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The Aryl Hydrocarbon Receptor, more than a xenobiotic-interacting protein

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ABSTRACT

The aryl hydrocarbon (dioxin) receptor (AhR) has been studied for several decades largely because of its critical role in xenobiotic-induced toxicity and carcinogenesis. Albeit this is a major issue in basic and clinical research, an increasing number of investigators are turning their efforts to try to understand the physiology of the AhR under normal cellular conditions. This is an exciting area that covers cell proliferation and differentiation, endogenous mechanisms of activation, gene regulation, tumor development and cell motility and migration, among others. In this review, we will attempt to summarize the studies supporting the implication of the AhR in those endogenous cellular processes.

Key words: aryl hydrocarbon receptor, proliferation, migration, cell migration

Abbreviations: AhR, aryl hydrocarbon (dioxin) receptor, ARNT, aryl hydrocarbon nuclear translocator; TGF β , transforming growth factor β ,

1. Introduction.

The AhR is a ligand-activated transcription factor known to mediate most of the toxic and carcinogenic effects of a wide variety of environmental contaminants such as dioxin (TCDD; 2,3,7,8-tetrachlorodibenzo-[p]-dioxin). This receptor belongs to the basic-helix-loop-helix (bHLH)/PAS (Period [Per]-Aryl hydrocarbon receptor nuclear translocator [Arnt]-Single minded [Sim]) family of heterodimeric transcriptional regulators. bHLH/PAS proteins are involved in the control of diverse physiological processes such as circadian rhythms, organ development, neurogenesis, metabolism and in the stress response to hypoxia (reviewed in [1-3]). The large degree of conservation of this receptor among species [4], its constitutive pattern of expression during development and in adult tissues [5] and the phenotypic alterations found in mice lacking AhR expression [6-8], have provided strong support for the involvement of the AhR in cell physiology independently of xenobiotic metabolism. AhR activation is followed by changes in its compartmentalization within the cell. Whereas a large fraction of the unliganded AhR resides in the cytosolic compartment bound to a molecular chaperone complex (Hsp90/XAP2/p23), upon ligand binding, the receptor translocates to the nucleus and heterodimerizes with ARNT. This heterodimer now binds to a partially characterized set of co-activators and/or co-repressors and the resulting complex interacts with consensus regulatory sequences (xenobiotic response elements, XREs) located upstream in the promoter of target genes (e.g. cytochromes P450 such as CYP1A1) [3]. Once transcriptional regulation has occurred, the AhR is exported to the cytosol and degraded by the proteasome [9-11] (Figure 1).

Although the molecular events leading to AhR activation in presence of xenobiotics are generally well understood, AhR signaling pathways in absence of

exogenous ligands remain largely unknown. Nevertheless, increasing experimental evidence suggests physiological roles for the AhR in cell proliferation and differentiation, in liver and immune system homeostasis and in tumor development. Molecular mechanisms for AhR activation in absence of xenobiotics remain elusive in part because no clear endogenous ligands have been identified. Yet, novel means of receptor activation could link the AhR to endogenous functions. Thus, this receptor can be activated by natural compounds such as indirubin and indigo [12], an AhR repressor regulates AhR activity by binding and sequestering ARNT [13], proteasome inhibition transcriptionally activates the AhR in mouse embryo primary fibroblasts in the absence of xenobiotics [11,14] and transient over-expression of AhR and ARNT in CV-1 cells promotes transcriptional activation of CYP1A1 in absence of exogenous ligand [15]. Recently, several studies have shown that shear stress can activate the AhR in different models resulting in target genes induction (McMillan BJ, Bradfield CA, PNAS 2007; Mufti BBRC 1995). This effect could be mediated by the release of an endogenous ligand. Moreover, characterization of different AhRs in invertebrates has provided evidence for the involvement of the protein in developmental processes and for its inability to bind xenobiotics in these species (Puga Biochem Pharma 2005 Review; Crews ST Genes Dev 2006 Review); this suggest that the xenobiotic-detoxifying function of the AhR is a late-acquired property and might have masked other “ancient” physiological functions.

In this short review we will try to outline how the AhR can promote or inhibit cell proliferation, provide clues about physiological processes that seem to require AhR activity and suggest a role for this receptor in cell migration and human cancer. Some of these issues have been the focus of excellent reviews discussing diversity and evolution of the AhR [4], cell cycle regulation and tumorigenesis [16,17], AhR cross-talk to

developmental signaling pathways [18] and dioxin-mediated toxicity as compared to AhR-mediated physiological functions [19].

2. Endogenous role of the AhR in cell proliferation.

Although most of the studies about AhR activation have been done in presence of exogenous ligands, an increasing body of evidence strongly suggest that this receptor can modulate cell proliferation in absence of xenobiotics, a conclusion that supports its role in normal cell physiology.

2.1. Activation in the absence of xenobiotics.

Early reports in cultured HeLa cells showed that a significant fraction of the AhR protein could be detected in the cell nucleus, in absence of exogenous ligand, forming an active transcriptional complex with ARNT [20]. Xenobiotic-independent AhR activation was also observed in AhR-deficient kidney CV-1 cells, in which transient transfection of AhR and ARNT induced nuclear translocation and reporter gene expression in absence of exogenous ligand [15]. Experiments using the nuclear export inhibitor leptomycin B have shown that the AhR shuttles between cytosol and nucleus under normal cellular conditions [21] and, in the immune system, activation of B lymphocytes with CD40 activated the AhR and induced CYP1A1 in absence of exogenous ligands [22]. Thus, these and other reports showed AhR activation in cultured cells by mechanisms not requiring the presence of exogenous ligands. Nevertheless, the question remained of whether, *in vivo*, changes in AhR levels would affect cell and tissue function.

2.2. Physiological clues about the endogenous roles of the AhR.

Homologous recombination in embryonic stem (ES) cells represents a major tool to decipher gene function *in vivo*. The production of mouse lines lacking AhR expression by gene targeting (gene knock-out) [6-8] has provided first-hand information about physiological processes requiring AhR activity. It was not only demonstrated that this

receptor is essential for dioxin-induced toxicity [23] and carcinogenesis [24], as presumable expected, but also the existence of an AhR-deficient phenotype. In addition to cardiac hypertrophy [25] and immune system and skin alterations [8,26], the AhR has a physiological role in ovary development that affects reproduction. AhR-null females showed difficulties in maintaining pregnancy and their pups had poor numbers of survival during lactation and weaning (independently of sex) [27]. Further studies revealed that this reproductive defect could be associated with alterations in the formation and number of primordial follicles in the mouse ovary [28].

A well conserved alteration in all AhR-null mouse models, significantly contributing to demonstrate the endogenous role of this receptor, is the hepatic phenotype. The livers of AhR mice are smaller in size and show portal fibrosis and early lipid accumulation [6,8]. Vascular defects are also present that include increased numbers of arteries and arterioles [26] and portosystemic shunting in adult animals [29]. Gene expression arrays have been used to compare the liver mRNA profile between wild type and AhR-null mice. In agreement with its endogenous role in hepatic homeostasis, absence of AhR alone resulted in changes in the expression pattern of 392 genes [30]. One of these genes, which appears as a potential candidate to help explain the hepatic phenotype, is transforming growth factor β (TGF β). Livers of AhR-null mice have increased levels of TGF β in the portal areas [31] that could contribute to the development of fibrosis and to a lower proliferation rate due to the profibrogenic and antiproliferative activities of this cytokine. Further, additional studies have shown that the increased levels of TGF β could, in fact, be related to retinoic acid accumulation and reduced retinoic acid metabolism resulting from downregulation of CYP2C39, a major cytochrome P450 able to catalyze retinoic acid 4-hydroxylase reactions in the liver [32,33]. Therefore, many different studies, in several organs and target tissues, strongly

support that the AhR is relevant to preserve normal cell physiology and gives a clue about its relevance in disease states.

2.3. The AhR as a positive regulator of cell proliferation.

One of the most intriguing and exciting aspects of AhR biology is its ability to promote or to inhibit cell proliferation depending on the cell phenotype. Reports have been published clearly showing that the properties of a particular cell profoundly affects whether it will grow or not upon AhR activation or over-expression. This has important implications if the AhR is to be considered a molecular marker in such human diseases as cancer.

The AhR cooperates with signaling molecules involved in survival pathways that promote cell proliferation. One such molecule is NF- κ B with which the AhR physically interacts through the p65/RelA subunit in human breast cancer MCF-7 cells [34]. The interaction between AhR and p65/RelA resulted in transactivation of the *c-myc* proto-oncogene, thus offering a mechanism whereby this receptor could contribute to increased proliferation and carcinogenesis in the breast. Cellular AhR protein content is also relevant to induce proliferation since its over-expression in human lung carcinoma A549 cells increased E2F/DP2 activation, proliferating cellular nuclear antigen (PCNA) levels and proliferation rates [35]. Interestingly, mouse models over-expressing a constitutively active form of the AhR also demonstrated the role of this receptor as proliferation promoter. Mice expressing a constitutively active AhR showed increased frequency of hepatocarcinomas (induced by N-nitrosodiethylamine) than wild type animals [36]. Moreover, this mouse model developed spontaneous tumors in the glandular stomach in the absence of xenobiotic treatment [37]. Thus, in defined cell types, AhR over-expression and/or activation stimulates proliferation and even carcinogenesis *in vivo* in

the absence of xenobiotics. From these reports, it can be proposed that this receptor has oncogenic activity.

2.4. The AhR as a negative regulator of cell proliferation.

There are also different reports demonstrating that the AhR has antiproliferative activity. An initial study showed that upon activation by exogenous ligand, the AhR induced transcriptional activation of the tumor suppressor p27^{Kip1} in the absence of cell proliferation in 5L hepatoma cells, and that p27^{Kip1}-induction by dioxin in fetal thymus occurred along with inhibition of cell proliferation [38]. A set of studies has later proposed that the antiproliferative activity of the AhR takes place, at least in part, through its physical interaction with the retinoblastoma (pRb) tumor suppressor protein, a process resulting in pRb-mediated inhibition of E2F-dependent transcription and cell cycle arrest. The AhR synergizes and interacts with pRb in presence of exogenous ligand under basal protein levels in MCF-7 human breast cancer cells and after transient transfection in SAOS-2 human osteosarcoma cells [39]. In addition, the cell cycle blockade at G₁ induced by dioxin in MCF-7 and mouse hepatoma Hepa-1 cells was dependent on the interaction of activated AhR with the p300 co-activator, which induced p300 displacement from E2F-dependent promoters and proliferation arrest [40].

Constitutive AhR over-expression has been also used to show an inhibitory role of this receptor in cell proliferation. Constitutively active AhR arrested cell cycle at G₁ and induced apoptosis in Jurkat T cells, in part by regulating the expression of proliferation- and apoptosis-related genes through XRE-dependent mechanisms [41]. In the immune system, transgenic mice containing a constitutively active AhR targeted to T cells by the CD2 promoter had lower thymocyte numbers and altered differentiation [42]. Interestingly, two recent reports have shown that sustained AhR activation inhibits the regenerative growth of the caudal fins in zebrafish [43] and liver regeneration after

2/3 partial hepatectomy in mice, probably by a mechanism that decreased cyclin-dependent kinase 2 (CDK-2) activity but did not affect p27^{Kip1} or p21^{Cip1} tumor suppressors [44]. Collectively, these studies argue for a tumor suppressor activity of the AhR in the control of cell proliferation.

This dual effect of the AhR on cell proliferation became also evident from a report in which receptor levels were downregulated by transient transfection of specific small interfering RNAs (siRNAs) [45]. In MCF-7 human breast cancer cells, AhR siRNAs promoted the G₁/S transition of the cell cycle and cell proliferation, suggesting a growth inhibitory role of the receptor. In contrast, in HepG2 human hepatoma cells, AhR siRNAs blocked the G₁/S transition of the cell cycle and downregulated cyclin D1, cyclin E and CDK-2/4, thus revealing a growth promoting activity of the receptor.

Therefore, it appears that cell phenotype is a critical parameter in determining whether the AhR will promote (oncogenic) or inhibit (tumor suppressor) cell growth and proliferation. Further studies are needed to clarify not only the specific signaling pathways involved but also the intermediate proteins interacting with the AhR.

3. Novel cellular functions of the AhR

3.1 Developmental functions in invertebrates

The AhR has been identified in several invertebrates such as the fly *Drosophila melanogaster* and the worm *Caenorhabditis elegans*. Spineless (ss) is a bHLH-PAS *Drosophila* protein and is considered to be an AhR ortholog. It plays a central role in defining the distal regions of both antenna and leg. Loss-of-function alleles of ss cause several developmental defects: transformation of distal antenna into leg, deletion of distal leg (tarsal) structures, and reduction in size of most bristles (Duncan DM Genes Dev 1998). The characterization of the target genes and then, of the gene network involved in those process, begins to be deciphered (Emerald BS, Development 2003;

Suzanne M., Development 2003; Adachi-Yamada T, Mol.Cell.Biol 2005; Kozu S, Dev. Biol 2006; Emmons RB, Dev. Biol 2006). Like AhR, Spineless functions as a heterodimer with the Drosophila ortholog of Arnt, Tango. Spineless-Tango heterodimers are very similar to Ahr-Arnt heterodimers in DNA-binding specificity and transcriptional activation ability. However, the interaction of Spineless with Tango does not seem to require the presence of classical “mammalian AhR ligands” (Emmons RB, Development 1999). Recent reports have also suggested that ss is also important in neuronal development, especially diversification of dendritic morphology of some specialized sensory neurons and as a major determinant of the Drosophila retinal mosaic eye (Wernet MF, Nature, 2006).

The importance of Spineless in fly neuron functions is interesting in regard of several recent publications on the AhR ortholog in another invertebrate, the nematode *Caenorhabditis elegans*; the corresponding proteins, AHR-1 and AHA-1 (orthologs of ARNT) also share biochemical properties with their mammalian cognates. However, like Spineless, AHR-1 does not appear to be activated by dioxin or beta-naphthoflavone (Powell-Coffman JA Development 2004). The expression pattern of AHR-1 is particularly interesting since it is expressed in GABAergic motor neurons (specifically named RMEL and RMER) and regulates their fate specification (Huang X, Development 2004). Moreover, another study showed that in *ahr-1*-deficient animals, the touch receptor neuron AVM, as well as other neuronal subtypes exhibit cell and axonal migration defects. The authors show the involvement of TGFbeta (UNC-129) in this process, a particularly relevant observation since TGFbeta and AhR signaling pathways crosstalk in numerous mammalian systems (Qin H, Dev. Biology 2004). Finally, another study has shown that loss-of-function mutations in *ahr-1* or *aha-1* suppress neuropeptide receptor-1 (*npr-1*)-related aggregation behavior and regulates cell-type-specific

expression of soluble guanylate cyclase genes that have key roles in aggregation behavior and hyperoxia avoidance (Qin H, Dev. Biology 2006).

All these results show that AhR has important physiological roles in invertebrates and regulates developmental processes. Remarkably, a common feature of these models is AhR implication in neuron differentiation and function. This particular aspect of the AhR function has not been extensively studied in mammalian models, probably because of the focus on its xenobiotic-detoxifying functions. Further studies are required since epidemiological observations have suggested that dioxin exposure was associated with neurodevelopmental, cognitive and behavioral defects (Mendola P, MRDDRR, 2002).

3.2 Functions related to cell adhesion and migration.

The contribution of the AhR to adhesion processes involving cell-cell and cell-substratum interactions is only beginning to emerge, and several laboratories are currently trying to determine at which level the AhR interacts with those signaling pathways. Since cell-cell and cell-substratum interactions have a profound impact in cell migration, and cell migration is directly related to the metastatic potential of tumor cells, these considerations make this issue worth of investigation.

Early work showed that when adherent normal human keratinocytes were induced to go into suspension in the absence of xenobiotics, the AhR became activated and CYP1A1 induced, suggesting that physiological control of cell adhesion in stratified squamous epithelia could, under certain conditions, activate this receptor [46]. Further, suspension of adherent mouse hepatoma Hepa-1c1 cells also induced nuclear translocation and transcriptional activation of the AhR in the absence of xenobiotics [47], again suggesting that this receptor could be activated during changes in the adhesion status of the cell.

Additional studies also point to the hypothesis that preventing cell-cell contacts activate the AhR. Suspension of C3H10T1/2 fibroblasts transfected with a reporter construct stimulated AhR activation and induced reporter expression to levels similar to those obtained by exogenous ligand [48]. However, an interesting difference between both mechanisms is that suspension-induced was more transient than xenobiotic-induced activation. The use of pharmacological inhibitors suggested that this transient effect could be due to differences in transcriptional activation rather than in nuclear translocation and/or DNA binding efficiency of the receptor. Further experiments performed at low cell density revealed that the absence of cell-cell contacts had a prominent role over the lack of cell-substratum interactions to induce AhR activation, suggesting that cell density could regulate AhR distribution within the cell, with the transcriptionally inactive receptor located in the cytosol at confluence and the transcriptionally active receptor present in the nucleus at sparse cell densities [48]. In agreement with these observations, the AhR was found to be mainly nuclear in low density cultures of the keratinocyte cell line HaCaT, both nuclear and cytoplasmic at subconfluence and predominantly cytosolic at confluence [49]. By introducing mutations within the region of nuclear localization of the AhR, this differential distribution was attributed to the different modulation of the nuclear export activity of the cell [49].

The correlation between AhR activity and cell migration is still diffuse at the present time. As mentioned above, work done analyzing the *Caenorhabditis elegans* AhR receptor (*ahr-1*) has shown that *ahr1*-null animals have defects in neuronal differentiation that are due, among other causes, to aberrant axonal migration [50]. The regulator of the epithelial-mesenchymal transition (EMT) Slug has been recently reported to be induced by the AhR in the keratinocyte cell line HaCaT [51]. From this study, it was found that transcriptional activation of Slug was coincident with nuclear

translocation of the AhR and that Slug and the AhR co-localized in the nucleus of cells located at the margin of the wound-healing model in culture. These results not only confirm AhR activation when cell-cell contacts are disrupted, but also open the possibility that the AhR could interact with specific cell adhesion molecules.

We have recently shown that immortalized mouse mammary fibroblasts from AhR-null mice had decreased migration in culture and that this phenotype was associated to a lower efficiency to induce lamellipodia [52]. Signaling pathways that regulate cell migration were also inhibited in AhR-null cells, which exhibited lower activation of focal adhesion kinase (FAK), protein kinase B/AKT (PKB/AKT), mitogen activated kinase ERK1 and Rac-1. Further, AhR-null immortalized fibroblasts, as opposed to wild type cells, had a much lower efficiency to induce tumors *in vivo* in immunodeficient NOD-SCID mice. From these data, we suggested that their lower migration ability compromised the potential of AhR-null immortalized fibroblasts to induce tumors *in vivo* and that, in this cell type, the AhR could have oncogenic activity.

The implication of AhR in cell plasticity and mobility is supported by studies using xenobiotic ligands of this receptor. Indeed, we have recently shown that TCDD-treated mammary carcinoma MCF-7 cells display cell scattering, increased cell surface, appearance of lamellipodia-like protusions and subsequently higher cell adherence and motility. Those morphological changes are associated with cytoskeleton reorganization, mainly vincullin and actin redistribution. These cellular effects are accompanied by the modification of the expression of several genes; notably, a decrease of E-Cadherin was observed, which is in line with the previously described cellular alterations. Concomitantly, TCDD was shown to activate Jun N-terminal Kinases (JNKs) which was found to be required for the dioxin effect on cell morphology and mobility (Transy 2006). A summary of these data is presented in Figure 2.

While those cellular effects of the AhR seem not to be related to adaptation to xenobiotic exposure, they may still be mechanistically connected to the induction of xenobiotic metabolizing enzymes. Indeed, CYP1A1 is the most potently induced gene following AhR activation. It has been shown that CYP1A1 activity is associated with the release of reactive oxygen species (ROS), which may account for the oxidative stress that is observed following exposure to AhR ligands (**Morel Y, Mol. Cell. Biol 1999; Barouki Bioch. Pharma 2001 Review; Baulig A 2003**). ROS display a wide variety of cellular effects, among which JNK activation and activation of cellular mobility. It is thus possible that some of the cellular effects of AhR activation may be related to the induction of CYP genes and to the consequent increase in ROS.

Thus, it appears plausible to hypothesize that the AhR participates in signaling pathways regulating cell-cell and cell-substratum adhesion and, by extension, that this receptor has a role in cell migration. Alteration of this signaling due to changes in AhR levels or activity could contribute to abnormal cell migration, possibly affecting the tumorigenic potential of the target cell, notably its invasive and metastatic properties.

4. Potential of the AhR in human tumor development in the absence of xenobiotics.

A major question in human cancer is the identification of molecular markers helpful not only for the early diagnosis of the disease but also to predict treatment efficacy and specificity in individual tumors and patients. Although this is a long-term goal in AhR research, some recent reports suggest that this receptor could be relevant in human cancer.

The AhR appears to have constitutive expression and activity in established cell lines from human adult T-cell leukemia (ATL) and in some primary ATL cell cultures [53], which suggest that this receptor has oncogenic activity in this tumor type. We have

recently shown that the human AhR promoter has CpG islands prone to be methylated, and that human tumor cell lines expressing AhR have unmethylated promoter whereas those cell lines not expressing this protein have a heavily methylated promoter [54]. In particular, the acute lymphoblastic leukemia (ALL) REH and the chronic myeloid leukemia (CML) K562 cell lines did not express AhR and had higher levels of AhR promoter methylation. Furthermore, the AhR was methylated in 33% of 21 human primary ALL tumors, which suggested that AhR silencing in this tumor type could be reflecting a tumor suppressor activity for this receptor. Interestingly, the frequency of AhR methylation in ALL was similar to that found for tumor suppressors p53, p73, p15 and p16 [54]. Therefore, it can be observed that depending on the phenotype of the human tumor, and as previously discussed, the AhR could correlate with increased or with decreased proliferation potential and, thus, behaves as an oncogen or as a tumor suppressor gene.

Because changes in AhR levels and activity could be relevant to human cancer, some studies have addressed whether specific AhR ligands can be useful as chemotherapeutic agents to modulate the activity of this receptor in human tumors (reviewed in [55]). In pancreatic cancer tissue samples, AhR protein was expressed at high levels and localized mainly in the cytosolic compartment of the cell. Interestingly, the proliferation and anchorage-independent growth of these cells were inhibited by several receptor agonists, suggesting that these or related compounds could be relevant in the therapy of pancreatic cancer [56].

Thus, although much more work has to be done, it appears that the AhR has a role in human cancer in a cell type-specific manner and that it could have diagnostic as well as prognostic value in the detection and treatment of this disease.

5. Concluding remarks

The AhR field is benefiting from intense investigation covering both xenobiotic-related and unrelated issues. From either perspective, new and exciting results are arising that propose this cellular receptor as a relevant target in human disease. The fact that this receptor remains active under physiological conditions is an important argument to screen for cellular processes where its activity may be required. Moreover, it does not seem to require any ligands in invertebrates and regulates important developmental process at least in two classical models. Its rather interesting behavior with regard to the control of cell proliferation makes this protein an attractive model and a potential target in human cancer. An additional aspect of AhR Biology, that it is also quite appealing, is its contribution to the regulation of cell adhesion and migration, two processes highly important in development and cancer. It is clear from the point of view of the endogenous role of the AhR that much more work is needed, probably in every available model. However, the studies performed so far are providing evidences about which cellular functions may be relevant to locate this receptor within physiological pathways controlling cell functioning in health and disease.

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Figure Legends.

Figure 1. Transcriptional regulation of target genes by the AhR. Ligand binding to the AhR in the cytosol induces the release of a molecular chaperone complex containing at least Hsp90, XAP2 and p23. This makes nuclear localization signals accessible for importins binding and nuclear translocation. Once in the nucleus, ARNT is bound to the receptor and a transcriptionally active heterodimer is formed that can bind to consensus regulatory sequences (XREs) located upstream in the promoter of target genes. After transcriptional regulation has occurred, the AhR dissociates from ARNT and from the DNA and translocates again to the cytosol through exportins binding. In the cytosol, the AhR is degraded by the proteasome.

Figure 2. The AhR interacts with signalling pathways controlling cell adhesion and migration. As a model, signalling regulating focal adhesion dynamics and lamellipodia formation is represented. From tyrosine kinase receptors (RTKs) signals are transduced to plasma membrane associated protein complexes containing, among others, focal adhesion kinase (FAK), paxillin, vinculin, p130^{CAS}. Other molecules such as phosphoinositide 3-kinase (PI3K) and small GTPases of the Rac and Rho family also associates to these protein structures. Whereas FAK is mainly involved in focal adhesion dynamics with the substratum, Rac/Rho are involved in actin cytoskeleton reorganization and lamellipodia/philopodia formation. Molecules altering these pathways are expected to change the adhesion/migration pattern of the cell. The AhR could act at different levels in these pathways and some possibilities (not excluding many others) are indicated by the red arrows and the question marks.

Figure 1

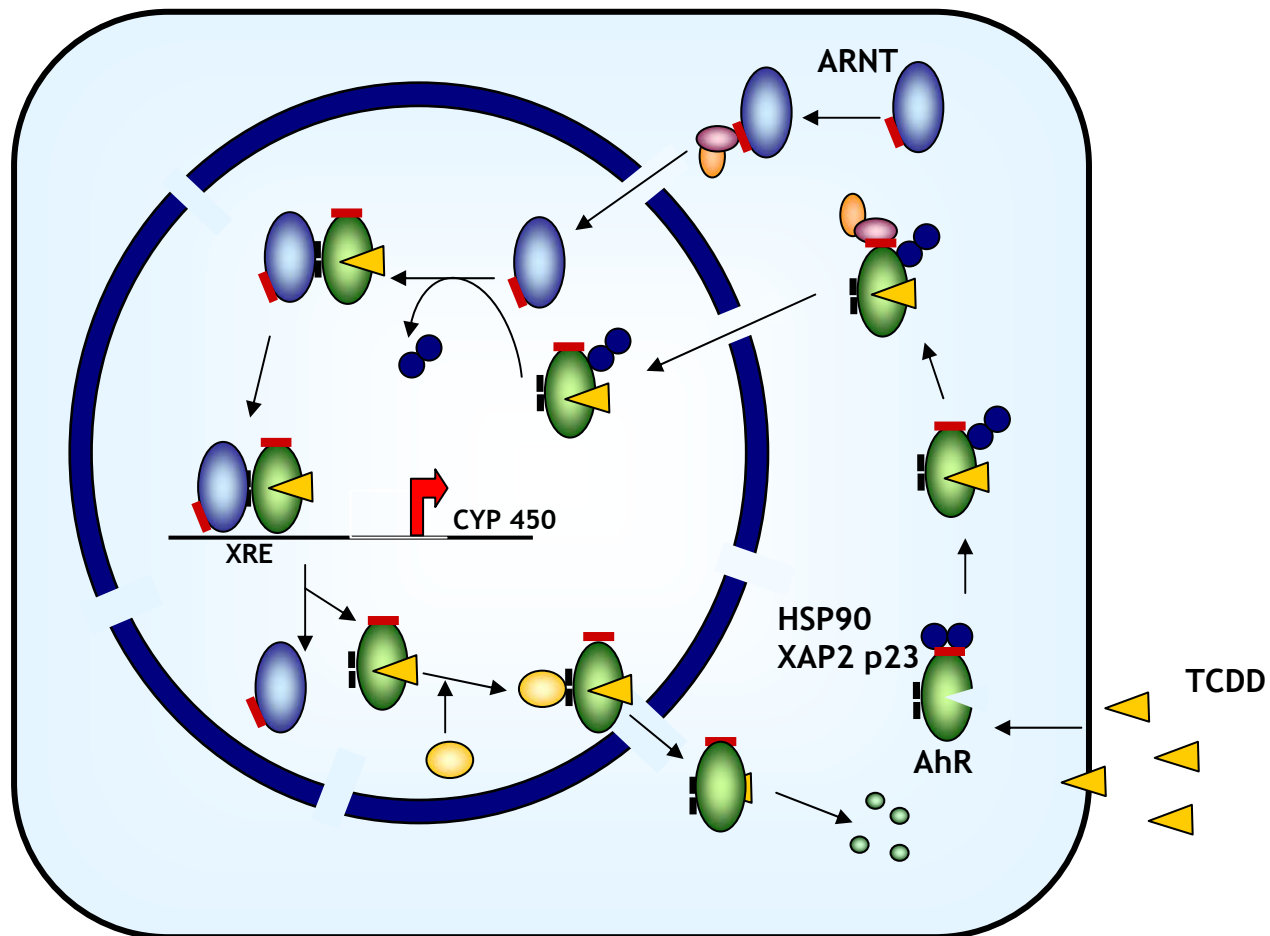
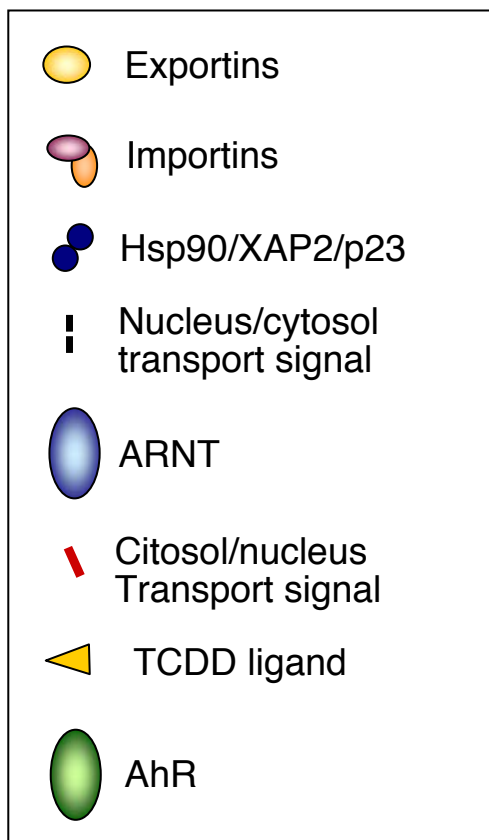


Figure 2

