Aryl Hydrocarbon Receptor: Molecular Mechanisms and Structural Determinants of Activation and Physiology

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1 INTRODUCTION

1.1 MOLECULAR MECHANISM OF THE AHR SIGNALING

The aryl hydrocarbon receptor (AHR) was discovered in the late 1970s as a cytosolic protein that can induce drug-metabolizing enzymes in response to lipophilic xenobiotic compounds (Okey et al. 1979; Poland et al. 1976). The first discovered high affinity ligands included several polycyclic aromatic compounds and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Okey et al. 1979; Poland et al. 1976). In the following years, the AHR received broader attention due to its relevance as an environmental sensor and due to the highly toxic potential of its ligands. The biology and physiological effects of this receptor have been extensively studied, since an understanding of the principle molecular setting of the AHR is crucial for comprehension of its functional pathways.

The AHR can be localized either in the cell cytoplasm or in the cell nucleus. In the basal unliganded state, the larger population of AHR is located in the cytoplasm, although a minor population is always present in the cell nucleus (Reyes et al. 1992). Depending on compartmentalization and functional state, the protein is interacting with different co-factors and associated proteins (FIG. 1). In the cytoplasm and prior to activation, the AHR is maintained in a protein complex which consists of molecular chaperone HSP90, the AHR interacting protein AIP (also known as hepatitis B virus X-associated protein XAP2) and co-chaperone p23 (Barouki et al. 2012; Beischlag et al. 2008). The AIP also interacts with HSP90, which enhances its binding to AHR (Bell and Poland 2000). After ligand binding, AHR translocates into the cell nucleus, however it is not completely clear how this affects the original complex. According to recent studies, the HSP90 binding is not affected, indicating that ligand-bound AHR is translocated to the nucleus in complex with HSP90 (Tsuji et al. 2014).

Inside the nuclear compartment, the AHR forms a heterodimer with the AHR nuclear receptor translocator (ARNT) (Probst et al. 1993). This new complex is able to recognize and bind to a specific DNA sequence - the so called “xenobiotic response element” (XRE), which is located in the upstream promoter region of several genes (Yao and Denison 1992). Therefore, binding of AHR-ARNT heterodimer to XRE is essential to trigger transcription of AHR-

specific downstream genes which include a series of xenobiotic metabolizing enzymes (Jones et al. 1985; Reyes et al. 1992) and also the AHR-regulating antagonistic AHR repressor (AHRR) protein. The AHRR competes with AHR for heterodimerization with ARNT as well as for binding to XRE and therefore mediates a negative feedback mechanism of AHR activation (Mimura et al. 1999).

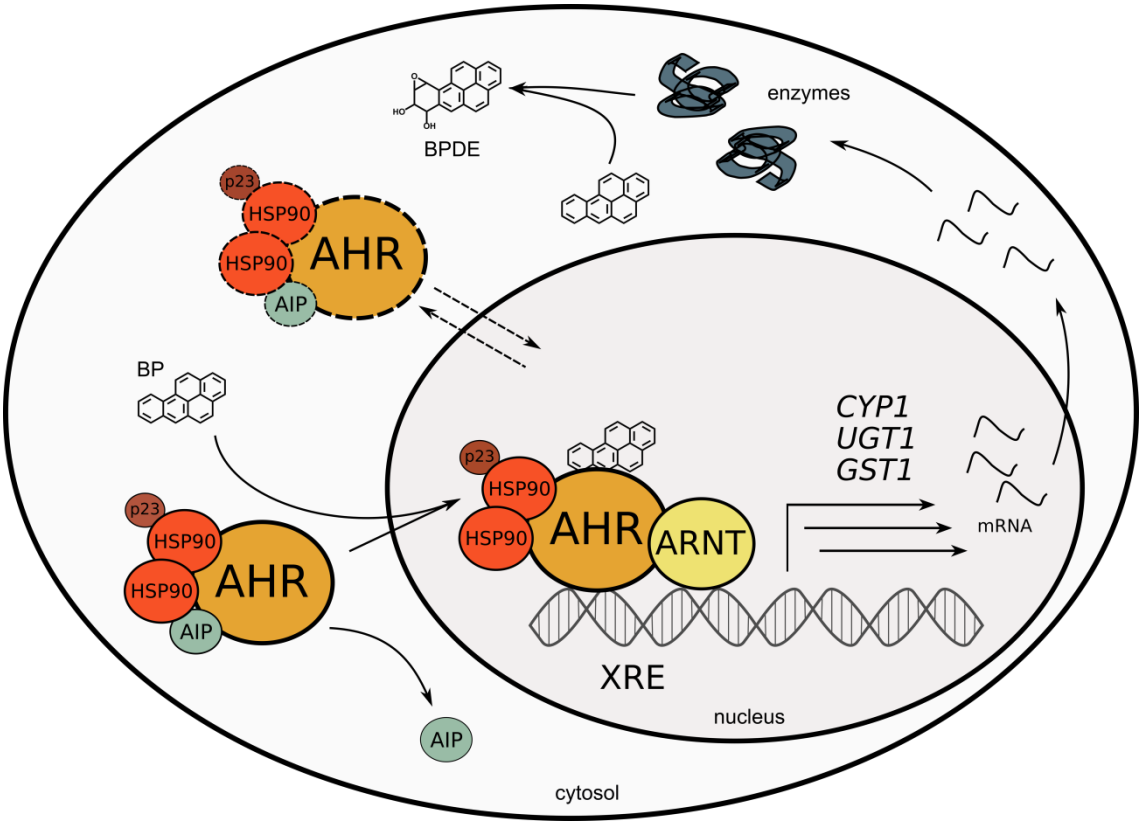

FIG. 1: Model of ligand-triggered activation mechanisms of the AHR, benzo[*a*]pyrene (BP) as an example. In the unliganded state the AHR is maintained in a cytosolic complex with HSP90, p23 and AIP. The AHR is subject to permanent nucleocytoplasmic shuttling (dashed lines). After ligand binding (solid lines), AIP dissociates while the remaining complex translocates into the cell nucleus. Nuclear AHR heterodimerizes with ARNT and binds to the XRE of the DNA. This is a basic prerequisite for transcription of several drug-metabolizing enzyme genes. After translation and protein folding, the enzymes are capable of metabolizing the inducer substance - BP is transformed through an epoxide to the BP-diol-epoxide (BPDE), a highly potent genotoxic agent.

Additionally to the ligand-dependent trafficking, the AHR protein complex is permanently shuttling between cell nucleus and cytoplasm (Ikuta et al. 2000). Even though the exact physiological function of nucleocytoplasmic shuttling is still unknown, the nuclear export of the AHR occurring as part of this process is discussed as putative mechanism to terminate transcription.

Importantly, the induction of the AHR nuclear translocation and the adjacent gene transcription should be regarded as two separate mechanisms, since the enrichment of the receptor within the cell nucleus does not necessarily lead to increased levels of target gene products (Pollenz et al. 2006). Therefore, the assessment of both steps is crucial for an adequate evaluation of AHR activation.

1.2 Physiological Relevance of the AHR

Since its discovery, the AHR has long been known as a cellular receptor which is primarily responsible for the binding of anthropogenic xenobiotic substances as for example TCDD or certain PAHs (FIG. 2). On the other hand, it should be considered, that an endogenous receptor is also engaged in physiological functions. In this context, an essential role of the AHR in immunity and development has been initially suggested by animal models. The AHR knock-out mice express an altered phenotype with an impaired immune system as well as reduced viability and fertility (Gonzalez et al. 1995). Plenty of additional research was done exploring the endogenous significance of AHR, as previously reviewed by Stockinger et al. (2014), Murray et al. (2014), Bock (2016), Mulero-Navarro and Fernandez-Salguero (2016). The AHR was found to play a significant role in regulation of immunity and development of the immune system (Esser et al. 2009; Stockinger et al. 2014). In particular it can affect the differentiation of B-cells (De Abrew et al. 2011), T-cells (Josefowicz et al. 2012) and immune interactions of mucosal tissues with the host-microbiome (Bessede et al. 2014). The receptor seems to also be involved in apoptosis (Stolpmann et al. 2012), skin differentiation (van den Bogaard et al. 2015) and endocrine signaling (Ohtake et al. 2003; Reen et al. 2002).

In agreement with the proposed endogenous functions multiple endogenous ligands were discovered (FIG. 2). Usually, these compounds are water-soluble and in contrast to xenobiotic ligands not associated with increased toxicological risks (Bergander et al. 2004; Murray et al. 2014). The most studied endogenous AHR ligands with high activity are indole derivatives 6-formylindolo-[3,2-*b*]-carbazole (Wincent et al. 2009) and indirubin (Adachi et al. 2001). Different tryptophan catabolites were also found to activate the AHR such as kynurenic

acid (DiNatale et al. 2010) and kynurenine (Mezrich et al. 2010). In contrast to xenobiotics, endogenous ligands were shown to be successfully metabolized by the induced AHR downstream genes (Bergander et al. 2004).

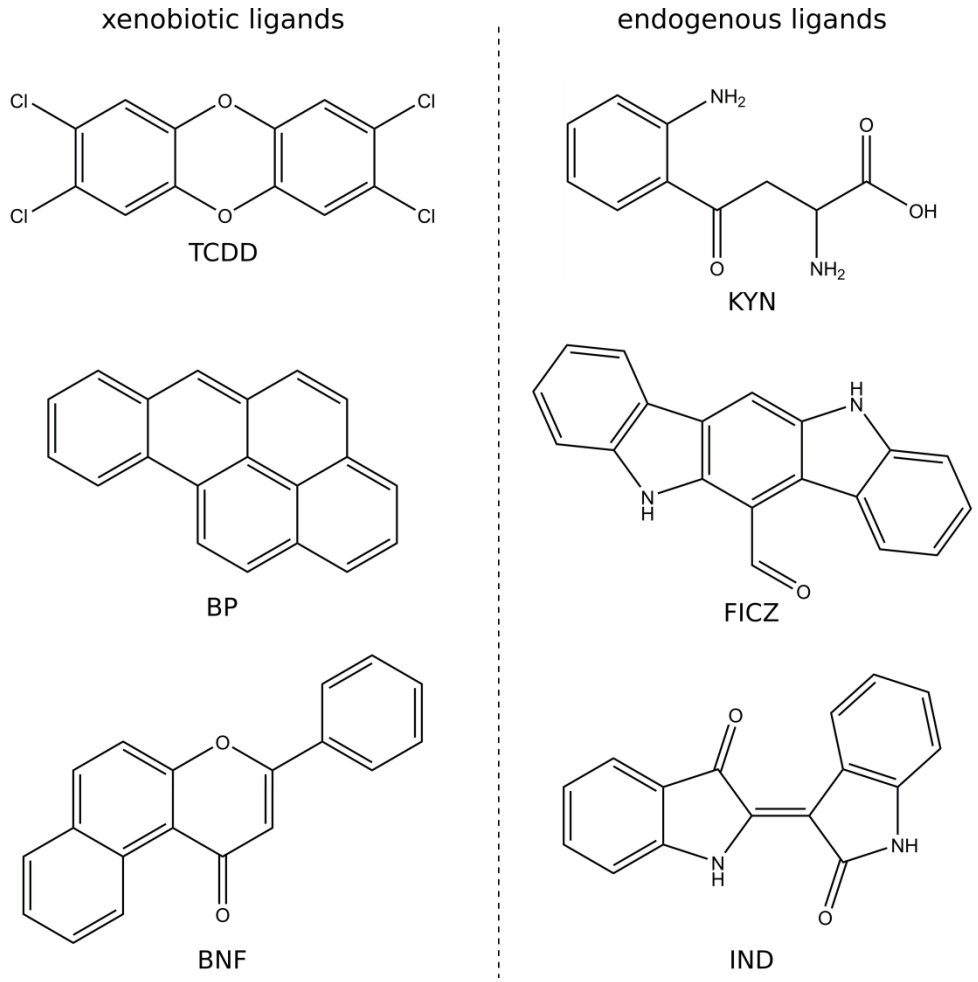

FIG. 2: Important xenobiotic and endogenous ligands of the AHR: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo[*a*]pyrene (BP), β-naphthoflavone (BNF), kynurenine (KYN), 6-formylindolo[3,2-*b*]carbazole (FICZ), indirubin (IND).

One much-discussed endogenous AHR mechanism that was originally related to the existence of endogenous AHR ligands was the nucleo-cytoplasmic shuttling of the receptor (Ikuta et al. 2000; Murray et al. 2014). Despite of several studies addressing the mechanism of this shuttling process (Pollenz et al. 2006; Ramadoss and Perdew 2005) its relation to the presence of endogenous AHR agonists could not be proven.

1.3 Therapeutic Potential of Targeting the AHR

Importantly, higher AHR expression levels as well as endogenously found AHR ligands have been shown to play a significant role in tumor promotion (Chen et al. 2013). Thus increased degradation of tryptophan to kynurenine was found in human brain tumors and higher levels of the catabolite were associated with a poor survival prognosis (Opitz et al. 2011). However, besides the clearly established pro-carcinogenic effects mediated by AHR, several anti-carcinogenic effects of certain AHR agonists were also discovered recently (Gaboriaud-Kolar et al. 2015; O'Donnell et al. 2014). For example, the endogenous AHR ligand indirubin is used in traditional Chinese medicine for leukemia treatment (Gaboriaud-Kolar et al. 2015; Hoessel et al. 1999). Based on such a wide involvement into carcinogenic pathways, the AHR is now regarded as an auspicious target for pharmaceutical research (Bock 2017; Kolluri et al. 2017; Mulero-Navarro and Fernandez-Salguero 2016; Murray et al. 2014). Treatment concepts concerning pro-carcinogenic effects include a general disruption of AHR activation by endogenous and xenobiotic ligands (Chen et al. 2013), but also a targeted AHR induction with specific agonists for anti-carcinogenic effects (O'Donnell et al. 2014). Another approach reaping attention is developing selective AHR modulators (Safe and McDougal 2002). A further application of blocking the receptor activity by AHR antagonists was suggested for promoting a therapeutic progenitor cell expansion (Boitano et al. 2010). AHR agonists could further find their application in supporting immune tolerance in autoimmune diseases (Bessede et al. 2014) and through its antagonization of the estrogen receptor reduce breast tumor development (Powell et al. 2013).

However, due to the multiple functions of AHR, a systemic treatment also bears risks and would involve the disadvantages of an extensive AHR shut-down or induction. The fact that AHR seems to be both - a pro-tumorigenic and a tumor suppressing factor (Fan et al. 2010) - is complicating the treatment approach. This underlines the importance of more detailed studies on the molecular activation mechanisms of the human AHR.

1.4 Role of the AHR in Xenobiotic Metabolism

The AHR was originally named “dioxin-receptor” because it was identified due to its high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Poland et al. 1976). Concomitantly with the discovery of other highly toxic ligands, such as polycyclic aromatic hydrocarbons (PAHs) and recognition of its function as transcription factor, the AHR emerged to one of the most important cellular receptors involved in the metabolism of xenobiotic substances (Hankinson 1995). After ligand binding and nuclear translocation, the AHR-ARNT complex induces a variety of xenobiotic metabolizing enzymes (FIG. 1). This includes enzymes, which are involved in phase I as well as in phase II xenobiotic metabolism (Nebert et al. 2000).

Most of the AHR-mediated functionalizational reactions (phase I reactions) are dependent on specific cytochrome P-450 monooxygenases 1- family (CYP1) proteins, notably CYP1A1, CYP1A2 and CYP1B1 (Guengerich 2006; Jones et al. 1985). CYPs are monooxygenases that transfer oxygen into inert organic compounds, leading to formation of more reactive intermediate substances. These metabolites then typically represent targets for phase II enzymes. The CYP1 family members are playing a crucial role in the enhancement of water solubility of lipophilic and insoluble xenobiotic AHR ligands (Batt et al. 1994; Guengerich 2006). Although called “detoxification enzymes”, CYP1 enzymes also provide highly critical toxification reactions in the form of metabolic activation of pro-carcinogens to genotoxic metabolites (Luch 2005; Shimada and Guengerich 2006). One prominent example of AHR inducing substance classes, which are undergoing this toxification pathway, are the PAHs. Some of these substances bind to AHR and as a result induce the CYP1 family enzymes, by which these compounds are converted to different intermediate metabolites. Through a series of oxidation and hydroxylation steps mediated by induced metabolizing enzymes, the PAHs are partly metabolized to diol-epoxides - highly reactive substances with direct genotoxic impact (Moorthy et al. 2015). For example, benzo[*a*]pyrene (BP), a classified carcinogen, is metabolized into benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE) (FIG. 1). This highly reactive electrophilic species was shown to produce stable DNA-adducts as a basis for its genotoxic and carcinogenic effects (Henkler et al. 2012).

Further, the non-genotoxic AHR ligand β-naphthoflavone (BNF) which is a strong CYP1 inducer, was also shown to display carcinogenic tumor-promoting properties *in vivo* (Shoda et al. 2000).

Beside a major regulation of phase I metabolism through CYPs or aldehyde dehydrogenases (ALDH3) (Asman et al. 1993), the AHR can also induce phase II enzymes. Generally, enzymes of the phase II xenobiotic metabolism are mediating conjugation reactions of functionalized xenobiotic metabolites from phase I reactions to charged endogenous molecules like glutathione (Higgins and Hayes 2011). This biotransformation facilitates excretion and decreases the pharmacological activity of the metabolized substance (Wang et al. 2016). Different phase II detoxification enzymes like glutathione S-transferases (GSTA1) (Nebert et al. 2000) and uridine 5'-diphospho-glucuronosyltransferases (UGT1) (Yueh et al. 2003) were shown to be AHR-dependent. Several phase II enzymes are also involved in degradation of endogenous AHR ligands meaning an autoregulation of endogenous AHR signaling (Bergander et al. 2004).

1.5 Protein Structure of the AHR

1.5.1 Domain Structure of the AHR

The human AHR is a comparatively large protein consisting of 848 amino acids (AA). The AHR AA sequence consists of different structural and functional domains; an overview is given in FIG. 3. The indicated residues are approximations that are used to define these domains in the literature.

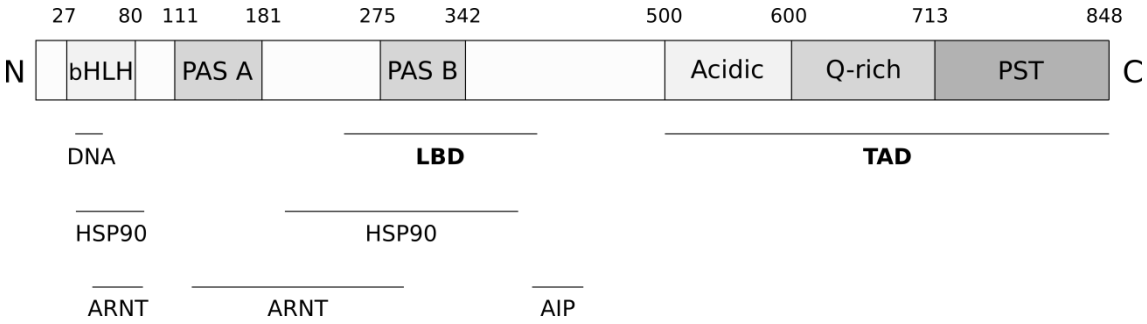

FIG. 3: Two-dimensional structure of the functional domains and corresponding associated structures of human AHR. AA numeration compiled from UniProt-data, Reen et al. 2002 and Murray et al. 2014. (N - amino-terminal end, C - carboxyl-terminal end)

The AHR protein is a member of the basic helix-loop-helix (bHLH) - PAS (period circadian protein (Per), aryl hydrocarbon receptor nuclear translocator (ARNT), single-minded protein (Sim)) family of DNA-binding proteins (Gu et al. 2000; Okey et al. 1994). The characteristic bHLH structural motif is located on the outer N-terminus of the protein and responsible for DNA binding and dimerization with co-factors like HSP90 and ARNT (Jones 2004). The adjacent bipartite PAS domain (PAS A / B) is also essential for protein dimerization (Zelzer et al. 1997). AHR partner proteins HSP90 and ARNT have a second interaction region in the area of the AHR PAS domain (Tsuji et al. 2014). The PAS area also contains the ligand binding domain (LBD), which associates with both endogenous and xenobiotic ligands (Beischlag et al. 2008). This functional domain of the human protein includes residues 230 - 397 and is overlapping with binding sites of HSP90, ARNT and AIP. However, the overlap with the latter is only marginal (FIG. 3) (Soshilov and Denison 2008). Extensive studies were done to investigate the murine AHR-LBD and several residues within were proposed to be essential for the ligand-mediated effects (Bessede et al. 2014; Bisson et al. 2009; Pandini et al. 2007; Soshilov and Denison 2014; Xing et al. 2012). However, this information is still lacking for the human AHR.

The situation within the ligand binding pocket of the human receptor appears to be strikingly different, since the AA sequence as well as ligand binding affinity of human and murine AHR exhibit momentous differences (Flaveny et al. 2009; Hubbard et al. 2016; Ramadoss and Perdew 2004). It was shown, that the murine AHR exhibits a tenfold higher affinity than the human counterpart to the dioxin TCDD due to a single homologous residue difference (murine A375, human V381), making the mice more sensitive to TCDD mediated effects (Ramadoss and Perdew 2004). *Vice versa* indigoid substances like indirubin have much higher affinity to the human AHR (Flaveny et al. 2009). Consequently, due to high species specificity of the AHR, the human LBD should be studied separately.

The consensus motifs which regulate intracellular trafficking of AHR are also located on the N-terminus of the protein. The well-characterized bipartite nuclear localization signal (NLS) is recognized by import proteins and contains the residues 13 - 39, whereas the nuclear export signal (NES) is targeted by CRM1 cargo proteins and spans over residues 63 - 73 (Ikuta et al. 1998). Both the NLS and the NES mediate the nuclear cytoplasmic shuttling and are crucial for the maintenance of compartment equilibrium in the AHR distribution. Treatment of cells with the unspecific CRM1 (nuclear export protein) blocker leptomycin B (LMB) and the subsequent accumulation of AHR in the cell nucleus visualizes the impact of a permanently active NES (Ikuta et al. 2000). Besides the direct import and export signals it is still not clear how the endogenous AHR shuttling is regulated and which additional structures or residues are critical for the process. Obviously the sole presence of NLS and NES is not sufficient to provide the classical unstimulated protein distribution within cellular compartments. Observations suggest that parts of the C-terminal domain contribute to shuttling processes in murine AHR, but they have to be confirmed and substantiated in human AHR (Pollenz et al. 2006; Ramadoss and Perdew 2005).

Whereas the N-terminal part of the AHR is generally highly conserved across different species, the C-terminal half exhibits a high degree of species specificity. Thus the N-terminal part of human and murine AHR show a homology of 85% and the C-terminal section a homology of only 58% (Murray

et al. 2014; Ramadoss and Perdew 2005). The C-terminal part of the protein contains an essential structural motif, which mediates the gene induction occurring in response to AHR ligand binding - the transactivation domain (TAD) (Ma et al. 1995). In human AHR, this domain roughly spans from AA 500 to 848 and can be divided into an acidic, a glutamine-rich (Q-rich) and a proline/serine/threonine-rich (PST) subdomain (Ramadoss and Perdew 2005). Explicitly the Q-rich subdomain is critical for the AHR transactivation potential. Importantly, it does recruit essential co-factors and components of the transcription system and facilitates transcriptional activation of target genes (Kumar et al. 2001).

1.5.2 Three-dimensional Structure of the AHR

Since the AHR is a large three-dimensional (3D) biomolecule, information on the 3D conformation is crucial for understanding its function. Unfortunately, knowing the amino acid sequence alone does not allow extrapolation to a solid 3D conformation of a protein. Whereas sequencing can nowadays be easily completed, the determination of a rough 3D shape demands a lot more effort. The covalently bound backbone of the protein results in a 3D conformation, which is determined by noncovalent interactions of residue side chains. This allows a protein to remain very flexible for adapting to different environments and functions and requires a successful crystallization with subsequent X-ray diffraction analysis for detailed structure representation (Palmer and Niwa 2003; Spence et al. 2012).

To date, there is no complete resolved X-ray or nuclear magnetic resonance structure of the human AHR protein, which impedes our knowledge about the 3D structure of the receptor. Nevertheless, it was possible to overexpress associated AHR-bHLH-PAS A domains and determine corresponding crystal structures with X-ray crystallography, despite results with relatively poor resolutions of 3.3 – 4.0 Å, which do not allow a clear assessment of atomic and molecular interactions (Schulte et al. 2017; Seok et al. 2017). However, one of the most interesting regions of the AHR receptor is the LBD within the PAS B domain, as it forms the 3D ligand-binding pocket and as a consequence defines the prerequisites for the ligand binding selectivity and mediates the first AHR

activation steps (Hubbard et al. 2016). Furthermore, the LBD is involved in interactions with different AHR partner proteins before and after ligand binding, which is crucial for receptor activation and subsequent target gene induction (FIG. 3) (Soshilov and Denison 2008). Hence, it is of great interest to depict the 3D structure of this functional unit, while investigating the functions of the LBD and for a mechanistic understanding of mutagenesis studies.

Unfortunately, there is no available crystal structure of the human AHR-PAS B domain. Nevertheless, it is possible, to derive an approximation of the unresolved target structure by homology modeling. While developing a homology model, it is essential to use an experimentally determined structure of a template with a maximum of structure similarity and residue identity (Bermudez et al. 2016). The resolution of the template structure should be 2.0 Å or better (Acharya and Lloyd 2005). In the case of the AHR-LBD the most suitable template structure is the PAS B domain of HIF2α protein PDB 3H82 (Key et al. 2009), which has a high resolution of 1.5 Å. Like AHR, HIF2 also belongs to the family of bHLH-PAS-proteins and therefore carries the critical PAS A and B domains (Gu et al. 2000). It shows the highest sequence similarity to the human AHR within the bHLH-PAS-family (ca. 30 % identical residues within PAS B) and was also used by different groups for homology modeling of AHR PAS B domain of the murine receptor (Bessede et al. 2014; Pandini et al. 2007; Soshilov and Denison 2014; Xing et al. 2012) and of the human AHR (Bisson et al. 2009; Perkins et al. 2014). Several of the publications specifically addressed the role of distinct residues in the ligand binding process (Bessede et al. 2014; Bisson et al. 2009; Pandini et al. 2007; Soshilov and Denison 2014; Xing et al. 2012). However, the modeling approaches performed on human AHR-LBD lack the corresponding experimental data.

2 AIM OF THIS WORK

The human AHR is known for its significant involvement in the xenobiotic metabolism and in the formation of highly reactive intermediates. Besides the resulting pathological relevance, a versatile physiological role of the AHR was uncovered, turning the receptor to an appealing pharmaceutical target.

One of the physiological characteristics of the AHR is the nucleocytoplasmic shuttling. Even though this endogenous process was addressed in several publications, the distinct mechanism and the interplay between the N-terminal NLS / NES and other functional domains of the human protein are still not clarified. Therefore, the first part of this work is aimed at the detailed analysis of nucleocytoplasmic shuttling of the human AHR in connection with ligand-induced receptor activation. In this context, the following questions were addressed:

1. What are the properties of nucleocytoplasmic shuttling of human AHR in living cells?
2. What subdomains or residues within the C-terminus of human AHR are essential for flawless function of nucleocytoplasmic shuttling and AHR export in general?
3. What is the conjunction of the earlier defined areas to the ligand-mediated activation of human AHR?

The interaction with xenobiotic and endogenous ligands is the central characteristic of the AHR, leading to receptor activation and multiple downstream-effects in relation to xenometabolism, immunity and other physiological functions. Therefore, investigation of the LBD as the essential structural element is required for understanding the AHR activation processes. The LBD and the role of distinct residues within were addressed in several studies, but the focus was placed on the murine AHR-LBD. Since the murine and human AHR differ in their amino acid sequences as well as in their binding affinities to several ligands, there is a clear need to investigate the human AHR-LBD specifically.

For this purpose, the two initial steps of ligand-dependent receptor activation were in the focus of the second part of this work. The following questions were addressed:

1. What are the dose-response properties of selected xenobiotic and endogenous ligands regarding the induction of nuclear import of human AHR and adjacent target gene transcription?
2. Which residues within the human AHR-LBD seem to be essential for ligand-induced effects provided by previously tested ligands?
3. Are these residues also indispensable for the nucleocytoplasmic shuttling of the human AHR?
4. What possible mechanistic explanation for the impact of LBD-residues on human AHR activation can be given through state-of-the-art three-dimensional molecular modeling methods?

Taken together, this work contributes to the understanding of essential molecular mechanisms, which are underlying the physiology of human AHR.

3 RESULTS

3.1 TKACHENKO, A. ET AL. (2016) "THE Q-RICH/PST DOMAIN OF THE AHR REGULATES BOTH LIGAND-INDUCED NUCLEAR TRANSPORT AND NUCLEOCYTOPLASMIC SHUTTLING"

Anna Tkachenko, Frank Henkler, Joep Brinkmann, Juliane Sowada, Doris Genkinger, Christian Kern, Tewes Tralau and Andreas Luch

The Q-rich/PST domain of the AHR regulates both ligand-induced nuclear transport and nucleocytoplasmic shuttling

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The authors contributions:

Project planning (60 %)
Experimental work (90 %)
Data analysis (80 %)
Preparation of manuscript (40 %).

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The Q-rich/PST domain of the AHR regulates both ligand-induced nuclear transport and nucleocytoplasmic shuttling

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The aryl hydrocarbon receptor (AHR) shuttles continuously between cytoplasm and nucleus, unless ligand-binding triggers association with the AHR nuclear translocator (ARNT) and subsequent binding to cognate DNA motifs. We have now identified Val 647 as mandatory residue for export from the nucleus and AHR-function. This residue prevents inactivation of the receptor as a consequence of nuclear sequestration *via* constitutive import. Concomitantly mutants lacking this residue are exclusively localised in the nucleus. Although ligands accelerate nuclear import transiently, stable nuclear transition depends on a motif adjacent to Val 647 that comprises residues 650–661. Together, this defined region within the Q-rich domain regulates intracellular trafficking of the AHR in context of both nucleocytoplasmic shuttling and receptor activation. Nuclear export therefore depends on the previously characterised N-terminal NES and the newly identified motif that includes V647. Nucleocytoplasmic distribution of full-length human AHR is further affected by a section of the PST domain that shows sequence similarities with nuclear export signals. In concert, these motifs maintain a predominant cytoplasmic compartmentalisation, receptive for ligand binding.

The AHR is a highly conserved protein belonging to the basic helix-loop helix (bHLH)-PAS family[1,2]. Originally identified for its association with xenobiotic ligands, particularly 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)[3], this receptor became quickly recognised as one of the major regulators for eukaryotic phase-I metabolism. Further xenobiotic ligands include β-naphthoflavone (βNF), polychlorinated biphenyls, as well as carcinogenic polycyclic aromatic hydrocarbons (PAHs)[4]. Amongst other effects receptor activation induces expression of key enzymes of oxidative phase-I metabolism, notably cytochrome P450-dependent monooxygenases (CYPs) 1A1 and 1B1, both of which convert benzo[*a*]pyrene and other carcinogenic PAHs into highly mutagenic diol-epoxide intermediates[5]. Since then, certain endogenous ligands[6,7] and additional properties of the AHR have been widely explored. These studies revealed important physiological functions in the immune response[8], autoimmunity[9,10], carcinogenesis[11], apoptosis[12], skin differentiation[13], as well as in endocrine[14,15] and host-microbiome[16] signalling. Yet, comparatively little is known about its activation mechanisms that are tightly related with intracellular trafficking of receptor complexes. Notably, functional regulation might involve both nuclear import and export pathways. Prior to activation the AHR receptor is maintained in a cytoplasmic chaperone complex which consists of Hsp90, XAP2 and p23[17]. According to the current model, the ligand-bound AHR shifts to the nucleus and forms a heterodimer with ARNT to initiate transcription[18]. Importantly, constitutive nuclear translocation also occurs in the absence of ligand binding. This process is driven by ligand-independent import and balanced by parallel export in order to maintain a predominant cytoplasmic compartmentalisation. Although nucleocytoplasmic shuttling was recognised as early as 1998[19], both the physiological relevance and molecular mechanisms are merely understood.

Activation and intracellular trafficking of the AHR are regulated by the N-terminal domain that contains a bipartite nuclear localisation signal (NLS)[19], as well as an adjacent nuclear export signal (NES)[20] and the DNA-binding domain[21]. The well-characterised NLS is both required and sufficient to mediate import of

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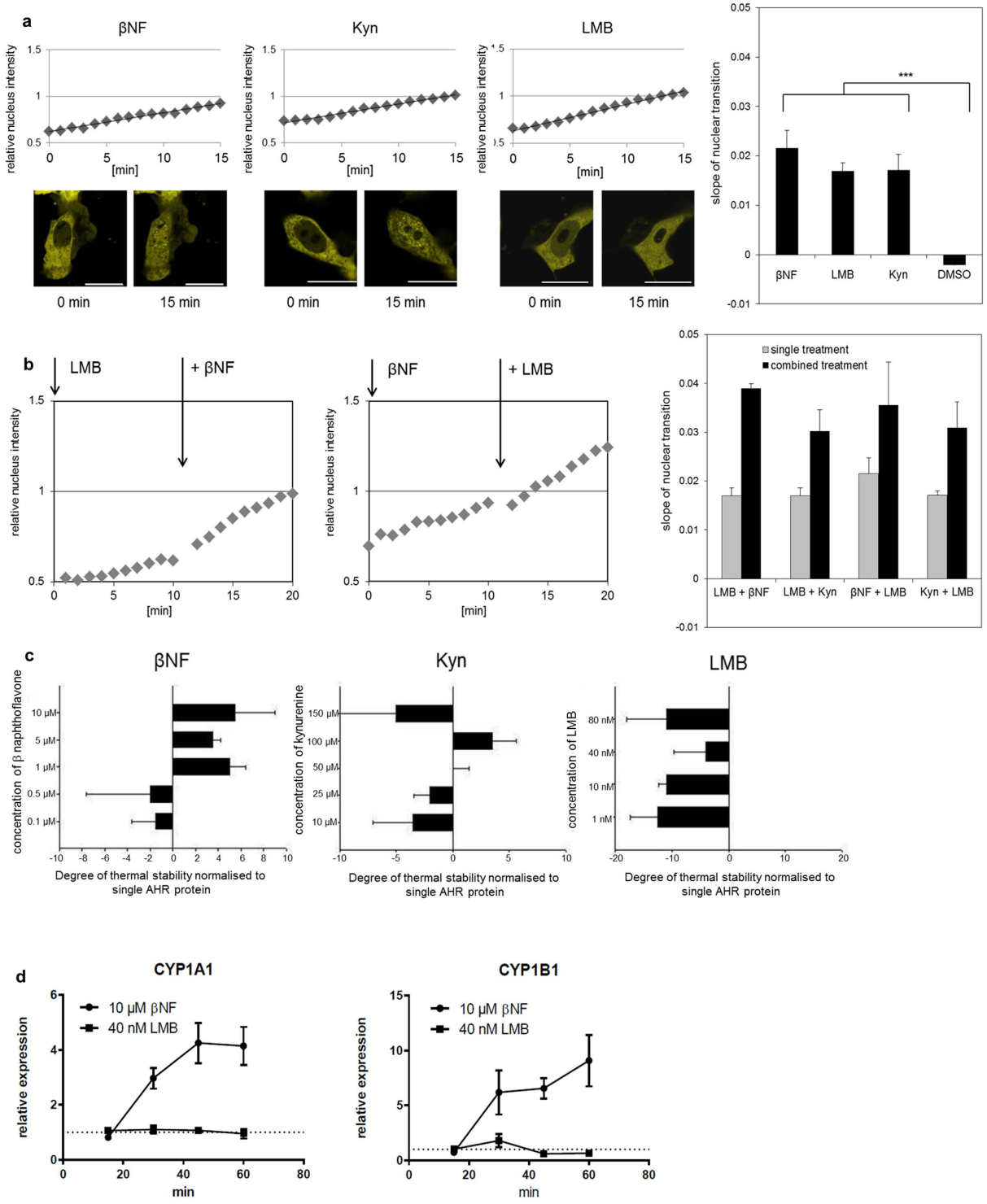

Figure 1. Constitutive nucleocytoplasmic shuttling of the AHR is accelerated by ligands. (**a**) Fluorescence images demonstrating the translocation of the full-length EYFP-AHR into the nucleus in HepG2 cells after treatment with 10 μM β-naphthoflavone (βNF), 100 μM kynurenine (Kyn) and 40 nM leptomycin B (LMB) for 15 min (scalebar = 20 μm). The selection of these concentrations was based on a dose-response analysis (Supplementary Fig. S2b). Graphs in the diagrams visualise the shift of nuclear staining in relation to total fluorescence of the analysed cells over 15 min. All three compounds triggered a comparable transition of EYFP-AHR into the nucleus, as indicated by comparable slopes of the graphs. Each bar (right diagram) represents the mean of at least 10 analysed cells +/− S.E.M. ***$p < 0.001$. (**b**) βNF accelerates the basal nucleocytoplasmic shuttling. Cells were treated for 10 min with 40 nM LMB, co-treated for another 10 min with 10 μM βNF (left side). Nuclear transition of EYFP-AHR was recorded and analysed as described above. Nucleocytoplasmic shuttling continues in the presence of ligand. Cells were treated with 10 μM βNF for 10 min, then co-treated with 40 nM LMB and analysed as described above (middle). Slopes of the recorded graphs were separately

determined for single and combined treatments as indicated (right side). Each bar represents the mean of at least 10 cells +/− S.E.M. (**c**) Thermal shift assay with purified recombinant human AHR (protein purification is summarised in Supplementary Fig. S4). Shown is the relative thermal stability of AHR in the presence of LMB, Kyn and βNF at different concentrations. Compared to unliganded AHR, βNF and Kyn increased thermal protein stability, while LMB apparently has a destabilising effect. A decrease of stability was noted using 150 μM Kyn, possibly triggered by precipitation. Shown are the means of three biological replicates +/− S.E.M. (**d**) Time-dependent transcriptional activation of CYP1A1 and CYP1B1 in HepG2 cells. Induction was only seen after treatment with 10 μM βNF but not with 40 nM LMB. Displayed values represent relative inductions of transcripts normalised to the solvent control. Values shown are means of three biological replicates +/− S.E.M.

AHR receptor complexes, leading either to receptor activation or to re-export into the cytoplasm, especially in the absence of ligands. Importantly, amino acid residues 63–73 comprise an export signal (NES)[19,20] which shows high sequence similarity to analogous viral and mammalian motifs[22]. This N-terminal NES overlaps with sequences that interact with the ARNT after ligand binding. It was hence postulated that nuclear export is blocked when this NES is masked by ARNT during receptor activation[23]. In consequence, AHR/ARNT complexes interact with xenobiotic response elements (XRE) to initiate expression of target genes[24], including CYPs 1A1 and 1B1[25]. Further, nuclear export of the AHR was discussed to terminate transcription and shown to trigger degradation *via* cytoplasmic proteasomes[23].

Several lines of evidence now suggest that nuclear export of the AHR depends on additional motifs or interacting factors. Notably, when the N-terminal NES was inactivated by mutagenesis, full-length AHR maintained a substantial cytoplasmic localisation in the absence of ligand[23]. In addition, several deletion mutants are exclusively localised in the nucleus[26] although the NES was not affected. It was speculated that these variant patterns are related to distinct interactions with XAP2, protein modifications or alternate conformations[26]. Moreover, the human receptor shifts from cytoplasmic staining towards a substantial nuclear staining pattern, when the C-terminal domain is replaced by its murine homolog[27]. Still, data on if and how the C-terminal domain contributes to nucleocytoplasmic shuttling and AHR compartmentalisation remain scarce. In this study, we have addressed the role of nucleocytoplasmic shuttling in relation to AHR activation and demonstrate an essential regulation of both receptor activation and intracellular trafficking by the C-terminal domain.

Results

Nucleocytoplasmic shuttling occurs as a constitutive and dynamic process. Previous investigations on the localisation of the AHR have mainly analysed cells that were fixed at defined time points and hence could only record cellular snap-shots. We therefore chose to investigate the cellular compartmentalisation of AHR in living HepG2 cells by live-cell fluorescence imaging using an expression construct that expresses the human matured protein (amino acids 11–848) fused to the C-terminus of enhanced yellow fluorescent protein (EYFP). The EYFP-tagged protein shows a shuttling behaviour similar to the wild-type protein, thus allowing continuous recording of functional AHR *in situ* (Fig. 1 and Supplementary Fig. S1). Our data confirm a constitutive and highly dynamic nucleocytoplasmic shuttling, since both, ligand binding of the xenobiotic βNF or endogenous kynurenine[6] (Kyn), as well as export inhibition by leptomycin B (LMB) resulted in a comparable accumulation of AHR in the nucleus (Fig. 1a,b). The latter compound was isolated from fungi and is known to affect CRM1-mediated nuclear export[19]. The binding of the ligands βNF and Kyn was confirmed by measuring AHR stabilisation in a thermal-protein shift assay, as was the absence of binding for LMB (Fig. 1c). The latter also failed to induce AHR-dependent target genes (Fig. 1d), further confirming its solely inhibitory function on nuclear export. The predominant cytoplasmic localisation of AHR is therefore maintained as a steady state by means of continuous parallel import and export, but shifts to nuclear accumulation after application of ligands. In addition the kinetic data show that while ligand binding accelerates the basal constitutive import, partial export continues to occur (Fig. 1b). As a consequence the ongoing export is likely to restrict the time frame for possible interactions of the respective receptor/ligand complexes with nuclear chromatin. It also indicates that corresponding molecular gene activation might actually require several passages of shuttling (Fig. 1b & see below).

Export of the AHR from the nucleus is strictly dependent on V647. Intracellular trafficking of the human AHR was further characterised using several deletion mutants. As previously observed for the murine protein by Pollenz and colleagues[26], extensive C-terminal deletion of murine AHR led to accumulation in the nucleus. In our experiments, an exclusive nuclear detection was observed for human $AHR^{\Delta 509}$ which lacks 339 amino acids at the C-terminus, as well as for the shorter variant $AHR^{\Delta 391}$ (Fig. 2). This observation confirms constitutive basal import of the AHR as key mechanism in shuttling. In contrast, the N-terminal NES acts not autonomously, but apparently requires either some additional factors or structural elements of the C-terminal domain. We explored the latter option by analysing several fluorescent mutants, namely $AHR^{\Delta 640}$, $AHR^{\Delta 644}$, $AHR^{\Delta 647}$, $AHR^{\Delta 650}$, $AHR^{\Delta 661}$ and $AHR^{\Delta 698}$. The data show a striking difference between $AHR^{\Delta 644}$ and $AHR^{\Delta 647}$. The inclusion of these three residues was sufficient to shift nuclear staining to a nearly exclusive cytoplasmic state (Figs 2 and 3). Further site directed mutagenesis of residues 645 to 647 (Fig. 2, see $AHR^{M645A,Q646A,V647A}$) led to an exclusive nuclear localisation of the full-length receptor. The respective locus seems therefore essential for any measurable export to happen. Further deletion mutagenesis confirmed V647 as critical residue (see $AHR^{\Delta 646}$, Figs 2 and 3).

We have applied additional site directed mutagenesis to confirm these findings (Fig. 2). While replacement of Q646 with alanine (AHR^{Q646A}) did not alter the cytoplasmic staining pattern of full-length AHR, replacement of V647 with alanine (AHR^{V647A}) did and led again to an exclusive nuclear staining. Interestingly, V647 is not conserved within in the AHR-sequence, but aligned to isoleucine in several species including mice. To test whether

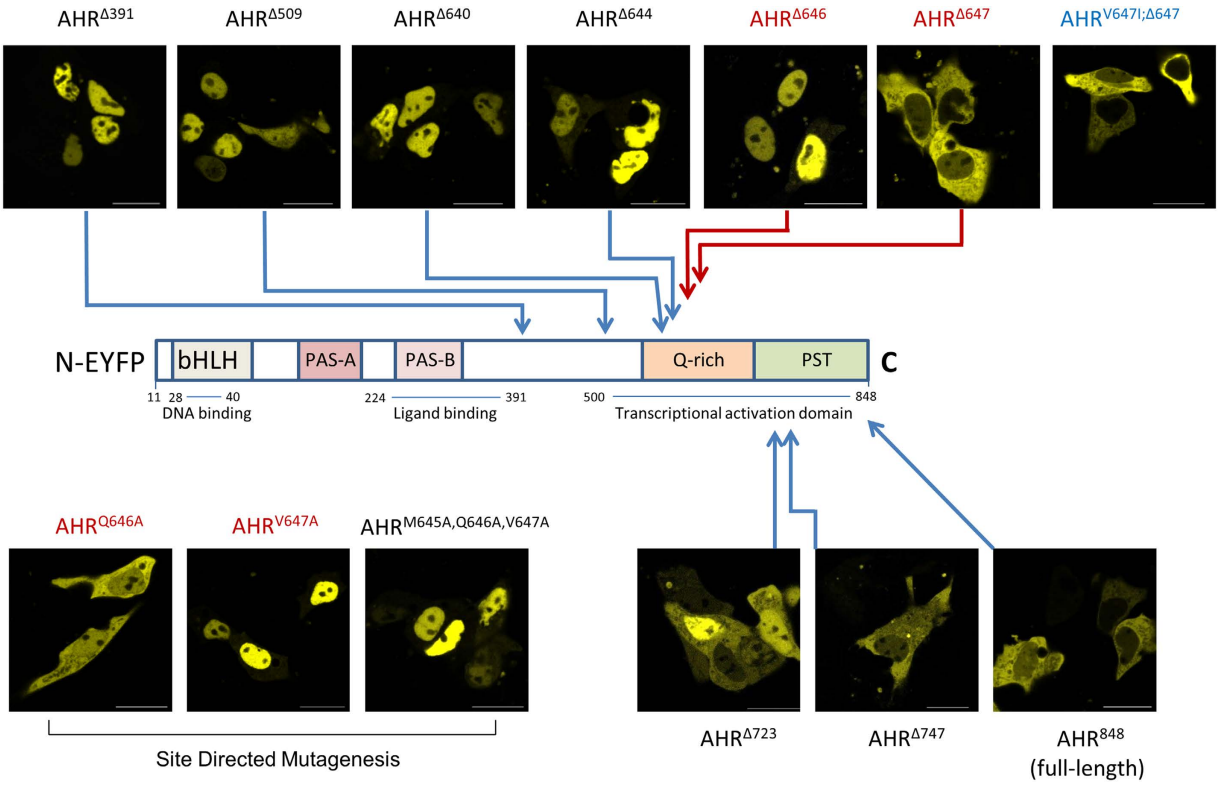

Figure 2. V647 determines the compartmentalisation of the AHR: expression of fluorescent AHR deletion mutants in HepG2 cells. Deletion mutants were derived from full-length human pEYFP-AHR-C1 (AHR^{848}). Mutants are named according to truncations sites, as defined by the last included residue. These sites are marked on the drafted full-length protein. Representative images that reflect the typical compartmentalisation are shown (scalebar = 20 μm). Mutants truncated after amino acid 391 ($AHR^{\Delta391}$), 509 ($AHR^{\Delta509}$), 640 ($AHR^{\Delta640}$) 644 ($AHR^{\Delta644}$) and 646 ($AHR^{\Delta646}$) show an exclusive nuclear staining, whereas the full-length protein (AHR^{848}) is predominantly located in the cytosol. $AHR^{\Delta647}$ is nearly exclusively detected in the cytoplasm. Inclusion of the Q-rich domain does increase nuclear association ($AHR^{\Delta723}$). This is balanced by a motif localised between Pro 728 and Leu 744 within in the PST domain. $AHR^{\Delta647}$ and full-length AHR^{848} show a similar predominantly cytoplasmic localisation. Replacing of residues M645, Q646 and V647 ($AHR^{M645A,Q646A,V647A}$), or V647 only (AHR^{V647A}) by alanines led to an exclusive nuclear staining, whereas mutant AHR^{Q646A} showed wild-type compartmentalisation (lower panel left). On the other side, replacement of V647 with isoleucine ($AHR^{V647I;\Delta647}$) did not affect the cytoplasmic staining pattern (upper panel right).

both amino acids are interchangeable, V647 was replaced with isoleucine in the $AHR^{\Delta647}$ mutant, thus creating $AHR^{V647I;\Delta647}$. In this experiment an exclusive cytoplasmic staining was confirmed similar to $AHR^{\Delta647}$. Therefore our data suggest equivalent properties of valine and isoleucine at residue 647 of the human AHR.

Activation-induced nuclear association of the AHR depends on the Q-rich domain. The C-terminal deletion mutant $AHR^{\Delta647}$ lacks essential parts of the Q-rich domain[28]. Notably this mutant showed an exclusive cytoplasmic staining that was markedly increased when compared with the wild-type receptor (Fig. 2). The deleted adjacent sequence is required for transcriptional activation of target genes *via* recruitment of co-activators and components of the basal transcription machinery[28]. Kinetic analysis of this mutant confirms its basal nuclear transition to be comparable to the full-length protein (Figs 1 and 4a). Similarly to the full-length receptor co-treatment with Kyn and the export inhibitor LMB accelerated the basal nuclear translocation, as did co-exposure to βNF and LMB. This was expected, since the deletion does neither affect the NLS, nor the ligand binding sites. Yet, contrastingly single substance exposure to Kyn or βNF failed to induce a stable or prolonged nuclear association of mutant $AHR^{\Delta647}$ (Fig. 4a). These data indicate that the Q-rich domain is required for ligand-induced export inhibition, likely involving further protein interactions. Consequently, interactions of ligand-bound AHR-complexes *via* the N-terminal domain are not sufficient to stabilise a nuclear fraction beyond the transient levels of shuttling. These findings propose the Q-rich domain as crucial switch to exit shuttling. Further analysis specified residues 648–661 as sufficient to maintain a stable nuclear fraction during activation (Fig. 4b,c). For these mutants, we have analysed the kinetics of nuclear transfer in the presence of agonists in detail. Our data demonstrate that the section between residues 648–661 is required and sufficient to facilitate nuclear accumulation of the AHR in response to ligands (Fig. 4c). Still, nuclear transition of the full-length receptor was slightly enhanced, pointing to the possibility that additional sites of the transactivation domain stabilise the nuclear fraction during ligand-induced activation.

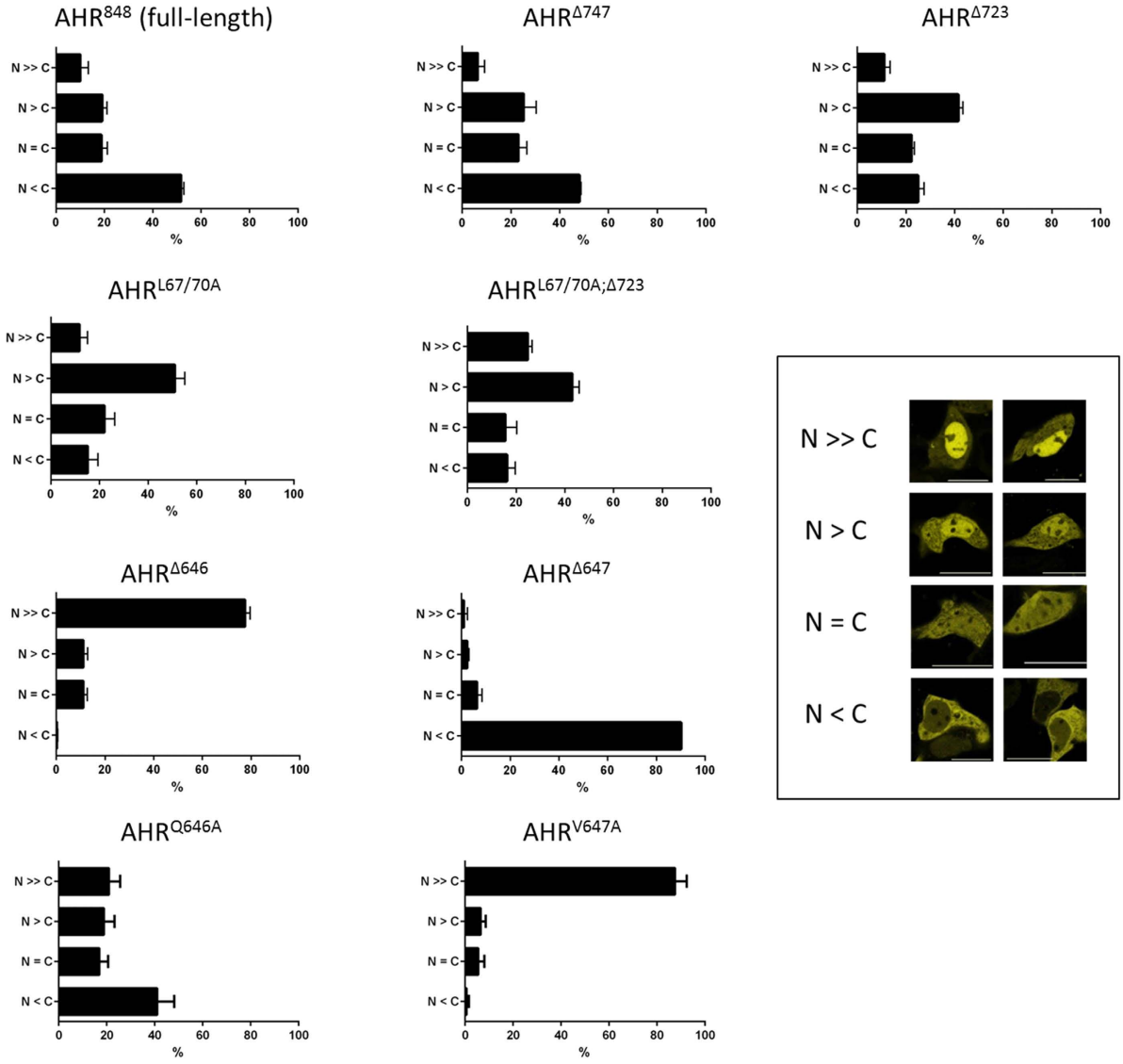

Figure 3. Compartmentalisation of EYFP-AHR mutants in transfected cells. A total of at least 300 positive cells that were found in randomly selected optical fields were analysed and classified after 24 h according to the defined staining patterns. Data represent the mean +/− S.D. out of three independent transfections. Insert: Staining patterns have been defined according to shown examples. N >> C exclusively nuclear; N > C predominantly nuclear; N = C equal distribution; N < C predominantly cytoplasmic.

The C-terminal PST domain affects the nucleocytoplasmic distribution of human full-length AHR. Microscopic analyses of $AHR^{\Delta 723}$ revealed a strongly enhanced nuclear staining in relation to $AHR^{\Delta 647}$ (Figs 2 and 3). Importantly, this staining was also distinguishable from the predominantly cytoplasmic pattern of the full-length AHR with its additional 125 amino acids. This finding raised the question on how the predominant cytoplasmic compartmentalisation is maintained. We analysed the C-terminal domain for relevant motifs, including potential export signals using LocNES predictor software[29]. The analysis yielded two high scoring hits that comprise two overlapping sequence motifs between Pro 728 and Leu 744 (Supplementary Fig. S3, PYPTTSSLEDFVTCLQL). Notably these motifs were not found in the partially homologous murine sequence, in which an alternate putative NES is predicted further upstream between Val 598 and Leu 612 by LocNES instead. Intriguingly, elongation of the human AHR protein to residue 747 ($AHR^{\Delta 747}$) restores the cytoplasmic compartmentalisation that is also seen with the wild-type receptor (AHR^{848}) (Fig. 2). Kinetic analysis of basal nucleocytoplasmic shuttling and ligand-induced nuclear translocation further confirmed the similarity to full-length AHR (Supplementary Fig. S2a). In contrast, the predominant nuclear compartmentalisation of $AHR^{\Delta 723}$ was further increased by concomitant inactivation of the N-terminal NES ($AHR^{L67/70A;\Delta 723}$). Deletion of both motifs doubled the proportion of cells that showed a nearly exclusive nuclear staining (Fig. 3). However, when this motif (i.e. Pro 728 – Leu 744) was fused to the C-terminal domain of EYFP, it did not shift the fusion protein towards an enhanced cytoplasmic distribution and no nuclear accumulation was observed after treatment with LMB

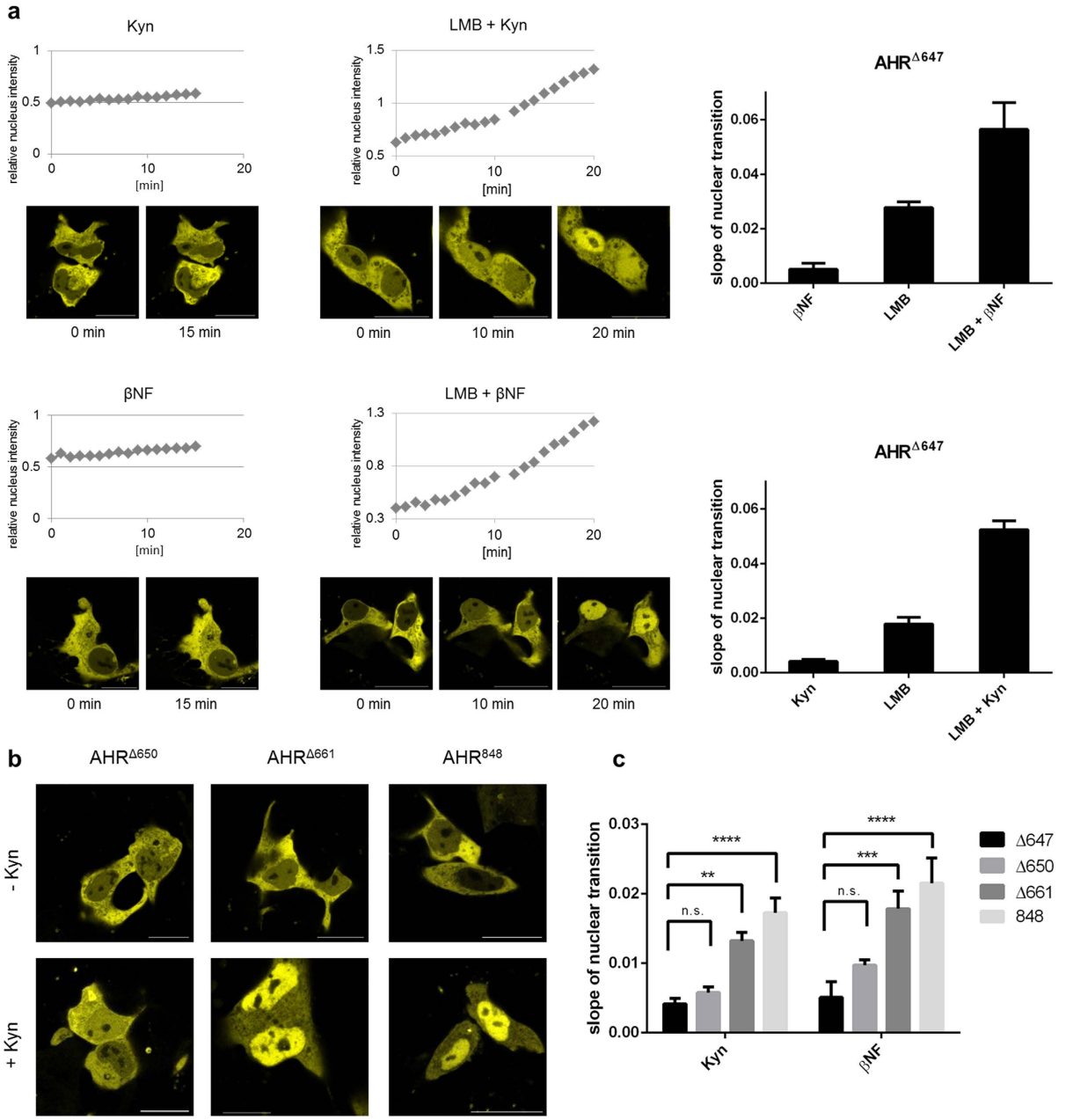

Figure 4. Ligand-induced nuclear association of the AHR depends on the Q-rich domain. (**a**) Translocation of EYFP-AHR$^{\Delta 647}$ into the nucleus in HepG2 cells after treatment with 100 μM Kyn or 10 μM βNF for 15 min (left). Translocation of AHR$^{\Delta 647}$ after exposure to 40 nM LMB for 10 min, followed by direct addition of 100 μM Kyn or 10 μM βNF for another 10 min (middle). Representative images of treated cells are shown for the indicated time points (scalebar = 20 μm). Nuclear transition was recorded and analysed as described in Fig. 1a. Slopes of the linear transition graphs have been separately determined for single and combined treatments (right). Each bar represents the mean +/− S.E.M. of 6 analysed cells. (**b**) Snapshots of cells imaged from transfected populations that were treated for 1 h with Kyn or left untreated. In response to ligand, AHR$^{\Delta 661}$ and full-length AHR^{848} showed a nearly exclusive nuclear staining pattern, while AHR$^{\Delta 650}$ remained predominantly cytoplasmic as in non-treated cells. Similar effects were observed after application of βNF. (**c**) Residues 648–661 are required for ligand-induced nuclear accumulation of the AHR. Cells expressing AHR$^{\Delta 647}$, AHR$^{\Delta 650}$, AHR$^{\Delta 661}$ or AHR^{848} (full-length) were treated with 100 μM Kyn or 10 μM βNF. Both, AHR$^{\Delta 661}$ and AHR^{848} showed significantly higher nuclear translocation rates than AHR$^{\Delta 647}$ (two-way ANOVA, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). No such significant differences were observed between AHR$^{\Delta 647}$ and AHR$^{\Delta 650}$. Values depicted represent the mean +/− S.E.M of at least 5 cells.

(data not shown). Accordingly, a possible function as autonomous CRM1-dependent export signal could not be substantiated in this study. The observed effects on the cytoplasmic fraction therefore might rather depend on upstream elements, especially the N-terminal NES and the Q-rich domain.

Discussion

Functions of the AHR are closely related to intracellular trafficking both in the context of shuttling and ligand-induced nuclear transfer. According to the classical nuclear transport model, the AHR is complexed with Hsp90 and co-chaperones. Ligand binding had previously been proposed to stimulate release of Hsp90 from the N-terminal bHLH domain thus exposing the NLS and inducing transport of the AHR into the nucleus[30,19]. In addition, Richter and co-workers (2001) demonstrated that nuclear import can occur in the absence of xenobiotic ligands, but less efficiently[31]. Our data are in agreement with these previous observations and confirm a substantial acceleration of basal import by Kyn and βNF. Import of the AHR into the nucleus might also depend on endogenous ligands that could be formed in cultured cells as well. It seems conceivable that this process operates continuously, although less efficient in the absence of interacting ligands. According to the model proposed by Lees and Whitelaw (1999) interactions between Hsp90 and the bHLH are comparatively weak[30]. Therefore it might be well possible that the NLS is transiently exposed in a steady state proportion of receptor molecules that enter the nucleus.

The interplay between parallel import and export pathways provides a new perspective on the activation of the AHR shifting the focus from a sequential model to kinetic shuttling as a means of highly complex and fine-tuned receptor regulation. Firstly, the exclusive nuclear detection of deletion mutants supports the concept of a constitutively active NLS, which is also in agreement with rapid nuclear accumulation after export inhibition in LMB-treated cells[19,20,31]. Continual activation-independent export is therefore required to maintain a receptive cytosolic fraction of AHR complexes in waiting to respond to endogenous or xenobiotic (exogenous) ligands. Secondly, during activation, shuttling might assert an element of kinetic control not only balancing the molecule pools of AHR between different intracellular compartments (nucleus *vs.* cytoplasm), but also by crucially limiting the time frame for nuclear interactions of both the N-terminal and Q-rich domains in a ligand-dependent manner. We have summarised our conclusions in accordance to the analysed AHR mutants in Fig. 5. Importantly, the N-terminal NLS acts autonomously. Although it might be masked by interacting factors, no particular conformation or regulation by other AHR domains is apparently required for its functions. According to our data, this is a marked difference to the NES that is also localised in the N-terminal domain. The capacity of the N-terminal NES to shift AHR complexes out of the nucleus depends on a C-terminal section within the Q-rich domain that comprises V647 as mandatory residue. Possibly, this residue stabilises a conformation required for nuclear export, but apparently not for import. Further, the same region of the Q-rich domain is essential to consolidate a stable nuclear association during receptor activation. Again, this does not necessarily involve interactions with transcription factors or other proteins, but might be alternatively related to structural properties of the full-length protein.

Taken together, we have defined a section of the Q-rich domain that regulates export and trafficking of the AHR in the context of both nucleocytoplasmic shuttling and receptor activation. In addition, export regulation that involves the essential N-terminal NES is also affected by a further C-terminal motif within the PST domain.

Methods

Reagents and plasmids. Dimethyl sulfoxide (DMSO), β-naphthoflavone (βNF), leptomycin B (LMB), and kynurenine (Kyn) were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Munich, Germany). Full-length or truncated cDNA of human AHR were subcloned into pEYFP-C1 (Clontech), using the Bgl*II* and Kpn*I* sites. A unique forward primer 5′-catgacagatctgccagtcgcaagcggcggaag-3′ was used. Constructs encode the processed human AHR starting at Ala 11.

As reverse primers the following oligonucleotides were used. Full-length AHR^{848} (11–848): 5′-catgacggtaccttacaggaatccactggatgtcaaatc-3′; Truncated AHR variants:

$AHR^{\Delta391}$: 5′-acacaggtaccttatccttcctcatctgttagtgg-3′;
$AHR^{\Delta509}$: 5′-acacaggtaccttacaggatagtatcatttcccatc-3′;
$AHR^{\Delta640}$: 5′-acacaggtaccttactgacacagctgttgctgtgg-3′;
$AHR^{\Delta644}$: 5′-acacaggtaccagatctttagtgcttcatcttctgacacagctg-3′;
$AHR^{\Delta646}$: 5′- acacaggtaccgaattcttattgcatgtgcttcatcttctgacac-3′;
$AHR^{\Delta647}$: 5′-acacaggtaccaagcttaaacttgcatgtgcttcatcttctgacac-3′;
$AHR^{\Delta650}$: 5-acaca ggtaccttacatgccattaacttgcatgtgc-3
$AHR^{\Delta661}$: 5-acacaggtaccagatcttaaggcacgaattggttagagttcc-3
$AHR^{\Delta698}$: 5′-acacaggtaccttactgtgtataaggcatagaatcc-3′
$AHR^{\Delta723}$: 5′-acacaagcttggtaccttaactccccatagggtagtccagctc-3′;
$AHR^{\Delta747}$: 5′-acacaggtaccttagttttcaggaagttgtaaacaagtg-3′;
$AHR^{\Delta808}$: 5′-acacaggtaccttaatttaaaactccattctgaaacttg-3′.
$AHR^{V647I;\Delta647}$: 5′-acacaggtaccaagcttaaatttgcatgtgcttcatcttctgacac-3′;
pEYFPAHR$^{\Delta NES}$ variants that carry mutations at certain amino acids ($AHR^{L67/70A}$, $AHR^{M645A,Q646A,V647A}$, AHR^{Q646A}, AHR^{V647A}) were generated and sequenced by MGW Eurofins (Ebersberg, Germany).

Tissue culture and treatments. The human hepatoma cell line HepG2 was cultured in RPMI. Human embryonic kidney 293 (HEK293) cells were cultured in DMEM. Both cell lines were maintained in 5% CO_2 at 37 °C in culture medium containing 10% fetal calf serum (v/v), L-glutamine (2 mM), and penicillin/streptomycin (100 U/ml). All media components were purchased from Pan-Biotech (Aidenbach, Germany). For stimulation of cells, the media were replaced with fresh media or HBSS (Gibco - Thermo Fisher, Waltham, MA, USA) containing

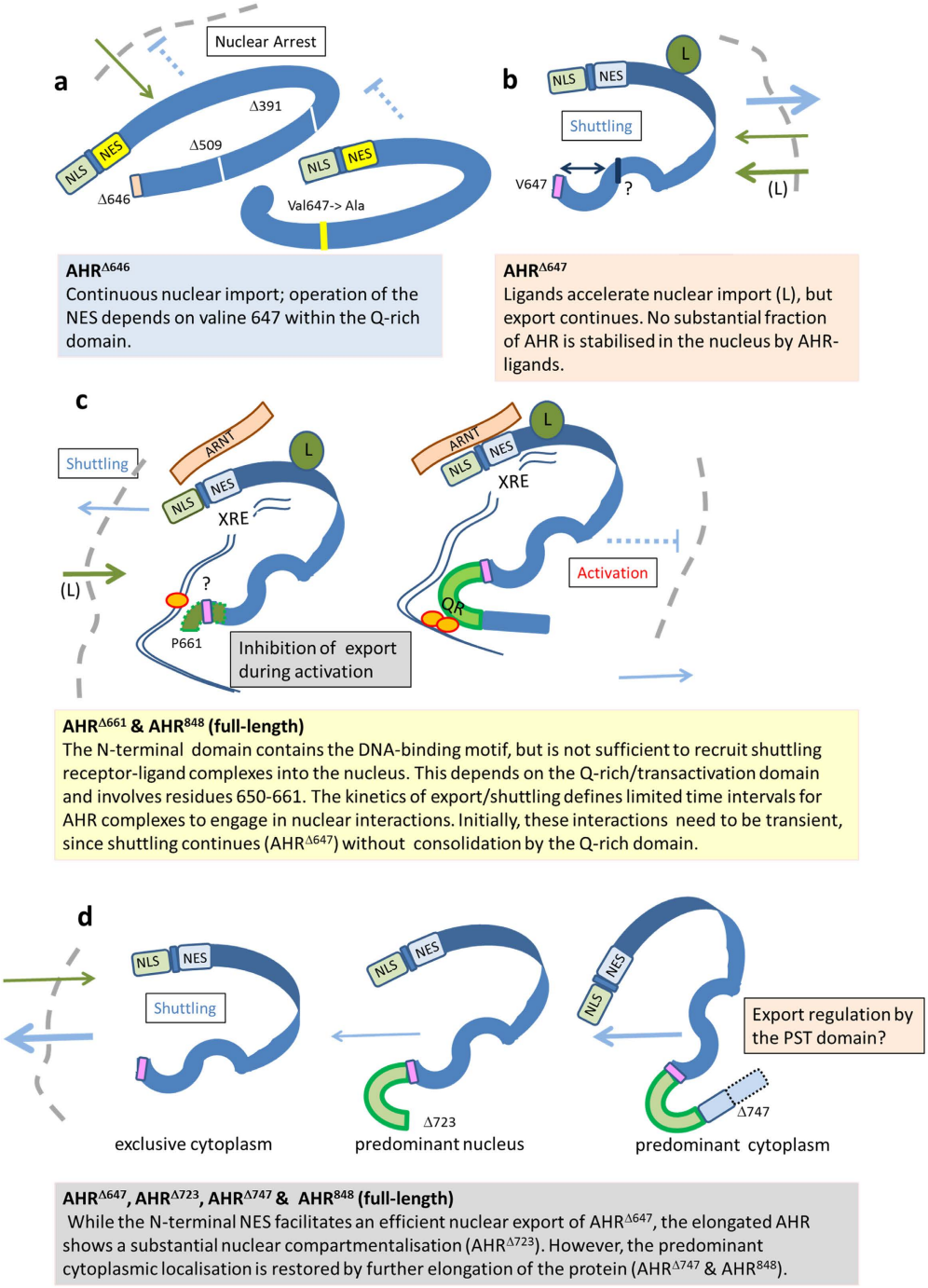

Figure 5. Nuclear export and intracellular trafficking of the human AHR are regulated by defined motifs. (**a**) The nuclear localisation signal (NLS) within the N-terminal domain triggers continuous basal import (green arrows) into the nucleus (shuttling). Contrary to this autonomous import mechanism, function of the adjacent nuclear export signal (NES) depends on C-terminal motifs, especially the mandatory residue V647. (**b**) Ligands (marked with L) accelerate import, while continued export (blue arrow) counteracts nuclear sequestration of the AHR, thus maintaining a predominant cytoplasmic fraction that is receptive for interactions with ligands. Notably, mutants that lack parts of the C-terminal domain (AHR$^{\Delta 647}$ and AHR$^{\Delta 650}$) do not efficiently accumulate in the nucleus, although nuclear transfer is accelerated by ligands. (**c**) Export of the AHR continues in the presence of ligands. Activation of the AHR might involve several passages of receptor molecules that need to engage in further associations with nuclear components during limited time intervals. Stable associations of the AHR with the nucleus likely require a defined section of the Q-rich domain (green, Pro 661 is indicated). However, it is as yet completely unknown how this motif stabilises nuclear compartmentalisation or whether it promotes interactions of the transactivation domain with transcription factors. (**d**) The N-terminal NES and the V647 motif facilitate an efficient nuclear export of AHR$^{\Delta 647}$, leading to a nearly exclusive cytoplasmic pattern. On the other side, the full-length AHR contains an additional motif within the PST domain to maintain a predominantly cytoplasmic compartmentalisation.

10 μM βNF, 100 μM Kyn or 40 nM LMB dissolved in DMSO/ethanol and further incubated for the indicated time period. Control cells were treated with solvent vehicle (0.1% DMSO) only.

Transient transfection. Depending on the further application, HepG2 cells and HEK293 cells were seeded either on 6-well plates (Techno Plastic Products AG, Trasadingen, Switzerland), glass-bottom dishes (*In Vitro* Scientific, Sunyvale, CA, USA), or on cover glasses (ThermoFisher Scientific, Loughborough, UK). At next day they were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and an appropriate DNA concentration according to the manufacturer's instructions. For HEK293 cells, the surfaces were coated with poly-L-lysine (Biochrom AG, Berlin, Germany).

RNA analysis. RNA was isolated using RNeasy Mini kit in connection with the QIAshredder (QIAGEN GmbH, Hilden, Germany). The purity and the concentration of each RNA sample were determined using a NanoDrop1000 device (Peqlab Biotechnologie GmbH, Erlangen, Germany). The isolated RNA was used for cDNA synthesis with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was carried out using an SYBR green assay (5Prime, Hamburg, Germany). Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as reference gene.

Western-blot analysis. Cells were lysed on ice in RIPA buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Igepal® and 0.25% sodium deoxycholate, with protease inhibitor cocktail (Calbiochem, San Diego, CA, USA). Equal amounts of proteins were applied to SDS-PAGE, transferred onto nitrocellulose membranes and immunoblotted according to the manufacturer's instructions. The primary antibody against AHR was used at 1:200 (sc-5579; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibody probed blots were visualised with appropriate horseradish peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology) using enhanced chemiluminescence (34078; Thermo Scientific, Waltham, MA, USA) for detection.

Immunofluorescence staining. For immunofluorescence staining the Inside Stain Kit (Miltenyi Biotech Ltd., Bisley, Surrey, UK; Miltenyi Biotech Inc., Auburn, CA, USA) was used with minor modifications. After the fixation step, the cover glasses were incubated with the anti-AHR primary antibody (1:100, Santa Cruz Biotechnology) overnight at 4 °C. The slides were then washed and incubated with the secondary antibody for 30 min at room temperature (1:500, anti-rabbit FITC, Bethyl Laboratories, Inc., Montgomery, TX, USA). The slides were mounted in the VECTASHIELD HardSet Mounting Medium with DAPI (VECTOR LABORATORIES, INC., Burlingame, CA, USA), and then analysed using a confocal microscope (see below).

On-line confocal microscopy. For live-cell fluorescence imaging microscopy, HepG2 cells were seeded on glass-bottom dishes and transfected with plasmid DNA. Twenty four hours post transfection, fresh medium was applied and cells were monitored by confocal microscope. For on-line investigations, representative cells or cell groups were selected and maintained in buffered medium at 37 °C. Concurrently with treatments, live-cell imaging was started at a rate of one picture per minute. Typically, single treatment experiments were finalised after 15 min and combined treatment experiments after 20 min. Microscopic analyses and image acquisitions were done on an LSM 700 confocal microscope (Carl Zeiss Jena GmbH, Jena, Germany), using ZEN 2012 blue edition and ZEN 2011 black edition software (Carl Zeiss Jena GmbH). Data were analysed and graphed using Microsoft Excel and Prism Software (Graph Pad, La Jolla, CA, USA). Statistical analysis was done using two-way ANOVA and either Dunnett's or Sidak's multiple comparisons test. $\alpha = 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Cloning and expression of AHR. The human AHR DNA sequence was cloned into the pTFCold vector system (Clonetech, Takara) using a synthesised and codon-optimised construct. Clone identity was confirmed by sequencing and the respective plasmids where subsequently used for protein expression in the Rosetta 2 (DE3) system (Novagen) using 2xYT-medium and standard conditions with 300 μM isopropyl β-D-1-thiogalactopyranoside (IPTG), as recommended by the manufacturer. Following overnight cold-expression cells were harvested, washed (20 mM PIPES, 300 mM NaCl, pH 7.8) and subjected to lysis by sonication. At least 70% of expressed AHR were recovered in the soluble fraction and purified using Talon superflow agarose (GE Healthcare, Freiburg, Germany) according to manufacturer's instructions. After elution the protein concentration was estimated by spectrophotometric analysis and the purification efficiency was checked using SDS-PAGE (refer to Supplementary Fig. S4 for an exemplary gel).

Thermal shift assay. Thermal stability of AHR without and with ligands was assessed fluorometrically as described[32]. Assays were performed in a 96-well format using SYPRO orange in conjunction with the melting curve feature of a real-time PCR cycler. Following background subtraction the melting point was calculated based on the maxima of the melting curves using the equation of Biggar and co-workers[32].

Matrix-assisted laser desorption/ionisation Time-of-Flight (MALDI-ToF). We used MALDI-ToF to verify the identity of the recombinant human AHR protein. Following electrophoretic separation gel slices containing recombinantly expressed and purified AHR (~150 kDa) were cut out, destained, trypsin digested and subjected to fragment-pattern analysis using the following protocol. Destained gel slices were first reduced for 15 min at 60 °C using 100 mM ammonium carbonate with 45 mM dithiothreitol (DTT), and then alkylated for

15 min using 100 mM iodoacetamide. Following alkylation the gel slice was washed for three times and equilibrated for another 30 min in equilibration buffer containing 50 mM ammonium bicarbonate and 5% acetonitrile before commencing with overnight 4 ng/μL trypsin digestion at 37 °C. Following digestion the samples were extracted with 60% acetonitrile/0.1% trifluoroacetic acid (TFA) and 100% acetonitrile. Subsequently, samples were purified and desalted using C18 ZipTips (Merck Millipore, Darmstadt, Germany) with TFA supplemented (0.1%, v/v) washing solution. Eluted samples were spotted with α-cyano-4-hydroxycinnamic acid matrix on AnchorChip targets (Bruker, Rheinstetten, Germany) and analysed using an UltrafleXtreme MALDI-ToF/ToF (Bruker, Rheinstetten, Germany). Data evaluation was performed with ProteinScape (MASCOT/Swissprot database). The following search parameters were used: 1 missed cleavage, carbamidomethyl (Cys) as fixed modification and oxidation (Met) as variable modifications. Taxonomy was set to *Homo sapiens*. MS tolerance and MS/MS tolerance were set to 50 ppm and to 0.7 Da, respectively.

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Author Contributions

A.T. carried out most parts of the experimental work and contributed towards planning and data processing and analysis. F.H. and A.L. designed the experimental program and contributed to data processing and analysis. C.K., J.B. and D.G. generated plasmids, contributed to the confocal microscopy studies, including data analysis. J.S. and T.T. planed and performed thermal shift assays and contributed to data analysis. The manuscript was written by F.H., A.T., T.T. and A.L.

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3.2 TKACHENKO, A. ET AL. (2017) "NUCLEAR TRANSPORT OF THE HUMAN ARYL HYDROCARBON RECEPTOR AND SUBSEQUENT GENE INDUCTION RELIES ON ITS RESIDUE HISTIDINE 291"

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Nuclear transport of the human aryl hydrocarbon receptor and subsequent gene induction relies on its residue histidine 291

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The authors contributions:

Project planning (80 %)
Experimental work (70 %)
Data analysis (80 %)
Preparation of manuscript (90 %).

4 Discussion

4.1 Role Of the Transactivation Domain in Nuclear AHR Transport

The Aryl Hydrocarbon Receptor (AHR) is a ubiquitous xenobiotic sensitive transcription factor, which had been known for more than 40 years as important toxicological mediator. Recent research indicates that the receptor is also involved in multiple physiological functions besides its role in toxicology. Due to its crucial impact on processes that affect human health, including cancer development, immune cell maturation and involvement in the formation of proper host-microbiome interactions, the AHR has also become a promising pharmacological target. In order to address potential functions of the AHR including elucidation of toxicity mechanisms as well as possible pharmacological interventions, it is crucial to understand the conformational interactions within the receptor molecule, as well as interactions with the relevant co-factors.

In the presented work, attention was given to different molecular and structural aspects as basis for the human AHR function, since most data published yet refer to the murine protein. The experiments were conducted using a fluorescent fusion construct of the human AHR gene, which allows studies in living cells as well as an assessment of kinetics and functional parameters. Foremost, as a methodological basis for live cell imaging, a convenient method appropriate for confocal microscopy was established. As part of this approach, cells were transfected with pEYFP-AHR, a yellow fluorescent construct, and treated with ligands or other substances of interest. The time-dependent alteration in fluorescence allocation within individual living cells was subsequently assessed in a semi-quantitative manner.

The first part of the study was focused on nucleocytoplasmic shuttling (Ikuta et al. 2000; Richter et al. 2001). Our data show that this process occurs permanently in living cells, but does not lead to receptor activation as previously discussed (Murray et al. 2014). In addition, we have demonstrated that ligands promote nuclear translocation of the AHR actively, meaning that the

endogenous import of the AHR is effectively accelerated by ligands leading to 2-3 fold increased net receptor import rates. Since the nuclear export of the receptor is not altered, ligands induce a nuclear compartmentalization of the AHR. The impact of interacting ligands demonstrated by our results is in agreement with the classical nuclear transport model, proposing ligands to dissociate HSP90 from the N-terminal bHLH domain which unmasks the NLS and facilitates an accelerated nuclear transport (Ikuta et al. 1998; Lees and Whitelaw 1999). In this context, the permanent basal nucleocytoplasmic shuttling may be a general mechanism to terminate transcription but also the premise for ligand independent functions of the AHR, which have also been recently suggested (Villa et al. 2016).

Furthermore, the critical segments responsible for receptor activation and ligand binding were analyzed by mutagenesis using both truncated mutants and point mutations within the C-terminal part of the receptor. Since AHR variants that lack major parts of the C-terminal domain are exclusively localized within the cell nucleus, the N-terminal NLS must be a constitutively active and autonomous signal, whereas the adjacent export motif (NES) requires additional motifs to operate. This conclusion is also reinforced by the rapid nuclear translocation of the AHR after export inhibition by LMB, which has been seen by other authors as well (Ikuta et al. 1998; Ikuta et al. 2000; Richter et al. 2001). Consequently, the predominant cytosolic compartmentalization of the human AHR depends on a dominant NES that is also permanently active in context of the full-length protein. This is an important prerequisite for receptor activation, since AHR ligands interact with the cytoplasmic fraction. Hence, in contrast to NLS, the likewise N-terminally located NES is not acting self-sufficiently and requires further regions within the C-terminal part of the AHR protein. Using additional truncated mutants lacking parts of the C-terminal domain, we were able to narrow down the region of interest to the Q-rich domain and identify a single residue which is essential for nuclear export of the AHR - the valine 647 (V647). The necessity of this residue for the AHR export was also confirmed by its point mutation to alanine (V647A). This mutation exhibited a strictly nuclear distribution in transfected cells, implying a complete lack of export processes.

Besides the importance of V647 for nuclear export, we did observe a further important effect. AHRΔ647 as well as AHRΔ650 failed to compartmentalize in the nucleus in response to both endogenous and xenobiotic ligands. However, these truncated variants showed nuclear import rates similar to the full-length AHR after treatment with LMB to inhibit exclusion from the nucleus. Similar to wild-type AHR, nuclear import of AHRΔ647 and AHRΔ650 is accelerated by ligands, but this does not lead to a stabilized nuclear fraction. This indicates that AHRΔ647-650/ligand complexes are re-exported, suggesting that parts of the C-terminal transactivation domain are required to trigger a nuclear accumulation of the AHR in response to ligands. Consistent with this hypothesis, a rapid ligand-induced nuclear compartmentalization, comparable with the full-length protein, was restored in the truncated variant AHRΔ661 as well as in longer AHR variants. Consequently, the region within the Q-rich domain protein spanning the residues 650 - 661 is mandatory for a stable nuclear association during receptor activation. This region might be engaged in interactions with other nuclear structures like chromatin; however, there are no experimental data yet to support this hypothesis.

4.2 The Ligand Binding Domain of Human AHR - Role of H291

The second part of the presented work was focused on the ligand binding of the AHR, as it is the central function of a ligand-dependent transcription factor. The corresponding structural unit of the AHR is named ligand binding domain (LBD) and is located within the PAS B domain. The role of distinct residues within the LBD was addressed in several studies before, but most of them were conducted on murine receptor forms. Further, the assessed endpoints of successful ligand binding were late reaction steps like DNA binding or gene induction. Additionally, the selection of tested ligands was quite limited despite of the wide range of AHR ligands and the fact that responses might differ for various ligands.

In this work, the focus was set on a systematic examination of various ligands in order to clarify initial steps of receptor activation. We have further addressed the relevance of selected residues within the ligand binding domain, as an attempt to identify the underlying molecular mechanisms. As the endogenous functions

of the AHR are attracting more attention, three endogenous (KYN, IND, FICZ) and one xenobiotic ligand (BNF) were selected for testing. The efficiencies of all investigated AHR ligands were shown before (Adachi et al. 2001; Bergander et al. 2004; Fritsche et al. 2007; Murray et al. 2014; Opitz et al. 2011; Wincent et al. 2009). Besides ligand mediated gene induction, which has been addressed before (Bessede et al. 2014; Xing et al. 2012), we also addressed the induction of nuclear translocation as initial step of receptor activation. Using both assays we could record proper dose-response curves with EC50 values similar to literature data (Nguyen and Bradfield 2008) for all tested substances, showing the general eligibility of our chosen test system. Analysis the dose-responses for all tested endogenous ligands after a short-term (5 h) and a long-term (24 h) treatment revealed that the exposure levels that induce transient gene induction and nuclear import are correlated. In contrast, xenobiotic BNF which triggered a rapid nuclear translocation did only induced a delayed expression of target genes as for example CYP1A1. Consequently, the effects of endogenous ligands are fast and specific, whereas BNF showed much flatter dose responses, but also a sustained activation. Metabolization of the endogenous AHR ligands by up-regulated enzymes was suggested as an important control mechanism to terminate activation, while some xenobiotics can persist as stable activating stimulus (Bergander et al. 2004; Hubbard et al. 2015; Nebert and Karp 2008). Hence, despite of its promiscuity, AHR might respond to endogenous ligands in a defined physiological way that differs from an unspecific activation in response to xenobiotics. The possibility of a specific AHR activation has also been in theory considered before (Quintana and Sherr 2013) and first hints were found for the murine protein (Bessede et al. 2014).

In order to investigate the human LBD at a functional level, this domain was modified by site-directed mutagenesis and the impact of single residue changes on both initial steps of AHR activation were analyzed subsequently. Optimal single concentrations of all substances were used, which were determined in previous dose-finding studies. The selected residue mutations were based on homologous amino acids within the murine AHR, which had been published in earlier studies as most important for an accurate LBD function (Bessede et al. 2014; Bisson et al. 2009; Goryo et al. 2007; Pandini et al. 2007; Soshilov and Denison 2014; Xing et al. 2012). Further, these residues showed proper

orientations towards aligned ligands within various models of the ligand binding pocket.

In these experiments, we did observe that the corresponding human homologs to high impact murine mutations C333A and Q383A had only minor consequences on ligand-mediated effects by the human AHR. Intriguingly, a mutation of Q383A (Bessede et al. 2014) even led to an opposite effect showing an enhanced gene induction in response to BNF compared with the wild-type protein. This discrepancy might be due to deviations between the murine and human AHR ligand-binding pockets, the most prominent of which is a single homologous residue difference (murine A375, human V381) within the LBD (Connor and Aylward 2006; Ramadoss and Perdew 2004). Consequently, murine data based on AHR responses to TCDD or other substances should be re-assessed for conclusions that refer to the human protein. However, as in the murine protein, residue H291 was essential for both nuclear AHR translocation and consequently, gene induction in response to all tested ligands. Mutation to alanine abolished all ligand-dependent AHR processes showing a consistency with the impact of murine mutation H285A on DNA binding of the AHR complex (Pandini et al. 2007; Soshilov and Denison 2014; Xing et al. 2012). This result demonstrates a partial consistency in the LBD function of human and murine AHR. Subsequent analysis of the H291A variant regarding the basal ligand-independent receptor import that occurs in the context of nucleocytoplasmic shuttling revealed no differences compared with the wild-type protein. Hence, as this histidine to alanine mutation had a significant impact on the triggering of AHR import by all tested ligands, but not on shuttling, it is clear, that these two processes are independently regulated. Consequently, nucleocytoplasmic shuttling is unlikely to be triggered by low exposure to endogenous ligands as previously discussed (Murray et al. 2014).

Thus, the crucial function of H291 in the ligand-induced activation of the human AHR becomes even more intriguing. For a first mechanistic explanation of the pivotal significance of H291, a range of computational methods was applied. The approach aimed to simulate the molecular environment within and around the ligand-binding pocket via 3D molecular modeling, which would allow

comparisons of wild-type and mutated protein forms as well as effects of different ligands.

Since no crystal structure of the AHR-PAS B domain was available at project initiation, a 3D homology model of the LBD was developed. The crystal structure of a protein from the same family as AHR and with the highest available sequence similarity with about 30% identical residues (PDB 3H82, (Key et al. 2009)) was applied as template. This structure was also used earlier by other authors for homology modeling (Bisson et al. 2009; Perkins et al. 2014), but in our case the homology model was built taking into account previous *in vitro* data. The derived AHR-LBD homology model was then validated and subsequently used in molecular docking experiments and molecular dynamics (MD) simulations.

Although the modeling results only predict possible protein structures or conformational alterations, we would like to propose two hypotheses of H291 involvement in ligand-mediated AHR response. First, this histidine may be involved in the direct ligand binding, as it is appropriately located within the ligand binding pocket as interaction partner. Also, the structural analysis of molecular docking simulations indicated direct interactions with all tested ligands, which were ceased in the H291A form. Second, the MD simulations predict a similar conformational effect within the PAS B domain after ligand-binding and as consequence of the H291A mutation. Both the ligand-binding of the wild-type AHR and the H291A mutation result in an orthogonal conformational change of a backbone loop, which was shown to directly interact with ARNT (Tsuji et al. 2014) and is also located on the AHR-ARNT protein-protein interface in our model. Therefore, this orthogonal switch within the AHR-PAS B backbone region may stabilize the AHR-ARNT complex interaction after ligand binding, which is required for durable nuclear receptor complex accumulation and consequently for ligand-mediated gene induction (Jones et al. 1985; Probst et al. 1993). Hence, as a mutation of the H291 to alanine leads to an altered conformation even before ligand exposure; it acts like a “closed lock” that prevents a stable AHR-ARNT interaction.

Since the AHR-ARNT interaction sites are not only limited to their PAS B domains, as shown recently in a co-crystal structure (Schulte et al. 2017; Seok

et al. 2017), we can assume a highly complex interplay within the protein-protein interface with multiple decisive structural elements. Nevertheless, an essential H291 role in the AHR-ARNT interaction is also conceivable due to presented data. It would be of high interest to obtain a crystal structure of at least the PAS A and PAS B domains of both interacting proteins of the AHR-ARNT-complex. The structural consequences of binding different ligands as well as of LBD residue mutation on the protein complex formation could then be assessed directly and eventually help elucidate the role of H291.

4.3 Further Conclusions and Consequences

Based on the results of both publications, novel aspects of AHR physiology and possible pharmacological intervention can be derived.

First, several experiments have shown that the ligand-mediated import and the endogenous import due to nucleocytoplasmic shuttling are two independent processes regulated by different functional elements. Hence, the earlier hypothesis of nucleocytoplasmic shuttling caused by endogenous AHR ligands was not confirmed (Murray et al. 2014). Further, as basal and ligand induced AHR import mechanisms are regulated independently, it is conceivable these pathways might be targeted separately. This could open up new options for pharmacological intervention, but also risks.

Consistent with earlier assumptions, it became apparent, that xenobiotic and endogenous agonistic ligands display differential effects on various AHR activation steps. Irrespective of the mechanistic basis for such a deviation, this circumstance possesses attractive potential in the therapeutic area of application. Depending on which effect characteristics are aspired, different ligand classes can be focused – xenobiotic agonists for slow but prolonged AHR activation, endogenous agonists for fast and short-term receptor induction.

The differences between human and murine AHR, previously recognized by other authors (Flaveny et al. 2009; Hubbard et al. 2016; Ramadoss and Perdew 2004) could be strengthened from an additional perspective and supplemented by new data. As we see in the comparison between murine and human AHR, homologous point mutations of residues within the LBD observed to date have

different consequences on the ligand-dependent AHR activation (Bessede et al. 2014). Similar experiments certainly have to be conducted with similarly generated murine and AHR constructs to obtain a reliable comparative analysis of the significance of individual residues for ligand binding between mice and humans. Nonetheless, the results presented in this work together with older data suggest that the species specificity concerns not only TCDD, but also other, structural dissimilar AHR ligands like indirubin or kynurenine. Importantly, *in vitro* and *in vivo* studies on mice are crucial parts of carcinogenicity testing, which plays a decisive role in pharmaceutical and chemical development (ICH 2012; Muller et al. 2006). As the AHR activation and the induction of its downstream genes like the *CYP1*-family are regarded as direct indications of genotoxic effects (Nebert et al. 2004), all potential AHR ligands should be examined with a focus on the species specificity.

5 SUMMARY

The aryl hydrocarbon receptor (AHR) is a ubiquitous ligand-dependent transcription factor from the bHLH-PAS-protein family. Discovered as the central toxicity mediator of xenobiotic substances like TCDD and several PAKs, the AHR gained recently further attention due to identification of various endogenous ligands and associated physiological functions. Despite longtime investigation especially of the murine AHR, some important questions about molecular receptor interactions are still open. The newly discussed possibilities for pharmacological intervention in pathological and physiological AHR-dependent processes additionally increase the interest in the detailed study of the human AHR.

In this thesis, different aspects of molecular physiology of the human AHR were addressed in detail. In the first presented publication, alongside the kinetics of ligand-dependent AHR nuclear import, the mechanism of nucleocytoplasmic shuttling was examined. Using a fluorescent AHR fusion construct and a newly developed live-cell imaging method, it was demonstrated that the ligand-independent endogenous nuclear import and export are occurring permanently and rapidly, while ligands accelerate the import process effectively. Further, in-depth mutagenesis studies have clearly shown that the nuclear localization signal acts autonomously and constitutively, while the export of the AHR is strictly dependent not only on the nuclear export signal, but also on an amino acid within the glutamine-rich domain of the protein (valine 647). Further, the adjacent region spanning residues 650 to 661 seems to be strictly required for ligand-induced nuclear accumulation of the AHR.

The second part of this work focused on the ligand binding of the human AHR. Both the gene induction and the previous step of ligand-induced nuclear import were assessed semi-quantitatively as endpoints for receptor activation. The endogenous ligands triggered a rapid and transient induction of the AHR, suggesting a specific mechanism of activation. In contrast, the analyzed xenobiotic ligand triggered a delayed, but persistent activation of the AHR, implying an unspecific mode of action. Using site-directed mutagenesis histidine 291 (H291) within the ligand binding domain (LBD) was identified as essential for ligand-dependent activation of the human AHR, but not for the

nucleocytoplasmic shuttling. This indicates that the basal import in context of shuttling does not depend on low level exposure against endogenous ligands. In contrast to the homologous murine mutant, the Q383A mutant did not show any impairment in the ligand response, providing additional evidence for the species specificity of the AHR. With computational methods a mechanistic explanation for the involvement of H291 in the mediation of ligand activity of human AHR was developed for the first time. On the one hand, it is conclusive that H291 plays a major role in the shaping of the ligand binding site including mediation of direct interactions with ligands. On the other hand, this residue might be crucial for the formation of ligand-induced AHR-ARNT heterodimer and consequently for further activation steps.

Taken together, this thesis offers a new perspective on the significance of nucleocytoplasmic shuttling and export regulation of the human AHR. In addition, detailed and novel insights into the molecular mechanisms of receptor activation and the role of individual amino acids within the human AHR LBD are summarized. The data are also a valid basis for further applied research.

6 ZUSAMMENFASSUNG

Der Arylhydrocarbon Rezeptor (AHR) ist ein ubiquitärer ligandenabhängiger Transkriptionsfaktor aus der Familie der bHLH-PAS-Proteine. Entdeckt als der zentrale Vermittler der Toxizität von xenobiotischen Substanzen wie TCDD und diversen PAKs, erlangte der AHR in den letzten Jahren immer mehr Aufmerksamkeit durch die Identifizierung seiner endogenen Liganden und damit zusammenhängenden physiologischen Funktionen. Trotz langjähriger Erforschung vor allem des murinen Rezeptors, sind noch wichtige Fragen bezüglich der molekularen Interaktionen offen. Die aktuell diskutierten Möglichkeiten zur pharmakologischen Intervention in pathologische und physiologische AHR-abhängige Prozesse steigern zusätzlich das Interesse an der ausführlichen Untersuchung des humanen AHR.

In dieser Arbeit wurden verschiedene Aspekte der molekularen Physiologie des humanen AHR im Detail analysiert. In der ersten vorgestellten Publikation wurden neben der Kinetik des ligandenabhängigen AHR-Kernimports auch die Mechanismen des nukleozytoplasmatischen Shuttlings untersucht. Mit einem fluoreszierenden AHR-Fusionskonstrukt und einer neu entwickelten Live-Cell-Imaging-Methode wurde gezeigt, dass der ligandenunabhängige endogene Kernimport und Rückexport permanent und hochdynamisch ablaufen, wobei die Liganden den Importprozess effektiv beschleunigen. Weiterhin haben eingehende Mutationsstudien verdeutlicht, dass das Kernlokalisationssignal autonom und konstitutiv aktiv ist, während der Export des AHR nicht nur vom Kernexportsignal, sondern auch von einer Aminosäure (Valin 647) innerhalb der glutaminreichen Domäne essentiell abhängig ist. Speziell für die ligandeninduzierte Anreicherung des Rezeptors im Zellkern scheint zusätzlich die angrenzende Proteinregion AA 650 - 661 verantwortlich zu sein.

Im zweiten Teil dieser Arbeit lag der Fokus auf der Ligandenbindung des humanen AHR. Dabei wurden als Endpunkte für die Aktivierung des Rezeptors sowohl die Geninduktion, als auch der vorhergehende ligandeninduzierte Kernimport semiquantitativ beurteilt. Es konnte gezeigt werden, dass endogene Liganden eine schnelle und zeitlich begrenzte Induktion des AHR bewirken, die auf eine spezifische Wirkung schließen lässt.

Der untersuchte xenobiotische Ligand induzierte dagegen eine verzögerte aber dauerhafte Rezeptoraktivierung, was eine unspezifische Ligandenwirkung nahe legt. Mittels ortsgerichteter Mutagenese wurde dann innerhalb der Ligandenbindungsdomäne (LBD) Histidin 291 (H291) als essentiell für die ligandenabhängige Aktivierung, aber nicht für das nukleozytoplasmatische Shuttling des humanen AHR identifiziert. Das zeigt, dass der basale Import nicht von einer Grundexposition gegenüber endogenen Liganden abhängig ist. Die Q383A Mutante wies im Gegensatz zur homologen murinen Mutante keine Beeinträchtigung in der Ligandenantwort auf, was einen zusätzlichen Hinweis auf die Speziesspezifität des AHR liefert. Mithilfe von computergestützten Methoden wurde dann erstmalig ein mechanistischer Erklärungsansatz für die Beteiligung des H291 an der Vermittlung der Ligandenwirkung im humanen AHR entwickelt. Einerseits ist es schlüssig, dass H291 eine wichtige Rolle in der Formung der Ligandenbindungstasche einschließlich der Vermittlung direkter Ligandeninteraktionen spielt. Andererseits könnte das Histidin entscheidend für die Entstehung des ligandeninduzierten AHR-ARNT-Heterodimers und damit auch für die weiteren Aktivierungsschritte sein.

Insgesamt bietet diese Arbeit eine neue Sichtweise auf die Bedeutung des nukleozytoplasmatischen Shuttlings und der Exportregulation des humanen AHR. Zusätzlich werden neue und detaillierte Einblicke in die molekularen Mechanismen der Rezeptoraktivierung und in die Rolle von einzelnen Aminosäuren innerhalb der humanen AHR-LBD zusammengefasst. Die Ergebnisse bieten viele Ansatzpunkte für weiterführende angewandte Forschungsarbeiten.

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8 LIST OF ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
A	alanine
Å	ångström
AA	amino acid
AHR	aryl hydrocarbon receptor
AHRR	aryl hydrocarbon receptor repressor
ALDH	aldehyde dehydrogenase
AIP	aryl hydrocarbon receptor interacting protein
ARNT	aryl hydrocarbon receptor nuclear translocator
bHLH	basic-helix-loop-helix
BNF/β-NF	β-naphthoflavone
BP	benzo[*a*]pyrene
BPDE	benzo[*a*]pyrene-7,8-diol-9,10-epoxide
C (-terminus)	carboxyl-terminal end
CRM1	chromosomal maintenance 1
CYP	cytochrome P-450 monooxygenase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC50	half maximal effective concentration
FICZ	6-formylindolo[3,2-*b*]carbazole
GST	glutathione S-transferase
H	histidine
HIF2α	hypoxia-inducible factor 2 alpha
HSP90	heat shock protein 90

ICH	International Conference on Harmonisation
IND	indirubin
KYN	kynurenine
LBD	ligand binding domain
LMB	leptomycin B
MD	molecular dynamics
mRNA	messenger ribonucleic acid
N (-terminus)	amino-terminal end
NES	nuclear export signal
NLS	nuclear localization signal
p23	protein 23
PAH	polycyclic aromatic hydrocarbon
PAS	Per/ aryl hydrocarbon receptor translocator / Sim
PDB	protein data base
pEYFP	enhanced yellow fluorescent protein expression vector
PST	proline/serine/threonine-rich
Q	glutamine
RNA	ribonucleic acid
TAD	transactivation domain
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
UGT	uridine 5'-diphospho-glucuronosyltransferase
V	valine
XRE	xenobiotic response element

9 LIST OF PUBLICATIONS

Peer-reviewed articles

Tkachenko A, Bermudez M, Irmer-Stooff S, Genkinger D, Henkler-Stephani F, Wolber G, Luch A. Nuclear transport of the human aryl hydrocarbon receptor and subsequent gene induction relies on its residue histidine 291. *Arch Toxicol* 2017. doi: 10.1007/s00204-017-2129-0. Epub 2017 Nov 21.

Paschke M, Tkachenko A, Ackermann K, Hutzler C, Henkler F, Luch A. Activation of the cold-receptor TRPM8 by low levels of menthol in tobacco products. *Toxicol Lett* 2017 Apr 5;271:50-57. doi: 10.1016/j.toxlet.2017.02.020. Epub 2017 Feb 24.

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

Book chapter

Henkler F, Tkachenko A, Paschke M, Hutzler C, Luch A. „Gesundheitliche Risiken von E-Zigaretten“ Die E-Zigarette, Geschichte - Gebrauch - Kontroversen, 2016, edited by H. Stöver, Fachhochschulverlag, pp. 41-57.

Conference contributions

Tkachenko A, Irmer-Stooff S, Bermudez M, Wolber G, Henkler F, Luch A. H291 of AHR is essential for ligand-mediated activation, but not for endogenous shuttling. Effects of xenobiotic versus endogenous ligands on the aryl hydrocarbon receptor. 2nd German Pharm-Tox Summit, 06 - 09 March 2017, Heidelberg, Germany.

Tkachenko A, Irmer-Stooff S, Bermudez M, Wolber G, Henkler F, Luch A. Accelerated ligand-induced nuclear transport of the AHR is strictly dependent on histidine 291. LS2 Annual Meeting 2017, 02 - 03 February 2017.

Tkachenko A, Luch A, Henkler F. On the nucleo-cytoplasmic shuttling of the aryl hydrocarbon receptor (AHR). 1st German Pharm-Tox Summit, 29 February - 03 March 2016, Berlin, Germany.

Tkachenko A, Kern C, Brinkmann J, Luch A, Henkler F. Xenobiotic agonists are not required for nuclear translocation of the aryl hydrocarbon receptor (AhR). 81th Congress of the German Society for Experimental and Clinical Pharmacology and Toxicology (DGPT), 10 - 12 March 2015, Kiel, Germany.

Paschke M, Hutzler C, Tkachenko A, Henkler F, Luch A. Effect of the menthol content of cigarettes on the cold - menthol receptor TRPM8. 81th Congress of the German Society for Experimental and Clinical Pharmacology and Toxicology (DGPT), 10 - 12 March 2015, Kiel, Germany.

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