# Arylhydrocarbon receptor assembles a ubiquitin ligase to target sex steroid receptors for destruction

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## Abstract

Arylhydrocarbon receptor (AhR) is a ligand-dependent transcription factor that mediates most of toxic effects of dioxins and related aromatic hydrocarbons. The ubiquitin-proteasome system mediates target protein-selective degradation, in which the ubiquitin ligase determines target-selectivity. Here we show that AhR is a ligand-dependent ubiquitin ligase, regulating degradation of sex steroid receptors. AhR is co-purified with cullin 4B (CUL4B)-based modular ubiquitin ligase complex through direct binding to CUL4B. The AhR-CUL4B complex promotes ubiquitination of estrogen receptor α (ERα) and androgen receptor (AR) and subsequent degradation of these receptors. Our findings define a novel role for AhR as an atypical component of a ubiquitin ligase complex, and propose that target-specific regulation in protein destruction, as well as gene expression, is modulated by environmental toxins. Moreover, constitutive-active AhR, which lacks ligand-binding domain and is active in the absence of ligands, directly modulates function and stability of ERα in the absence of AhR-ligands, indicating direct regulation of ERα by AhR.

## Introduction

Arylhydrocarbon receptor (AhR) is a member of bHLH/PAS domain family transcription factors that mediates toxic effects of dioxins such as tetrachloro-dibenzo-*p*-dioxin (TCDD)[1,2,8], including disruption of estrogen and androgen signaling. AhR translocates into nucleus upon binding of dioxins-type ligands, and dimerizes with AhR nuclear translocator (Arnt), then recognizing xenobiotics responsive element (XRE) and recruiting co-activators such as p300/CBP and DRIP/TRAP/mediator to activate transcription[1].

AhR/Arnt is also known to modulate function of other transcription factors[2]. It has been reported that ligand-activated AhR/Arnt associates with estrogen receptor alpha and beta (ERα and ERβ)[3-6], which are members of nuclear receptor superfamily transcription factors. By means of this association, liganded AhR potentiates transactivation function of 17β-estradiol ($E_2$)-unbound ERα, and represses $E_2$-bound ERα–mediated transcription through estrogen-responsive element (ERE)[3]. Reciprocally, $E_2$-bound ERα has also been

reported to be recruited to XRE-bound AhR to either potentiate or repress AhR-mediated transcription[5, 6].

Studying the inhibitory cross-talk of AhR-ERα, here we show that liganded AhR has a ubiquitin ligase activity and promotes ubiquitination and degradation of ERα and AR by assembling a ubiquitin ligase complex, $CUL4B^{AhR}$ [7].

## Methods

*Biochemical purification and separation of AhR-associated complexes*

Stable-transformed HeLa cells were incubated for one hour with either 3MC (1 μM) or α-naphthoflavone (1 μM), and the nuclear extracts were prepared[7]. The extracts were loaded onto M2 anti-FLAG agarose gel (Sigma), the bound proteins were eluted by incubation with Flag peptide [7].

*Cell culture and transfection*

Cells were cultured in phenol-red-free DMEM containing 0.2% charcoal-stripped FBS, transfected with the receptor expression vectors and the luciferase reporter plamids[3,7], and were treated with $E_2$ (10 nM), DHT (10 nM), tamoxifen (100 nM), 3MC (1 μM), α-naphthoflavone (1 μM; 100 nM where indicated), β-naphthoflavone (1 μM), benzo[a]pyrene (100 nM), TCDD (10 nM), indirubin (10 nM), MG132 (10 μM), and cycloheximide (5 μM) either for 24 hrs (Luciferase assays), for 6 hrs (ubiquitination assays), for 3 hrs (Western blotting), or for 90 mins (immunoprecipitation).

*Luciferase assays*

Cells at 40-50% confluence were transfected with the indicated plasmids (0.25 μg reporter plasmids, 0.025 μg ERα, 0.05 or 0.2 μg AhR, CA-AhR, Arnt,) using Lipofectamine reagent (Gibco BRL)[3,7]. Luciferase activity was determined with the luciferase assay system (Promega). As a reference plasmid to normalize transfection efficiency, 25 ng pRL-CMV plasmid (Promega) was co-transfected.

## Results

*AhR promotes proteasomal degradation of sex steroid receptors*

Exploring the functions of AhR in sex hormone signaling, we found that protein levels of endogenous ERα (in mammary tumor-derived MCF-7 cells), ERβ (in ovarian tumor-derived KGN cells), and AR (in prostate cancer-derived LNCaP cells), were drastically decreased upon activation of AhR by binding of AhR-agonists, 3MC, βNF and benzo(a)pyrene (BaP). 3MC-enhanced degradation of sex steroid receptors was clearly attenuated in the presence

of a proteasome inhibitor MG132, and 3MC-enhanced poly-ubiquitination of ERα was consistently observed.

*AhR assembles a ligand-dependent ubiquitin ligase complex*

Next, AhR-containing complexes from the HeLa cells stably expressing FLAG-AhR in the presence of 3MC or an AhR-antagonist α-naphthoflavone (αNF) were purified. By a mass-spectrometric analysis, one of the isolated complexes was shown to be composed of the ubiquitin ligase core components, cullin 4B (CUL4B), damaged-DNA binding protein 1 (DDB1), Roc1(Rbx1), and related components. This suggests that AhR is a ligand-dependent component to form a CUL4B complex.

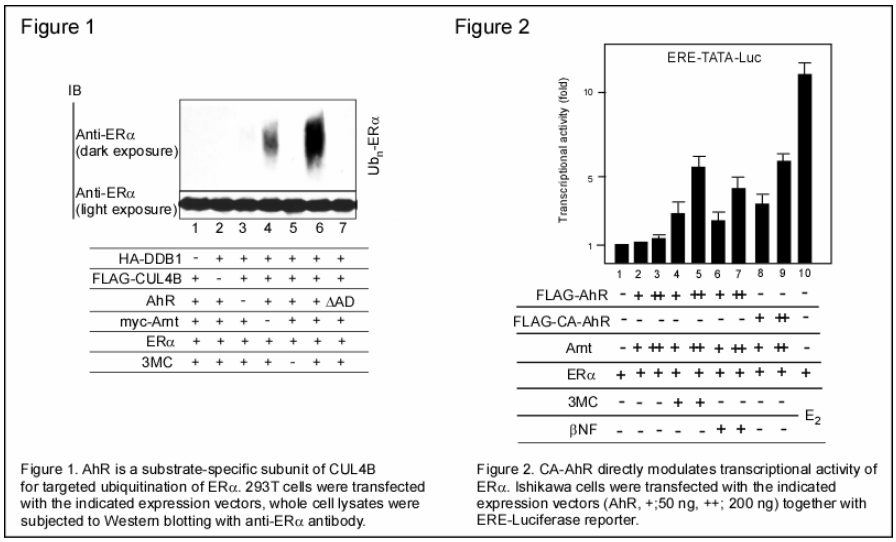


Figure 1. AhR is a substrate-specific subunit of CUL4B for targeted ubiquitination of ERα. 293T cells were transfected with the indicated expression vectors, whole cell lysates were subjected to Western blotting with anti-ERα antibody.

Figure 2. CA-AhR directly modulates transcriptional activity of ERα. Ishikawa cells were transfected with the indicated expression vectors (AhR, +;50 ng, ++; 200 ng) together with ERE-Luciferase reporter.

*AhR is an adapter component of ubiquitin ligase complex*

ERα co-immunoprecipitated with DDB1 only when AhR/Arnt was bound to DDB1 in the presence of CUL4B and 3MC. In contrast, CUL4B appeared to stably associate with DDB1. Strikingly, in the same cell lysates, co-expression of the full components in the presence of 3MC significantly promoted ubiquitination of ERα (Fig. 1). As expected from the *in vitro* observations, the AhR ΔAD mutants that lack interactions with CUL4B, failed to associate with DDB1 or induce ERα ubiquitination (Fig. 1).

*Constitutive-active AhR modulates function and stability of unliganded ERα*

To obtain the pure 'active-state' of AhR, we used a constitutive-active AhR mutant (CA-AhR) which lacks ligand-binding PAS-B domain[8]. As this mutant is transcriptionally active in the absence of ligands, we thought that this mutant is appropriate for studying intrinsic function of AhR, irrespective of ligand-type-specific differences.

A reporter plasmid containing estrogen-responsive element (ERE) was co-transfected with

different amounts of either wild-type AhR or CA-AhR into Ishikawa cells. CA-AhR activated ERE-Luciferase activity in a dosage-dependent manner in the absence of AhR-ligands (Figure 2). The activation function of CA-AhR for ERα-mediated transcription appears comparable with that of ligand-bound wild-type AhR (Figure 2). We have obtained similar results on CA-AhR modulation of ERα function in MCF-7 cells (data not shown). Consistently, the transcriptional activity of an ERα mutant [ERα ΔLBD] that lacks C-terminal ligand-binding domains (E/F regions)[3] was likewise activated by CA-AhR in the absence of ligands.

Moreover, co-transfection of CA-AhR with FLAG-ERα promotes proteasomal degradation of FLAG-ERα in MCF-7 cells in the absence of AhR-ligands.

## Discussion

Although it is well established that AhR is the key factor in mediating adverse effects of dioxin-type compounds[1], the underlying mechanisms still remain elusive. The present study proposes that adverse effects of AhR-ligands in sex hormone signaling are, at least in part, attributed to enhanced degradation of sex steroid receptors by $CUL4B^{AhR}$. Based on distinct domain requirements of AhR for its transcription and ubiquitination functions, it is possible to speculate that these two functions of AhR are independent and are responsible for a distinct set of biological events caused by AhR ligands. As substrate-specific adapters of ubiquitin ligase complexes are known to generally recognize a number of proteins, identification of other $CUL4B^{AhR}$ substrate proteins would reveal molecular links of AhR-mediated signaling with other signaling pathways and cellular events. This would lead to a greater understanding of the wide variety of biological actions induced by endogenous and exogenous AhR ligands.

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