHR and ER to interact with ARNT[21].

The highly conserved bHLH domain within the N-terminal domain contains a bipartite nuclear localisation signal (NLS), adjacent to a nuclear export signal (NES). These highly conserved motifs regulate the intracellular

[1]Department of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany. [2]Department of Biology, Chemistry and Pharmacy, Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany. ✉email: Rashad.Haidar@bfr.bund.de

trafficking of the AHR. Notably, the DNA-binding motif of the AHR overlaps with the NLS, more accurately with the amino acids Histidine 39 (H39) and Arginine 40 (R40) in the second NLS[22–24].

Activation of the AHR is strongly linked to intracellular transport. Prior to binding to inducing ligands, the AHR is mainly located in the cytoplasm[25] as a part of a chaperon complex consisting of HSP90, XAP2 and p23[26]. The HSP90 masks the NLS to prevent nuclear import[26]. The current model of the AHR signalling pathway describes ligand-binding as key event that triggers a conformational change[27], which unmasks the NLS and exposes it to importin-α. The latter binds to importin-β to pass the nuclear protein import[28]. However, continuous shuttling of the AHR between cytoplasm and nucleus is confirmed in the absence of ligands[23,29]. In the context of shuttling, basal import is efficiently compensated by NES-dependent export that maintains a predominantly cytoplasmic compartmentalization. Notably, this shuttling does not lead to any activation[29] and its import related-mechanism, with regard to the NLS, is still not well understood.

During activation, AHR is released from its chaperon complex in the nucleus and forms a heterodimer with ARNT[30]. The AHR-ARNT dimer binds to a cognate sequence in the promoter region of AHR target genes, the xenobiotic response elements (XRE)[31]. This binding is accelerated by numerous coactivators that associate with the AHR-ARNT dimer to enhance the promoter accessibility[32]. In order to initiate transcription, receptor-ligand complexes need to engage with several co-factors and DNA, involving both the N- and C-terminal domains. Importantly, this needs to occur within the limited time interval of nuclear transit, since the export of the AHR from the nucleus continues in the presence of ligands[29]. The nuclear mechanisms involved in terminating export and subsequently stabilizing the nuclear population are important steps towards activation. However, these steps are not yet fully understood.

In principle, associations with ARNT as well as binding to the DNA and other nuclear factors might anchor the AHR in the nucleus, thus facilitating further steps towards transcription. However, this theory has not yet been tested. As alternative option, ligand-binding might inactivate export into the cytoplasm, possibly due to conformational changes that masks the NES. This could terminate endogenous shuttling, leading to a constitutive nuclear population to engage in various functional interactions. The NES is also postulated to terminate transcription by re-exporting the receptor into the cytoplasm[29], where it can be degraded by the proteasome[33].

In this study, we have investigated whether the ligand-induced nuclear accumulation of the AHR depends on its heterodimerization with ARNT and its DNA-binding domain that associates with the cognate XRE motif. Our data indicate the nuclear compartmentalization is not affected or stabilized by consecutive association with either DNA or ARNT. Instead, the alternative concept of an inhibited NES-dependent export during receptor activation is supported.

Results

Using the AHR crystal structure to generate AHR-mutants that are deficient for DNA-binding and/or dimerization with ARNT. To design AHR mutants that are incapable of interacting with ARNT or the XRE, we took reference to the AHR crystal structure model as defined by[34]. The quoted crystal structure refers to an AHR-ARNT complex, containing only the bHLH and PAS-A domains of both proteins, bound to a 12mer double-stranded DNA. Crucial residues that are involved in both interactions with ARNT or the XRE have been identified or confirmed in agreement with previous functional studies[35,36].

According to the crystal structure, the interaction of AHR with the XRE motif relies on three amino acids: Ser36 (S36), His39 (H39) and Arg40 (R40). While S36 and R40 form hydrogen bonds with cytosine and guanine on the DNA strand, H39 builds a contact to the phosphate backbone of the thymine base[34]. For an entire characterization profile of the DNA-binding motif, we examined different AHR-mutants covering the whole DNA-binding motif as well as the individually mutated amino acids (Fig. 1b).

The dimerization of AHR and ARNT includes three interaction interfaces in the bHLH as well as the PAS-A domain. In accordance with this information the amino acids L50, A79, F82, L118, A121, L122 have been identified as crucial for the formation of a stable AHR-ARNT heterodimer[34,36]. For a precise description of the role of the AHR-ARNT heterodimer, we generated an AHR mutant in which all amino acids described above were mutated (Fig. 1c).

In order to verify the generated AHR mutants, we analyzed their transcriptional activity as functional output in MCF-7$^{\Delta AHR}$ cells. *CYP1A1* mRNA levels were determined after induction of the AHR or the AHR mutants with IND, respectively (Fig. 2a–c). A mutation in the DNA-binding motif leads to a loss of transcriptional activity of the AHR. While AHR^{H39G} and AHR^{R40D} inhibit *CYP1A1* induction completely, AHR^{S36G} still shows a minor induction (Fig. 2b). Further analysis examined the XRE driven luciferase activity after treating MCF-7$^{\Delta AHR}$ cells with an increased concentration of TCDD or IND. In contrast to wild-type AHR, which activates luciferase in a concentration-dependent manner, the AHR DNA-binding deficient mutants do not elicit any reaction to increased concentrations of the ligands (Fig. 2d).

The transcriptional activity of ARNT-binding deficient mutants confirms that mutations in the bHLH domain (L50D.A79D.F82D) are sufficient to inhibit the induction of *CYP1A1* (Fig. 2c). Nevertheless, the induction of *CYP1A1* is not inhibited completely through the mutation in the PAS-A domain (L118E.A121D.L122E) (Fig. 2c). Based on these results, we decided to solely consider the mutation L50D.A79D.F82D.L118E.A121D.L122E (AHR$^{\Delta ARNT}$), which covers the ARNT-binding motif in both domains. Furthermore, a loss of the ARNT-dimerization ability of AHR$^{\Delta ARNT}$ has been validated with co-immunoprecipitation (Co-IP) (Supplementary Fig. 1).

The DNA-binding motif alters the nucleo-cytoplasmic distribution of AHR through the overlap with the second NLS. To investigate the cellular compartmentalization of the AHR in living HepG2 cells, human AHR was expressed as a fusion protein, linked to the enhanced yellow fluorescent protein (EYFP) as

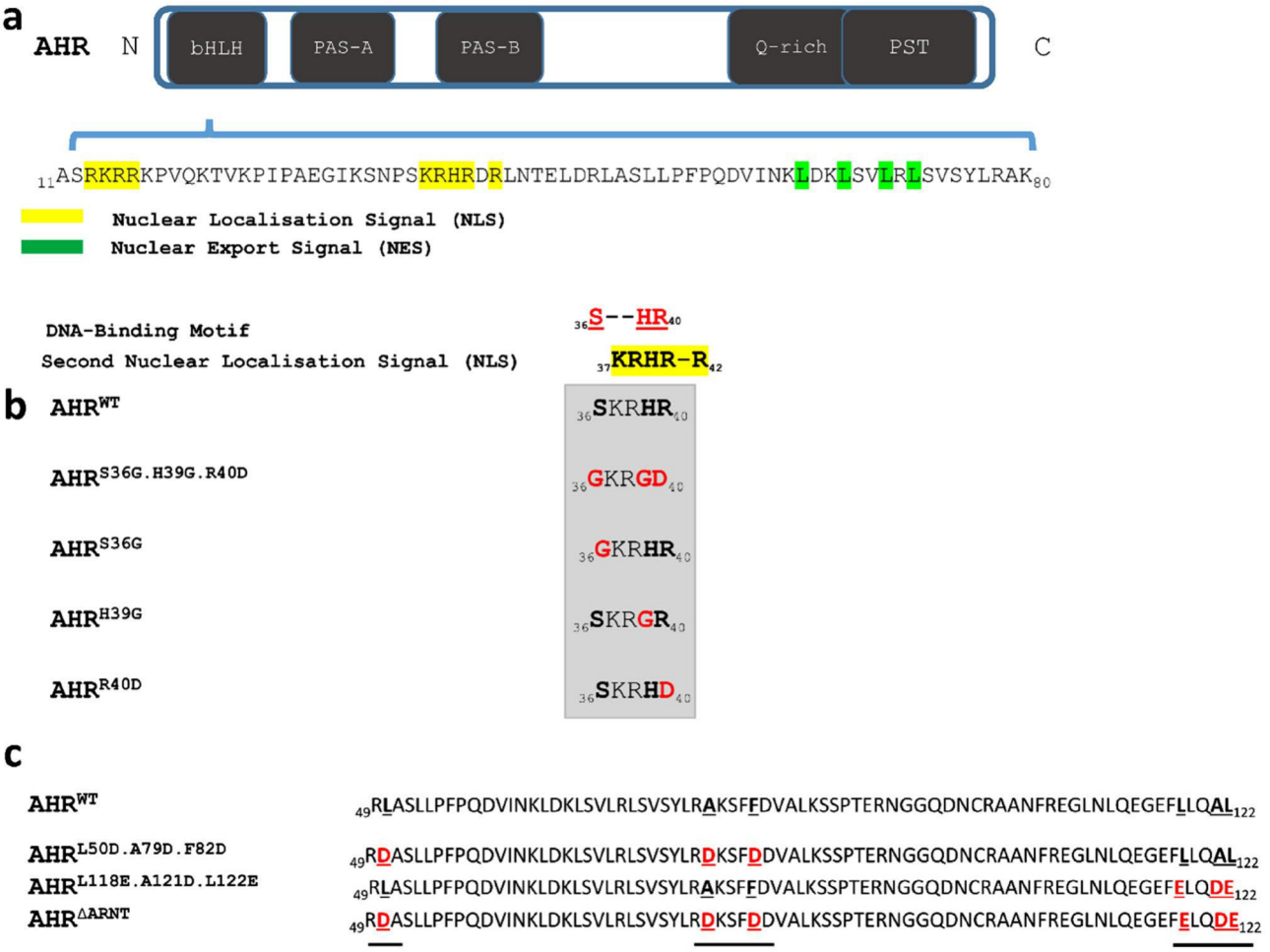


Figure 1. Design of AHR mutants to study DNA-binding and ARNT dimerization. (**a**) Domain architecture of the AHR. The bipartite nuclear localization signal (NLS) and a nuclear export signal (NES) are indicated as amino acid sequence. Graphical representation of mutated amino acids in the DNA-binding motif (**b**) and ARNT-binding domain (**c**) of the AHR.

described previously (Tkachenko et al., 2016). The EYFP-tagged AHR or AHR mutants allowed us to perform online fluorescence imaging in living cells.

The DNA-binding motif includes two positive charged amino acids H39 and R40 that are considered to be a part of the 2nd NLS (Fig. 1a). Changing the weakly alkaline H39 to the neutral glycine (AHR^{H39G}) did lead to a compartmentalization similar to the wild-type receptor (Fig. 3a). In contrast to AHR^{H39G}, the distribution of the AHR^{R40D} mutant was shifted to an exclusively cytoplasmic distribution (Fig. 3a), suggesting that the basal nuclear import was abolished due to inactivation of the second NLS motif. From a technical perspective, AHR^{H39G} was confirmed as AHR mutant that is selectively deficient for DNA-binding, but contains a fully functional NLS.

On the other hand, AHR^{R40G} allows to address specifically the impact of the 2nd NLS on nuclear import. Interestingly, in response to the endogenous ligand (IND), nuclear accumulation was not delayed, but even accelerated when compared to wild-type AHR (Fig. 3b–d). Consistently, the xenobiotic ligand (BNF) did induce similar import profiles for AHR^{R40D} and wild-type receptor (Fig. 3b,c,h). These data suggest that the 2nd NLS has no apparent function in the ligand induced nuclear import.

To verify this hypothesis, we compared the basal import of AHR^{R40D} with the wild-type after blocking the protein export with leptomycin B (LMB). The subcellular distribution of AHR was observed until 30 min after treatment. Our results reveal that both the nuclear accumulation and the slope of nuclear transition of AHR^{R40D} is significantly reduced when compared with the wild-type (Fig. 3e–h), thus confirming a function of the 2nd NLS in the basal, but not in a ligand-induced nuclear import.

Activation related nuclear retention of the AHR neither requires dimerization with ARNT nor binding to XRE. AHR heterodimerization with ARNT is the key to form a stable complex with the XRE motif. As ARNT is a nuclear protein that mediates AHR activation, we decided to investigate the requirement of the ARNT-AHR heterodimer for AHR nucleo-cytoplasmic translocation. The AHR mutant AHR^{H39G}, containing a mutation within the DNA-binding domain, failed to activate *CYP1A1* expression (Fig. 2b). We used the

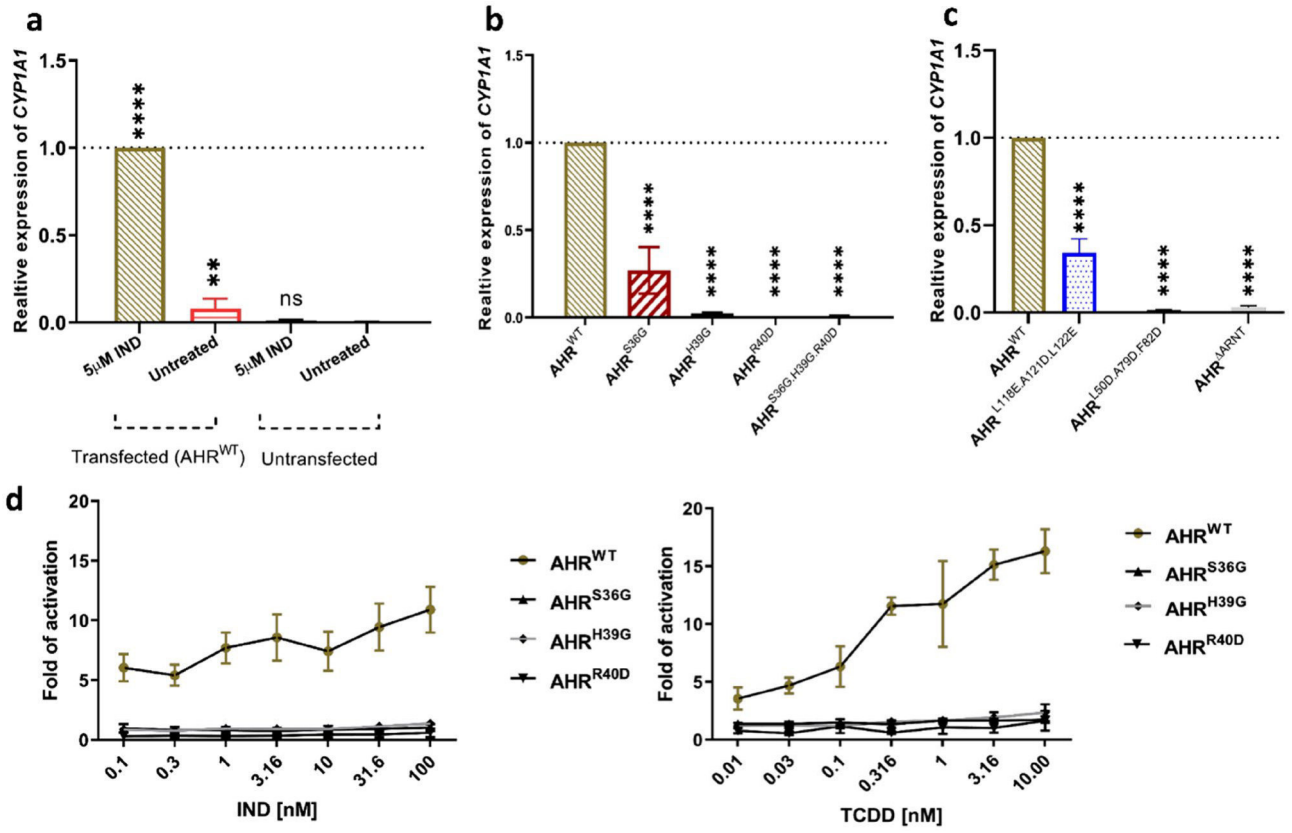


Figure 2. Transcriptional activity of the AHR and AHR mutants. Relative *CYP1A1* mRNA level determined by qPCR in MCF-7$^{\Delta AHR}$ cells. Values were standardized against *HPRT* and normalized against AHR^{WT} transfected cells treated with 5 µM indirubin (IND) for three hours. Depicted are the results for a comparison of treatment and AHR^{WT} transfection (**a**), AHR DNA-binding deficient mutants (**b**) and AHR mutants deficient for ARNT dimerization (**c**). (**d**) Luciferase activity driven by an XRE-promoter after stimulation with increasing concentrations of IND and TCDD for twenty-four hours. (**a**–**c**) Each bar represents the mean of three biological replicates ± S.D., One way Anova (ns: not significant, ** $P < 0.01$, **** $P < 0.0001$). (**d**) Presented are the means of three biological replicates + /- S.D.

mutants AHR$^{H39G.\Delta ARNT}$and AHR^{H39G} to test, whether DNA-binding of the AHR or the formation of the corresponding transcription complex is a prerequisite for the maintenance of stable nuclear localization.

Analyzing intracellular trafficking of the AHR mutants after treatment with IND or BNF showed that both mutants AHR^{H39G} and AHR$^{H39G.\Delta ARNT}$ exhibited a stronger nuclear accumulation after stimulation with the endogenous IND, as compared with the xenobiotic ligand BNF (Fig. 4b–d). Furthermore, the mutant AHR$^{H39G.\Delta ARNT}$ shows significantly accelerated import after stimulation with IND (Fig. 4a).

This data indicates that the formation of the AHR-ARNT heterodimer and subsequent binding to the XRE is not crucial to stabilize the ligand-dependent nuclear retention of the AHR.

Transient knockdown of ARNT does not change the AHR nucleo-cytoplasmic profile in MCF-7 cells. MCF-7 $^{\Delta AHR}$ cells show a significant decrease in *ARNT* mRNA at 24 and 48 h after transfection with siRNA against *ARNT* (Fig. 5a). In the basal state, AHR^{WT} is predominantly located in the nucleus and knockdown of ARNT does not change the intracellular distribution (Fig. 5b,e). Interestingly, neither the ligand dependent nuclear accumulation nor the nuclear import after stimulation with IND or BNF are changed through ARNT knockdown (Fig. 5c,d,f). In conclusion, live tracking of AHR^{WT} in ARNT knockdown cells clearly indicates that nuclear import and accumulation caused by ligand binding is independent from ARNT mediated interactions.

Discussion

In the absence of exogenous ligands, the AHR shuttles continuously between the cytoplasm and the nucleus while a predominantly cytoplasmic compartmentalization is maintained due to an efficient nuclear export. This "shuttling" has been previously reported as a constitutive process that does not lead to any transcriptional activity. Ligand binding accelerates nuclear import and sequesters the receptor in the nucleus. Importantly, this is linked to an exit from the shuttling cycle and to a deactivated export, which determines the cytoplasmic localization prior to activation. However, the molecular mechanisms that stabilize the nuclear AHR population have not been characterized yet.

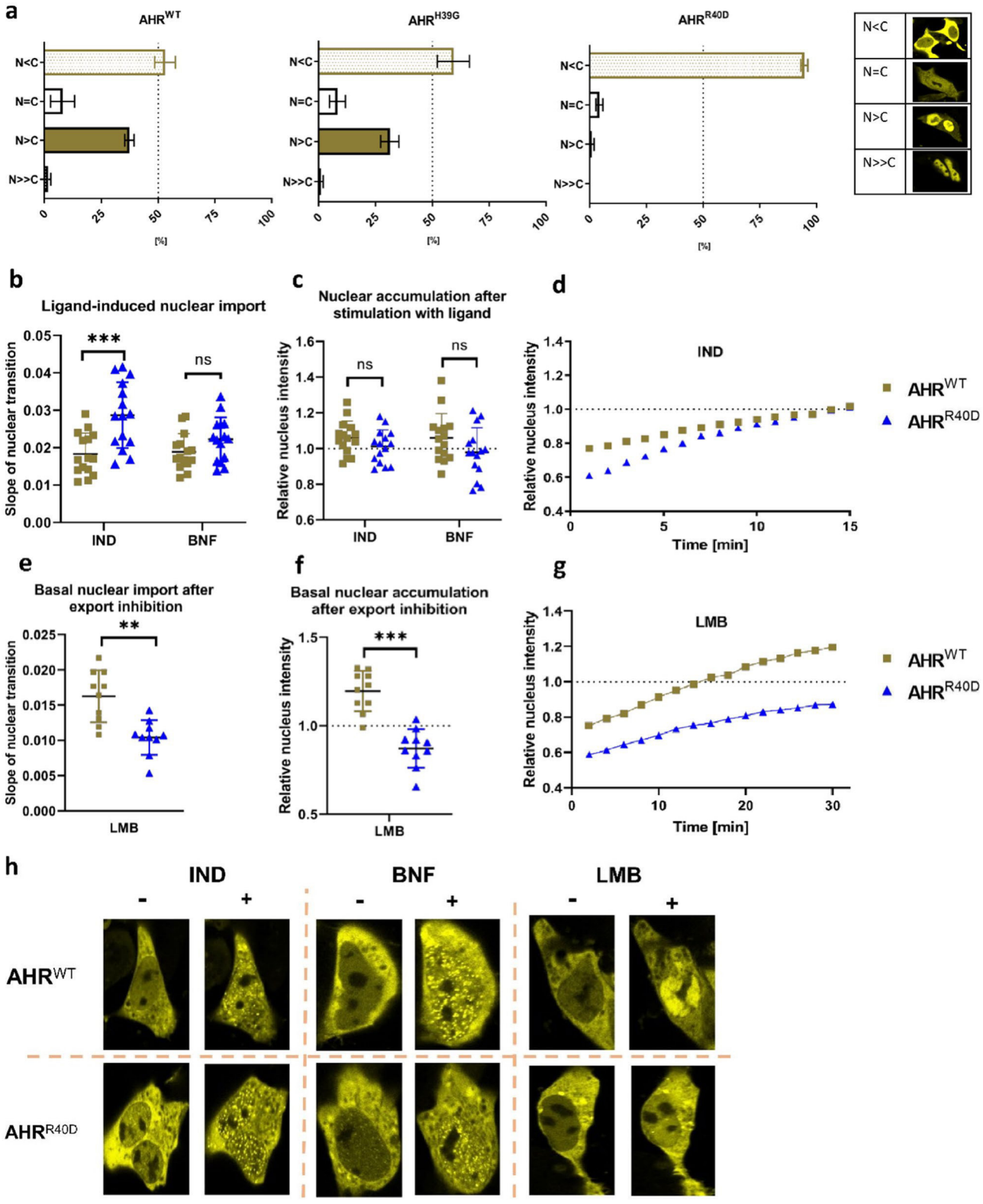


Figure 3. The DNA-binding motif determines the intracellular distribution of the AHR through the overlap with the second nuclear localization signal (NLS). (**a**) Distribution of the EYFP-tagged AHR and AHR mutants in HepG2 cells are shown. More than 300 cells were randomly selected and classified according to the given distribution pattern. Data represents mean ± S.D from three biological replicates. (**b**,**e**) Slopes of nuclear transition the AHR^{WT} and AHR^{R40D} (Arg → Asp) after treatment with 10 μM indirubin (IND), 10 μM β-naphthoflavone (BNF) or 200 nM leptomycin B (LMB). (**c**,**f**) Nuclear accumulation of the AHR^{WT} and AHR^{R40D} 15 min or 30 min after treatment with IND, BNF or LMB, respectively. Individual values and the mean ± S.D. of 15 (**b**,**c**) or 10 (**e**,**f**) cells are presented (two way ANOVA, Dunnett's multiple comparisons test, *** $p < 0.001$). (**d**,**g**) Representative measurements of time-lapse experiments after stimulation with IND (**d**) or LMB (**g**) of indicated AHR variants in HepG2 cells. Relative nuclear intensity denotes the intensity of the nucleus against the intensity of the entire cell. (**h**) Representative images of HepG2 cells transfected with AHR^{WT} or AHR^{R40D} before and after 15 min of treatment with IND, BNF, and LMB.

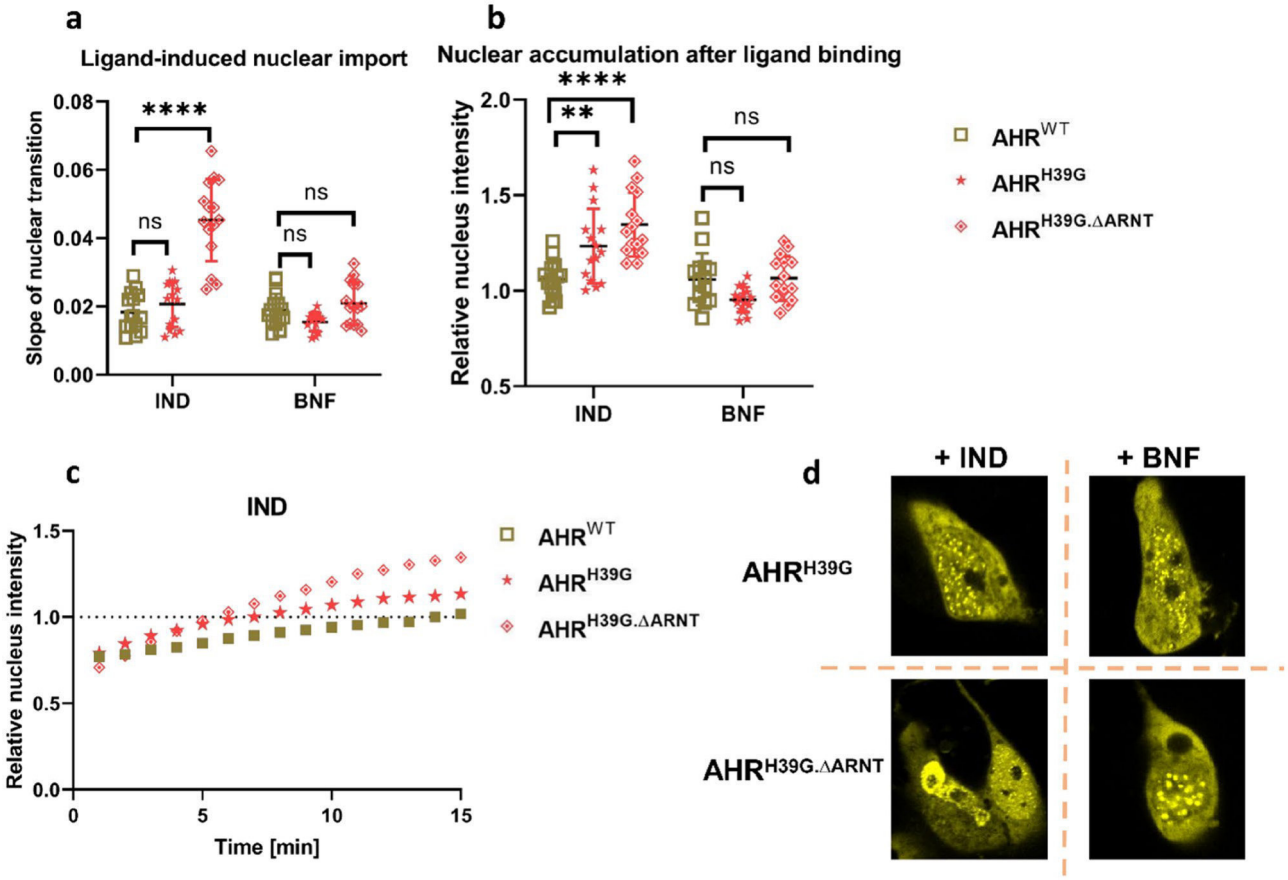


Figure 4. Forming the AHR-ARNT heterodimer and the active binding to the XRE is not decisive for stable ligand-dependent nuclear retention. (**a**) Slopes of nuclear transition of the AHR^{WT}, AHR^{H39G} and $AHR^{H39G.\Delta ARNT}$ after treatment with10μM indirubin (IND) or 10 μM β-naphthoflavone (BNF). (**b**) Nuclear accumulation of the AHR^{WT}, AHR^{H39G} and $AHR^{H39G.\Delta ARNT}$ 15 min after treatment with IND or BNF, respectively. Data display the individual values and the mean + /- S.D. of 15 cells (two way ANOVA, Dunnett's multiple comparisons test, ** $p < 0.01$, *** $p < 0.001$). (**c**) Representative measurements of time-lapse experiments after stimulation with IND of indicated AHR variants in HepG2 cells. Relative nucleus intensity denotes the intensity of the nucleus against the intensity of the entire cell. (**d**) Snapshots of HepG2 cells 15 min after stimulating with IND or BNF.

A prior study of our lab defined the Q-rich/PST domain in the C-terminus as a crucial component for regulating both the shuttling and the ligand-induced import[29]. In particular, the Q-rich/PST domain is essential for regulating intracellular trafficking and nuclear accumulation after ligand binding. The C-terminal AHR deletion mutant $AHR^{\Delta 647}$, lacking 201 residues after valine 647, did not show any nuclear accumulation in response to ligands due to increased export. Intriguingly, point mutations of valine 647 (AHR^{V647A}), or C-terminal deletions that exclude this residue ($AHR^{\Delta 646}$ or shorter) display consistently an exclusive nuclear staining. These previous data implied a mandatory role of valine 647 to maintain a conformation that is capable to undergo export. Further, the adjacent C-terminal domain is required to switch off export during activation, possibly in concert with the well-studied N-terminal trafficking motifs.

In this manuscript, we have addressed the impact of the N-terminal domain on both shuttling and ligand-induced import. Besides the well characterized nuclear transport signals, this domain harbors the DNA-binding XRE motif and participates in the heterodimerization with ARNT that is regarded as crucial step towards activation. There are now two hypotheses for the molecular mechanism of the AHR activation cascade. Firstly, ligand binding might trigger a conformational change that accelerates import and blocks nuclear export in parallel. This would enable all consecutive interactions that lead to transcription of target genes and other nuclear effects. Alternatively, termination of export could be linked to molecular interactions of the AHR that are formed during activation. In order to distinguish both options, we have analyzed whether the DNA-binding motif or the interaction with ARNT are required to stabilize the nuclear AHR fraction during activation. In our experiments, we have used IND as an endogenous ligand, as well as BNF as a xenobiotic agonist. Further, we verified that all AHR mutants, deficient of DNA or ARNT-binding, did not induce transcription of *CYP1A1*.

As the DNA-binding motif overlaps with the 2nd NLS, mutagenesis might also affect nuclear import. Indeed, partial inactivation of nuclear import was indicated for AHR^{R40D} by an absence of basal nuclear staining. We have aimed to separate both functions, generating a mutant that is deficient to bind the XRE without compromising the second section of the bipartite NLS. AHR^{H39G} met the requirement of a comparable basal staining as the wild-type receptor and was used to address the impact of the DNA-binding domain on ligand-induced

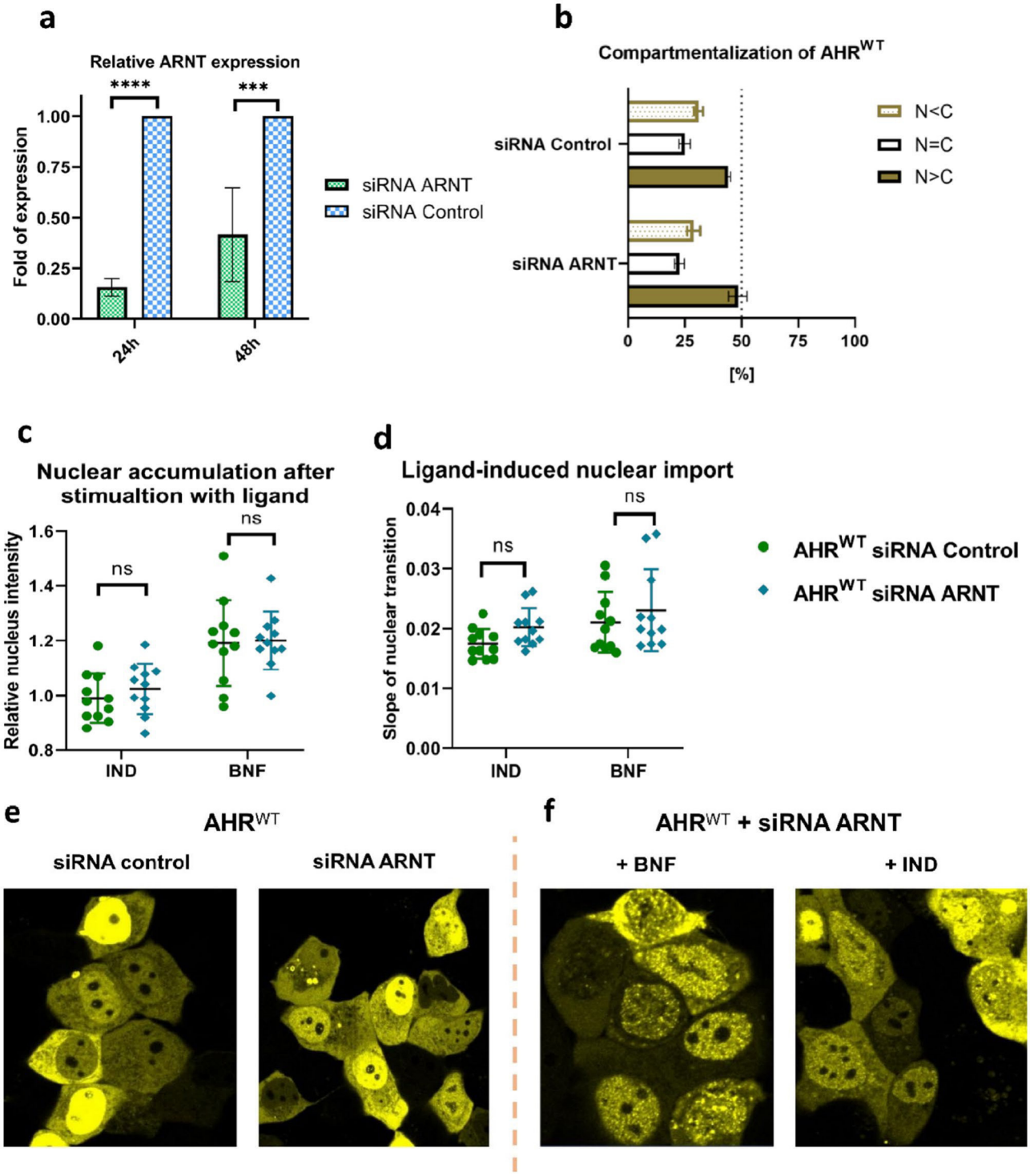


Figure 5. Down-regulation of ARNT is not critical for the AHR nucleo-cytoplasmic translocation in MCF-7 cells. (**a**) Relative *ARNT* mRNA level determined by qPCR in MCF-7$^{\Delta AHR}$ cells after transfection with siRNA against ARNT and control siRNA, respectively. Values were standardized against *HPRT* and normalized against control siRNA transfected cells. Each bar depicts the mean of three biological replicates ± S.D. (two way ANOVA, Dunnett's multiple comparisons test, *** $p < 0.001$,**** $P < 0.0001$). (**b**) Intracellular distribution pattern of EYFP-tagged AHR^{WT} in MCF-7 $^{\Delta AHR}$ cells are shown. At least 300 transfected cells are randomly selected and sorted according to the following classification: N > C predominantly nuclear; N = C equal distribution; N < C predominantly cytoplasmic. Each bar represents the mean + /- S.D. of three biological experiments. Depicted are relative nuclear intensity (**c**) and slope (**d**) of time-lapse measurements after stimulation with 10 μM indirubin (IND) or 10 μM β-naphthoflavone (BNF) in control siRNA and ARNT siRNA transfected MCF-7$^{\Delta AHR}$ cells. The presented data contains individual values and the mean + /- S.D. of 11 cells. (**e**) Snapshots of MCF-7 $^{\Delta AHR}$ cells before and after knocking down the ARNT gene. (**f**) Snapshots of the MCF-7 cells after stimulation with BNF and IND.

nuclear import. Importantly, the kinetics of nuclear accumulation were similar for both to the wild-type AHR and AHR^{H39G}. Surprisingly, this was also true for AHR^{R40D}, suggesting that neither DNA-binding nor the second section of the NLS affect the ligand-induced nuclear retention.

We further used the mutant AHR^{R40D} to characterize the 2nd NLS in more detail. Interestingly, this mutant failed to accumulate in the nucleus after protein export was inhibited using LMB. Our data indicate a specific role for this trafficking motif in the basal (i.e. ligand-independent) nuclear import that occurs in the context of nucleo-cytoplasmic shuttling. In contrast, this motif has no impact on the ligand induced nuclear transfer. These observations underscore the idea that the ligand induced and the basal import constitute two independent transport mechanisms. The basal import might take place due to a direct binding to importin-β through the arginine rich NLS. Findings by Pterulis et al. further support this hypothesis by showing that the murine AHR is able to perform a stable binding with importin-β[26]. In conclusion, our results propose that the NLS stays partially unmasked in the absence of exogenous ligands, thereby enabling binding with importin-β and allowing the basal nuclear import. However, this theory has to be addressed in a next study.

As second question, we have studied whether dimerization with ARNT is required to retain the AHR in the nucleus. The mutant $AHR^{H39G.\Delta ARNT}$ is an inactive AHR variant with a similar compartmentalization as the wild type, that is incapable to dimerize with ARNT or bind the DNA. Data indicate that ligand-induced nuclear retention of the AHR does not depend on functional interactions with ARNT. This was also confirmed after inhibition of ARNT expression using siRNA. The transient knockdown of ARNT reveals that heterodimerization is not required for the AHR to maintain a stable nuclear localization.

Notably in comparison with wild type AHR, both $AHR^{H39G.\Delta ARNT}$and AHR^{H39G} exhibit a strong increase in the nuclear accumulation after stimulation with the endogenous IND but not with the xenobiotic ligand BNF. For $AHR^{H39G.\Delta ARNT}$ treated with IND, we also detected an increased slope of nuclear transition, which directly leads to higher nuclear accumulation. The reason for the change in nuclear transition, meaning nuclear import and parallel re-export in general, through IND opposed to BNF cannot be elucidated with our experiments. For AHR^{H39G}, the nuclear transition was not significantly altered in comparison to wild type AHR and increase in nuclear accumulation was less pronounced. However, nuclear import and induction of target gene expression (*CYP1A1*) correlates for IND but less for BNF[4]. Based on this, one might hypothesize that nuclear transition and functional effectivity of AHR within the nucleus might be linked. Therefore, the effect is stronger for the more potent ligand IND than for BNF.

Taken together, our results indicate that the stabilized nuclear population of ligand-bound or ligand-free AHR is neither dependent on interactions with DNA nor the ARNT. Therefore, the kinetic of nuclear accumulation is not related to the molecular interactions conducted by the activated receptor in the nucleus. In conclusion to our data, we propose that the conformational change followed by ligand binding promotes the nuclear import and blocks the export autonomously.

Methods

Plasmids. The plasmid pEYFP-AHR-C1 used by[29] encodes the human AHR (start at Ala 11). All pEYFP-AHR variants, which carry mutations at certain amino acids (AHR^{H39G}, AHR^{R40D}, AHR^{S36G}, $AHR^{S36G.H39G.R40D}$, $AHR^{L118E.A121D.L122E}$, $AHR^{L50D.A79D.F82D}$ and $AHR^{H39G.L50D.A79D.F82D.L118E.A121D.L122E.}$) were generated by GenScript (GenScript Biotech, Leiden, Netherlands).

Cell culture. The human hepatoma cell line HepG2 was purchased from DSMZ (Braunschweig, Germany). HepG2 cells were grown in RPMI 1640. MCF-$7^{\Delta AHR}$ cells were cultured in DMEM. Both cell lines were maintained in 5% CO_2 at 37 °C in culture medium containing 10% (v/v) FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. All media components were purchased from Pan-Biotech (Aidenbach, Germany). AHR-deficient variant of MCF-7 cells were kindly provided by Dr. P. Tarnow[37].

Transient transfection. HepG2 and MCF-$7^{\Delta AHR}$ cells were seeded either on 10-cm dishes, 6-well plates (Techno Plastic Products AG, Trasadingen, Switzerland), 96-well plates (Techno Plastic Products AG, Trasadingen, Switzerland), or on glass bottom dishes (In VitroScientific, Sunyvale, CA, USA). On the next day, cells on glass bottom dishes were transfected by using Xfect (Takara Bio Europe SAS, Saint-Germain-en-Laye, France) and an appropriate DNA amount according to the manufacturer's instructions. Cells on 10-cm dishes or on multiwell plates were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and an appropriate DNA amount as stated by the manufacturer's instructions. After 4 h incubation, transfection medium was removed and replaced by new RPMI or DMEM medium, respectively.

Reagents. β-naphthoflavone (BNF) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Munich, Germany). Leptomycin B (LMB) was acquired from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., Texas, U.S.A). Indirubin (IND) was provided from Enzo Life Sciences (Enzo Life Sciences GmbH, Lörrach, Germany) and 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD) from LGC Standards (LGC Standards GmbH, Wesel, Germany). All chemicals were purchased at the highest purity available.

RNA analysis. RNA was isolated form cells by using RNeasy Midi kit in conjunction with QIAshredder (QIAGEN GmbH, Hilden, Germany). The purity and the concentration of RNA were determined using a plate reader device (Infinite M200 PRO, Tecan Trading AG, Männedorf, Switzerland). Reverse transcription was performed with the high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA USA).

Gene	Forward primer	Reverse primer	Product size (bp)
CYP1A1	5′- CCAAGAGTCCACCCTTCCCAGCT -3′	5′- GAGGCCAGAAGAAACTCCGTGGC -3′	371
HPRT	5′-GTTCTGTGGCCATCTGCTTAG-3′	5′-GCCCAAAGGGAACTGATAGTC-3′	144

Table 1. Primer sequences used.

Quantitative PCR (qPCR) was performed on a 7500 Fast Real-Time PCR instrument using FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Hypoxanthine–guanine phosphoribosyl transferase (*HPRT*) was used as reference gene.

The primer sequences are listed in Table 1.

Luciferase assay. Cells used for the luciferase assay were seeded in 96-well plates (Merck KGaA, Darmstadt, Germany) at a concentration of 4.5×10^4 and 150 µL per well. Cell were transfected with pGL4.26-XRE provided by Dr. P. Tarnow[38] together with AHR plasmids (WT or mutants). 24 h after stimulation cells were washed with PBS and lysed with lysis-puffer (10 mM Tris 0.1 mM, 2 mM EDTA, 1% Triton™ X-100) for 15 min. 50 µl luciferin solution and 150 µl assay puffer (Gly-Gly puffer, DTT and ATP) were injected per well. Luminescence was measured in a Synergy Neo2 plate reader (BioTek, Bad Friedrichshall, Germany).

RNA-silencing. For RNA silencing, cells were transfected with 80 pmol of siRNA (sc-29734) or the control siRNA (sc-37007) (all from Santa Cruz Biotechnology, Heidelberg, Germany) by using Lipofectamine 2000 according to the manufacturer's instructions. The expression of the *ARNT* gene was measured by using the ARNT primer (sc-29734-PR) provided from Santa Cruz Biotechnology.

On-line confocal microscopy. HepG2 and MCF-$7^{\Delta AHR}$ cells are seeded and transfected as described above. Cells were monitored by confocal microscope (LSM 700, Carl Zeiss Jena GmbH, Jena, Germany) twenty four hours post transfection. For live cell imaging, cells were maintained in buffered medium in 5% CO_2 at 37 °C. Simultaneously with the treatments, the imagining was initiated at a rate of one picture per minute. Microscopic image acquisition was done by using ZEN 2012 black edition software (Carl Zeiss Jena GmbH). For data analysis ZEN 2012 blue edition was used (Carl Zeiss Jena GmbH). The nucleus and the whole cell were defined and outlined as a region of interest (ROI), for which the fluorescence intensity was extracted.

Statistics. Data were analysed and graphed using GraphPad Prism (Graph Pad, La Jolla, CA, USA). Statistical analysis was done using paired Dunnett's multiple comparisons test, one-way or two-way ANOVA, * $p < 0.01$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Received: 24 June 2021; Accepted: 24 August 2021
Published online: 14 September 2021

References

1. Beischlag, T. V. The aryl hydrocarbon receptor complex and the control of gene expression. *Crit. Rev. Eukaryot. Gene Exp.* **18**(3), 207–250 (2008).
2. Chen, G. & Bunce, N. J. Interaction between halogenated aromatic compounds in the Ah receptor signal transduction pathway. *Environ. Toxicol.* **19**(5), 480–489 (2004).
3. Luch, A. Nature and nurture—lessons from chemical carcinogenesis. *Nat. Rev. Cancer* **5**(2), 113–125 (2005).
4. Tkachenko, A. *et al.* Nuclear transport of the human aryl hydrocarbon receptor and subsequent gene induction relies on its residue histidine 291. *Arch. Toxicol.* **92**(3), 1151–1160 (2018).
5. Puga, A., Ma, C. & Marlowe, J. L. The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. *Biochem. Pharmacol.* **77**(4), 713–722 (2009).
6. Tarnow, T. & Luch, A. Chemical activation of estrogen and aryl hydrocarbon receptor signaling pathways and their interaction in toxicology and metabolism. *Exp. Opin. Drug Metab. Toxicol.* **15**(3), 219–229 (2019).
7. Carreira, V. S. *et al.* Ah receptor signaling controls the expression of cardiac development and homeostasis genes. *Toxicol. Sci.* **147**(2), 425–435 (2015).
8. Walisser, J. A., Glover, E., Pande, K., Liss, A. L. & Bradfield, C. A. Aryl hydrocarbon receptor-dependent liver development and hepatotoxicity are mediated by different cell types. *Proc. Natl. Acad. Sci. U.S.A.* **102**(49), 17858–17863 (2005).
9. Neavin, D. R., Liu, D., Ray, B. & Weinshilboum, R. M. The role of the aryl hydrocarbon receptor (AHR) in immune and inflammatory diseases. *Int. J. Mol. Sci.* **19**, 12 (2018).
10. Guerrina, N., Traboulsi, H., Eidelman, D. H. & Baglole, C. J. The aryl hydrocarbon receptor and the maintenance of lung health. *Int. J. Mol. Sci.* **19**, 12 (2018).
11. van den Bogaard, E. H. *et al.* Genetic and pharmacological analysis identifies a physiological role for the AHR in epidermal differentiation. *J. Invest. Dermatol.* **135**(5), 1320–1328 (2015).
12. Rannug, A. How the AHR became important in intestinal homeostasis-a diurnal FICZ/AHR/CYP1A1 feedback controls both immunity and immunopathology. *Int. J. Mol. Sci.* **21**, 16 (2020).
13. Zelante, T. *et al.* Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity* **39**(2), 372–385 (2013).
14. Nguyen, L. P. & Bradfield, C. A. The search for endogenous activators of the aryl hydrocarbon receptor. *Chem. Res. Toxicol.* **21**(1), 102–116 (2008).

15. Adachi, J. *et al.* Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. *J. Biol. Chem.* **276**(34), 31475–31478 (2001).
16. Moura-Alves, P. *et al.* AhR sensing of bacterial pigments regulates antibacterial defence. *Nature* **512**(7515), 387–392 (2014).
17. Murray, I. A., Patterson, A. D. & Perdew, G. H. Aryl hydrocarbon receptor ligands in cancer: Friend and foe. *Nat. Rev. Cancer* **14**(12), 801–814 (2014).
18. Wu, D., Potluri, N., Lu, J., Kim, Y. & Rastinejad, F. Structural integration in hypoxia-inducible factors. *Nature* **524**(7565), 303–308 (2015).
19. Kolonko, M. & Greb-Markiewicz, B. bHLH-PAS proteins: Their structure and intrinsic disorder. *Int. J. Mol. Sci.* **20**, 15 (2019).
20. Brunnberg, S. *et al.* The basic helix-loop-helix-PAS protein ARNT functions as a potent coactivator of estrogen receptor-dependent transcription. *Proc. Natl. Acad. Sci. U.S.A.* **100**(11), 6517–6522 (2003).
21. Rüegg, J. *et al.* The transcription factor aryl hydrocarbon receptor nuclear translocator functions as an estrogen receptor beta-selective coactivator, and its recruitment to alternative pathways mediates antiestrogenic effects of dioxin. *Mol. Endocrinol.* **22**(2), 304–316 (2008).
22. Ikuta, T., Eguchi, H., Tachibana, T., Yoneda, Y. & Kawajiri, K. Nuclear localization and export signals of the human aryl hydrocarbon receptor. *J. Biol. Chem.* **273**(5), 2895–2904 (1998).
23. Ikuta, T. *et al.* Nucleocytoplasmic shuttling of the aryl hydrocarbon receptor. *J. Biochem.* **127**(3), 503–509 (2000).
24. Lange, A. *et al.* Classical nuclear localization signals: Definition, function, and interaction with importin alpha. *J. Biol. Chem.* **282**(8), 5101–5105 (2007).
25. Rowlands, J. C. & Gustafsson, J. A. Aryl hydrocarbon receptor-mediated signal transduction. *Crit. Rev. Toxicol.* **27**(2), 109–134 (1997).
26. Petrulis, J. R., Kusnadi, A., Ramadoss, P., Hollingshead, B. & Perdew, G. H. The hsp90 Co-chaperone XAP2 alters importin beta recognition of the bipartite nuclear localization signal of the Ah receptor and represses transcriptional activity. *J. Biol. Chem.* **278**(4), 2677–2685 (2003).
27. Murray, I. A. *et al.* Evidence that ligand binding is a key determinant of Ah receptor-mediated transcriptional activity. *Arch. Biochem. Biophys.* **442**(1), 59–71 (2005).
28. Moroianu, J. Nuclear import and export pathways. *J. Cell. Biochem.* **75**(S32), 76–83 (1999).
29. Tkachenko, A. *et al.* The Q-rich/PST domain of the AHR regulates both ligand-induced nuclear transport and nucleocytoplasmic shuttling. *Sci. Rep.* **6**, 32009 (2016).
30. Tsuji, N. *et al.* The activation mechanism of the aryl hydrocarbon receptor (AhR) by molecular chaperone HSP90. *FEBS Open Bio* **4**, 796–803 (2014).
31. Swanson, H. I., Chan, W. K. & Bradfield, C. A. DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins. *J. Biol. Chem.* **270**(44), 26292–26302 (1995).
32. Hestermann, E. V. & Brown, M. Agonist and chemopreventative ligands induce differential transcriptional cofactor recruitment by aryl hydrocarbon receptor. *Mol. Cell. Biol.* **23**(21), 7920–7925 (2003).
33. Davarinos, N. A. & Pollenz, R. S. Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytosplasmic proteasome following nuclear export. *J. Biol. Chem.* **274**(40), 28708–28715 (1999).
34. Schulte, K. W., Green, E., Wilz, A., Platten, M. & Daumke, O. Structural basis for aryl hydrocarbon receptor-mediated gene activation. *Structure* **25**(7), 1025-1033.e3 (2017).
35. Seok, S. H. *et al.* Structural hierarchy controlling dimerization and target DNA recognition in the AHR transcriptional complex. *Proc. Natl. Acad. Sci. U.S.A.* **114**(21), 5431–5436 (2017).
36. Wu, D., Potluri, N., Kim, Y. & Rastinejad, F. Structure and dimerization properties of the aryl hydrocarbon receptor PAS-A domain. *Mol. Cell. Biol.* **33**(21), 4346–4356 (2013).
37. Tarnow, P. *et al.* Characterization of quinoline yellow dyes as transient aryl hydrocarbon receptor agonists. *Chem. Res. Toxicol.* **33**(3), 742–750 (2020).
38. Tarnow, P. *et al.* A novel dual-color luciferase reporter assay for simultaneous detection of estrogen and aryl hydrocarbon receptor activation. *Chem. Res. Toxicol.* **30**(7), 1436–1447 (2017).

Author contributions

R.H., F.H., J.K., A.L. designed the experimental program. R.H. performed the experimental work, A.R. contributed to the confocal microscopy and D.G. generated plasmids. Data analysis and figures creation were done by R.H. Manuscript writing was done by R.H. with contributions from F.H., A.L., P.L. and J.K. The final version was approved by all authors.

Funding

Open Access funding enabled and organized by Projekt DEAL.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-97507-w.

Correspondence and requests for materials should be addressed to R.H.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

3.2 Paper 2

The nuclear entry of the aryl hydrocarbon receptor (AHR) relies on the first nuclear localization signal and can be negatively regulated through IMPα/β specific inhibitors

Rashad Haidar, Reneh Shabo, Marie Moeser, Andreas Luch & Josephine Kugler

This chapter was published on 11 November 2023.

Scientific Reports, volume 13, Article number: 19668 (2023)

DOI: 10.1038/s41598-023-47066-z.

Link: https://doi.org/10.1038/s41598-023-47066-z

Author contributions within this chapter:

Project planning (80%)

Experimental work (85%)

Data analysis (85%)

Writing of the manuscript (80%).

Supplementary materials for the following publication are detailed in Annex II.

scientific reports

Check for updates

OPEN

The nuclear entry of the aryl hydrocarbon receptor (AHR) relies on the first nuclear localization signal and can be negatively regulated through IMPα/β specific inhibitors

Rashad Haidar[1,2]✉, Reneh Shabo[1], Marie Moeser[1], Andreas Luch[1,2] & Josephine Kugler[1]

The human aryl hydrocarbon receptor (AHR) undergoes continuous shuttling between nucleus and cytoplasm. Binding to exogenous or endogenous ligands promotes its rapid nuclear import. The proposed mechanism for the ligand-dependent import is based on exposing the bipartite nuclear localisation signal (NLS) to members of the importin (IMP) superfamily. Among this, the molecular interactions involved in the basal import still need to be clarified. Utilizing fluorescently fused AHR variants, we recapitulated and characterized AHR localization and nucleo-cytoplasmic shuttling in living cells. Analysis of AHR variants carrying NLS point mutations demonstrated a mandatory role of first ($_{13}$RKRRK$_{17}$) and second ($_{37}$KR-R$_{40}$) NLS segments on the basal import of AHR. Further experiments indicated that ligand-induced import is mainly regulated through the first NLS, while the second NLS is supportive but not essential. Additionally, applying IMPα/β specific inhibitors, ivermectin (IVM) and importazole (IPZ), slowed down the ligand-induced import and, correspondingly, decreased the basal nuclear accumulation of the receptor. In conclusion, our data show that ligand-induced and basal nuclear entry of AHR rely on the same mechanism but are controlled uniquely by the two NLS components.

The aryl hydrocarbon receptor (AHR) is a ligand-dependent transcription factor, which was initially discovered due to its contribution in mediating the toxicity of exogenous environmental toxicants, like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)[1] and benzo[*a*]pyrene (B[*a*]P)[2]. Exposure to AHR ligands, as for example the synthetic flavonoid β-naphthoflavone (BNF)[3], induces the expression of xenobiotic metabolizing enzymes (XMEs). These include phase I XMEs such as cytochrome P450-dependent monooxygenase 1A1 (*CYP1A1*) and phase II XMEs such as glutathione *S*-transferase α (*GST-α*)[4], indicating AHR's role in xenobiotic metabolism[5].

More current studies discovered essential physiological roles of AHR by demonstrating that many compounds from endogenous origins, including indole, heme, and tryptophan metabolites[6,7], are able to bind and activate the receptor. As for example, the endogenous indirubin (IND) is generated from dietary tryptophan by the intestinal and urinary microbiome[8]. It is one of the most potent AHR ligands and exhibits anti-inflammatory effects in vivo[9]. Furthermore, current research reveals that AHR has a more complex role in cell biology. In fact, AHR interacts with other signaling pathways involved in proliferation, apoptosis, and cell cycle regulation[10,11].

AHR activation is firmly linked with nuclear transition that depends on a sequence of positively charged amino acids, the nuclear localization signal (NLS)[12]. The NLS comprises two segments (bipartite NLS) and is located within the conserved bHLH (basic helix–loop–helix) domain in the N-terminal part of the protein[13]. According to the classical AHR pathway, unliganded AHR is mainly located in the cytoplasm bound by a chaperon complex consisting of two molecules of HSP90[14] and single molecules of co-chaperone p23[15] and hepatitis x-associated protein-2 (XAP2)[16]. Most AHR ligands are able to cross the cell membrane through simple diffusion

[1]Department of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany. [2]Department of Biology, Chemistry and Pharmacy, Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany. ✉email: Rashad.Haidar@bfr.bund.de

due to their hydrophobicity[17]. Ligand binding to cytosolic AHR triggers a conformational change that exposes the NLS to a nuclear transporter in order to permit nuclear import[18]. On the molecular level, members of the importin (IMP) superfamily IMPβ1 or its adaptor protein IMPα can recognize the revealed NLS[18,19]. Thereafter, AHR along with the IMPα/β1 heterodimer reaches the nucleus through the nuclear pore complexes (NPCs). On the nuclear side of NPCs, IMPβ1 binds to RanGTP (Ras-related nuclear protein) subsequently leading to a release of the NLS-cargo[20].

In the nucleus, AHR releases its chaperons[21] and builds a stable dimer with the AHR nuclear translocator (ARNT)[22,23]. The AHR/ARNT heterodimer recruits several co-factors[24], binds its target sequences, called XRE (xenobiotic response element)[25], and regulates the expression of AHR target genes. Ultimately, AHR is exported from the nucleus to the cytoplasm to undergo proteasomal degradation marking the end of activation[26]. This nuclear export is established through CRM1 (chromosome region maintenance 1 also known as exportin) that binds the nuclear export signal (NES) of the receptor[27]. In a previous report, we showed that the molecular interactions performed by the receptor in the nucleus, ARNT dimerization and DNA binding, do not alter the nuclear retention after ligand binding[28].

Analyzing AHR translocation in vitro, however, discloses many aspects that are still not completely understood. For example, AHR undergoes constitutive shuttling between the nucleus and cytoplasm[29,30], which neither leads to any activation[31], nor demands HSP90 dissociation[32]. Although most of the effort given on AHR trafficking intends to clarify the ligand-induced transition, the basal shuttling requests further investigation. On this stage, the research done in the last decade revealed many nuclear import inhibitors targeting IMPs[33]. The best characterized ones are importazole (IPZ)[34] and ivermectin (IVM)[35,36]. The former revealed interferences with the IMPα/β1 mediated-nuclear entry of NF-κB[33] and the latter showed import suppressing properties on the hypoxia induced factor (HIF)[37]. Both import inhibitors differ markedly in their mechanism. While IVM inhibits the binding between IMPα and the NLS[38,39], IPZ interferes with IMPβ1 and RanGTP binding[40].

In the current study, we aimed to elucidate the mechanism of AHR translocation in the basal and ligand-induced state in living cells. We confirmed faithful recapitulation of endogenous AHR localization for our EYFP-AHR overexpression system, independent of cell cycle, expression level, or fusion with EYFP. Through point mutations over the entire NLS, importance of both parts of the bipartite NLS for AHR localization has been shown. But surprisingly, these effects are not transferable on the ligand-induced import, which can still occur with 2nd NLS mutants, confirming that the 1st NLS of AHR is mandatory for the nuclear import. Application of the nuclear transport inhibitors IVM and IPZ resulted in a clear reduction of nuclear accumulation of ligand-free and ligand-bound AHR. In summary, our data demonstrated that ligand-induced and basal nuclear entry are two different processes that rely on the same molecular mechanism.

Results

EYFP-AHR in MCF-7$^{\Delta AHR}$ faithfully recapitulates AHR localization

In our study, we aimed to investigate the nucleo-cytoplasmic translocation of unliganded and ligand bound AHR. To avoid any confounding effects through endogenous AHR, AHR-knockout MCF-7 cells (MCF-7$^{\Delta AHR}$) have been used[41]. Transient transfection of MCF-7$^{\Delta AHR}$ cells with plasmids coding for EYFP-AHR^{WT} or EYFP-AHR^{mut} allowed real-time tracking of AHR's subcellular localization in living cells as described previously[28,31].

Notably, localization of AHR^{WT} in MCF-7$^{\Delta AHR}$ cells can be defined in three different states: accumulated in the nucleus, equally distributed or predominantly cytoplasmic (Figs. 1a, 2h and Supplementary Fig. S1). Antibody staining of endogenous AHR in fixed MCF-7^{WT} revealed a similar distribution (Fig. 1b). Treating cells for 30 min with 10 µM IND, however, captured only predominantly nuclear accumulated AHR in both cases (Fig. 1c,d). To assure functional output of EYFP-AHR^{WT} translocation, we verified *CYP1A1* and *CYP1B1* mRNA induction after IND treatment with qPCR (Supplementary Fig. S2).

To exclude an influence of the cell cycle on AHR localization, we determined the cell cycle phase of EYFP-AHR^{WT} transfected MCF-7$^{\Delta AHR}$ cells by measuring DNA content. After 48 h of cultivation, roughly 65% of MCF 7$^{\Delta AHR}$ cells were in G1 phase (Fig. 1e). With nocodazole as positive control for cell cycle analysis, we shifted the population to mainly G2 phase (Fig. 1f). Interestingly, cells that have been transfected, but were negative for EYFP-AHR expression, exhibited the same distribution as untransfected cells (Fig. 1i). On the other side, the great majority of EYFP-AHR positive cells were in G1 phase (Fig. 1g,h). To further elucidate whether changed cell cycle progression is a result of EYFP-AHR overexpression, we similarly analyzed the cell cycle of cells transiently transfected with EYFP alone. Interestingly, these cells exhibited changed cell cycle distribution as well, leading to the hypothesis, that transfection itself alters cell cycle progression (Supplementary Fig. S3). Overall, transfection of EYFP-AHR^{WT} in MCF-7$^{\Delta AHR}$ faithfully recapitulates AHR localization, independent of expression level and cell cycle.

Impact of individual amino acids of the NLS on the basal import of AHR

Since we sought to investigate the nuclear transition of AHR, we decided to address the impact of NLS components on the basal import of AHR first. Therefore, we mutated every positively charged amino acid of the bipartite NLS individually and generated mutants for the first and the second part of the NLS as well (Fig. 2a–c).

We evaluated the effect of individual amino acids on the intracellular distribution of AHR in MCF-7$^{\Delta AHR}$ cells by categorizing at least 100 cells into one of the three described states (Fig. 2h and Supplementary Fig. S4). Consistently, switching the positively charged NLS amino acid arginine to aspartate (AHR^{R13D}, AHR^{K14D}, AHR^{R15D}, AHR^{R16D}, AHR^{K17D}, AHR$^{1st.NLS}$, AHR^{K37D}, AHR^{R38D}, AHR^{R40D}, and AHR$^{2nd.NLS}$) shifted the AHR compartmentalization to be solely cytoplasmic. On the other hand, similar substitution of the other two positions (AHR^{H39D} and AHR^{R42D}) did not alter the typical cellular distribution (Fig. 2d and Supplementary Figs. S5 and S6).

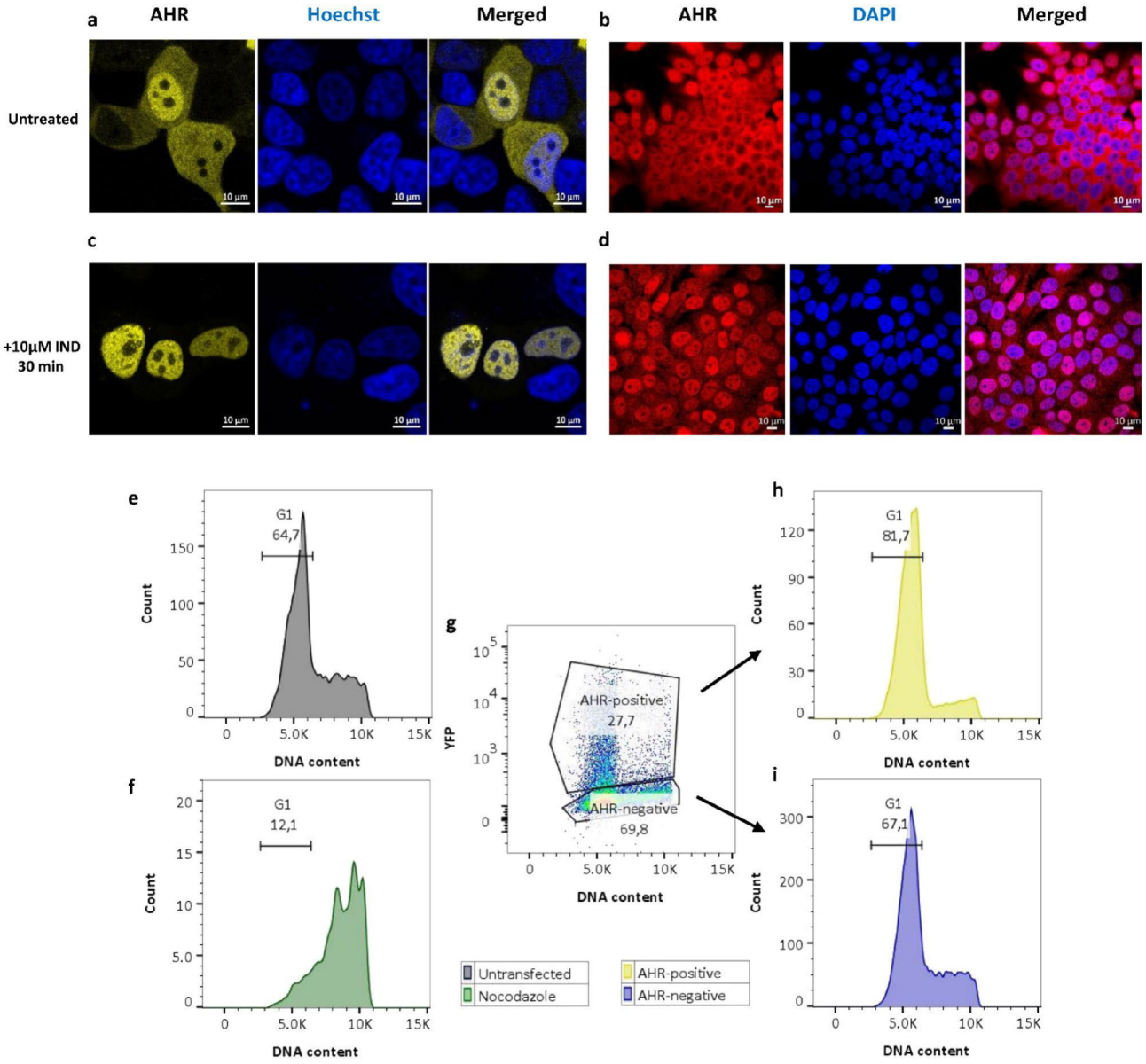


Figure 1. AHR intracellular distribution in MCF-7 cells. Representative images of EYFP-AHR^{WT} transiently transfected in MCF-7$^{\Delta AHR}$ cells before (**a**) and after (**c**) 30 min treatment with 10 µM indirubin (IND). Immunofluorescence images of AHR in MCF-7^{WT} cells before (**b**) and after (**d**) 30 min treatment with 10 µM IND. Nuclei are marked either with Hoechst (**a**,**c**) or with DAPI (**b**,**d**). Cell cycle analysis of control cells without (**e**) and with nocodazole (f). EYFP-AHR transfected cells were sorted for expression level (**g**) and subpopulations individually analyzed for cell cycle for EYFP-AHR-positive (**h**) and for EYFP-AHR-negative cells (**i**). Total number of analyzed cells: 7624 (**e**), 1149 (**f**), 18,192 (**g**), 4441 (**h**) and 11,181 (**i**).

Next, we analyzed the relative nuclear intensity (Fig. 2h) of basal EYFP-AHR^{WT} and EYFP-AHR^{mut}, respectively, thus informing about the nuclear retention of AHR in the absence of ligands (Fig. 2e). Our results exhibited a significant decrease in the relative nuclear intensity of NLS mutants compared with primary cytosolic EYFP-AHR^{WT} cells (Fig. 2e and Supplementary Fig. S5). Interestingly, mutations in the first part of the NLS seemed to have a higher impact on the nuclear retention than mutations on the second part of the NLS. Again, EYFP-AHR^{H39D} and EYFP-AHR^{R42D} revealed a comparable nuclear intensity to the wild type (Fig. 2e).

Moreover, we tested whether the basal import can still occur in first and second NLS full mutants after inhibiting nuclear export using leptomycin B (LMB) by measuring the change of relative nuclear intensity over time (Supplementary Fig. S7). Remarkably, the basal import for both mutants was completely repressed after treating the cells with 200 nM LMB for 30 min (Fig. 2f,g and supplementary Fig. S8).

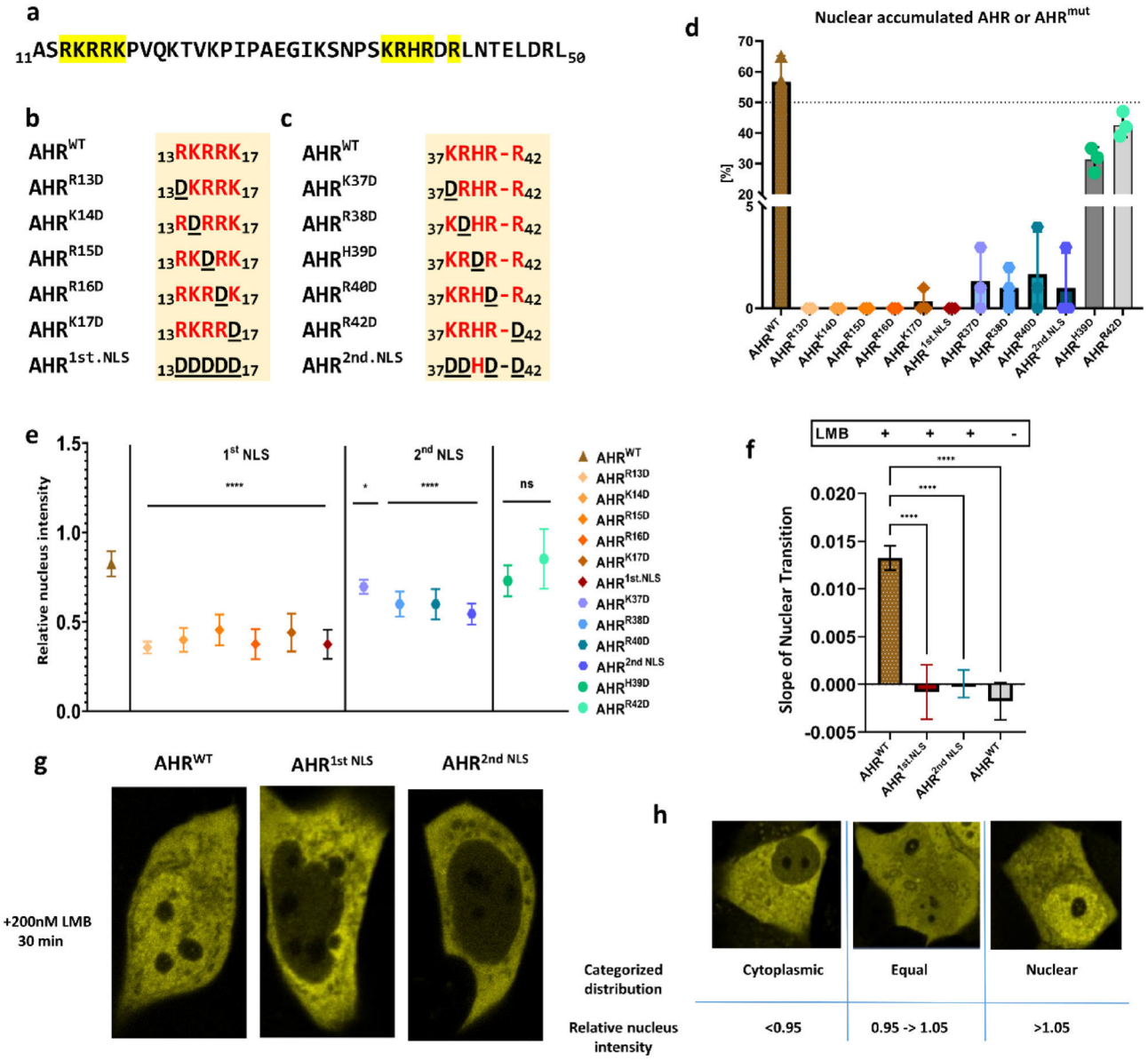


Figure 2. Role of the nuclear localization signal (NLS) on the basal shuttling of AHR. The N-terminal bHLH domain of AHR is indicated as amino acid sequence (**a**). Graphical representation of mutated amino acids in 1st NLS (b) and 2nd NLS (**c**). The percentage of cells with nuclear accumulated AHR^{WT} or AHR^{mut} for 100 randomly chosen cells in the basal state (**d**) according to our classification (**h**). The ratio of the mean fluorescence intensity within the nucleus and the mean fluorescence intensity of the cells in the cytoplasmic state is given as relative nuclear intensity (**e**). Slopes of the nuclear transition of AHR^{WT} or AHR^{mut} after treatment with 200 nM leptomycin B (LMB) for 30 min (**f**). Values represent the mean ± S.D of n = 100 (**d**), n = 10 (**e**), n = 14 for AHR^{WT} and n = 5 for AHR^{mut} or unstimulated AHR^{WT} (**f**). Statistical analysis was performed with a one-way ANOVA, Dunnett's post-test, $^{*}p < 0.05$, $^{****}p < 0.0001$.

The ligand-induced nuclear entry of AHR depends primarily on the first part of NLS

Next, we studied the ligand-induced nuclear import of NLS mutants. After treatment with either IND or BNF, we tracked the subcellular location of fluorescent fusion proteins over 15 min.

In response to ligands, all mutants with point mutations in the 1st NLS, except EYFP-AHR^{K17D}, revealed a clear reduction of nuclear transition slopes compared to the wild type protein (Fig. 3a–d). In contrast, mutants with point mutations in the 2nd NLS resulted only in a slight reduction of nuclear import after stimulation with IND and showed no effect on the import rate for BNF treatment (Fig. 3a–d). When mutating the entire first or second NLS, respectively, the effect on the nuclear transition slope was similar: mutating the 1st NLS blocked the nuclear transition entirely, whereas a mutation of the 2nd NLS only reduced the rate of nuclear import (Fig. 3a–f). EYFP-AHR^{H39D} and EYFP-AHR^{R42D} had no to very limited effect on the nuclear transition for IND and BNF, respectively, supporting the results from the basal shuttling that these amino acids do not contribute to NLS function (Fig. 3a–e). Interestingly, independent of import rate, nuclear accumulation can take place of

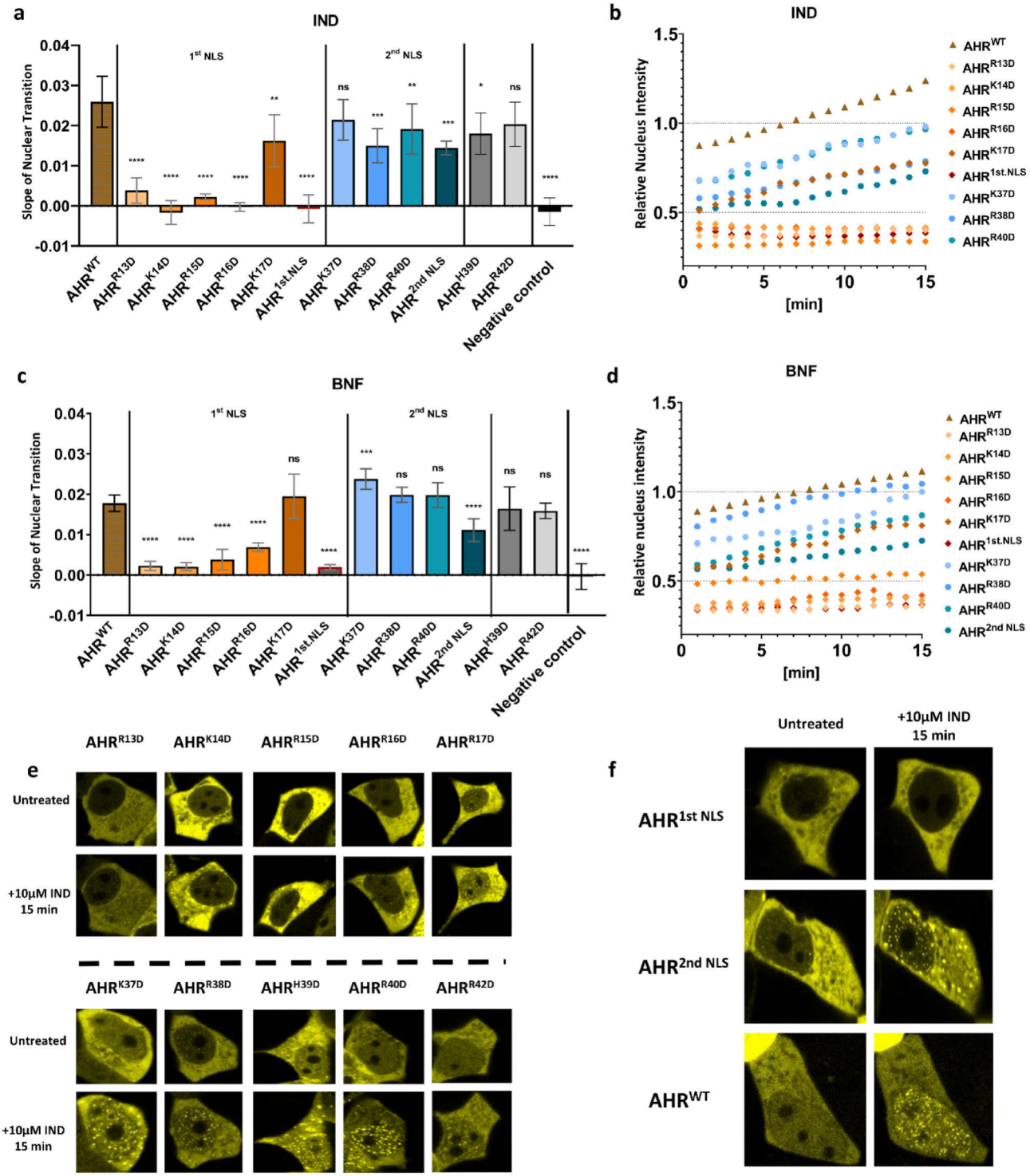


Figure 3. The nuclear entry of AHR depends primarily on the first part of nuclear localization signal (NLS). Slopes of the nuclear transition of EYFP-AHR^{WT} or EYFP-AHR^{mut} after treatment with 10 μM indirubin (IND) (**a**) or 10 μM β-naphthoflavone (BNF) (**c**) for 15 min. Negative control refers to untreated sample transfected with AHR^{WT}. Mean of time-lapse measurements after stimulation with 10 μM IND (**b**) or 10 μM BNF (**d**) for 15 min. Representative images of 1st and 2nd NLS point mutants before and after treatment with IND (**e**). Snapshots of AHR^{WT}, AHR$^{1st.NLS}$ and AHR$^{2nd.NLS}$ before and after treatment with IND (**f**). Data show the mean ± S.D of n = 12 for AHR^{WT}, n = 5 for AHR^{mut} and NC (**a**–**c**). Statistical analysis was performed with a one-way ANOVA, Dunnett's post-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

ligand activated EYFP-AHR^{WT} or EYFP-AHR^{mut}, leading to the formation of protein clusters within the nucleus (Fig. 3e, f and supplementary Fig. S3).

IPZ and IVM reduce nuclear accumulation of AHR

Taken together, the results of basal and ligand-dependent nuclear import for the individual amino acids of the NLS indicate different roles for the two parts of the NLS and we hypothesized a difference in import mechanism for unliganded and ligand-bound AHR. We next intended to study the influence of importin specific inhibitors IPZ and IVM on the nucleo-cytoplasmic translocation of AHR.

MCF-7$^{\Delta AHR}$ cells transiently transfected with EYFP-AHR^{WT} were incubated either with IPZ or IVM for 90 min. Image analysis elicited a significant reduction of the nuclear EYFP-AHR^{WT} staining. This was also accompanied with an increase of cytoplasmic EYFP-AHR^{WT} in studied cells. Therefore, the relative nuclear intensity diminished to 80% during this treatment (Fig. 4a–c and Supplementary Fig. S9).

Further analysis examined the influence of IPZ and IVM on the ligand-induced nuclear import of AHR. In response to IND stimulation, EYFP-AHR^{WT} live tracking exhibited remarkable deceleration of around 50% of nuclear import for both IPZ and IVM compared with the control (Fig. 4d,e). Taken together, these results indicate that basal and ligand-induced nuclear import are both affected by application of both import inhibitors.

To evaluate whether suppressing the AHR nuclear entry will influence AHR activity, we determined the functional output of the AHR after ligand stimulation either with or without preincubation with import inhibitors.

Compared to ligand stimulated control without import inhibitors, IVM treatment resulted in a notable decrease of *CYP1A1* mRNA levels and a slight decrease of *CYP1B1* mRNA expression (Fig. 4f). On the other hand, IPZ resulted in a significant decrease in the expression of both *CYP1A1* and *CYP1B1* (Fig. 4f).

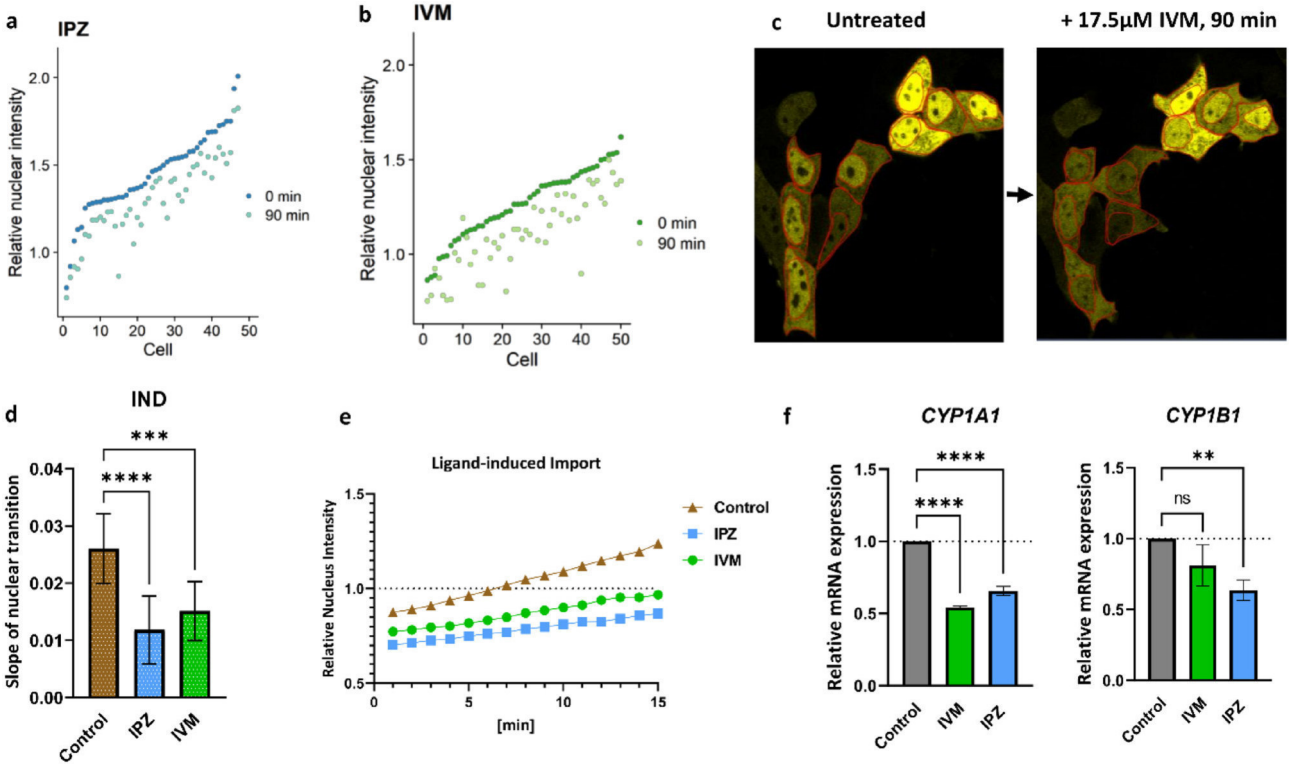


Figure 4. The inhibitors of importin (IMP) α/β mediated import importazole (IPZ) and ivermectin (IVM) reduce the nuclear accumulation of AHR. MCF-7$^{\Delta AHR}$ cells were transfected with EYFP-AHR^{WT} and treated with IPZ or IVM for 90 min at concentrations of 10 µM and 17.5 µM, respectively. Relative nuclear intensity of around 50 cells (sorted according to 0 min value) at the time of treatment (0 min) and after 90 min of treatment with IPZ (**a**) and IVM (**b**), respectively. Representative images of cells before and after incubation with 17.5 µM IVM for 90 min (**c**). Slopes of nuclear transition (**d**) and mean of time-lapse measurements (**e**) after incubation with 10 µM IPZ or 7.5 µM IVM for 22 h followed by co-treatment with 10 µM indirubin (IND) for 15 min. Data is expressed as mean + /− S.D. for 12 cells. Control implies to cells treated with ligand only. One-way ANOVA, Dunnett's post-test, ***$p < 0.001$, ****$p < 0.0001$. MCF-7^{WT} cells were treated with IPZ or IVM for 24 h at concentrations of 10 µM and 7.5 µM, respectively. Thereafter, cells were co-treated with 2.5 µM IND for 2 h. Relative *CYP1A1* and *CYP1B1* mRNA levels determined by qPCR (**f**). Values were standardized against HPRT and normalized to a sample treated with DMSO and ligand (mean + /− S.D.; one-way ANOVA, Dunnett's post-test, **$p < 0.01$, ****$p < 0.0001$).

Discussion

For import and export through the nuclear envelope, proteins larger than 40 kDa require specific signals in the form of amino acid sequences[42]. These signals permit recognition by nuclear transporters like the IMPα/β1 heterodimer for nuclear import or CRM1 for nuclear export. AHR possesses NLS and NES clusters, which unsurprisingly results in the ability of dynamic shuttling. Certainly, in the literature AHR is repeatedly defined as a cytoplasmic protein, while the nuclear entry requests ligand binding that induces a conformational change unmasking the NLS[43,44,45]. This theory ignores the nuclear retention of unliganded receptor detected in various in vitro systems confirming the existence of basal shuttling. Additionally, a nuclear trapped AHR shares a similar ability to bind ligands and induce *CYP1A1* expression in comparison to wild type AHR[29]. From our point of view, a mainly cytoplasmic localization could be attributed to high export efficiency or impeded import in individual cells. One popular theory links the basal nuclear accumulation of AHR with the presence of endogenous ligands in cell culture medium[46]. Nevertheless, a study of our lab showed that a part of ligand binding domain of AHR, namely the amino acid H291, is essential to bind endogenous ligands but is not mandatory for the nucleo-cytoplasmic shuttling[47]

Our first results confirmed that AHR subcellular localization of endogenous or overexpressed AHR varies between neighboring cells. From a technical aspect, cells in 2D cell culture are normally not synchronized and follow their own cell cycle progression. This offers the possibility that the noticed receptor staining might be related to various cell cycle stages. Especially since the cell cycle influences many physiological processes. In fact, in MCF-7 cells it has been shown that AHR physically interacts with proteins implicated in the cell cycle, like cyclin-dependent kinase 4 (CDK4)[48] or the inhibitor of CDK2, p27[49]. More interesting, knocking out p27 resulted in increasing level of nuclear AHR[50]. In comparison to our results, it is interesting to note that the increase of detected cells in G1 phase in AHR positive cells might be related to AHR function. However, transfecting the MCF-7 cells with EYFP leads to a similar increase in the G1 phase, referring to a transfection-related effect on the cell cycle in general. Concisely, in our experiments typical distribution of AHR has been observed independent of cell cycle, expression level, or fusion with EYFP. Based on these data, we could make sure that we our system is suitable to study AHR localization.

In a previous study of our lab, we examined the DNA binding motif which has one overlapping amino acid with the 2nd NLS (EYFP-AHR^{R40D}). This substitution impairs DNA binding and basal import, thereby changing the intracellular distribution[28]. Interestingly, this point mutation does not interfere with the ligand-induced import, which led us to hypothesize that two different import mechanisms for the AHR nuclear entry exist[28].

Initially, Ikuta and co-workers identified the minimal NLS of AHR as $_{13}$RKRR$_{16}$ and $_{37}$KRH$_{39}$ by analyzing the intracellular distribution[12]. Our results expand the NLS sequence to include Arg17 along with Arg40 considering the localization of unliganded AHR. At the same time, His39 and Arg42 do not seem to influence the nuclear import in general. Technically, the methods used about 2 decades ago covered only short AHR variants involving the NLS and neighboring amino acids[12,30]. But this is not sufficient for full characterization of the AHR, since the C-terminus has an effect on its translocalization[31]. In particular, our experiments highlight a central role of the 1st NLS for the basal nuclear accumulation and the ligand-induced import of the receptor. Point mutations in the 2nd NLS lead to predominant cytoplasmic AHR staining, pointing to a diminished basal import, with only a slight reduction of the ligand-induced import.

Nuclear import can occur through IMPβ1 alone or with help of the adaptor protein IMPα[51,52]. According to Petrulis et al., murine AHR can be bound by IMPβ1 directly[18]. In combination with the apparently differing mechanisms for basal and ligand-induced import, we wanted to elucidate whether IMPα is mandatory for AHR translocation. IVM and IPZ are both specific inhibitors for IMP mediated import. IVM impedes binding between IMPα and NLS, thereby disrupting IMPα/β1 mediated transport[38,39]. IPZ disrupts IMPβ1 RanGTP binding, through which all IMPβ1 associated imports are inhibited[40]. Both inhibitors clearly reduce the basal nuclear accumulation of EYFP-AHR and diminish the ligand-induced import rate indicating similar mechanisms for both import processes.

According to our results, we suggest the following hypothesis: In the unliganded state, blocking of both NLS parts is only partial, thereby allowing IMPα binding only at a diminished rate. The part of the AHR chaperone complex that is responsible for the shielding still needs to be determined. After ligand binding, the conformational change facilitates rapid IMPα recruitment without impediments. Interestingly, ligand-induced import is mainly implemented through the 1st NLS. The 2nd NLS is complementary but not essential. In conclusion, we show that ligand-independent nuclear import is mediated by the same molecular mechanism as the ligand-dependent import (Fig. 5).

Methods

Plasmids

The plasmid pEYFP-AHR-C1 has been used previously[28] and encodes the human AHR starting with the amino acid alanine from position 11. GenScript (GenScript Biotech, Leiden, Netherlands) generated the modified variants of pEYFP-AHR that contain mutations at specific amino acids AHR^{R13D}, AHR^{K14D}, AHR^{R15D}, AHR^{R16D}, AHR^{K17D}, AHR$^{1st.NLS}$, AHR^{K37D}, AHR^{R38D}, AHR^{H39D}, AHR^{R40D}, AHR^{R42D} and AHR$^{2nd.NLS.}$). AHR^{WT} and AHR^{mut} have been validated by sequencing (Eurofins, Germany).

Cell culture

MCF-7 cell line was purchased from ATCC (Manassas, VA, USA). AHR knockout MCF-7 cells (MCF-7$^{\Delta AHR}$) were generated in our lab using a CRISPR-Cas9 based approach[41]. Both cell lines were cultured in DMEM supplemented with 10% (v/v) FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. All cell culture media and supplements were bought from Pan-Biotech (Aidenbach, Germany). Cells were maintained

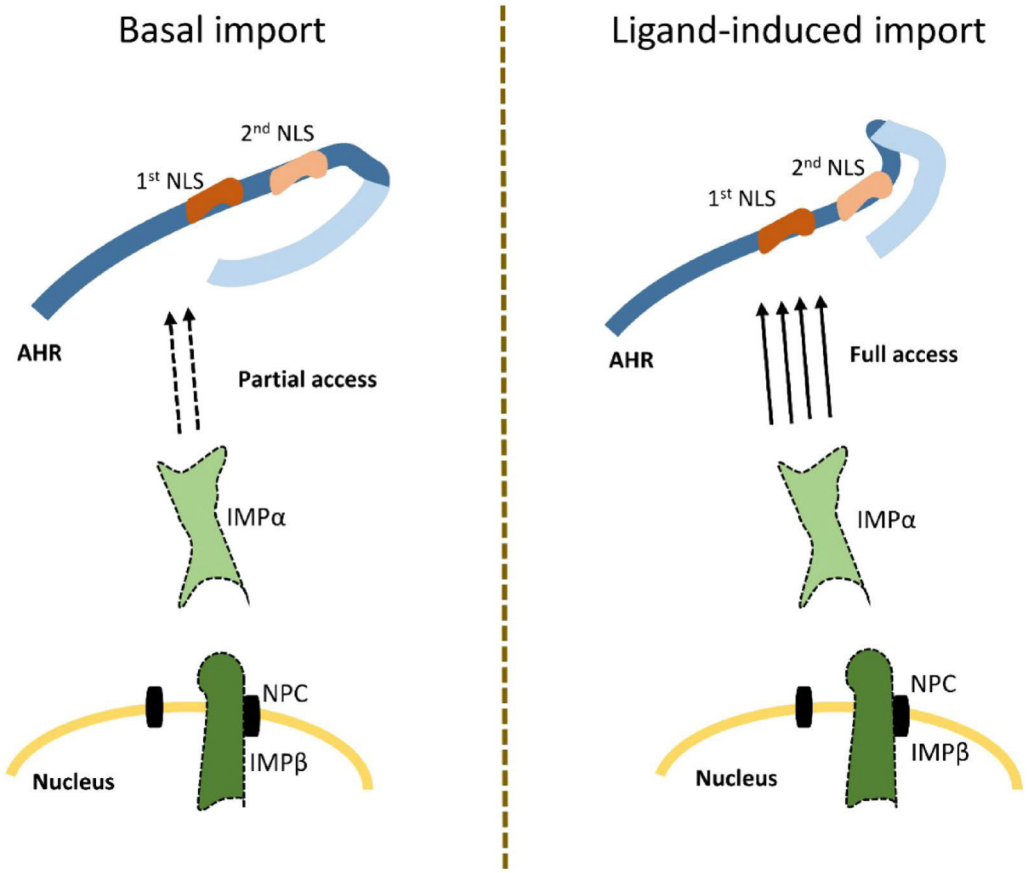


Figure 5. Nuclear import of AHR is mediated by importin (IMP) α/β1. The basal import occurs at a diminished rate because of a partial blocking of the 1st and 2nd nuclear localisation signal (NLS) through the c-terminal part of AHR or parts of the chaperon complex. Upon ligand binding, a conformational change grants full access for IMPα to the 1st NLS, thereby increasing import rate drastically. NPC, nuclear pore complex.

in 5% CO_2 at 37 °C and humidified atmosphere. Cells were routinely inspected for absence of mycoplasma contamination.

Reagents

B-naphthoflavone (BNF), dimethyl sulfoxide (DMSO) and ivermectin (IVM) were acquired from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Munich, Germany). Importazole (IPZ) was obtained from BIOZOL (Biozol Diagnostica Vertrieb GmbH, Eching, Germany). Indirubin (IND) was purchased from Enzo Life Sciences (Enzo Life Sciences GmbH, Lörrach, Germany). Leptomycin B (LMB) was deliviered from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., TX, USA). DMSO was used as vehicle for BNF, IND, IPZ and IVM, while ethanol (Carl Roth, Germany) was used as a vehicle for LMB. The end-concentration does not exceed 0.1% when using DMSO or ethanol. All chemicals were ordered at the highest available purity.

Transient transfection

MCF-7 or MCF-$7^{\Delta AHR}$ cells were seeded either on 6-well plates (both from Techno Plastic Products AG, Trasadingen, Switzerland) or on glass bottom dishes (In VitroScientific, Sunyvale, CA, USA). After 24 h, cells on multiwell plates were transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and an appropriate DNA amount as indicated by the manufacturer's instructions. Meanwhile, cells on glass bottom dishes were transfected with Xfect (Takara Bio Europe SAS, Saint-Germain-en-Laye, France) and an appropriate DNA amount as stated by the manufacturer's instructions. In both cases, transfection medium was refreshed after incubation for 4 h.

Cell treatment with import inhibitors

To investigate the influence of import inhibitors on the basal localization of AHR, EYFP-AHR^{WT} transiently transfected MCF-$7^{\Delta AHR}$ cells were incubated with IPZ or IVM at concentration of 10 µM and 17.5 µM, respectively, for 90 min. To study the impact on ligand induced import, cells were incubated with 10 µM IPZ or 7.5 µM IVM after medium change after transfection for nearly 22 h and then co-treated with 10 µM IND for 15 min. To measure the effect of import inhibitors on the transcriptional activity of AHR, total RNA levels of MCF-7^{WT} cells were gathered after incubating the cells for 22 h with either IPZ or IVM at concentration of 10 µM and 7.5 µM, respectively, and afterwards stimulated with 2.5 µM IND for 2 h.

Gene	Forward primer	Reverse primer
CYP1A1	5′-CCAAGAGTCCACCCTTCCCAGCT-3′	5′-GAGGCCAGAAGAAACTCCGTGGC-3′
CYP1B1	5′-TGGATTTGGAGAACGTACCG-3′	5′-CCACGACCTGATCCAATTCT-3’
HPRT	5′-GTTCTGTGGCCATCTGCTTAG-3′	5′-GCCCAAAGGGAACTGATAGTC-3′

Table 1. Primer sequences used.

Immunofluorescence staining

Cover glasses were coated with Poly-L-Lysin 0.01% solution (Sigma-Aldrich, Germany) according to the manufacturer's instructions. Cells were grown on cover glasses with a cell density of 2.5×10^5/ml for 48 h and then fixed in 3.7% formaldehyde (15 min), permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, Germany) in PBS (10 min). Finally, cells were blocked with 5% fetal bovine serum (FBS) in PBS for 1 h at room temperature. After blocking, the coverslips were incubated with primary antibody for 90 min, washed and then incubated with the secondary antibody for 1 h. The antibodies were diluted in 1.5% FBS in PBS solution. Slides were mounted in Vectashield HardSet Mounting Medium with DAPI (VECTOR LABORATORIES, INC., Burlingame, CA, USA). The used primary antibody is anti-AHR (sc-5579, Santa Cruz Biotechnology, 1:25) and secondary antibody is goat anti rabbit, Alexa Fluor™ 594 (A-11012, 1:400), purchased from Invitrogen, Thermo Fisher Scientific, Germany.

Cell cycle analysis by flow cytometry

Cells grown on glass bottom dishes were transfected using Xfect as described above. After 24 h, cells were detached from culture flasks by using trypsin (0.5%-EDTA 0.2%; Pan-Biotech (Aidenbach, Germany)) solution for 20 min. After cell fixation with ice cold 80% ethanol for 1 h, cells were washed with PBS and stained with propidium iodide (PI)/RNAse solution (Thermo Fisher Scientific, Germany) according to the manufacturer's protocol. Stained cells were analyzed by fluorescence-activated cell-sorting (FACS) (BD FACSAria III). For data analysis, FlowJo (V.10.7.1, BD Biosciences) was used. For analysis, samples were analyzed to discriminate doublets (FSC-A versus FSC-W). Cell cycle analysis was performed using the PI-A intensity on a linear scale of either AHR-YFP-positive or -negative cells. To determine the intensity of G2-phase and verify cell cycle analysis, nocodazole (0.6 µg/ml; Sigma-Aldrich, Germany) synchronized cells were analyzed as positive control.

Gene expression analysis

Total RNA was isolated form cells using RNeasy Midi kit and QIAshredder (both from QIAGEN GmbH, Hilden, Germany). Thereafter, purity and concentration of isolated RNA were determined using a plate reader device (Infinite M200 PRO, Tecan Trading AG, Männedorf, Switzerland). 1 µg or 500 ng of the extracted RNA was reversely transcribed into cDNA by using the high-capacity cDNA Reverse transcription kit (Applied Biosystems, Foster City, CA USA).

Transcript levels were determined with fast SYBR Green mix (Applied Biosystems, Foster City, CA, USA) and quantitative PCR (qPCR) device 7500 Fast Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA). Hypoxanthine–guanine phosphoribosyl transferase (*HPRT*) was used as reference gene.

The primer sequences are listed in Table 1.

On-line confocal microscopy

As described in[28], for cell monitoring the confocal microscope LSM 700 (Carl Zeiss Jena GmbH, Jena, Germany) was used. During the measurement of living cells, suitable cell culture conditions like 5% CO_2 and 37 °C were maintained. The relative nuclear intensity refers to the mean fluorescence intensity of the nucleus divided by the mean fluorescence intensity of the whole cell. This was determined by outlining the nucleus and whole cells as region of interest (ROI) by using ZEN 2012 blue edition (Carl Zeiss Jena GmbH). The slope of nuclear transition was measured after plotting the relative nucleus intensity against time of the treatment. Representative examples of the analysis of AHR localization, time-lapse measurements and relative nuclear intensity are in (supplementary Figs. S1, S4 and S7).

Statistics

Data analysis and graphing were performed with GraphPad Prism (Graph Pad, La Jolla, CA, USA) and R-Studio (R-Tools Technology Inc, Canada). Statistical analysis was done using paired Dunnett's multiple comparisons test, one-way ANOVA, $^{*}p < 0.01$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Received: 23 May 2023; Accepted: 8 November 2023
Published online: 11 November 2023

References

1. Beischlag, T. V., Luis Morales, J., Hollingshead, B. D. & Perdew, G. H. The aryl hydrocarbon receptor complex and the control of gene expression. *Crit. Rev. Eukaryot. Gene Expr.* **18**(3), 207–250 (2008).

2. Luch, A. Nature and nurture: Lessons from chemical carcinogenesis. *Nat. Rev. Cancer* **5**(2), 113–125 (2005).
3. Zhou, X. *et al.* β-naphthoflavone activation of the Ah receptor alleviates irradiation-induced intestinal injury in mice. *ANTIGE* **9**(12), 1264 (2020).
4. Nebert, D. W. *et al.* Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochem. Pharmacol.* **59**(1), 65–85 (2000).
5. Esser, C. & Rannug, A. The aryl hydrocarbon receptor in barrier organ physiology, immunology, and toxicology. *Pharmacol. Rev.* **67**(2), 259–279 (2015).
6. Nguyen, L. P. & Bradfield, C. A. The search for endogenous activators of the aryl hydrocarbon receptor. *Chem. Res. Toxicol.* **21**(1), 102–116 (2008).
7. Wei, G. Z. *et al.* Tryptophan-metabolizing gut microbes regulate adult neurogenesis via the aryl hydrocarbon receptor. *Proc. Natl. Acad. Sci. U.S.A.* **118**(27), e2021091118 (2021).
8. Meijer, L., Guyard, N., Skaltousnis, L. A. & Eisenbrand, G. *Indirubin, the Red Shade of Indigo* (Life in Progress Edition, 2006).
9. Qi, T., Li, H. & Li, S. Indirubin improves antioxidant and anti-inflammatory functions in lipopolysaccharide-challenged mice. *Oncotarget* **8**(22), 36658–36663 (2017).
10. Tappenden, D. M., Hwang, H. J., Yang, L., Thomas, R. S. & LaPres, J. J. The Aryl-hydrocarbon receptor protein interaction network (AHR-PIN) as identified by tandem affinity purification (TAP) and mass spectrometry. *J. Toxicol.* **2013**, 279829 (2013).
11. Denison, M. S., Soshilov, A. A., He, G., DeGroot, D. E. & Zhao, B. Exactly the same but different: Promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol. Sci.* **124**(1), 1–22 (2011).
12. Ikuta, T., Eguchi, H., Tachibana, T., Yoneda, Y. & Kawajiri, K. Nuclear localization and export signals of the human aryl hydrocarbon receptor. *J. Biol. Chem.* **273**(5), 2895–2904 (1998).
13. Greb-Markiewicz, B. & Kolonko, M. Subcellular localization signals of bHLH-PAS proteins: Their significance, current state of knowledge and future perspectives. *Int. J. Mol. Sci.* **20**(19), 4746 (2019).
14. Perdew, G. H. Association of the Ah receptor with the 90-kDa heat shock protein. *J. Biol. Chem.* **263**(27), 13802–13805 (1988).
15. Kazlauskas, A., Poellinger, L. & Pongratz, I. Evidence that the co-chaperone p23 regulates ligand responsiveness of the dioxin (Aryl hydrocarbon) receptor. *J. Biol. Chem.* **274**(19), 13519–13524 (1999).
16. Meyer, B. K., Pray-Grant, M. G., Vanden Heuvel, J. P. & Perdew, G. H. Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. *Mol. Cell. Biol.* **18**(2), 978–988 (1998).
17. Stevens, E. A., Mezrich, J. D. & Bradfield, C. A. The aryl hydrocarbon receptor: a perspective on potential roles in the immune system. *Immunology.* **127**(3), 299–311 (2009).
18. Petrulis, J. R., Kusnadi, A., Ramadoss, P., Hollingshead, B. & Perdew, G. H. The hsp90 co-chaperone XAP2 alters Importin β recognition of the bipartite nuclear localization signal of the Ah receptor and represses transcriptional activity. *J. Biol. Chem.* **278**(4), 2677–2685 (2003).
19. Ikuta, T., Kobayashi, Y. & Kawajiri, K. Phosphorylation of nuclear localization signal inhibits the ligand-dependent nuclear import of aryl hydrocarbon receptor. *Biochem. Biophys. Res. Commun.* **317**(2), 545–550 (2004).
20. Kosyna, F. K. & Depping, R. Controlling the gatekeeper: Therapeutic targeting of nuclear transport. *Cells* **7**(11), 221 (2018).
21. Tsuji, N. *et al.* The activation mechanism of the aryl hydrocarbon receptor (AhR) by molecular chaperone HSP90. *FEBS Open Bio.* **4**, 796–803 (2014).
22. Barouki, R., Aggerbeck, M., Aggerbeck, L. & Coumoul, X. The aryl hydrocarbon receptor system. *Drug. Metabol. Drug. Interact.* **27**(1), 3–8 (2012).
23. Schulte, K. W., Green, E., Wilz, A., Platten, M. & Daumke, O. Structural basis for aryl hydrocarbon receptor-mediated gene activation. *Structure* **25**(7), 1025-1033.e3 (2017).
24. Hestermann, E. V. & Brown, M. Agonist and chemopreventative ligands induce differential transcriptional cofactor recruitment by aryl hydrocarbon receptor. *Mol. Cell. Biol.* **23**(21), 7920–7925 (2003).
25. Swanson, H. I., Chan, W. K. & Bradfield, C. A. DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins. *J. Biol. Chem.* **270**(44), 26292–26302 (1995).
26. Davarinos, N. A. & Pollenz, R. S. Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytosplasmic proteasome following nuclear export. *J. Biol. Chem.* **274**(40), 28708–28715 (1999).
27. Marlowe, J., & Puga, A., Novel AHR Interactions, in *Comprehensive Toxicology (Second Edition)*. 93–115 (Elsevier. 2010)
28. Haidar, R. *et al.* The role of DNA-binding and ARNT dimerization on the nucleo-cytoplasmic translocation of the aryl hydrocarbon receptor. *Sci. Rep.* **11**(1), 18194 (2021).
29. Richter, C. A., Tillitt, D. E. & Hannink, M. Regulation of subcellular localization of the aryl hydrocarbon receptor (AhR). *Arch. Biochem. Biophys.* **389**(2), 207–217 (2001).
30. Ikuta, T. *et al.* Nucleocytoplasmic shuttling of the aryl hydrocarbon receptor. *J. Biochem.* **127**(3), 503–509 (2000).
31. Tkachenko, A. *et al.* The Q-rich/PST domain of the AHR regulates both ligand-induced nuclear transport and nucleocytoplasmic shuttling. *Sci. Rep.* **6**, 32009 (2016).
32. Heid, S. E., Pollenz, R. S. & Swanson, H. I. Role of heat shock protein 90 dissociation in mediating agonist-induced activation of the aryl hydrocarbon receptor. *Mol. Pharmacol.* **57**(1), 82–92 (2000).
33. Jans, D. A., Martin, A. J. & Wagstaff, K. M. Inhibitors of nuclear transport. *Curr. Opin. Cell. Biol.* **58**, 50–60 (2019).
34. Kuusisto, H. V. & Jans, D. A. Hyper-dependence of breast cancer cell types on the nuclear transporter Importin β1. *Biochim. Biophys. Acta Mol. Cell Res.* **1853**(8), 1870–1878 (2015).
35. Tay, M. Y. F. *et al.* Nuclear localization of dengue virus (DENV) 1–4 non-structural protein 5; protection against all 4 DENV serotypes by the inhibitor Ivermectin. *Antiviral Res.* **99**(3), 301–306 (2013).
36. Götz, V. *et al.* Influenza A viruses escape from MxA restriction at the expense of efficient nuclear vRNP import. *Sci. Rep.* **6**(1), 23138 (2016).
37. Kosyna, F. K., Nagel, M., Kluxen, L., Kraushaar, K. & Depping, R. The importin α/β-specific inhibitor ivermectin affects HIF-dependent hypoxia response pathways. *Biol. Chem.* **396**(12), 1357–1367 (2015).
38. Wagstaff, K. M., Rawlinson, S. M., Hearps, A. C. & Jans, D. A. An AlphaScreen®-based assay for high-throughput screening for specific inhibitors of nuclear import. *J. Biomol. Screen.* **16**(2), 192–200 (2011).
39. Wagstaff, K. M., Sivakumaran, H., Heaton, S. M., Harrich, D. & Jans, D. A. Ivermectin is a specific inhibitor of importin α/β-mediated nuclear import able to inhibit replication of HIV-1 and dengue virus. *Biochem. J.* **443**(3), 851–856 (2012).
40. Soderholm, J. F. *et al.* Importazole, a small molecule inhibitor of the transport receptor importin-β. *ACS Chem. Biol.* **6**(7), 700–708 (2011).
41. Tarnow, P. *et al.* Characterization of Quinoline yellow dyes as transient aryl hydrocarbon receptor agonists. *Chem. Res. Toxicol.* **33**(3), 742–750 (2020).
42. Cautain, B., Hill, R., de Pedro, N. & Link, W. Components and regulation of nuclear transport processes. *Febs j.* **282**(3), 445–462 (2015).
43. Choudhary, M. & Malek, G. The aryl hydrocarbon receptor: a mediator and potential therapeutic target for ocular and non-ocular neurodegenerative diseases. *Int. J. Mol. Sci.* **21**(18), 6777 (2020).
44. Guerrina, N., Traboulsi, H., Eidelman, D. H. & Baglole, C. J. The aryl hydrocarbon receptor and the maintenance of lung health. *Int. J. Mol. Sci.* **19**(12), 3882 (2018).

45. Shivanna, B., Chu, C. & Moorthy, B. The aryl hydrocarbon receptor (AHR): A novel therapeutic target for pulmonary diseases?. *Int. J. Mol. Sci.* **23**(3), 1516 (2022).
46. Veldhoen, M., Hirota, K., Christensen, J., O'Garra, A. & Stockinger, B. Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. *J. Exp. Med.* **206**(1), 43–49 (2009).
47. Tkachenko, A. *et al.* Nuclear transport of the human aryl hydrocarbon receptor and subsequent gene induction relies on its residue histidine 291. *Arch. Toxicol.* **92**(3), 1151–1160 (2018).
48. Barhoover, M. A., Hall, J. M., Greenlee, W. F. & Thomas, R. S. Aryl hydrocarbon receptor regulates cell cycle progression in human breast cancer cells via a functional interaction with cyclin-dependent kinase 4. *Mol. Pharmacol.* **77**(2), 195–201 (2010).
49. Kolluri, S. K., Weiss, C., Koff, A. & Göttlicher, M. p27(Kip1) induction and inhibition of proliferation by the intracellular Ah receptor in developing thymus and hepatoma cells. *Genes Dev.* **13**(13), 1742–1753 (1999).
50. Elson, D. J. *et al.* The cyclin-dependent kinase inhibitor p27(Kip1) interacts with the aryl hydrocarbon receptor and negatively regulates its transcriptional activity. *FEBS Lett.* **596**(16), 2056–2071 (2022).
51. Lu, J. *et al.* Types of nuclear localization signals and mechanisms of protein import into the nucleus. *Cell Commun. Signal.* **19**(1), 60 (2021).
52. Palmeri, D. & Malim, M. H. Importin beta can mediate the nuclear import of an arginine-rich nuclear localization signal in the absence of importin alpha. *Mol Cell Biol.* **19**(2), 1218–1225 (1999).

Author contributions

R.H., J.K. designed the experimental program. R.H. performed the experimental work, R.S. contributed to the confocal microscopy and J.K., M.M. planned and contributed to the FACS analysis. Data analysis and figures creation were done by R.H. and J.K. Manuscript writing was done by R.H. with contributions from A.L. and J.K. The final version was approved by all authors.

Funding

Open Access funding enabled and organized by Projekt DEAL.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-023-47066-z.

Correspondence and requests for materials should be addressed to R.H.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

4 Discussion

It is notable that the research on the AHR is still expanding, even with the comprehensive description of its role in xenobiotic-metabolism. This certainly resulted from the involvement in various physiological processes, like mediating the degradation of sex steroid receptors (Ohtake *et al.*, 2007) (Wormke *et al.*, 2003) or regulating immune responses (Gutiérrez-Vázquez & Quintana, 2018). Recently, AHR gained more attention due to association with host-microbiome interaction (Perdew *et al.*, 2023) and SARS-CoV-2 pathology (Turski *et al.*, 2020). Beyond all, current research on the AHR seeks to discover novel natural ligands or relations to other signaling pathways. At the same time, localization of the AHR is still not fully understood due to many unanswered questions regarding the basal import. In this context, analyzing the translocation helps understanding an important step in the signaling pathway of the AHR and empowering research towards potential therapeutic applications.

In the current work, I aimed to achieve a better understanding of the AHR translocation. Thus, a fluorescent-labelled AHR was utilized. This allowed real-time tracking in living cells in both the basal state or after treatment with different substances. In contrast to the majority of data published in this field, I used the full-length variant of hAHR. In our lab, the performed transient transfection experiments of *EYFP-AHR*^{WT} in HepG2 and MCF-7$^{\Delta AHR}$ cells did not show only predominant cytoplasmic AHR, which contradicts the classic description of the AHR as a cytoplasmic receptor. Nonetheless, snapshots of the AHR in living cells demonstrate the intracellular distribution to be one of three states: predominantly nuclear, predominantly cytoplasmic, or equally distributed. Notably, staining the endogenous AHR in wildtype MCF-7 cells exhibits comparable localization with the overexpressed EYFP-AHR^{WT}. In fact, the three mentioned states of localization were observed in neighbored cells similar to the overexpressed AHR. This means that our established system recapitulates the localization of the endogenous AHR.

It might come to mind, that the three distribution states are linked to different cell cycle phases, especially since it has been shown that the AHR interacts physically with proteins involved in the cell cycle, like cyclin-dependent kinase 4 (Barhoover *et al.*, 2010). In my experiments, the utilized cells are not synchronized and follow their own cell cycle progression. To verify that this does not influence the AHR localization, I analyzed the cell cycle stages of MCF-7 cells after transfection with *EYFP-AHR*^{WT} or *EYFP* alone as control plasmid. My results showed that the transfection itself stops the progression of cell cycle in the G1 phase (Haidar *et al.*, 2023). Hence, the EYFP-AHR positive cells share the same cell cycle stage.

Furthermore, observing nuclear AHR in the basal state might be linked to the presence of endogenous ligands (Murray *et al.*, 2014). Cell culture medium presents a possible source for endogenous ligands. This idea is supported by a study that detected IND in the fetal bovine serum (FBS). FBS is a widely used growth supplement in cell culture that improves cell proliferation. Notably, the concentration of IND

in FBS is enough to activate the AHR (Adachi *et al.*, 2001). Nevertheless, the results of many studies contradict a possible correlation between the AHR localization and the presence of endogenous ligands. In a previous study of our lab, mutational analysis in the ligand binding domain of AHR identified His291 as a crucial amino acid for AHR activation through the endogenous ligands IND, Kyn, and FICZ. A substitution of His with Ala on the position 291 generates an AHR variant that cannot be imported into the nucleus after IND treatment. Surprisingly, this mutation, which leads to a functional deficit, does not impair the basal shuttling, since a nuclear accumulation occurs with LMB treatment (picture not shown). And overall, this means that basal shuttling and ligand induced import are independent from each other (Tkachenko *et al.*, 2018).

Another source of endogenous ligands might be the media itself, but the cell culture medium used to incubate HepG2 cells, was examined particularly to reveal its content of natural AHR agonists. Indeed, this medium, namely RPMI, exhibits much less content of endogenous ligands compared to other cell culture media. Additionally, no AHR activity is detected under the influence of natural (endogenous) ligands contained in RPMI medium (Veldhoen *et al.*, 2009). Hence, there should be no risk of endogenous ligands altering localization and functional behavior of the AHR, when incubating the cells in RPMI medium. In the experiments I performed, even with RPMI, the basal AHR^{WT} was detected in the nucleus in almost half of the analyzed cells. More interesting, similar intracellular distribution of the AHR^{WT} was observed when investigating MCF-7 cells, which are cultured in different medium. It is also noteworthy, that the AHR localization changes to be completely nuclear, after treatment with ligands. This further points to the notion that endogenous ligands in the cell culture medium are not the reason for the basal nuclear accumulation of the AHR.

Additionally, in the literature, localization of the AHR in the basal state was linked to interactions with XAP2 in the cytoplasmic chaperon complex. First, XAP2 was supposed to stabilize the AHR in the cytoplasm. In particular, the localization of a short variant of the AHR comprising PAS and TAD domains was investigated in monkey kidney epithelial cells (COS-1 cells). Co-expression with XAP2 increased the levels of cytosolic AHR (LaPres *et al.*, 2000). Examining the same cell line in a different lab revealed that overexpression of XAP2 can shift the localization of full-length mouse AHR to the cytoplasm as well. This cytoplasmic localization was not affected by inhibiting the nuclear export with LMB, which gives a hint at an impaired basal import of the AHR (Petrulis *et al.*, 2003). Further investigation supposed that the correlation between XAP2 and the AHR localization is specific to the mouse AHR (Ramadoss *et al.*, 2004). At the end, the study of Pollenz *et al.,* 2006 diminished all the potential of XAP2 as a regulator of the AHR localization. They demonstrated that neither overexpression nor downregulation of endogenous XAP2 can influence the subcellular distribution of AHR (Pollenz *et al.*, 2006). Thus, the role of the chaperone complex in regulating the localization stays unresolved.

Up to this point, the three distribution states of AHR localization are neither result from endogenous ligands, nor from interaction with XAP2 in the chaperon complex. A possible clarification for the intracellular distribution might rely on the nucleo-cytoplasmic shuttling, which is supported in the present work.

Originally, the nucleo-cytoplasmic shuttling of AHR was characterized *in vitro* with help of LMB as a specific inhibitor of the nuclear export. Treating cells with LMB alone is sufficient for AHR to accumulate in the nucleus, indicating a continuous shuttling of the AHR (Tkachenko *et al.*, 2016). In a different study, cells are incubated with LMB at first to promote a nuclear accumulation of the AHR and then co-treated with TCDD. This treatment regime increases the AHR activity compared to TCDD only treated control significantly. Additionally, this observation implies that TCDD is also able to reach the nuclear AHR (Pollenz & Barbour, 2000). From a logical point of view, the protective role of the AHR should not only be limited to its cytoplasmic localization and shuttling might aim to maintain this protective role in the entire cell. However, this theory was neither tested nor verified in the current thesis and would request further verification of ligands entering the nucleus where they bind and activate the AHR.

In my published work, shuttling of the AHR is further characterized but for the first time by using import inhibitors instead of LMB. The application of import inhibitors was not possible in earlier studies, since their characterization was carried out only a few years ago (Kosyna & Depping, 2018). Again, the shuttling of AHR is proven as a permanent process in the basal state since both import inhibitors IVM and IPZ increase the cytoplasmic and reduce the nuclear localization (Haidar *et al.*, 2023).

In order to examine all possible states of the AHR localization, the present thesis sought to clarify all processes that stabilize the AHR in the nucleus after ligand binding. The molecular interactions performed by the AHR after ligand activation might stabilize the nuclear localization. Logically, these interactions are based on a strong binding with the nuclear located components, DNA and ARNT. Previous studies gave several supporting information, (1) a previous study found that a functional ARNT is crucial for the induction of a ligand-dependent AHR degradation occurring in the cytoplasm after AHR activation (Q. Ma & Baldwin, 2000). (2) In another study, a point mutation on ARNT reduced the degradation rate of the AHR (Numayama-Tsuruta *et al.*, 1997). (3) More important, the cellular availability of ARNT can direct AHR to work as a transcription factor or serve as an E3 ubiquitin ligase (Luecke-Johansson *et al.*, 2017). Thus, it is not clear how the AHR localization can be influenced when ARNT dimerization is not possible.

Therefore, I decided to study the influence of ARNT dimerization and DNA binding on the nucleo-cytoplasmic translocation. Interestingly, the responsible region in the AHR to interact with ARNT and DNA is relevant to the regulatory motives in localization as well. Indeed, the AHR dimerizes with ARNT through amino acids located before and after the NES sequence. Furthermore, the AHR binds the XRE

through three amino acids: Ser36, His39, and Arg40. His39 was identified primarily as a part of the second NLS. Likewise, Ser36, which is a protein kinase c phosphorylation site, supposed to have an impact on ligand-induced import of AHR (Ikuta *et al.*, 2004b).

Through applying point mutations on the full-length hAHR, I intended to generate variants of the AHR that cannot maintain a stable binding with ARNT, XRE, or both. The chosen amino acids for the mutational analysis are based on the crystal structure of AHR/ARNT-XRE (Seok *et al.*, 2017) (Schulte *et al.*, 2017). The AHR variants that carry XRE or ARNT binding-deficient mutations do not demonstrate impaired translocation pattern after ligand treatment, although the functional output was entirely abolished. Additionally, in the basal state AHR^{WT} shares a similar intracellular distribution with ARNT and XRE binding-deficient mutants (Haidar *et al.*, 2021). Based on these data, neither ARNT nor XRE binding are essential for the nuclear accumulation of AHR. Overall, these findings support the notion that the mechanism of stabilization of the AHR within the nucleus is similar for the ligand bound and the ligand free AHR.

Although His39 was originally considered as part of the second NLS, AHR^{H39G} unexpectedly does not show any impairment in the level of nuclear accumulation or activation pattern compared to the wildtype.

Finally, AHR^{R40D} showed a completely cytoplasm localization in the basal state, which exhibits characteristics associated to impaired NLS. In other words, Arg40, but not His39, is part of the second NLS. This guided me to examine the effect of the second NLS on translocation, especially since I proved that XRE-binding motive does not influence its localization (Haidar *et al.*, 2021).

The NLS of the AHR was previously analyzed but its characterization was performed by using a short variant of the AHR (Ikuta *et al.*, 1998). More particularly, deletion mutants from N- or C-terminus or single point mutants were examined to identify the functional parts of NLS. Briefly, GST-AHR (13–42)-GFP or its related mutants were purified and microinjected in the cytoplasm of HeLa cells. Thirty minutes post microinjection, nuclear or cytoplasmic staining of the AHR variants was assessed to classify whether the deleted or mutated amino acids have been an essential part of the NLS. According to this method, the sequence of the NLS was published as: 1^{st} part: 13–16 (RKRR) and 2^{nd} part: 37–39 (KRH) (Ikuta *et al.*, 1998). However, later research recognized the C-terminal part of the AHR as a key player in the AHR localization (Ramadoss & Perdew, 2005). More precisely, the amino acid valine on the position 647 in the Q-rich domain is determining the AHR-localization. Single point mutation on this position (AHR^{V647A}) shifts the compartmentalization of the AHR to be predominantly nuclear. More interesting, the deletion mutant ($AHR^{\Delta 647}$) is completely cytoplasmic in the basal state and does not react to ligand treatment but to the export inhibitor LMB. This points to a continuous export of $AHR^{\Delta 647}$ (Tkachenko *et al.*, 2016). These studies gave the insight on a probable interaction between the C-

terminus and the NLS to regulate AHR localization, which was, unfortunately, not considered when the NLS of the AHR was primarily identified.

In order to characterize the NLS, a series of mutants of the full length hAHR were generated. In these mutants, the positively charged amino acids in the region (13-17) and (37-42) were substituted for the negatively charged aspartic acid (Haidar *et al.*, 2023). In contravention of the studies done in this field, I examined the intracellular distribution of the basal AHR variants and their basal import. Additionally, I analyzed the activation pattern of these mutants after treatment with either BNF or IND. Overall, an impaired NLS expresses itself in a predominantly cytoplasmic localization in the absence of ligands. According to this, the two NLS segments are validated to be: ($_{13}$RKRRK$_{17}$) and ($_{37}$KR-R$_{40}$). In fact, AHR^{H39D} and AHR^{R42D} show a similar intracellular distribution like the AHR^{WT} (Haidar *et al.*, 2023).

When analyzing the NLS mutants, it was very surprising that the ligand-induced import can still occur on mutants of the second part NLS, while mutating the first NLS blocks the ligand-induced import entirely or impairs it significantly. Prior to ligand binding, mutating the first part of NLS leads to a stronger cytoplasmic localization accompanied with weak nuclear localization compared with mutations on the second part. Based on these data, we came to the result that the first part of NLS is the main regulator for the AHR nuclear import, while the second part NLS is just supportive and complementary but not crucial (Haidar *et al.*, 2023).

After characterizing the intrinsic factors responsible for AHR localization, it was required to examine the cellular components involved in nuclear entry. In this matter, it is interesting to note that only one published paper dealt with the nuclear transporters of AHR. Indeed, with the help of a thermal shift assay it was found that the AHR can bind IMPβ directly, unfortunately, a direct binding of the AHR and IMPα has not been tested (Petrulis *et al.*, 2003). Therefore, the nuclear import was supposed to be mediated by IMPβ directly and not IMPα.

To examine if the import of the AHR is mediated through IMPα generally, I used the well-characterized import inhibitors IVM and IPZ. Studies on IVM showed that IVM targets specifically the nuclear import mediated by IMPα, while IMPβ recognized cargoes are not affected (Wagstaff *et al.*, 2011) (Wagstaff *et al.*, 2012). Of note, these studies were performed on virus molecules and might not be transferable on AHR. Meanwhile, IPZ disturbs the binding between IMPβ and RanGTP, thereby inhibiting both IMPα and IMPβ mediated nuclear translocation (Soderholm *et al.*, 2011). Nevertheless, my results showed that both import inhibitors IPZ and IVM can slow down the rates of nuclear import after ligand-binding. In comparable manner, nuclear localization of the basal AHR^{WT} was significantly decreased after incubation with IPZ and IVM. Based on our data, it is more reasonable to assume that the AHR binds to IMPα to undergo a nuclear import for both basal and ligand induced import (Haidar *et al.*, 2023).

Taken together, this thesis offers comprehensive description of the intracellular distribution of the AHR and its nuclear import with a main focus on the responsible residues and the differences between basal

and ligand-induced import. An *in vitro* system is established and validated to track an overexpressed EYFP-AHR. The intracellular distribution of the basal AHR varies between neighbored cells but not due to transfection, cell cycle progression, or endogenous ligands in the cell culture medium. A permanent shuttling of the AHR causes different localization pattern between a mainly cytoplasmic and a mainly nuclear localization.

The AHR possesses a bipartite NLS, whereby the first part ($_{13}$RKRRK$_{17}$) is the main regulator and the second ($_{37}$KR-R$_{40}$) is supportive but not essential for nuclear import. In contrast to the wildtype receptor, the NLS mutated AHR is solely located in the cytoplasm prior to ligand treatment. Further, the ligand-induced import and basal import are two different processes since they can occur separately and independent from each other. However, both import processes rely on the same particular mechanism, as they cannot be diminished or decelerated separately. Finally, import inhibitors like IPZ or IVM can be utilized in order to regulate or slow down the nuclear transport of AHR after ligand-binding.

It is essential to recognize the limitations in the current work, as shuttling was only investigated in two malignant cell lines without including non-malignant cell lines from the same tissue type. In other words, it is still not clear if shuttling occurs in a tissue and cell type specific manner. In addition, evidence on nucelo-cytoplasmic shuttling occurring *in vivo* is still missing due to many technical restrictions.

Moreover, the influence of the protein chaperon complex on the AHR translocation should not be completely ignored and is still to be addressed. Indeed, a report on the HSP90 in the protein complex of glucocorticoid receptor demonstrated that the nuclear transporter, IMPβ, interacts with HSP90 and the co-chaperon p23. Beside this, the glucocorticoid receptor is able to bind parts of the NPC in the nuclear envelope more efficiently when associated with HSP90 (Echeverría *et al.*, 2009). These results might be transferable to the AHR-HSP90 complex and need further examination.

The present thesis supports that AHR recruits IMPα to ensure a nuclear import. However, IMPα has seven identified isoforms in human. These diverse isoforms interact with NLS containing cargoes specifically, which means they have different target proteins (Pumroy & Cingolani, 2015). The specific IMPα isoforms have been identified for many important cellular components and virus molecules, but not for the AHR. This includes for example STAT1 (McBride *et al.*, 2002), Notch1 (Huenniger *et al.*, 2010), herpes simplex virus (Döhner *et al.*, 2018), and influenza virus (Gabriel *et al.*, 2011). Of note, the cellular levels of IMPα isoforms are not defined for all cell types and might differ according to the cell type and origin. For this reason, identifying the specific IMPα isoforms for the nuclear transport of AHR can clarify the reason of observed differences in the AHR localization between cell lines, between different type of tissues, or between cancer and non-malignant cells. Lastly, characterizing the nuclear import of a protein should also cover identifying the involved type of NPC component, expressly

nucleoporin, as done for the human telomerase (Frohnert *et al.*, 2014). In summary, the type of IMPα and nucleoporin in the AHR nuclear import might be an important factor in the AHR translocation and should be examined in future studies.

5 Summary

The AHR is a ligand dependent transcription factor, which belongs to the bHLH-PAS superfamily of proteins. Originally, the AHR was discovered for mediating the toxicity of environmental toxicants like TCDD and different PAHs. Advanced research showed that the AHR can be bound and activated by a wide range of agonists, including various exogenous and endogenous compounds. According to the classical description of the AHR pathway, the AHR is mainly located in the cytoplasm prior to ligand-binding. Most ligands own hydrophobic characteristics allowing them to enter the cell via diffusion. Ligand-binding to the AHR triggers a nuclear import followed by building a dimer with a protein called ARNT. This heterodimer recruits many co-factors, thereby getting access and subsequently binding to a special sequence in the DNA, the XRE. The AHR/ARNT complex bound to XRE induces the expression of xenobiotic metabolizing enzymes, including phase I and phase II enzymes, thus pointing to a key role of the AHR in xenobiotic metabolism. Examining the intracellular distribution of the AHR in living cells reveals that the AHR undergoes a shuttling between cytoplasm and nucleus. This shuttling occurs continuously and does not lead to any activation. Despite the huge number of studies examining the AHR, the localization remains scarcely investigated.

In the presented thesis, the localization of the AHR was investigated thoroughly after transient transfection of *EYFP-AHR* leading to expression of a fluorescent fusion protein that can be detected in real-time in living cells. I validated this system by comparing the localization of the overexpressed AHR fusion protein with the endogenous AHR with and without the presence of ligands. First of all, in depth mutagenesis analyses on the crucial amino acids to dimerize with ARNT and bind to XRE were carried out. This aimed to characterize if these two significant steps might have an impact on maintaining the activated AHR in the nucleus after ligand-binding or generally affect its nucelo-cytoplasmic translocation in the basal state. Later, the regulatory motive responsible for intracellular distribution and nuclear import, namely NLS, was characterized using point mutations. Thereby, the intracellular distribution of the AHR variants that carry an impaired NLS was detected in the basal state or after ligand treatment. My results demonstrated that neither ARNT dimerization nor DNA binding have an impact on stabilizing the AHR in the nucleus prior or post ligand-binding. Moreover, the sequence of NLS was identified as ($_{13}$RKRRK$_{17}$) and ($_{37}$KR-R$_{40}$), whereby the first part represents the main regulator, while the second one has only a supportive and complementary role. Besides that, I examined both nuclear import processes of the AHR: basal and ligand-induced. Thereafter, the effect of novel import inhibitors IPZ and IVM was tested on the mentioned nuclear import of the AHR. I found that ligand-induced import can occur when the basal import is diminished. At the same time, trying to block the nuclear import using import inhibitors showed comparable influence on both import pathways. My results proposed that ligand-induced and basal import happen autonomously and independent of each other, but they rely on the same particular mechanism. Taken together, this thesis expressed novel

findings on the AHR's sequence and the molecular components involved in its nucleo-cytoplasmic translocation using advanced methods.

6 Zusammenfassung

Der AHR ist ein Liganden-abhängiger Transkriptionsfaktor aus der bHLH-PAS-Proteinfamilie. Ursprünglich wurde der AHR bei der Untersuchung bezüglich der Toxizität von Umweltschadstoffen, wie TCDD und B[*a*]P entdeckt. Weitere Studien zeigten, dass der AHR von einer Vielzahl an Agonisten gebunden und aktiviert werden kann. Dazu gehören verschiedene exogene und endogene Stoffe. Bei dem klassischen AHR-Signalweg befindet sich der AHR primär im Zytoplasma in Abwesenheit von Liganden. Die meisten Liganden können aufgrund ihrer hydrophoben und planaren Eigenschaften in die Zelle diffundieren, woraufhin eine Bindung zwischen AHR und Ligand initiiert werden kann. Diese Interaktion führt zum nuklearen Import des AHR-Liganden-Komplexes. Im Nukleus erfolgt die Dimerisierung mit ARNT, ein weiteres Protein aus der bHLH-PAS Proteinfamilie. Anschließend werden anhand des AHR-ARNT-Heterodimers mehrere Cofaktoren rekrutiert, wodurch der Zugang zu einem spezifischen Motiv in der DNA (XRE) ermöglicht wird. Die Bindung des AHR-ARNT-Komplexes an XRE induziert die Expression von Xenobiotika metabolisierenden Enzymen, einschließlich Phase-I- und Phase-II-Enzymen. Diese weisen auf die Schlüsselrolle des AHR in der Metabolisierung von Xenobiotika hin. Untersuchungen zur intrazellulären Verteilung des AHR in lebenden Zellen zeigte, dass der AHR zwischen dem Zytoplasma und dem Zellkern pendelt. Dieses „Shuttling“ erfolgt kontinuierlich und führt zu keiner Aktivierung des AHR. Trotz vieler bereits erschienener Studien zum AHR ist die Lokalisation vergleichsweise wenig erforscht und lässt viele Fragen unbeantwortet.

In der vorliegenden Arbeit wurde die Lokalisation des AHR umfangreich untersucht. Nach der transienten Transfektion von EYFP-AHR in HepG2- und MCF7-Zellen wurde die Lokalisation und Liganden-induzierte Translokation des fluoreszierenden Fusionsproteins in lebenden Zellen charakterisiert. Das dieses System dabei der endogenen, physiologischen Lokalisierung entspricht, konnte unter anderem mithilfe von Immunfärbung belegt werden.

In den dabei entstandenen Publikationen wurde der Einfluss molekularer Interaktionen im Zellkern auf die intrazelluläre Verteilung des AHR untersucht. Dafür wurden AHR-Varianten generiert, bei denen essenzielle Aminosäuren sowohl zur ARNT-Dimerisierung als auch zur XRE-Bindung mutiert wurden. Die Ergebnisse zeigten, dass weder die Dimerisierung mit ARNT noch die DNA-Bindung die Stabilisierung des AHR im Zellkern vor oder nach Liganden-Bindung beeinflusst. Weiterhin wurde das NLS, das für die intrazelluläre Verteilung und den nukleären Import verantwortliche regulatorische Motiv, unter Nutzung von Punktmutationen charakterisiert. Dabei wurde die intrazelluläre Verteilung der AHR-Varianten mit mutierter NLS im basalen Zustand und nach Liganden-Behandlung beobachtet. Anhand dessen konnte die NLS-Sequenz als ($_{13}$RKRRK$_{17}$) und ($_{37}$KR-R$_{40}$) identifiziert werden, wobei der erste Teil als Hauptregulator gilt, während der zweite nur eine unterstützende und ergänzende Rolle

hat. Zusätzlich wurden im Rahmen dieser Arbeit die beiden nukleären Importprozesse des AHR untersucht: der basale und der Liganden-induzierte Import. Die dabei generierten Ergebnisse zeigten, dass der Liganden-induzierte Import erfolgte, auch wenn der basale gehemmt wurde. Des Weiteren wurde die Wirkung der Importinhibitoren IPZ und IVM bezüglich des nukleären AHR-Imports getestet. Das Blockieren des nuklearen Imports mit Importinhibitoren führte zu einem vergleichbaren Einfluss auf beide Importwege. Die Ergebnisse dieser Arbeit deuteten darauf hin, dass der Liganden-induzierte und basale Import autonom und unabhängig voneinander erfolgten, jedoch auf dem gleichen spezifischen Mechanismus beruhten. Zusammenfassend bietet die vorliegende Arbeit unter Anwendung moderner Methoden neue Erkenntnisse über die intrinsischen Eigenschaften des AHR sowie über die molekularen Komponenten, die an der nukleo-zytoplasmatischen Translokalisation beteiligt sind.

7 Acknowledgements

First of all, I would like to express my deepest gratitude to Professor Dr. Dr. Andreas Luch for giving me the opportunity to conduct my research in his department. Thank you for having faith in my work and for motivating me to reach my fullest potential. I would also like to extend my sincere thanks to Professor Dr. Gerhard Wolber for reviewing my dissertation.

A special thank goes to my supervisor, Dr. Josephine Kugler, for her invaluable guidance, constructive feedback, and thoughtful suggestions. I deeply appreciate all her support including proofreading this thesis. I am also grateful to Dr. Frank Henkler for his trust in me and for the scientific support he provided for my first publication.

My heartfelt thanks go to Doris Genkinger for creating a wonderful atmosphere in the lab and for her assistance with technical and organizational challenges.

I would like to thank all the students I supervised, especially Reneh Shabo, for their significant contributions to the success of this thesis. I am thankful to all my colleagues for the enjoyable times and the pleasant moments. In particular, I want to thank my colleagues from the “Großraumbüro”: Lisa, Florian, Tessa, Selina, Rico, and Falk for the great time and humorous conversations.

Finally, I want to thank my family for always believing in me and cheering me up, also for providing the financial means to continue my education in Germany. Special thanks to Aline for being a constant source of strength and happiness.

8 List of publications

Peer-reviewed articles

Haidar, R., Henkler, F., Kugler, J., Rosin, A., Genkinger, D., Laux, P., & Luch, A. (2021). The role of DNA-binding and ARNT dimerization on the nucleo-cytoplasmic translocation of the aryl hydrocarbon receptor. Sci. Rep., 11(1), 18194. doi:10.1038/s41598-021-97507-w.

Haidar, R., Shabo, R., Moeser, M., Luch, A., & Kugler, J. (2023). The nuclear entry of the aryl hydrocarbon receptor (AHR) relies on the first nuclear localization signal and can be negatively regulated through IMPα/β specific inhibitors. Sci. Rep., 13(1), 19668. doi:10.1038/s41598-023-47066-z.

Conference contributions

Haidar, R., Kugler, J. (2023). Analyzing the molecular mechanism of AHR nuclear entry. Toxicol. Lett., 384(3):S308. DOI: 10.1016/S0378-4274(23)00969-4. Congress of the European Societies of Toxicology (EUROTOX 2023), 10-13 September 2023, Ljubljana, Slovenia

9 Annex I: Supplementary material for chapter 3.1

Supplementary Data

Supplementary information to:

The Role of DNA-Binding and ARNT Dimerization on the Nucleo-Cytoplasmic Translocation of the Aryl Hydrocarbon Receptor

Rashad Haidar[1-2]*, Frank Henkler[1], Josephine Kugler[1], Aline Rosin[1], Doris Genkinger[1], Peter Laux[1], Andreas Luch[1-2]

[1] German Federal Institute for Risk Assessment (BfR), Department of Chemical and Product Safety, Berlin, Germany

[2] Institute of Pharmacy, Department of Biology, Chemistry and Pharmacy, Freie Universität Berlin, Berlin, Germany

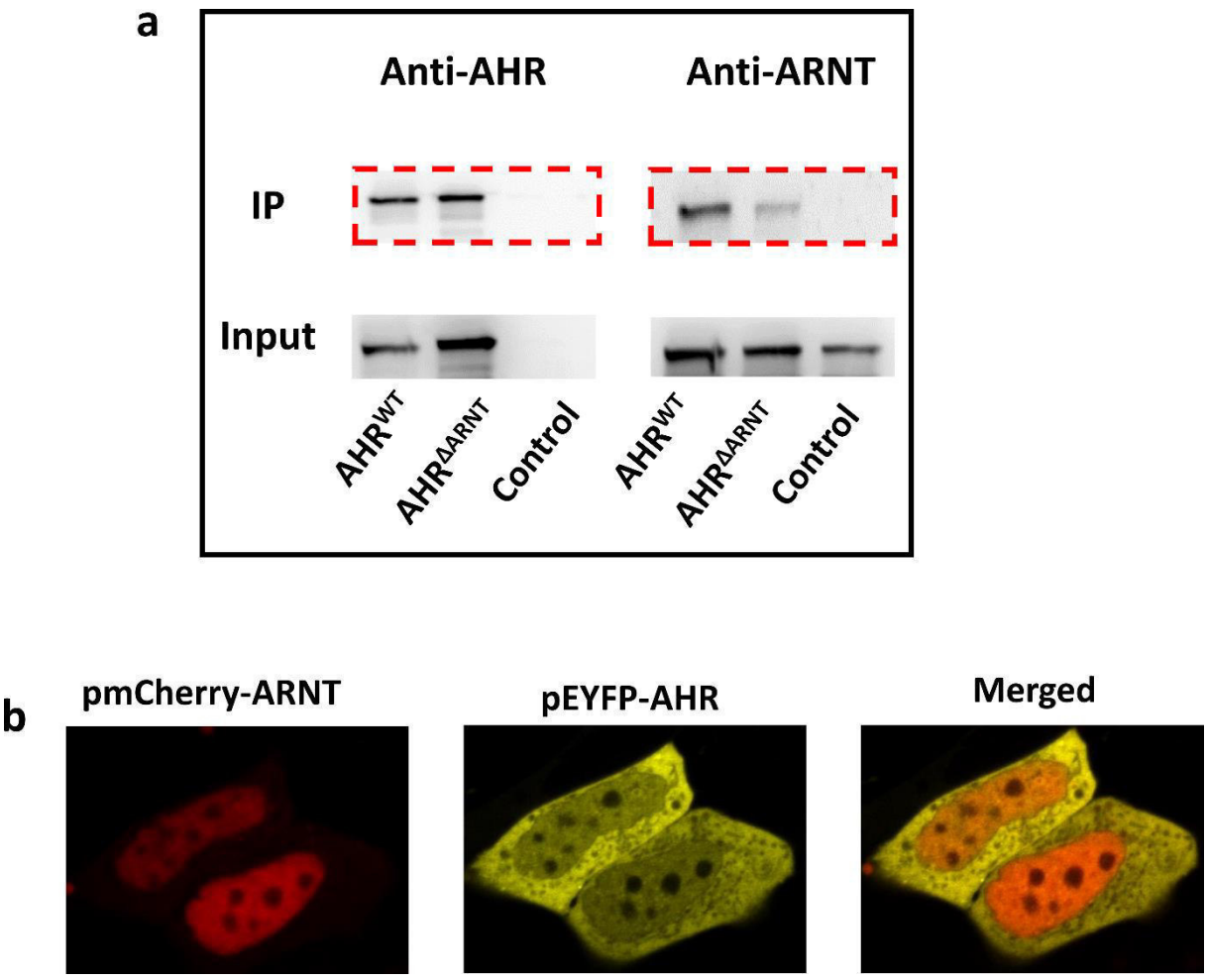
* Correspondence should be addressed to Rashad.Haidar@bfr.bund.de

Supplementary materials and methods

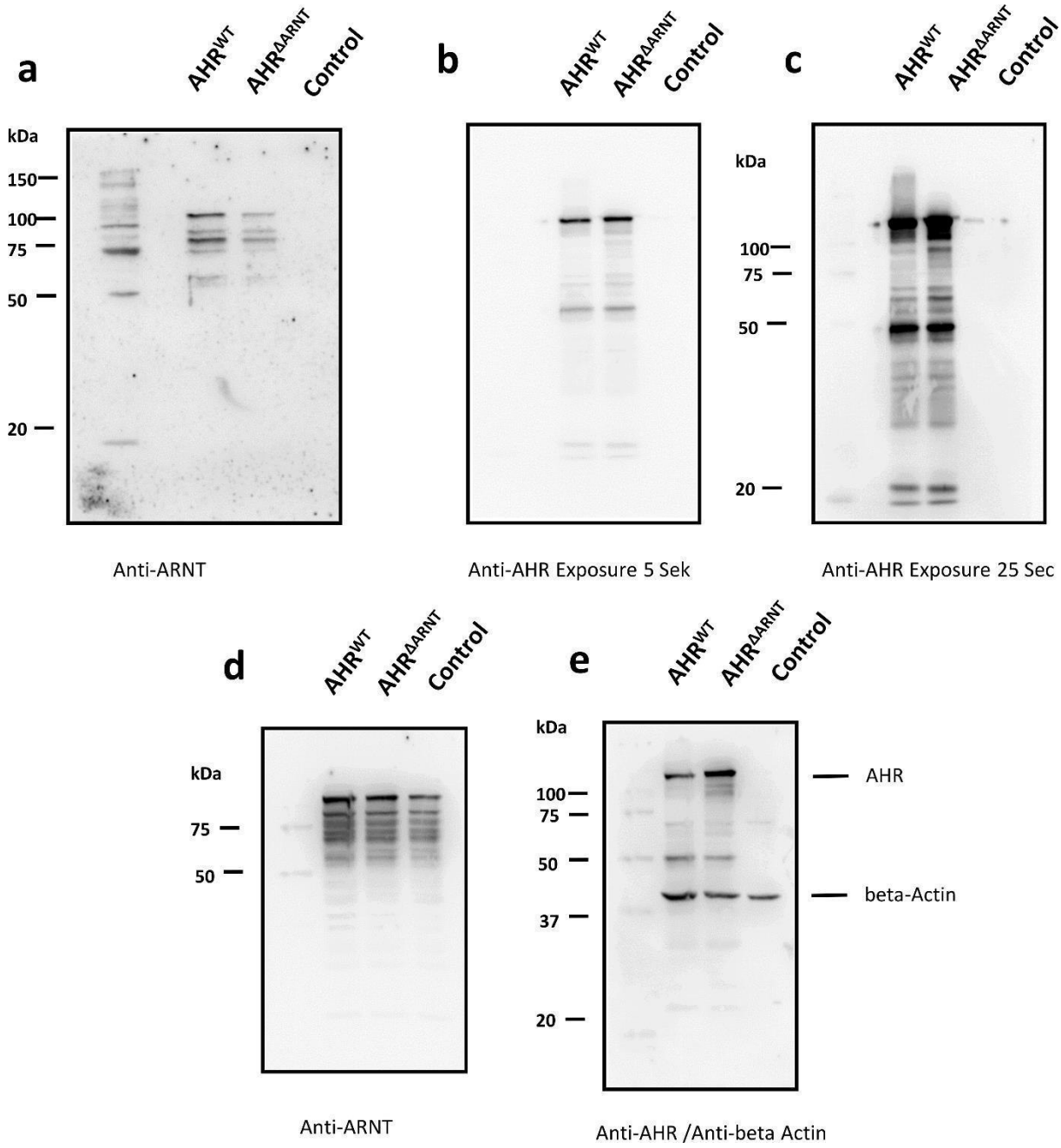
Co-Immunoprecipitation. MCF-7$^{\Delta AHR}$ cells were seeded on 10-cm dishes (Techno Plastic Products AG, Trasadingen, Switzerland). On the next day, cells were transfected by using 24 µg pEYFP-AHR (WT or mutant) and 16 µg pmCherry-ARNT with 40 µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For the negative control, cells were transfected with pmCherry-ARNT alone. After 4 hours incubation, transfection medium was removed and replaced by new cultivation medium. After 24 hours, cells were treated with 5 µM Indirubin for three hours. For co-immunoprecipitation the GFP-Trap® Magnetic Agarose (Chromotek, Planegg, Germany) was used according to the manufacturer's instructions. Cell extracts were incubated with GFP-trap beads for two hours at 4°C. For each sample, 15 µg supernatant was saved as input for Western blots. YFP protein were eluted with SDS-sample buffer and then tested by western blotting.

Western-blot. Cells were lysed on ice in RIPA buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1 % SDS, 1 % Triton™ X-100, 1 % deoxycholate) with protease inhibitor cocktail (Calbiochem, San Diego, CA, USA) and DNase (New England Biolabs, Ipswich; England). Protein concentration was measured with the Pierce™ BCA Protein Assay Kit (Thermo Scientifc, Waltham, MA, USA). Equal amounts of proteins were applied to SDS-PAGE, transferred onto nitrocellulose membranes and immunoblotted according to the manufacturer's instructions. The primary antibodies were, anti-AHR used at 1:300 (sc-133088) and anti-ARNT used at 1:600 (sc-17811) (all from Santa Cruz Biotechnology, Heidelberg, Germany). Primary antibodies were labelled with appropriate horseradish peroxidase (HRP)-coupled secondary antibodies (Santa Cruz Biotechnology) and visualized with Pierce ECL Substrate (Thermo Fisher Scientifc, Waltham, MA, USA). As a loading control, HRP conjugated anti-beta Actin was used at 1:12,500 (ab 49900, Abcam, Cambridge, UK).

Supplementary Figures



Suppl Fig 1. Co-Immunoprecipitation: (a) Cropped Western Blot images after co-immunoprecipitation of input (lower lane) and precipitate (upper row) for AHR (left) and ARNT (right) for indicated constructs and pmCherry-ARNT. Full-length blots are presented in Suppl Fig 2 **(b)** Representative image of MCF-7$^{\Delta AHR}$ cells co-transfected with pEYFP-AHR (yellow) and pmCherry-ARNT (red).



Suppl Fig 2.Full length blots of the cropped Western Blot images from Suppl Fig 1 after co-immunoprecipitation of precipitate for ARNT **(a)**, AHR after 5 / 25 seconds exposure **(b)/(c)** and input for ARNT **(d)**, AHR and beta Actin **(e)**.

10 Annex II: Supplementary material for chapter 3.2

Supplementary Data

Supplementary information to

The nuclear entry of the aryl hydrocarbon receptor (AHR) relies on the first nuclear localization signal and can be negatively regulated through IMPα/β specific inhibitors

Rashad Haidar[1-2]*, Reneh Shabo[1], Marie Moeser[1], Andreas Luch[1-2] and Josephine Kugler[1]

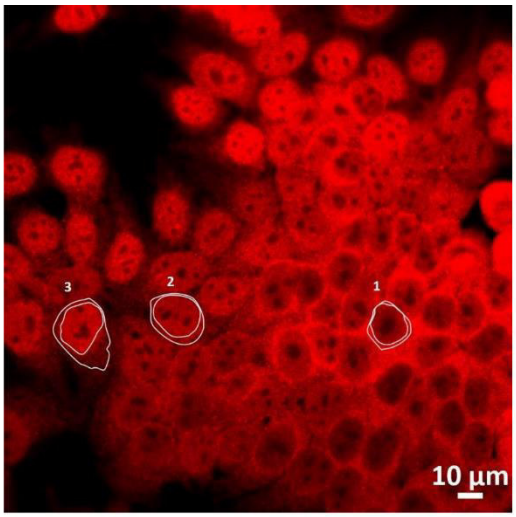
[1] German Federal Institute for Risk Assessment (BfR), Department of Chemical and Product Safety, Berlin, Germany

[2] Institute of Pharmacy, Department of Biology, Chemistry and Pharmacy, Freie Universität Berlin, Berlin, Germany

*** Correspondence should be addressed to Rashad.Haidar@bfr.bund.de**

Supplementary Figures

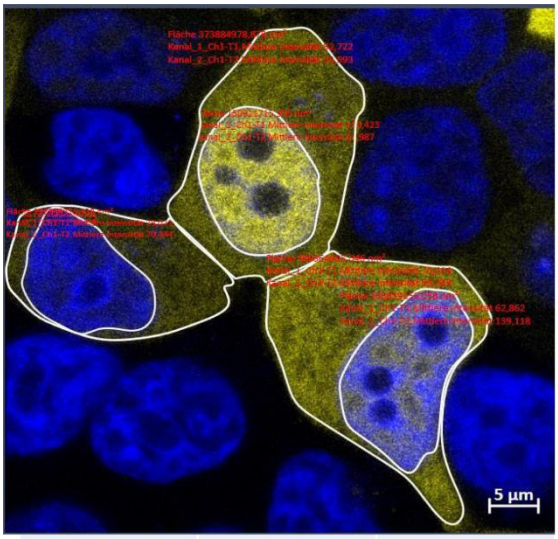
a

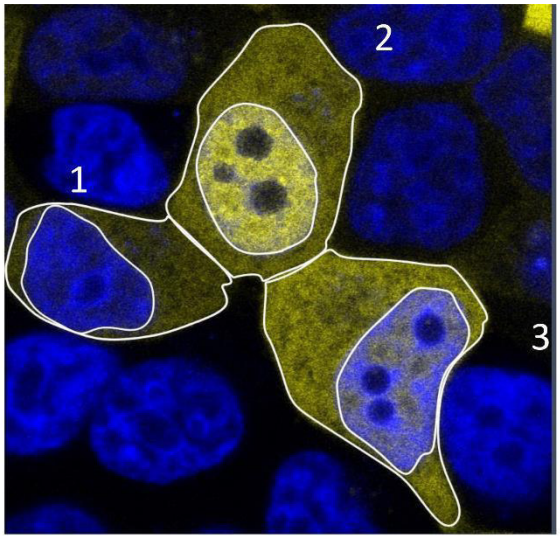


Name	Area	Fluorescence Intensity
	nm²	
Nucleus (1)	1,79735E+08	28,40085
Whole Cell (1)	2,30214E+08	32,49268
Nucleus (2)	2,43866E+08	30,58789
Whole Cell (2)	3,41581E+08	29,94433
Nucleus (3)	2,44534E+08	34,81347
Whole Cell (3)	4,52608E+08	29,06058

Cell	Nucleus	Whole Cell	Relative nucleus intensity
1	28,40085	32,49268	0,874069
2	30,58789	29,94433	1,021492
3	34,81347	29,06058	1,197962

b



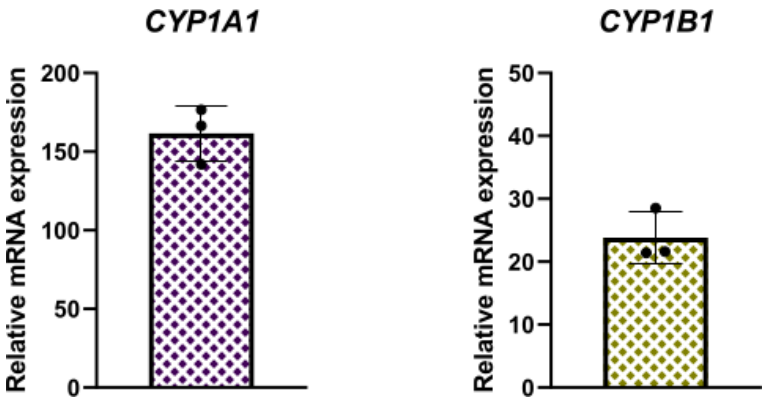


Name	Area nm²	EYFP Intensity
Nucleus (1)	125434680	17,9864225
Whole Cell (1)	252944319	29,0213524
Nucleus (2)	150911716	110,42257
Whole Cell (2)	373884979	82,7222964
Nucleus (3)	389364605	70,4138841
Whole Cell (3)	166839155	62,8621562

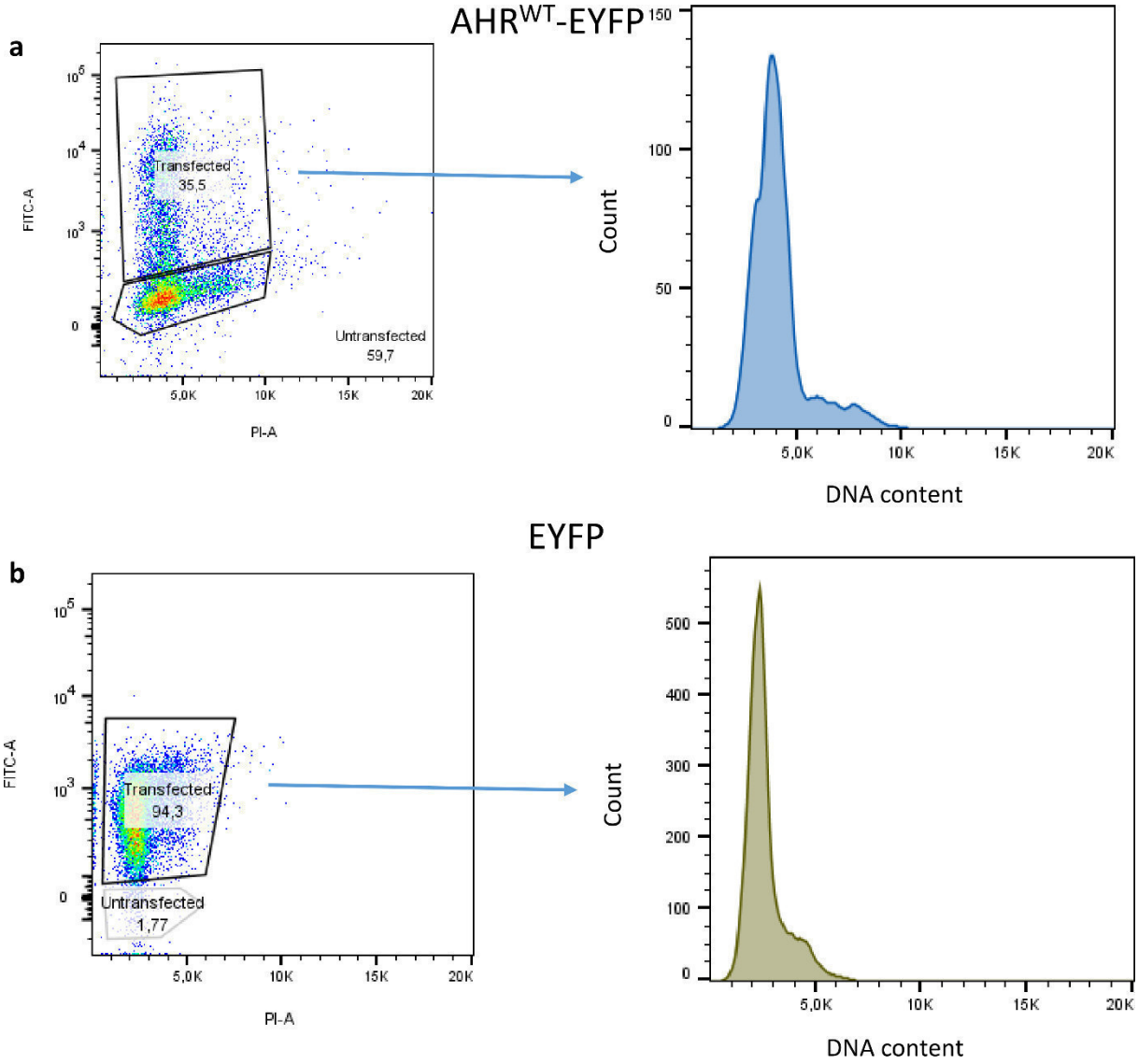
Cell	Nucleus	Whole Cell	Relative nucleus intensity
1	17,9864225	29,0213524	0,61976514
2	110,42257	82,7222964	1,33485862
3	62,8621562	70,4138841	0,89275229

Supplementary Figure S1: Representative example of the assessment of relative nucleus intensity. The nucleus and the whole cells are marked as region of interest (ROI) by using ZEN 2012 blue edition. The relative nuclear intensity is

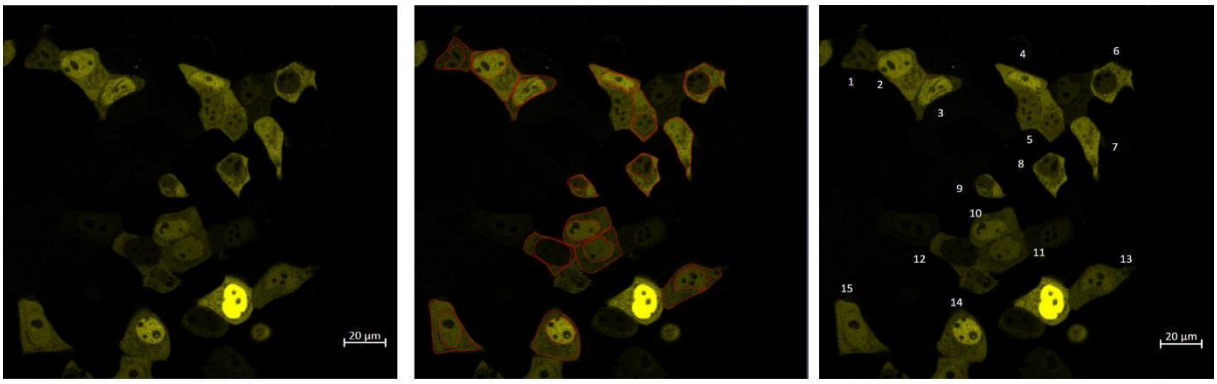
determined by the mean fluorescence intensity of the nucleus divided by the mean fluorescence intensity of the whole cell (a) Immunofluorescence images of AHR in MCF 7^{WT}. (b) EYFP-AHR^{WT} in transiently transfected in MCF $7^{\Delta AHR}$ cells.



Supplementary Figure S2: Relative *CYP1A1* and *CYP1B1* in EYFP-AHR^{WT} transfected MCF-$7^{\Delta AHR}$ cells. Cells were treated with 2.5 µM IND or 0.1% DMSO for 2 h. Relative *CYP1A1* and *CYP1B1* mRNA levels determined by qPCR. Values were standardized against HPRT and normalized to sample treated with DMSO only.

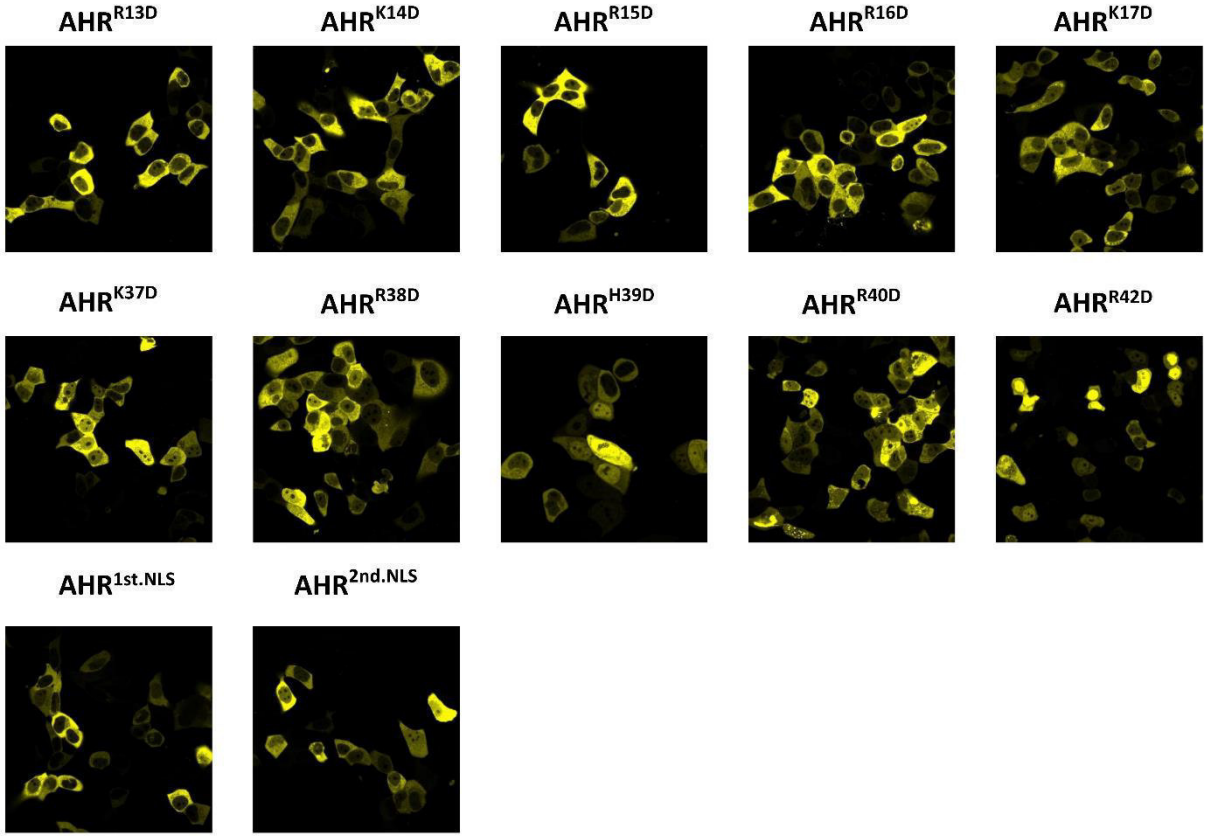


Supplementary Figure S3: Cell cycle analysis of (a) EYFP-AHR transfected and (b) EYFP-transfected cells. Total number of analyzed cells: 3573(a), 9715(b).

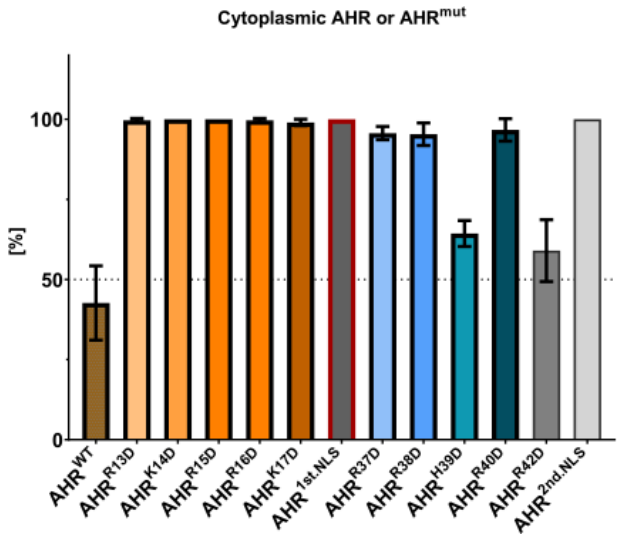


Cell	Nucleus	Whole Cell	Relative nucleus intensity	Localization
1	32,60259089	31,00435768	1,051548664	Equal distributed
2	94,34107402	79,82384993	1,181865747	Nuclear
3	92,80375147	72,89917012	1,273042633	Nuclear
4	112,2188771	95,00307161	1,181213146	Nuclear
5	48,92213533	60,3714155	0,81035263	Cytoplasmic
6	31,65004156	52,62605932	0,601413862	Cytoplasmic
7	88,76074435	86,60516028	1,024889788	Equal distributed
8	36,46220615	55,885867	0,652440556	Cytoplasmic
9	34,46927374	50,1973051	0,686675782	Cytoplasmic
10	58,87929677	48,69960793	1,209030201	Nuclear
11	47,60108884	38,58784858	1,233577164	Nuclear
12	13,51727057	20,60690953	0,655958165	Cytoplasmic
13	50,89350269	44,7212931	1,138015007	Nuclear
14	104,8081382	67,04128846	1,56333717	Nuclear
15	55,51982571	52,75499381	1,052408913	Equal distributed

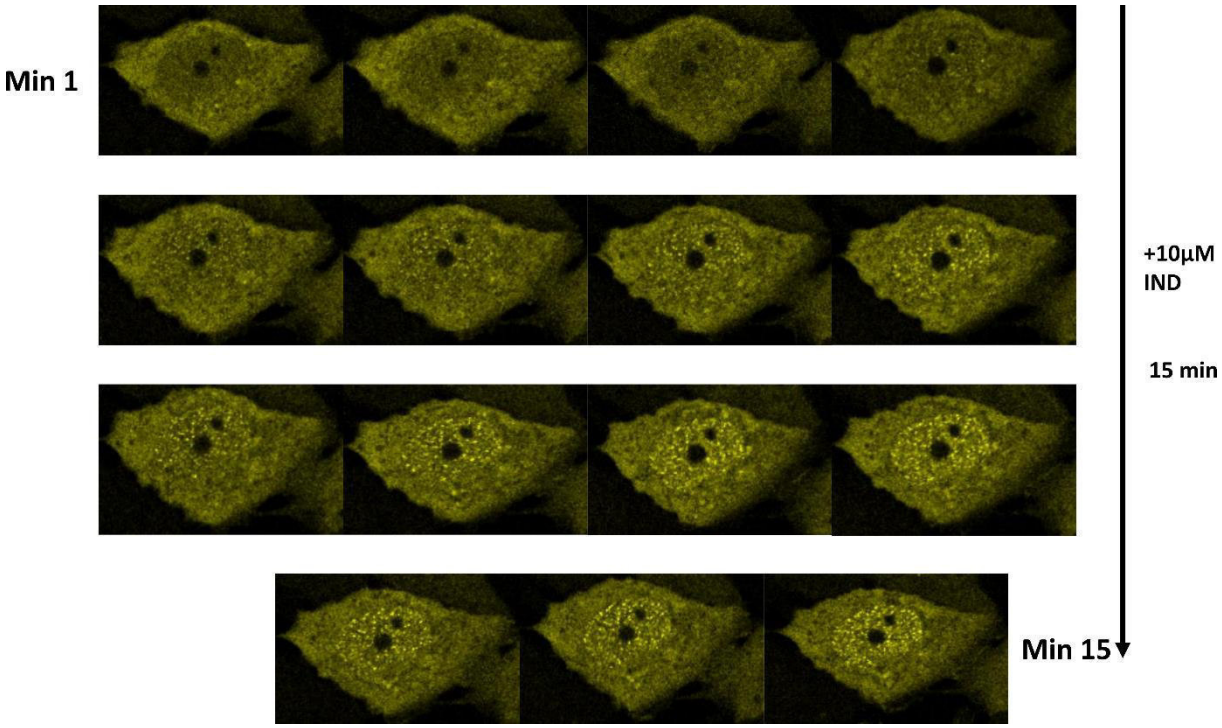
Supplementary Figure S4: Representative example of determining the localization of EYFP-AHR^{WT}. MCF7$^{\Delta AHR}$ cells transfected with EYFP-AHR^{WT}. In the basal state, fifteen cells were selected and the relative intensity of the nucleus of each cell was measured. The localization of AHR was assigned according to our classification in Fig2h.



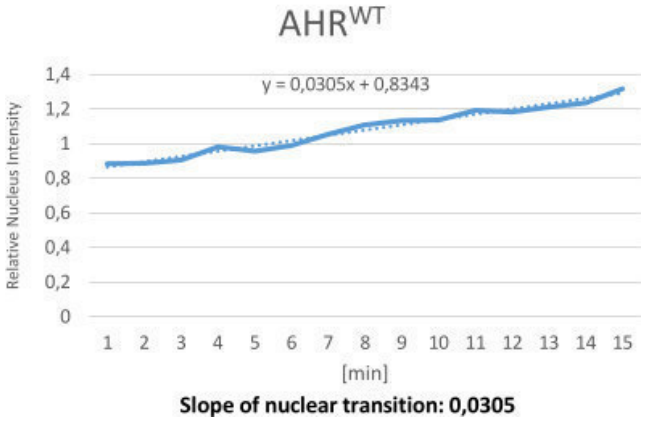
Supplementary Figure S5: Representative images of MCF7$^{\Delta AHR}$ cells transfected with EYFP-AHR^{mut} in the basal state.



Supplementary Figure S6: The percentage of cells with cytoplasmic AHR^{WT} or AHR^{mut} for hundred randomly chosen cells according to our classification in Fig S2 and in Fig2h.

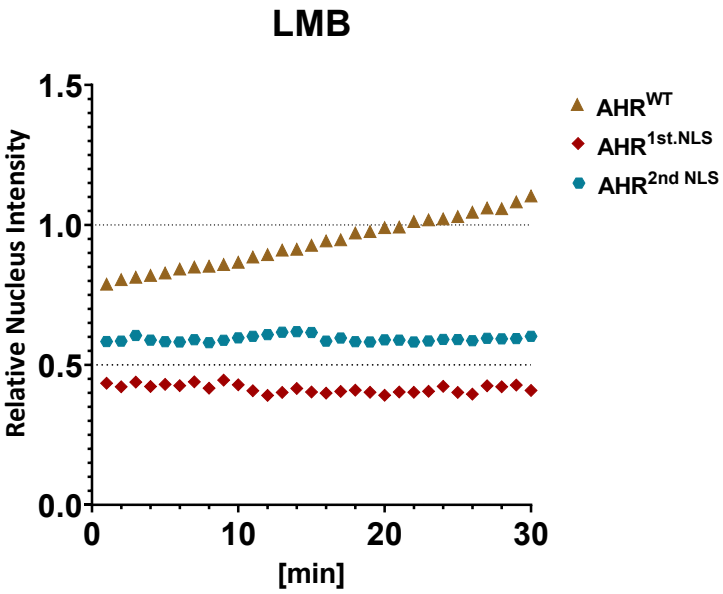


Time	Nucleus	Whole Cell	Relative Nucleus Intensity
1	9,21553719	10,4335161	0,88326285
2	9,01619835	10,1706648	0,88649056
3	8,28793388	9,15421522	0,90536804
4	10,7345455	10,9389993	0,98130964
5	10,1682645	10,6222298	0,9572627
6	10,7061157	10,8275074	0,98878858
7	11,8244628	11,2050491	1,05527987
8	12,6136489	11,3827964	1,1081327
9	12,2879339	10,8430432	1,13325509
10	12,7309091	11,2062828	1,13605102
11	13,911405	11,6710075	1,19196264
12	14,0634711	11,8881654	1,18298077
13	13,5110744	11,1554718	1,21116118
14	14,0502479	11,3701165	1,23571715
15	15,2241322	11,5685858	1,31598905

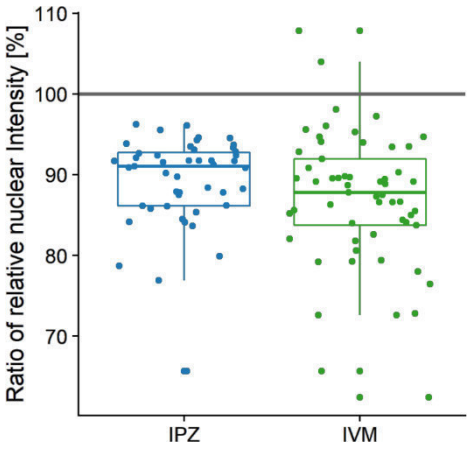


Slope of nuclear transition: 0,0305

Supplementary Figure S7: Calculation of the slope of nuclear transition after treatment. The representative example demonstrates the increase of relative nucleus intensity after cells treatment with 10 µM indirubin (IND) for 15 minutes. Fifteen snapshots of the cell are taken in ratio of one image per minute after treatment.



Supplementary Figure S8: Mean of time-lapse measurements after incubating cells transfected with AHR^{WT} or AHR^{mut} with 200 nM LMB for 30 min.



Supplementary Figure S9: Changes in the relative nuclear intensity after import inhibitors treatment. MCF7$^{\Delta AHR}$ cells were transfected with EYFP-AHR^{WT} and treated with importazole (IPZ) or ivermectin (IVM) for 90 min at concentrations of 10 µM and 17.5 µM, respectively. Changes in relative nuclear intensity of around 50 cells after 90 min of treatment are represented.

11 References

Adachi, J., Mori, Y., Matsui, S., Takigami, H., Fujino, J., Kitagawa, H., . . . Matsuda, T. (2001). Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. *J. Biol. Chem., 276*(34), 31475-31478. doi:10.1074/jbc.C100238200

Anderson, G., Beischlag, T. V., Vinciguerra, M., & Mazzoccoli, G. (2013). The circadian clock circuitry and the AHR signaling pathway in physiology and pathology. *Biochem. Pharmacol., 85*(10), 1405-1416. doi:https://doi.org/10.1016/j.bcp.2013.02.022

Arabnezhad, M. R., Montazeri-Najafabady, N., Chatrabnous, N., Ghafarian Bahreman, A., & Mohammadi-Bardbori, A. (2020). Anti-androgenic effect of 6-formylindolo[3,2-b]carbazole (FICZ) in LNCaP cells is mediated by the aryl hydrocarbon-androgen receptors cross-talk. *Steroids., 153*, 108508. doi:10.1016/j.steroids.2019.108508

Avilla, M. N., Malecki, K. M. C., Hahn, M. E., Wilson, R. H., & Bradfield, C. A. (2020). The Ah Receptor: Adaptive Metabolism, Ligand Diversity, and the Xenokine Model. *Chem. Res. Toxicol., 33*(4), 860-879. doi:10.1021/acs.chemrestox.9b00476

Bacsi, S. G., & Hankinson, O. (1996). Functional characterization of DNA-binding domains of the subunits of the heterodimeric aryl hydrocarbon receptor complex imputing novel and canonical basic helix-loop-helix protein-DNA interactions. *J. Biol. Chem., 271*(15), 8843-8850. doi:10.1074/jbc.271.15.8843

Bacsi, S. G., Reisz-Porszasz, S., & Hankinson, O. (1995). Orientation of the heterodimeric aryl hydrocarbon (dioxin) receptor complex on its asymmetric DNA recognition sequence. *Mol. Pharmacol., 47*(3), 432-438.

Badawy, A. A. (2017). Kynurenine Pathway of Tryptophan Metabolism: Regulatory and Functional Aspects. *Int. J. Tryptophan. Res., 10*, 1178646917691938. doi:10.1177/1178646917691938

Baker, J. R., Sakoff, J. A., & McCluskey, A. (2020). The aryl hydrocarbon receptor (AhR) as a breast cancer drug target. *Med. Res. Rev., 40*(3), 972-1001. doi:10.1002/med.21645

Barhoover, M. A., Hall, J. M., Greenlee, W. F., & Thomas, R. S. (2010). Aryl hydrocarbon receptor regulates cell cycle progression in human breast cancer cells via a functional interaction with cyclin-dependent kinase 4. *Mol. Pharmacol., 77*(2), 195-201. doi:10.1124/mol.109.059675

Beischlag, T. V., Luis Morales, J., Hollingshead, B. D., & Perdew, G. H. (2008). The aryl hydrocarbon receptor complex and the control of gene expression. *Crit. Rev. Eukaryot. Gene. Expr., 18*(3), 207-250. doi:10.1615/critreveukargeneexpr.v18.i3.20

Bjeldanes, L. F., Kim, J. Y., Grose, K. R., Bartholomew, J. C., & Bradfield, C. A. (1991). Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol in vitro and in vivo: comparisons with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Proc. Natl. Acad. Sci. U.S.A., 88*(21), 9543-9547. doi:10.1073/pnas.88.21.9543

Boobis, A. R., Nebert, D. W., & Felton, J. S. (1977). Comparison of beta-naphthoflavone and 3-methylcholanthrene as inducers of hepatic cytochrome(s) P-448 and aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity. *Mol. Pharmacol., 13*(2), 259-268.

Bradfield, C. A., Glover, E., & Poland, A. (1991). Purification and N-terminal amino acid sequence of the Ah receptor from the C57BL/6J mouse. *Mol. Pharmacol., 39*(1), 13-19.

Brauze, D., Crow, J. S., & Malejka-Giganti, D. (1997). Modulation by beta-naphthoflavone of ovarian hormone dependent responses in rat uterus and liver in vivo. *Can. J. Physiol. Pharmacol., 75*(8), 1022-1029. doi:10.1139/cjpp-75-8-1022

Burbach, K. M., Poland, A., & Bradfield, C. A. (1992). Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl. Acad. Sci. U.S.A., 89*(17), 8185-8189. doi:10.1073/pnas.89.17.8185

Busbee, P. B., Rouse, M., Nagarkatti, M., & Nagarkatti, P. S. (2013). Use of natural AhR ligands as potential therapeutic modalities against inflammatory disorders. *Nutr. Rev., 71*(6), 353-369. doi:10.1111/nure.12024

Çağatay, T., & Chook, Y. M. (2018). Karyopherins in cancer. *Curr. Opin. Cell Biol., 52*, 30-42. doi:10.1016/j.ceb.2018.01.006

Cai, H., Katoh-Kurasawa, M., Muramoto, T., Santhanam, B., Long, Y., Li, L., . . . Devreotes, P. N. (2014). Nucleocytoplasmic shuttling of a GATA transcription factor functions as a development timer. *Science, 343*(6177), 1249531. doi:10.1126/science.1249531

Caly, L., Druce, J. D., Catton, M. G., Jans, D. A., & Wagstaff, K. M. (2020). The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 in vitro. *Antiviral. Res., 178*, 104787. doi:10.1016/j.antiviral.2020.104787

Campbell, W. C., Fisher, M. H., Stapley, E. O., Albers-Schönberg, G., & Jacob, T. A. (1983). Ivermectin: a potent new antiparasitic agent. *Science, 221*(4613), 823-828. doi:10.1126/science.6308762

Campesato, L. F., Budhu, S., Tchaicha, J., Weng, C. H., Gigoux, M., Cohen, I. J., . . . Wolchok, J. D. (2020). Blockade of the AHR restricts a Treg-macrophage suppressive axis induced by L-Kynurenine. *Nat. Commun., 11*(1), 4011. doi:10.1038/s41467-020-17750-z

Chadli, A., Bouhouche, I., Sullivan, W., Stensgard, B., McMahon, N., Catelli, M. G., & Toft, D. O. (2000). Dimerization and N-terminal domain proximity underlie the function of the molecular chaperone heat shock protein 90. *Proc. Natl. Acad. Sci. U.S.A., 97*(23), 12524-12529. doi:10.1073/pnas.220430297

Cheng, X., & Merz, K. H. (2016). The Role of Indirubins in Inflammation and Associated Tumorigenesis. *Adv. Exp. Med. Biol., 929*, 269-290. doi:10.1007/978-3-319-41342-6_12

Cheong, J. E., & Sun, L. (2018). Targeting the IDO1/TDO2-KYN-AhR Pathway for Cancer Immunotherapy - Challenges and Opportunities. *Trends. Pharmacol. Sci., 39*(3), 307-325. doi:10.1016/j.tips.2017.11.007

Choi, J. M., Cho, E., Lee, B., Jeong, D., Choi, Y., Yu, J. H., & Jung, S. (2016). Enhancing bio-availability of β-naphthoflavone by supramolecular complexation with 6,6'-thiobis(methylene)-β-cyclodextrin dimer. *Carbohydr. Polym., 151*, 40-50. doi:10.1016/j.carbpol.2016.05.046

Ciolino, H. P., Daschner, P. J., & Yeh, G. C. (1998). Resveratrol inhibits transcription of CYP1A1 in vitro by preventing activation of the aryl hydrocarbon receptor. *Cancer. Res., 58*(24), 5707-5712.

Crews, S. T. (1998). Control of cell lineage-specific development and transcription by bHLH-PAS proteins. *Genes. Dev., 12*(5), 607-620. doi:10.1101/gad.12.5.607

Daujat, M., Peryt, B., Lesca, P., Fourtanier, G., Domergue, J., & Maurel, P. (1992). Omeprazole, an inducer of human CYP1A1 and 1A2, is not a ligand for the Ah receptor. *Biochem. Biophys. Res. Commun., 188*(2), 820-825. doi:10.1016/0006-291x(92)91130-i

Davarinos, N. A., & Pollenz, R. S. (1999). Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytosplasmic proteasome following nuclear export. *J. Biol. Chem., 274*(40), 28708-28715. doi:10.1074/jbc.274.40.28708

De Juan, A., & Segura, E. (2021). Modulation of Immune Responses by Nutritional Ligands of Aryl Hydrocarbon Receptor. *Front. Immunol., 12*, 645168. doi:10.3389/fimmu.2021.645168

Denison, M. S., & Heath-Pagliuso, S. (1998). The Ah receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals. *Bull. Environ. Contam. Toxicol., 61*(5), 557-568. doi:10.1007/pl00002973

Denison, M. S., & Nagy, S. R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol., 43*, 309-334. doi:10.1146/annurev.pharmtox.43.100901.135828

Deuster, E., Mayr, D., Hester, A., Kolben, T., Zeder-Göß, C., Burges, A., . . . Czogalla, B. (2019). Correlation of the Aryl Hydrocarbon Receptor with FSHR in Ovarian Cancer Patients. *Int. J. Mol. Sci., 20*(12). doi:10.3390/ijms20122862

DiNatale, B. C., Schroeder, J. C., Francey, L. J., Kusnadi, A., & Perdew, G. H. (2010). Mechanistic insights into the events that lead to synergistic induction of interleukin 6 transcription upon activation of the aryl hydrocarbon receptor and inflammatory signaling. *J. Biol. Chem., 285*(32), 24388-24397. doi:10.1074/jbc.M110.118570

Döhner, K., Ramos-Nascimento, A., Bialy, D., Anderson, F., Hickford-Martinez, A., Rother, F., . . . Sodeik, B. (2018). Importin α1 is required for nuclear import of herpes simplex virus proteins and

capsid assembly in fibroblasts and neurons. *PLoS. Pathog., 14*(1), e1006823. doi:10.1371/journal.ppat.1006823

Döhr, O., & Abel, J. (1997). Transforming growth factor-beta1 coregulates mRNA expression of aryl hydrocarbon receptor and cell-cycle-regulating genes in human cancer cell lines. *Biochem. Biophys. Res. Commun., 241*(1), 86-91. doi:10.1006/bbrc.1997.7773

Dolwick, K. M., Schmidt, J. V., Carver, L. A., Swanson, H. I., & Bradfield, C. A. (1993). Cloning and expression of a human Ah receptor cDNA. *Mol. Pharmacol., 44*(5), 911-917.

Dong, F., & Perdew, G. H. (2020). The aryl hydrocarbon receptor as a mediator of host-microbiota interplay. *Gut. Microbes., 12*(1), 1859812. doi:10.1080/19490976.2020.1859812

Duan, Z., Li, Y., & Li, L. (2018). Promoting epithelial-to-mesenchymal transition by D-kynurenine via activating aryl hydrocarbon receptor. *Mol. Cell. Biochem., 448*(1-2), 165-173. doi:10.1007/s11010-018-3323-y

Dumbrava, E. E., Cecchini, M., Zugazagoitia, J., Lopez, J. S., Jager, D., Oliva, M., . . . Papadopoulos, K. P. (2023). Initial results from a first-in-human, phase I study of immunomodulatory aryl hydrocarbon receptor (AhR) inhibitor BAY2416964 in patients with advanced solid tumors. *J. Clin. Oncol., 41*(16_suppl), 2502-2502. doi:10.1200/JCO.2023.41.16_suppl.2502

Dzeletovic, N., McGuire, J., Daujat, M., Tholander, J., Ema, M., Fujii-Kuriyama, Y., . . . Poellinger, L. (1997). Regulation of dioxin receptor function by omeprazole. *J. Biol. Chem., 272*(19), 12705-12713. doi:10.1074/jbc.272.19.12705

Echeverría, P. C., Mazaira, G., Erlejman, A., Gomez-Sanchez, C., Piwien Pilipuk, G., & Galigniana, M. D. (2009). Nuclear import of the glucocorticoid receptor-hsp90 complex through the nuclear pore complex is mediated by its interaction with Nup62 and importin beta. *Mol. Cell. Biol., 29*(17), 4788-4797. doi:10.1128/mcb.00649-09

Eguchi, H., Hayashi, S., Watanabe, J., Gotoh, O., & Kawajiri, K. (1994). Molecular cloning of the human AH receptor gene promoter. *Biochem. Biophys. Res. Commun., 203*(1), 615-622. doi:10.1006/bbrc.1994.2227

Eguchi, H., Ikuta, T., Tachibana, T., Yoneda, Y., & Kawajiri, K. (1997). A nuclear localization signal of human aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor 1beta is a novel bipartite type recognized by the two components of nuclear pore-targeting complex. *J. Biol. Chem., 272*(28), 17640-17647. doi:10.1074/jbc.272.28.17640

Elson, D. J., & Kolluri, S. K. (2023). Tumor-Suppressive Functions of the Aryl Hydrocarbon Receptor (AhR) and AhR as a Therapeutic Target in Cancer. *Biology, 12*(4). doi:10.3390/biology12040526

Esser, C., & Rannug, A. (2015). The aryl hydrocarbon receptor in barrier organ physiology, immunology, and toxicology. *Pharmacol. Rev., 67*(2), 259-279. doi:10.1124/pr.114.009001

Fagerberg, L., Hallström, B. M., Oksvold, P., Kampf, C., Djureinovic, D., Odeberg, J., . . . Uhlén, M. (2014). Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol. Cell. Proteomics., 13*(2), 397-406. doi:10.1074/mcp.M113.035600

Fernández-Gallego, N., Sánchez-Madrid, F., & Cibrian, D. (2021). Role of AHR Ligands in Skin Homeostasis and Cutaneous Inflammation. *Cells, 10*(11). doi:10.3390/cells10113176

Fernandez-Salguero, P., Hilbert, D. M., Rudikoff, S., Ward, J. M., & Gonzalez, F. J. (1996). Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity. *Toxicol. Appl. Pharmacol., 140*(1), 173-179. doi:10.1006/taap.1996.0210

Fernandez-Salguero, P., Pineau, T., Hilbert, D. M., McPhail, T., Lee, S. S., Kimura, S., . . . Gonzalez, F. J. (1995). Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science, 268*(5211), 722-726. doi:10.1126/science.7732381

Fitzgerald, C. T., Nebert, D. W., & Puga, A. (1998). Regulation of mouse Ah receptor (Ahr) gene basal expression by members of the Sp family of transcription factors. *DNA. Cell. Biol., 17*(9), 811-822. doi:10.1089/dna.1998.17.811

Freitas, N., & Cunha, C. (2009). Mechanisms and signals for the nuclear import of proteins. *Curr. Genomics., 10*(8), 550-557. doi:10.2174/138920209789503941

Fritsche, E., Schäfer, C., Calles, C., Bernsmann, T., Bernshausen, T., Wurm, M., . . . Krutmann, J. (2007). Lightening up the UV response by identification of the arylhydrocarbon receptor as a

cytoplasmatic target for ultraviolet B radiation. *Proc. Natl. Acad. Sci. U.S.A., 104*(21), 8851-8856. doi:10.1073/pnas.0701764104

Frohnert, C., Hutten, S., Wälde, S., Nath, A., & Kehlenbach, R. H. (2014). Importin 7 and Nup358 Promote Nuclear Import of the Protein Component of Human Telomerase. *PLOS ONE, 9*(2), e88887. doi:10.1371/journal.pone.0088887

Fujii-Kuriyama, Y., & Kawajiri, K. (2010). Molecular mechanisms of the physiological functions of the aryl hydrocarbon (dioxin) receptor, a multifunctional regulator that senses and responds to environmental stimuli. *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci., 86*(1), 40-53. doi:10.2183/pjab.86.40

Gabriel, G., Klingel, K., Otte, A., Thiele, S., Hudjetz, B., Arman-Kalcek, G., . . . Klenk, H.-D. (2011). Differential use of importin-α isoforms governs cell tropism and host adaptation of influenza virus. *Nat. Commun., 2*(1), 156. doi:10.1038/ncomms1158

Garg, R., Gupta, S., & Maru, G. B. (2008). Dietary curcumin modulates transcriptional regulators of phase I and phase II enzymes in benzo[a]pyrene-treated mice: mechanism of its anti-initiating action. *Carcinog., 29*(5), 1022-1032. doi:10.1093/carcin/bgn064

Garrison, P. M., Rogers, J. M., Brackney, W. R., & Denison, M. S. (2000). Effects of histone deacetylase inhibitors on the Ah receptor gene promoter. *Arch. Biochem. Biophys., 374*(2), 161-171. doi:10.1006/abbi.1999.1620

Ghotbaddini, M., Cisse, K., Carey, A., & Powell, J. B. (2017). Simultaneous inhibition of aryl hydrocarbon receptor (AhR) and Src abolishes androgen receptor signaling. *PLOS ONE, 12*(7), e0179844. doi:10.1371/journal.pone.0179844

Gillam, E. M., Notley, L. M., Cai, H., De Voss, J. J., & Guengerich, F. P. (2000). Oxidation of indole by cytochrome P450 enzymes. *Biochemistry, 39*(45), 13817-13824. doi:10.1021/bi001229u

Gomez, A., Bindesbøll, C., Satheesh, S. V., Grimaldi, G., Hutin, D., MacPherson, L., . . . Matthews, J. (2018). Characterization of TCDD-inducible poly-ADP-ribose polymerase (TIPARP/ARTD14) catalytic activity. *Biochem. J., 475*(23), 3827-3846. doi:10.1042/bcj20180347

Gonzalez, F. J., & Fernandez-Salguero, P. (1998). The aryl hydrocarbon receptor: studies using the AHR-null mice. *Drug. Metab. Dispos., 26*(12), 1194-1198.

Götz, V., Magar, L., Dornfeld, D., Giese, S., Pohlmann, A., Höper, D., . . . Schwemmle, M. (2016). Influenza A viruses escape from MxA restriction at the expense of efficient nuclear vRNP import. *Sci. Rep., 6*(1), 23138. doi:10.1038/srep23138

Greb-Markiewicz, B., & Kolonko, M. (2019). Subcellular Localization Signals of bHLH-PAS Proteins: Their Significance, Current State of Knowledge and Future Perspectives. *Int. J. Mol. Sci., 20*(19). doi:10.3390/ijms20194746

Grishanova, A. Y., & Perepechaeva, M. L. (2022). Aryl Hydrocarbon Receptor in Oxidative Stress as a Double Agent and Its Biological and Therapeutic Significance. *Int. J. Mol. Sci., 23*(12). doi:10.3390/ijms23126719

Gruszczyk, J., Grandvuillemin, L., Lai-Kee-Him, J., Paloni, M., Savva, C. G., Germain, P., . . . Bourguet, W. (2022). Cryo-EM structure of the agonist-bound Hsp90-XAP2-AHR cytosolic complex. *Nat. Commun., 13*(1), 7010. doi:10.1038/s41467-022-34773-w

Guerrina, N., Traboulsi, H., Eidelman, D. H., & Baglole, C. J. (2018). The Aryl Hydrocarbon Receptor and the Maintenance of Lung Health. *Int. J. Mol. Sci., 19*(12). doi:10.3390/ijms19123882

Gutiérrez-Vázquez, C., & Quintana, F. J. (2018). Regulation of the Immune Response by the Aryl Hydrocarbon Receptor. *Immunity, 48*(1), 19-33. doi:10.1016/j.immuni.2017.12.012

Hahn, M. E., Karchner, S. I., & Merson, R. R. (2017). Diversity as Opportunity: Insights from 600 Million Years of AHR Evolution. *Curr. Opin. Toxicol., 2*, 58-71. doi:10.1016/j.cotox.2017.02.003

Haidar, R., Henkler, F., Kugler, J., Rosin, A., Genkinger, D., Laux, P., & Luch, A. (2021). The role of DNA-binding and ARNT dimerization on the nucleo-cytoplasmic translocation of the aryl hydrocarbon receptor. *Sci. Rep., 11*(1), 18194. doi:10.1038/s41598-021-97507-w

Haidar, R., Shabo, R., Moeser, M., Luch, A., & Kugler, J. (2023). The nuclear entry of the aryl hydrocarbon receptor (AHR) relies on the first nuclear localization signal and can be negatively regulated through IMPα/β specific inhibitors. *Sci. Rep., 13*(1), 19668. doi:10.1038/s41598-023-47066-z

Hamamoto, T., Gunji, S., Tsuji, H., & Beppu, T. (1983). Leptomycins A and B, new antifungal antibiotics. I. Taxonomy of the producing strain and their fermentation, purification and characterization. *J Antibiot (Tokyo), 36*(6), 639-645. doi:10.7164/antibiotics.36.639

Hanson, K., Robinson, S. D., Al-Yousuf, K., Hendry, A. E., Sexton, D. W., Sherwood, V., & Wheeler, G. N. (2018). The anti-rheumatic drug, leflunomide, synergizes with MEK inhibition to suppress melanoma growth. *Oncotarget, 9*(3), 3815-3829. doi:10.18632/oncotarget.23378

Hayashibara, T., Yamada, Y., Mori, N., Harasawa, H., Sugahara, K., Miyanishi, T., . . . Tomonaga, M. (2003). Possible involvement of aryl hydrocarbon receptor (AhR) in adult T-cell leukemia (ATL) leukemogenesis: constitutive activation of AhR in ATL. *Biochem. Biophys. Res. Commun., 300*(1), 128-134. doi:10.1016/s0006-291x(02)02793-6

Hilliard, M., Frohnert, C., Spillner, C., Marcone, S., Nath, A., Lampe, T., . . . Kehlenbach, R. H. (2010). The anti-inflammatory prostaglandin 15-deoxy-delta(12,14)-PGJ2 inhibits CRM1-dependent nuclear protein export. *J. Biol. Chem., 285*(29), 22202-22210. doi:10.1074/jbc.M110.131821

Hoessel, R., Leclerc, S., Endicott, J. A., Nobel, M. E., Lawrie, A., Tunnah, P., . . . Meijer, L. (1999). Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. *Nat. Cell. Biol., 1*(1), 60-67. doi:10.1038/9035

Hoffman, E. C., Reyes, H., Chu, F. F., Sander, F., Conley, L. H., Brooks, B. A., & Hankinson, O. (1991). Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science, 252*(5008), 954-958. doi:10.1126/science.1852076

Huenniger, K., Krämer, A., Soom, M., Chang, I., Köhler, M., Depping, R., . . . Kaether, C. (2010). Notch1 signaling is mediated by importins alpha 3, 4, and 7. *Cell. Mol. Life. Sci., 67*(18), 3187-3196. doi:10.1007/s00018-010-0378-7

Ikuta, T., Eguchi, H., Tachibana, T., Yoneda, Y., & Kawajiri, K. (1998). Nuclear localization and export signals of the human aryl hydrocarbon receptor. *J. Biol. Chem., 273*(5), 2895-2904. doi:10.1074/jbc.273.5.2895

Ikuta, T., Kobayashi, Y., & Kawajiri, K. (2004a). Cell density regulates intracellular localization of aryl hydrocarbon receptor. *J. Biol. Chem., 279*(18), 19209-19216. doi:10.1074/jbc.M310492200

Ikuta, T., Kobayashi, Y., & Kawajiri, K. (2004b). Phosphorylation of nuclear localization signal inhibits the ligand-dependent nuclear import of aryl hydrocarbon receptor. *Biochem. Biophys. Res. Commun., 317*(2), 545-550. doi:10.1016/j.bbrc.2004.03.076

Ikuta, T., Tachibana, T., Watanabe, J., Yoshida, M., Yoneda, Y., & Kawajiri, K. (2000). Nucleocytoplasmic shuttling of the aryl hydrocarbon receptor. *J. Biochem., 127*(3), 503-509. doi:10.1093/oxfordjournals.jbchem.a022633

Jans, D. A., Martin, A. J., & Wagstaff, K. M. (2019). Inhibitors of nuclear transport. *Curr. Opin. Cell. Biol., 58*, 50-60. doi:10.1016/j.ceb.2019.01.001

Jeschke, U., Zhang, X., Kuhn, C., Jalaguier, S., Colinge, J., Pfender, K., . . . Cavaillès, V. (2019). The Prognostic Impact of the Aryl Hydrocarbon Receptor (AhR) in Primary Breast Cancer Depends on the Lymph Node Status. *Int. J. Mol. Sci., 20*(5). doi:10.3390/ijms20051016

Jones, S. (2004). An overview of the basic helix-loop-helix proteins. *Genome Biol., 5*(6), 226. doi:10.1186/gb-2004-5-6-226

Juricek, L., & Coumoul, X. (2018). The Aryl Hydrocarbon Receptor and the Nervous System. *Int. J. Mol. Sci., 19*(9). doi:10.3390/ijms19092504

Karlsson, M., Zhang, C., Méar, L., Zhong, W., Digre, A., Katona, B., . . . Lindskog, C. (2021). A single-cell type transcriptomics map of human tissues. *Sci. Adv., 7*(31). doi:10.1126/sciadv.abh2169

Kazlauskas, A., Poellinger, L., & Pongratz, I. (1999). Evidence that the co-chaperone p23 regulates ligand responsiveness of the dioxin (Aryl hydrocarbon) receptor. *J. Biol. Chem., 274*(19), 13519-13524. doi:10.1074/jbc.274.19.13519

Kazlauskas, A., Poellinger, L., & Pongratz, I. (2000). The immunophilin-like protein XAP2 regulates ubiquitination and subcellular localization of the dioxin receptor. *J. Biol. Chem., 275*(52), 41317-41324. doi:10.1074/jbc.M007765200

Keam, S. J. (2022). Tapinarof Cream 1%: First Approval. *Drugs, 82*(11), 1221-1228. doi:10.1007/s40265-022-01748-6

Keminer, O., & Peters, R. (1999). Permeability of single nuclear pores. *Biophys. J., 77*(1), 217-228. doi:10.1016/s0006-3495(99)76883-9

Kennedy, L. H., Sutter, C. H., Leon Carrion, S., Tran, Q. T., Bodreddigari, S., Kensicki, E., . . . Sutter, T. R. (2013). 2,3,7,8-Tetrachlorodibenzo-p-dioxin-mediated production of reactive oxygen species is an essential step in the mechanism of action to accelerate human keratinocyte differentiation. *Toxicol. Sci., 132*(1), 235-249. doi:10.1093/toxsci/kfs325

Kikuchi, H., Hossain, A., Yoshida, H., & Kobayashi, S. (1998). Induction of cytochrome P-450 1A1 by omeprazole in human HepG2 cells is protein tyrosine kinase-dependent and is not inhibited by alpha-naphthoflavone. *Arch. Biochem. Biophys., 358*(2), 351-358. doi:10.1006/abbi.1998.0869

Kimura, A., Naka, T., Nakahama, T., Chinen, I., Masuda, K., Nohara, K., . . . Kishimoto, T. (2009). Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. *J. Exp. Med., 206*(9), 2027-2035. doi:10.1084/jem.20090560

Kimura, A., Naka, T., Nohara, K., Fujii-Kuriyama, Y., & Kishimoto, T. (2008). Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proc. Natl. Acad. Sci. U.S.A., 105*(28), 9721-9726. doi:10.1073/pnas.0804231105

Kolluri, S. K., Jin, U. H., & Safe, S. (2017). Role of the aryl hydrocarbon receptor in carcinogenesis and potential as an anti-cancer drug target. *Arch. Toxicol., 91*(7), 2497-2513. doi:10.1007/s00204-017-1981-2

Kolonko, M., & Greb-Markiewicz, B. (2019). bHLH-PAS Proteins: Their Structure and Intrinsic Disorder. *Int. J. Mol. Sci., 20*(15). doi:10.3390/ijms20153653

Komeili, A., & O'Shea, E. K. (2000). Nuclear transport and transcription. *Curr. Opin. Cell. Biol., 12*(3), 355-360. doi:10.1016/s0955-0674(00)00100-9

Koper, J. E., Troise, A. D., Loonen, L. M., Vitaglione, P., Capuano, E., Fogliano, V., & Wells, J. M. (2022). Tryptophan Supplementation Increases the Production of Microbial-Derived AhR Agonists in an In Vitro Simulator of Intestinal Microbial Ecosystem. *J. Agric. Food. Chem., 70*(13), 3958-3968. doi:10.1021/acs.jafc.1c04145

Kosyna, F. K., & Depping, R. (2018). Controlling the Gatekeeper: Therapeutic Targeting of Nuclear Transport. *Cells, 7*(11). doi:10.3390/cells7110221

Kosyna, F. K., Nagel, M., Kluxen, L., Kraushaar, K., & Depping, R. (2015). The importin α/β-specific inhibitor Ivermectin affects HIF-dependent hypoxia response pathways. *Biol. Chem., 396*(12), 1357-1367. doi:10.1515/hsz-2015-0171

Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E. P., Wolff, B., . . . Horinouchi, S. (1999). Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc. Natl. Acad. Sci. U.S.A., 96*(16), 9112-9117. doi:10.1073/pnas.96.16.9112

Kumar, M. B., & Perdew, G. H. (1999). Nuclear receptor coactivator SRC-1 interacts with the Q-rich subdomain of the AhR and modulates its transactivation potential. *Gene. Expr., 8*(5-6), 273-286.

Kumar, M. B., Ramadoss, P., Reen, R. K., Vanden Heuvel, J. P., & Perdew, G. H. (2001). The Q-rich subdomain of the human Ah receptor transactivation domain is required for dioxin-mediated transcriptional activity. *J. Biol. Chem., 276*(45), 42302-42310. doi:10.1074/jbc.M104798200

Kuusisto, H. V., & Jans, D. A. (2015). Hyper-dependence of breast cancer cell types on the nuclear transporter Importin β1. *Biochim. Biophys. Acta. Mol. Cell. Res., 1853*(8), 1870-1878. doi:https://doi.org/10.1016/j.bbamcr.2015.05.002

Lahvis, G. P., & Bradfield, C. A. (1998). Ahr null alleles: distinctive or different? *Biochem. Pharmacol., 56*(7), 781-787. doi:10.1016/s0006-2952(98)00134-8

Lahvis, G. P., Pyzalski, R. W., Glover, E., Pitot, H. C., McElwee, M. K., & Bradfield, C. A. (2005). The aryl hydrocarbon receptor is required for developmental closure of the ductus venosus in the neonatal mouse. *Mol. Pharmacol., 67*(3), 714-720. doi:10.1124/mol.104.008888

Langtry, H. D., & Wilde, M. I. (1998). Omeprazole. A review of its use in Helicobacter pylori infection, gastro-oesophageal reflux disease and peptic ulcers induced by nonsteroidal anti-inflammatory drugs. *Drugs, 56*(3), 447-486. doi:10.2165/00003495-199856030-00012

LaPres, J. J., Glover, E., Dunham, E. E., Bunger, M. K., & Bradfield, C. A. (2000). ARA9 modifies agonist signaling through an increase in cytosolic aryl hydrocarbon receptor. *J. Biol. Chem., 275*(9), 6153-6159. doi:10.1074/jbc.275.9.6153

Larigot, L., Benoit, L., Koual, M., Tomkiewicz, C., Barouki, R., & Coumoul, X. (2022). Aryl Hydrocarbon Receptor and Its Diverse Ligands and Functions: An Exposome Receptor. *Annu. Rev. Pharmacol. Toxicol., 62*, 383-404. doi:10.1146/annurev-pharmtox-052220-115707

Lin, T. M., Ko, K., Moore, R. W., Simanainen, U., Oberley, T. D., & Peterson, R. E. (2002). Effects of aryl hydrocarbon receptor null mutation and in utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure on prostate and seminal vesicle development in C57BL/6 mice. *Toxicol. Sci., 68*(2), 479-487. doi:10.1093/toxsci/68.2.479

Liu, Y., Zhou, N., Zhou, L., Wang, J., Zhou, Y., Zhang, T., . . . Huang, B. (2021). IL-2 regulates tumor-reactive CD8(+) T cell exhaustion by activating the aryl hydrocarbon receptor. *Nat. Immunol., 22*(3), 358-369. doi:10.1038/s41590-020-00850-9

Lo, W. Y., & Chang, N. W. (2013). An indirubin derivative, indirubin-3'-monoxime suppresses oral cancer tumorigenesis through the downregulation of survivin. *PLOS ONE, 8*(8), e70198. doi:10.1371/journal.pone.0070198

Lubet, R. A., Heckman, B. M., De Flora, S. L., Steele, V. E., Crowell, J. A., Juliana, M. M., & Grubbs, C. J. (2011). Effects of 5,6-benzoflavone, indole-3-carbinol (I3C) and diindolylmethane (DIM) on chemically-induced mammary carcinogenesis: is DIM a substitute for I3C? *Oncol. Rep., 26*(3), 731-736. doi:10.3892/or.2011.1316

Luch, A. (2005). Nature and nurture – lessons from chemical carcinogenesis. *Nat. Rev. Cancer., 5*(2), 113-125. doi:10.1038/nrc1546

Luecke-Johansson, S., Gralla, M., Rundqvist, H., Ho, J. C., Johnson, R. S., Gradin, K., & Poellinger, L. (2017). A Molecular Mechanism To Switch the Aryl Hydrocarbon Receptor from a Transcription Factor to an E3 Ubiquitin Ligase. *Mol. Cell. Biol., 37*(13). doi:10.1128/mcb.00630-16

Ma, J., Goryaynov, A., Sarma, A., & Yang, W. (2012). Self-regulated viscous channel in the nuclear pore complex. *Proc. Natl. Acad. Sci. U.S.A., 109*(19), 7326-7331. doi:10.1073/pnas.1201724109

Ma, Q., & Baldwin, K. T. (2000). 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced degradation of aryl hydrocarbon receptor (AhR) by the ubiquitin-proteasome pathway. Role of the transcription activaton and DNA binding of AhR. *J Biol Chem, 275*(12), 8432-8438. doi:10.1074/jbc.275.12.8432

Marlowe, J., & Puga, A. (2010). Novel AHR Interactions. In *Comprehensive Toxicology (Second Edition)* (Vol. 8, pp. 93-115). Oxford: Elsevier.

Massoomi, F., Savage, J., & Destache, C. J. (1993). Omeprazole: a comprehensive review. *Pharmacotherapy, 13*(1), 46-59.

Masuda, K., Kimura, A., Hanieh, H., Nguyen, N. T., Nakahama, T., Chinen, I., . . . Kishimoto, T. (2011). Aryl hydrocarbon receptor negatively regulates LPS-induced IL-6 production through suppression of histamine production in macrophages. *Int. Immunol., 23*(10), 637-645. doi:10.1093/intimm/dxr072

Matsumoto, S., Akahane, M., Kanagawa, Y., Kajiwara, J., Mitoma, C., Uchi, H., . . . Imamura, T. (2015). Unexpectedly long half-lives of blood 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) levels in Yusho patients. *Environ. Health., 14*(1), 76. doi:10.1186/s12940-015-0059-y

McBride, K. M., Banninger, G., McDonald, C., & Reich, N. C. (2002). Regulated nuclear import of the STAT1 transcription factor by direct binding of importin-alpha. *Embo. J., 21*(7), 1754-1763. doi:10.1093/emboj/21.7.1754

Meijer, L., Guyard, N., Skaltousnis, L. A., & Eisenbrand, G. (2006). *Indirubin, the red shade of indigo*: Life in Progress Edition.

Mengoni, M., Braun, A. D., Gaffal, E., & Tüting, T. (2020). The aryl hydrocarbon receptor promotes inflammation-induced dedifferentiation and systemic metastatic spread of melanoma cells. *Int. J. Cancer., 147*(10), 2902-2913. doi:10.1002/ijc.33252

Merchant, M., Krishnan, V., & Safe, S. (1993). Mechanism of action of alpha-naphthoflavone as an Ah receptor antagonist in MCF-7 human breast cancer cells. *Toxicol. Appl. Pharmacol., 120*(2), 179-185. doi:10.1006/taap.1993.1101

Meyer, B. K., & Perdew, G. H. (1999). Characterization of the AhR-hsp90-XAP2 core complex and the role of the immunophilin-related protein XAP2 in AhR stabilization. *Biochemistry, 38*(28), 8907-8917. doi:10.1021/bi982223w

Meyer, B. K., Pray-Grant, M. G., Vanden Heuvel, J. P., & Perdew, G. H. (1998). Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. *Mol. Cell. Biol., 18*(2), 978-988. doi:10.1128/mcb.18.2.978

Meyer, T., & Vinkemeier, U. (2004). Nucleocytoplasmic shuttling of STAT transcription factors. *Eur. J. Biochem., 271*(23-24), 4606-4612. doi:10.1111/j.1432-1033.2004.04423.x

Micka, J., Milatovich, A., Menon, A., Grabowski, G. A., Puga, A., & Nebert, D. W. (1997). Human Ah receptor (AHR) gene: localization to 7p15 and suggestive correlation of polymorphism with CYP1A1 inducibility. *Pharmacogenetics, 7*(2), 95-101. doi:10.1097/00008571-199704000-00002

Mimura, J., Ema, M., Sogawa, K., & Fujii-Kuriyama, Y. (1999). Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. *Genes. Dev., 13*(1), 20-25. doi:10.1101/gad.13.1.20

Mobini, K., Tamaddon, G., Fardid, R., Keshavarzi, M., & Mohammadi-Bardbori, A. (2019). Aryl hydrocarbon-estrogen alpha receptor-dependent expression of miR-206, miR-27b, and miR-133a suppress cell proliferation and migration in MCF-7 cells. *J. Biochem. Mol. Toxicol., 33*(6), e22304. doi:10.1002/jbt.22304

Moretti, S., Nucci, N., Menicali, E., Morelli, S., Bini, V., Colella, R., . . . Puxeddu, E. (2020). The Aryl Hydrocarbon Receptor Is Expressed in Thyroid Carcinoma and Appears to Mediate Epithelial-Mesenchymal-Transition. *Cancers (Basel), 12*(1). doi:10.3390/cancers12010145

Mulero-Navarro, S., Carvajal-Gonzalez, J. M., Herranz, M., Ballestar, E., Fraga, M. F., Ropero, S., . . . Fernandez-Salguero, P. M. (2006). The dioxin receptor is silenced by promoter hypermethylation in human acute lymphoblastic leukemia through inhibition of Sp1 binding. *Carcinogenesis, 27*(5), 1099-1104. doi:10.1093/carcin/bgi344

Murray, I. A., Patterson, A. D., & Perdew, G. H. (2014). Aryl hydrocarbon receptor ligands in cancer: friend and foe. *Nat. Rev. Cancer., 14*(12), 801-814. doi:10.1038/nrc3846

Murray, I. A., & Perdew, G. H. (2020). How Ah Receptor Ligand Specificity Became Important in Understanding Its Physiological Function. *Int. J. Mol. Sci., 21*(24). doi:10.3390/ijms21249614

Nebert, D. W. (2017). Aryl hydrocarbon receptor (AHR): "pioneer member" of the basic-helix/loop/helix per-Arnt-sim (bHLH/PAS) family of "sensors" of foreign and endogenous signals. *Prog. Lipid. Res., 67*, 38-57. doi:10.1016/j.plipres.2017.06.001

Nebert, D. W., & Gelboin, H. V. (1968a). Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. *J. Biol. Chem., 243*(23), 6242-6249.

Nebert, D. W., & Gelboin, H. V. (1968b). Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. II. Cellular responses during enzyme induction. *J. Biol. Chem., 243*(23), 6250-6261.

Nebert, D. W., & Gielen, J. E. (1972). Genetic regulation of aryl hydrocarbon hydroxylase induction in the mouse. *Fed. Proc., 31*(4), 1315-1325.

Nebert, D. W., Roe, A. L., Dieter, M. Z., Solis, W. A., Yang, Y., & Dalton, T. P. (2000). Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochem. Pharmacol., 59*(1), 65-85. doi:https://doi.org/10.1016/S0006-2952(99)00310-X

Nguyen, L. P., & Bradfield, C. A. (2008). The Search for Endogenous Activators of the Aryl Hydrocarbon Receptor. *Chem. Res. Toxicol., 21*(1), 102-116. doi:10.1021/tx7001965

Novikov, O., Wang, Z., Stanford, E. A., Parks, A. J., Ramirez-Cardenas, A., Landesman, E., . . . Sherr, D. H. (2016). An Aryl Hydrocarbon Receptor-Mediated Amplification Loop That Enforces Cell Migration in ER-/PR-/Her2- Human Breast Cancer Cells. *Mol. Pharmacol., 90*(5), 674-688. doi:10.1124/mol.116.105361

Numayama-Tsuruta, K., Kobayashi, A., Sogawa, K., & Fujii-Kuriyama, Y. (1997). A point mutation responsible for defective function of the aryl-hydrocarbon-receptor nuclear translocator in

mutant Hepa-1c1c7 cells. *Eur J Biochem, 246*(2), 486-495. doi:10.1111/j.1432-1033.1997.00486.x

O'Donnell, E. F., Saili, K. S., Koch, D. C., Kopparapu, P. R., Farrer, D., Bisson, W. H., . . . Kolluri, S. K. (2010). The anti-inflammatory drug leflunomide is an agonist of the aryl hydrocarbon receptor. *PLOS ONE, 5*(10). doi:10.1371/journal.pone.0013128

Ohtake, F., Baba, A., Takada, I., Okada, M., Iwasaki, K., Miki, H., . . . Kato, S. (2007). Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. *Nature, 446*(7135), 562-566. doi:10.1038/nature05683

Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., . . . Kato, S. (2003). Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature, 423*(6939), 545-550. doi:10.1038/nature01606

Okey, A. B., Bondy, G. P., Mason, M. E., Kahl, G. F., Eisen, H. J., Guenthner, T. M., & Nebert, D. W. (1979). Regulatory gene product of the Ah locus. Characterization of the cytosolic inducer-receptor complex and evidence for its nuclear translocation. *J. Biol. Chem., 254*(22), 11636-11648.

Oshima, M., Mimura, J., Sekine, H., Okawa, H., & Fujii-Kuriyama, Y. (2009). SUMO modification regulates the transcriptional repressor function of aryl hydrocarbon receptor repressor. *J. Biol. Chem., 284*(17), 11017-11026. doi:10.1074/jbc.M808694200

Pappas, B., Yang, Y., Wang, Y., Kim, K., Chung, H. J., Cheung, M., . . . Chan, W. K. (2018). p23 protects the human aryl hydrocarbon receptor from degradation via a heat shock protein 90-independent mechanism. *Biochem. Pharmacol., 152*, 34-44. doi:10.1016/j.bcp.2018.03.015

Paris, A., Tardif, N., Galibert, M. D., & Corre, S. (2021). AhR and Cancer: From Gene Profiling to Targeted Therapy. *Int. J. Mol. Sci., 22*(2). doi:10.3390/ijms22020752

Parks, A. J., Pollastri, M. P., Hahn, M. E., Stanford, E. A., Novikov, O., Franks, D. G., . . . Sherr, D. H. (2014). In silico identification of an aryl hydrocarbon receptor antagonist with biological activity in vitro and in vivo. *Mol. Pharmacol., 86*(5), 593-608. doi:10.1124/mol.114.093369

Perdew, G. H. (1988). Association of the Ah receptor with the 90-kDa heat shock protein. *J. Biol. Chem., 263*(27), 13802-13805.

Perdew, G. H., Esser, C., Snyder, M., Sherr, D. H., van den Bogaard, E. H., McGovern, K., . . . Patterson, A. D. (2023). The Ah Receptor from Toxicity to Therapeutics: Report from the 5th AHR Meeting at Penn State University, USA, June 2022. *Int. J. Mol. Sci., 24*(6). doi:10.3390/ijms24065550

Petrulis, J. R., Kusnadi, A., Ramadoss, P., Hollingshead, B., & Perdew, G. H. (2003). The hsp90 Co-chaperone XAP2 Alters Importin β Recognition of the Bipartite Nuclear Localization Signal of the Ah Receptor and Represses Transcriptional Activity. *J. Biol. Chem., 278*(4), 2677-2685. doi:https://doi.org/10.1074/jbc.M209331200

Petrulis, J. R., & Perdew, G. H. (2002). The role of chaperone proteins in the aryl hydrocarbon receptor core complex. *Chem. Biol. Interact., 141*(1), 25-40. doi:10.1016/S0009-2797(02)00064-9

Phillips, J. L., Löhr, C. V., Nguyen, B. D., Buermeyer, A. B., & Kolluri, S. K. (2022). Loss of the aryl hydrocarbon receptor increases tumorigenesis in p53-deficient mice. *Toxicol. Appl. Pharmacol., 454*, 116191. doi:10.1016/j.taap.2022.116191

Piwarski, S. A., Thompson, C., Chaudhry, A. R., Denvir, J., Primerano, D. A., Fan, J., & Salisbury, T. B. (2020). The putative endogenous AHR ligand ITE reduces JAG1 and associated NOTCH1 signaling in triple negative breast cancer cells. *Biochem. Pharmacol., 174*, 113845. doi:10.1016/j.bcp.2020.113845

Poland, A., Glover, E., & Kende, A. S. (1976). Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. *J. Biol. Chem., 251*(16), 4936-4946.

Pollenz, R. S., & Barbour, E. R. (2000). Analysis of the complex relationship between nuclear export and aryl hydrocarbon receptor-mediated gene regulation. *Mol. Cell. Biol., 20*(16), 6095-6104. doi:10.1128/mcb.20.16.6095-6104.2000

Pollenz, R. S., Wilson, S. E., & Dougherty, E. J. (2006). Role of endogenous XAP2 protein on the localization and nucleocytoplasmic shuttling of the endogenous mouse Ahb-1 receptor in the presence and absence of ligand. *Mol. Pharmacol., 70*(4), 1369-1379. doi:10.1124/mol.106.027672

Pongratz, I., Mason, G. G., & Poellinger, L. (1992). Dual roles of the 90-kDa heat shock protein hsp90 in modulating functional activities of the dioxin receptor. Evidence that the dioxin receptor functionally belongs to a subclass of nuclear receptors which require hsp90 both for ligand binding activity and repression of intrinsic DNA binding activity. *J. Biol. Chem., 267*(19), 13728-13734.

Popken, P., Ghavami, A., Onck, P. R., Poolman, B., & Veenhoff, L. M. (2015). Size-dependent leak of soluble and membrane proteins through the yeast nuclear pore complex. *Mol. Biol. Cell., 26*(7), 1386-1394. doi:10.1091/mbc.E14-07-1175

Popp, M., Reis, S., Schießer, S., Hausinger, R. I., Stegemann, M., Metzendorf, M. I., . . . Weibel, S. (2022). Ivermectin for preventing and treating COVID-19. *Cochrane. Database. Syst. Rev., 6*(6), Cd015017. doi:10.1002/14651858.CD015017.pub3

Pumroy, R. A., & Cingolani, G. (2015). Diversification of importin-α isoforms in cellular trafficking and disease states. *Biochem. J., 466*(1), 13-28. doi:10.1042/bj20141186

Quimby, B. B., & Dasso, M. (2003). The small GTPase Ran: interpreting the signs. *Curr. Opin. Cell. Biol., 15*(3), 338-344. doi:10.1016/s0955-0674(03)00046-2

Ramadoss, P., & Perdew, G. H. (2005). The transactivation domain of the Ah receptor is a key determinant of cellular localization and ligand-independent nucleocytoplasmic shuttling properties. *Biochemistry, 44*(33), 11148-11159. doi:10.1021/bi050948b

Ramadoss, P., Petrulis, J. R., Hollingshead, B. D., Kusnadi, A., & Perdew, G. H. (2004). Divergent roles of hepatitis B virus X-associated protein 2 (XAP2) in human versus mouse Ah receptor complexes. *Biochemistry, 43*(3), 700-709. doi:10.1021/bi035827v

Rannug, A., Rannug, U., Rosenkranz, H. S., Winqvist, L., Westerholm, R., Agurell, E., & Grafström, A. K. (1987). Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah receptor and are likely to be endogenous signal substances. *J. Biol. Chem., 262*(32), 15422-15427.

Rebl, H., Sawade, M., Hein, M., Bergemann, C., Wende, M., Lalk, M., . . . Nebe, B. (2022). Synergistic effect of plasma-activated medium and novel indirubin derivatives on human skin cancer cells by activation of the AhR pathway. *Sci. Rep., 12*(1), 2528. doi:10.1038/s41598-022-06523-x

Reyes, H., Reisz-Porszasz, S., & Hankinson, O. (1992). Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science, 256*(5060), 1193-1195. doi:10.1126/science.256.5060.1193

Richter, C. A., Tillitt, D. E., & Hannink, M. (2001). Regulation of subcellular localization of the aryl hydrocarbon receptor (AhR). *Arch. Biochem. Biophys., 389*(2), 207-217. doi:10.1006/abbi.2001.2339

Rijo, M. P., Diani-Moore, S., Yang, C., Zhou, P., & Rifkind, A. B. (2021). Roles of the ubiquitin ligase CUL4B and ADP-ribosyltransferase TiPARP in TCDD-induced nuclear export and proteasomal degradation of the transcription factor AHR. *J. Biol. Chem., 297*(2), 100886. doi:10.1016/j.jbc.2021.100886

Rothhammer, V., & Quintana, F. J. (2019). The aryl hydrocarbon receptor: an environmental sensor integrating immune responses in health and disease. *Nat. Rev. Immunol., 19*(3), 184-197. doi:10.1038/s41577-019-0125-8

Sampaio, G. R., Guizellini, G. M., da Silva, S. A., de Almeida, A. P., Pinaffi-Langley, A. C. C., Rogero, M. M., . . . Torres, E. (2021). Polycyclic Aromatic Hydrocarbons in Foods: Biological Effects, Legislation, Occurrence, Analytical Methods, and Strategies to Reduce Their Formation. *Int. J. Mol. Sci., 22*(11). doi:10.3390/ijms22116010

Santostefano, M., Merchant, M., Arellano, L., Morrison, V., Denison, M. S., & Safe, S. (1993). alpha-Naphthoflavone-induced CYP1A1 gene expression and cytosolic aryl hydrocarbon receptor transformation. *Mol. Pharmacol., 43*(2), 200-206.

Schaldach, C. M., Riby, J., & Bjeldanes, L. F. (1999). Lipoxin A4: a new class of ligand for the Ah receptor. *Biochemistry, 38*(23), 7594-7600. doi:10.1021/bi982861e

Schecter, A., Birnbaum, L., Ryan, J. J., & Constable, J. D. (2006). Dioxins: an overview. *Environ. Res., 101*(3), 419-428. doi:10.1016/j.envres.2005.12.003

Schulte, K. W., Green, E., Wilz, A., Platten, M., & Daumke, O. (2017). Structural Basis for Aryl Hydrocarbon Receptor-Mediated Gene Activation. *Structure, 25*(7), 1025-1033.e1023. doi:10.1016/j.str.2017.05.008

Seok, S. H., Lee, W., Jiang, L., Molugu, K., Zheng, A., Li, Y., . . . Xing, Y. (2017). Structural hierarchy controlling dimerization and target DNA recognition in the AHR transcriptional complex. *Proc. Natl. Acad. Sci. U.S.A., 114*(21), 5431-5436. doi:10.1073/pnas.1617035114

Shivanna, B., Chu, C., & Moorthy, B. (2022). The Aryl Hydrocarbon Receptor (AHR): A Novel Therapeutic Target for Pulmonary Diseases? *Int. J. Mol. Sci., 23*(3). doi:10.3390/ijms23031516

Sinal, C. J., & Bend, J. R. (1997). Aryl hydrocarbon receptor-dependent induction of cyp1a1 by bilirubin in mouse hepatoma hepa 1c1c7 cells. *Mol. Pharmacol., 52*(4), 590-599. doi:10.1124/mol.52.4.590

Sinal, C. J., Webb, C. D., & Bend, J. R. (1999). Differential in vivo effects of alpha-naphthoflavone and beta-naphthoflavone on CYP1A1 and CYP2E1 in rat liver, lung, heart, and kidney. *J. Biochem. Mol. Toxicol., 13*(1), 29-40. doi:10.1002/(sici)1099-0461(1999)13:1<29::aid-jbt4>3.0.co;2-x

Smirnova, A., Wincent, E., Vikström Bergander, L., Alsberg, T., Bergman, J., Rannug, A., & Rannug, U. (2016). Evidence for New Light-Independent Pathways for Generation of the Endogenous Aryl Hydrocarbon Receptor Agonist FICZ. *Chem. Res. Toxicol., 29*(1), 75-86. doi:10.1021/acs.chemrestox.5b00416

Smith, S. H., Jayawickreme, C., Rickard, D. J., Nicodeme, E., Bui, T., Simmons, C., . . . Cote-Sierra, J. (2017). Tapinarof Is a Natural AhR Agonist that Resolves Skin Inflammation in Mice and Humans. *J. Invest. Dermatol., 137*(10), 2110-2119. doi:10.1016/j.jid.2017.05.004

Soderholm, J. F., Bird, S. L., Kalab, P., Sampathkumar, Y., Hasegawa, K., Uehara-Bingen, M., . . . Heald, R. (2011). Importazole, a small molecule inhibitor of the transport receptor importin-β. *ACS Chem. Biol., 6*(7), 700-708. doi:10.1021/cb2000296

Sojka, K. M., Kern, C. B., & Pollenz, R. S. (2000). Expression and subcellular localization of the aryl hydrocarbon receptor nuclear translocator (ARNT) protein in mouse and chicken over developmental time. *Anat. Rec., 260*(4), 327-334. doi:10.1002/1097-0185(200012)260:4<326::Aid-ar10>3.0.Co;2-u

Sondermann, N. C., Faßbender, S., Hartung, F., Hätälä, A. M., Rolfes, K. M., Vogel, C. F. A., & Haarmann-Stemmann, T. (2023). Functions of the aryl hydrocarbon receptor (AHR) beyond the canonical AHR/ARNT signaling pathway. *Biochem. Pharmacol., 208*, 115371. doi:10.1016/j.bcp.2022.115371

Soshilov, A., & Denison, M. S. (2008). Role of the Per/Arnt/Sim domains in ligand-dependent transformation of the aryl hydrocarbon receptor. *J. Biol. Chem., 283*(47), 32995-33005. doi:10.1074/jbc.M802414200

Spink, B. C., Hussain, M. M., Katz, B. H., Eisele, L., & Spink, D. C. (2003). Transient induction of cytochromes P450 1A1 and 1B1 in MCF-7 human breast cancer cells by indirubin. *Biochem. Pharmacol., 66*(12), 2313-2321. doi:10.1016/j.bcp.2003.08.019

Springs, A. E., & Rice, C. D. (2006). The Effects of Indirubin-3'-Monoxime, A Novel AHR Ligand, on Stress and Toxicity-Related Gene/Protein Expression in Human U937 Cells Undergoing Differentiation and Activation. *J. Immunotoxicol., 3*(1), 1-10. doi:10.1080/15476910500468627

Stanford, E. A., Ramirez-Cardenas, A., Wang, Z., Novikov, O., Alamoud, K., Koutrakis, P., . . . Sherr, D. H. (2016). Role for the Aryl Hydrocarbon Receptor and Diverse Ligands in Oral Squamous Cell Carcinoma Migration and Tumorigenesis. *Mol. Cancer. Res., 14*(8), 696-706. doi:10.1158/1541-7786.Mcr-16-0069

Stejskalova, L., Dvorak, Z., & Pavek, P. (2011). Endogenous and exogenous ligands of aryl hydrocarbon receptor: current state of art. *Curr. Drug. Metab., 12*(2), 198-212. doi:10.2174/138920011795016818

Stevens, E. A., Mezrich, J. D., & Bradfield, C. A. (2009). The aryl hydrocarbon receptor: a perspective on potential roles in the immune system. *Immunology, 127*(3), 299-311. doi:10.1111/j.1365-2567.2009.03054.x

Stobbe-Maicherski, N., Wolff, S., Wolff, C., Abel, J., Sydlik, U., Frauenstein, K., & Haarmann-Stemmann, T. (2013). The interleukin-6-type cytokine oncostatin M induces aryl hydrocarbon receptor

expression in a STAT3-dependent manner in human HepG2 hepatoma cells. *FEBS. J., 280*(24), 6681-6690. doi:10.1111/febs.12571

Strambio-De-Castillia, C., Niepel, M., & Rout, M. P. (2010). The nuclear pore complex: bridging nuclear transport and gene regulation. *Nat. Rev. Mol. Cell. Biol., 11*(7), 490-501. doi:10.1038/nrm2928

Su, J. M., Lin, P., & Chang, H. (2013). Prognostic value of nuclear translocation of aryl hydrocarbon receptor for non-small cell lung cancer. *Anticancer. Res., 33*(9), 3953-3961.

Sugihara, K., Kitamura, S., Yamada, T., Okayama, T., Ohta, S., Yamashita, K., . . . Matsuda, T. (2004). Aryl hydrocarbon receptor-mediated induction of microsomal drug-metabolizing enzyme activity by indirubin and indigo. *Biochem. Biophys. Res. Commun., 318*(2), 571-578. doi:10.1016/j.bbrc.2004.04.066

Swanson, H. I., Chan, W. K., & Bradfield, C. A. (1995a). DNA Binding Specificities and Pairing Rules of the Ah Receptor, ARNT, and SIM Proteins. *J. Biol. Chem., 270*(44), 26292-26302. doi:https://doi.org/10.1074/jbc.270.44.26292

Swanson, H. I., Chan, W. K., & Bradfield, C. A. (1995b). DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins. *J Biol Chem, 270*(44), 26292-26302. doi:10.1074/jbc.270.44.26292

Tanaka, G., Kanaji, S., Hirano, A., Arima, K., Shinagawa, A., Goda, C., . . . Izuhara, K. (2005). Induction and activation of the aryl hydrocarbon receptor by IL-4 in B cells. *Int. Immunol., 17*(6), 797-805. doi:10.1093/intimm/dxh260

Tay, M. Y. F., Fraser, J. E., Chan, W. K. K., Moreland, N. J., Rathore, A. P., Wang, C., . . . Jans, D. A. (2013). Nuclear localization of dengue virus (DENV) 1–4 non-structural protein 5; protection against all 4 DENV serotypes by the inhibitor Ivermectin. *Antiviral. Res., 99*(3), 301-306. doi:https://doi.org/10.1016/j.antiviral.2013.06.002

Timney, B. L., Raveh, B., Mironska, R., Trivedi, J. M., Kim, S. J., Russel, D., . . . Rout, M. P. (2016). Simple rules for passive diffusion through the nuclear pore complex. *J. Cell. Biol., 215*(1), 57-76. doi:10.1083/jcb.201601004

Tkachenko, A., Bermudez, M., Irmer-Stooff, S., Genkinger, D., Henkler-Stephani, F., Wolber, G., & Luch, A. (2018). Nuclear transport of the human aryl hydrocarbon receptor and subsequent gene induction relies on its residue histidine 291. *Arch. Toxicol., 92*(3), 1151-1160. doi:10.1007/s00204-017-2129-0

Tkachenko, A., Henkler, F., Brinkmann, J., Sowada, J., Genkinger, D., Kern, C., . . . Luch, A. (2016). The Q-rich/PST domain of the AHR regulates both ligand-induced nuclear transport and nucleocytoplasmic shuttling. *Sci. Rep., 6*, 32009. doi:10.1038/srep32009

Tsuji, N., Fukuda, K., Nagata, Y., Okada, H., Haga, A., Hatakeyama, S., . . . Itoh, H. (2014). The activation mechanism of the aryl hydrocarbon receptor (AhR) by molecular chaperone HSP90. *FEBS Open Bio, 4*, 796-803. doi:10.1016/j.fob.2014.09.003

Turski, W. A., Wnorowski, A., Turski, G. N., Turski, C. A., & Turski, L. (2020). AhR and IDO1 in pathogenesis of Covid-19 and the "Systemic AhR Activation Syndrome:" a translational review and therapeutic perspectives. *Restor. Neurol. Neurosci., 38*(4), 343-354. doi:10.3233/rnn-201042

Vacher, S., Castagnet, P., Chemlali, W., Lallemand, F., Meseure, D., Pocard, M., . . . Perrot-Applanat, M. (2018). High AHR expression in breast tumors correlates with expression of genes from several signaling pathways namely inflammation and endogenous tryptophan metabolism. *PLOS ONE, 13*(1), e0190619. doi:10.1371/journal.pone.0190619

van der Watt, P. J., Chi, A., Stelma, T., Stowell, C., Strydom, E., Carden, S., . . . Leaner, V. D. (2016). Targeting the Nuclear Import Receptor Kpnβ1 as an Anticancer Therapeutic. *Mol. Cancer. Ther., 15*(4), 560-573. doi:10.1158/1535-7163.Mct-15-0052

Veldhoen, M., Hirota, K., Christensen, J., O'Garra, A., & Stockinger, B. (2009). Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. *J. Exp. Med., 206*(1), 43-49. doi:10.1084/jem.20081438

Vogel, C. F., & Haarmann-Stemmann, T. (2017). The aryl hydrocarbon receptor repressor - More than a simple feedback inhibitor of AhR signaling: Clues for its role in inflammation and cancer. *Curr. Opin. Toxicol., 2*, 109-119. doi:10.1016/j.cotox.2017.02.004

Vogel, C. F., Sciullo, E., Li, W., Wong, P., Lazennec, G., & Matsumura, F. (2007a). RelB, a new partner of aryl hydrocarbon receptor-mediated transcription. *Mol. Endocrinol., 21*(12), 2941-2955. doi:10.1210/me.2007-0211

Vogel, C. F., Sciullo, E., & Matsumura, F. (2007b). Involvement of RelB in aryl hydrocarbon receptor-mediated induction of chemokines. *Biochem. Biophys. Res. Commun., 363*(3), 722-726. doi:10.1016/j.bbrc.2007.09.032

Vogel, C. F., Van Winkle, L. S., Esser, C., & Haarmann-Stemmann, T. (2020). The aryl hydrocarbon receptor as a target of environmental stressors - Implications for pollution mediated stress and inflammatory responses. *Redox. Biol., 34*, 101530. doi:10.1016/j.redox.2020.101530

Vogeley, C., Sondermann, N. C., Woeste, S., Momin, A. A., Gilardino, V., Hartung, F., . . . Haarmann-Stemmann, T. (2022). Unraveling the differential impact of PAHs and dioxin-like compounds on AKR1C3 reveals the EGFR extracellular domain as a critical determinant of the AHR response. *Environ. Int., 158*, 106989. doi:10.1016/j.envint.2021.106989

Wagstaff, K. M., Rawlinson, S. M., Hearps, A. C., & Jans, D. A. (2011). An AlphaScreen®-based assay for high-throughput screening for specific inhibitors of nuclear import. *J. Biomol. Screen., 16*(2), 192-200. doi:10.1177/1087057110390360

Wagstaff, K. M., Sivakumaran, H., Heaton, S. M., Harrich, D., & Jans, D. A. (2012). Ivermectin is a specific inhibitor of importin α/β-mediated nuclear import able to inhibit replication of HIV-1 and dengue virus. *Biochem. J., 443*(3), 851-856. doi:10.1042/bj20120150

Waller, C. L., & McKinney, J. D. (1995). Three-dimensional quantitative structure-activity relationships of dioxins and dioxin-like compounds: model validation and Ah receptor characterization. *Chem. Res. Toxicol., 8*(6), 847-858. doi:10.1021/tx00048a005

Wang, C., Xu, C. X., Bu, Y., Bottum, K. M., & Tischkau, S. A. (2014). Beta-naphthoflavone (DB06732) mediates estrogen receptor-positive breast cancer cell cycle arrest through AhR-dependent regulation of PI3K/AKT and MAPK/ERK signaling. *Carcinogenesis, 35*(3), 703-713. doi:10.1093/carcin/bgt356

Wang, Q., VonHandorf, A., & Puga, A. (2016). Aryl Hydrocarbon Receptor. In S. Choi (Ed.), *Encyclopedia of Signaling Molecules* (pp. 1-15). New York, NY: Springer New York.

Wang, R., & Brattain, M. G. (2007). The maximal size of protein to diffuse through the nuclear pore is larger than 60kDa. *FEBS Lett, 581*(17), 3164-3170. doi:10.1016/j.febslet.2007.05.082

Wang, Z., Snyder, M., Kenison, J. E., Yang, K., Lara, B., Lydell, E., . . . Sherr, D. H. (2020). How the AHR Became Important in Cancer: The Role of Chronically Active AHR in Cancer Aggression. *Int. J. Mol. Sci., 22*(1). doi:10.3390/ijms22010387

Wattenberg, L. W., & Leong, J. L. (1968). Inhibition of the Carcinogenic Action of 7,12-Dimethylbenz(a)anthracene by Beta-Naphthoflavone. *Proc. Soc. Exp., 128*(4), 940-943. doi:10.3181/00379727-128-33161

Wen, W., Meinkoth, J. L., Tsien, R. Y., & Taylor, S. S. (1995). Identification of a signal for rapid export of proteins from the nucleus. *Cell, 82*(3), 463-473. doi:10.1016/0092-8674(95)90435-2

Williams, S. N., Shih, H., Guenette, D. K., Brackney, W., Denison, M. S., Pickwell, G. V., & Quattrochi, L. C. (2000). Comparative studies on the effects of green tea extracts and individual tea catechins on human CYP1A gene expression. *Chem. Biol. Interact., 128*(3), 211-229. doi:10.1016/s0009-2797(00)00204-0

Wolfe, W. H., Michalek, J. E., Miner, J. C., Pirkle, J. L., Caudill, S. P., Patterson, D. G., Jr., & Needham, L. L. (1994). Determinants of TCDD half-life in veterans of operation ranch hand. *J. Toxicol. Environ. Health., 41*(4), 481-488. doi:10.1080/15287399409531858

Wolff, B., Sanglier, J. J., & Wang, Y. (1997). Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. *Chem. Biol., 4*(2), 139-147. doi:10.1016/s1074-5521(97)90257-x

Wolff, S., Harper, P. A., Wong, J. M., Mostert, V., Wang, Y., & Abel, J. (2001). Cell-specific regulation of human aryl hydrocarbon receptor expression by transforming growth factor-beta(1). *Mol. Pharmacol., 59*(4), 716-724. doi:10.1124/mol.59.4.716

Wormke, M., Stoner, M., Saville, B., Walker, K., Abdelrahim, M., Burghardt, R., & Safe, S. (2003). The aryl hydrocarbon receptor mediates degradation of estrogen receptor alpha through activation of proteasomes. *Mol. Cell. Biol., 23*(6), 1843-1855. doi:10.1128/mcb.23.6.1843-1855.2003

Wühr, M., Güttler, T., Peshkin, L., McAlister, G. C., Sonnett, M., Ishihara, K., . . . Gygi, S. P. (2015). The Nuclear Proteome of a Vertebrate. *Curr. Biol., 25*(20), 2663-2671. doi:10.1016/j.cub.2015.08.047

Xie, G., Peng, Z., & Raufman, J. P. (2012). Src-mediated aryl hydrocarbon and epidermal growth factor receptor cross talk stimulates colon cancer cell proliferation. *Am. J. Physiol. Gastrointest. Liver. Physiol., 302*(9), G1006-1015. doi:10.1152/ajpgi.00427.2011

Xue, P., Fu, J., & Zhou, Y. (2018). The Aryl Hydrocarbon Receptor and Tumor Immunity. *Front. Immunol., 9*, 286. doi:10.3389/fimmu.2018.00286

Yang, Y., Wang, N., Xu, L., Liu, Y., Huang, L., Gu, M., . . . Sun, H. (2023). Aryl hydrocarbon receptor dependent anti-inflammation and neuroprotective effects of tryptophan metabolites on retinal ischemia/reperfusion injury. *Cell. Death. Dis., 14*(2), 92. doi:10.1038/s41419-023-05616-3

Yu, J., Feng, Y., Wang, Y., & An, R. (2018). Aryl hydrocarbon receptor enhances the expression of miR-150-5p to suppress in prostate cancer progression by regulating MAP3K12. *Arch. Biochem. Biophys., 654*, 47-54. doi:10.1016/j.abb.2018.07.010

Yueh, M. F., Huang, Y. H., Hiller, A., Chen, S., Nguyen, N., & Tukey, R. H. (2003). Involvement of the xenobiotic response element (XRE) in Ah receptor-mediated induction of human UDP-glucuronosyltransferase 1A1. *J. Biol. Chem., 278*(17), 15001-15006. doi:10.1074/jbc.M300645200

Zhao, B., Degroot, D. E., Hayashi, A., He, G., & Denison, M. S. (2010). CH223191 is a ligand-selective antagonist of the Ah (Dioxin) receptor. *Toxicol. Sci., 117*(2), 393-403. doi:10.1093/toxsci/kfq217

Zhou, X., Li, D., Xu, W., Zhang, H., Wang, H., & Perdew, G. H. (2020). β-Naphthoflavone Activation of the Ah Receptor Alleviates Irradiation-Induced Intestinal Injury in Mice. *Antioxidants, 9*(12). doi:10.3390/antiox9121264

Zhou, Y., Li, S., Huang, L., Yang, Y., Zhang, L., Yang, M., . . . Yang, Z. (2018). A splicing mutation in aryl hydrocarbon receptor associated with retinitis pigmentosa. *Hum. Mol. Genet., 27*(14), 2563-2572. doi:10.1093/hmg/ddy165

Zhu, Y., Bi, F., Li, Y., Yin, H., Deng, N., Pan, H., . . . Xiao, B. (2017). α- and β-Naphthoflavone synergistically attenuate H(2)O(2)-induced neuron SH-SY5Y cell damage. *Exp. Ther. Med., 13*(3), 1143-1150. doi:10.3892/etm.2017.4045