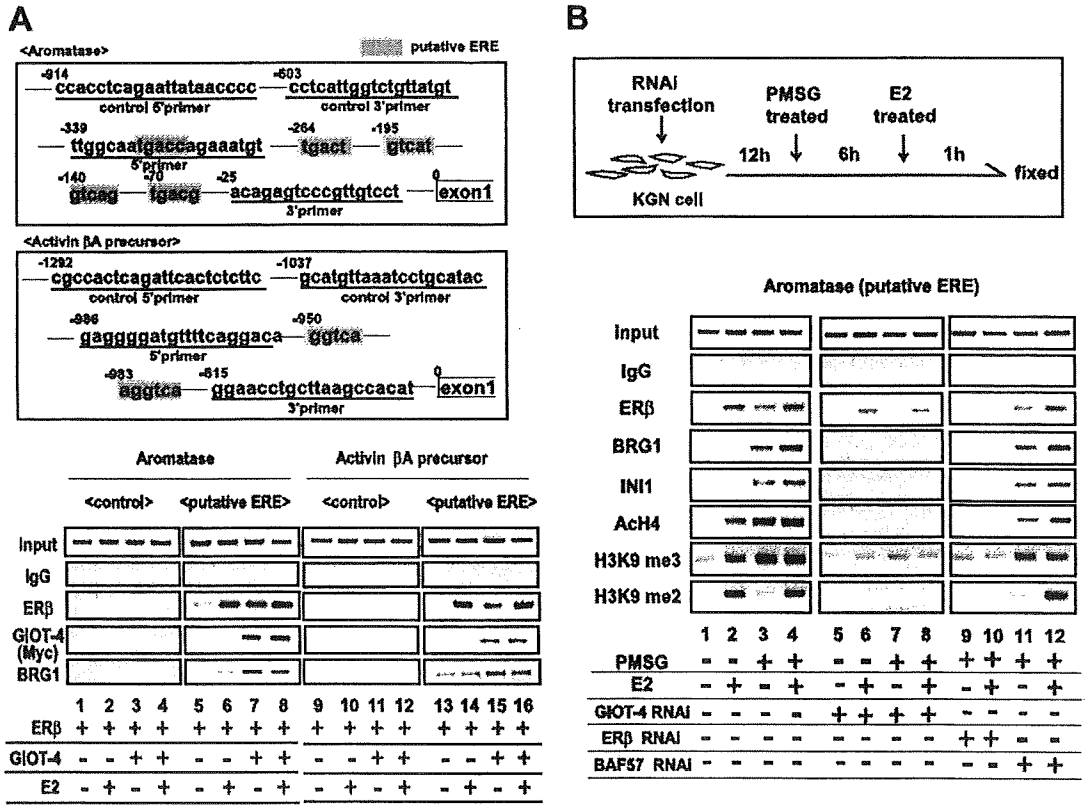

FIG. 6. GIOT-4 and human SWI/SNF complex components were recruited to consensus ERE-like elements in ERβ target gene promoters in a PMSG-dependent manner. (A, upper panels) Schematic diagrams of the aromatase promoter and the activin βA precursor promoter with putative EREs. (Lower panels) ERβ and GIOT-4 were overexpressed in KGN cells. Cells were then treated with E2 for 1 h and subjected to a ChIP assay. ERβ, GIOT-4, and BRG1 were recruited to promoters of the indicated genes. IgG, immunoglobulin G; +, present; −, absent. (B, upper panel) Protocol for PMSG treatment and transfection of KGN cells with siRNA. (Lower panels) ChIP assays were performed with or without siRNA against GIOT-4, ERβ, or BAF57. In the presence of PMSG, ERβ and components of the SWI/SNF complex were recruited to the aromatase promoter. The recruitment of SWI/SNF complex components and subsequent histone modifications, including the hyperacetylation of H4, were inhibited by the knockdown of GIOT-4 or ERβ but not by BAF57 knockdown. AcH4, acetylated H4.

ondary, and antral follicles after PMSG treatment was observed (Fig. 7B). Consistently, the expression levels of ERβ target genes were altered during ovulation (Fig. 7C). Moreover, by a ChIP analysis, PMSG-induced recruitment of these factors (GIOT-4, Brg1, and ERβ) to the ERβ target gene promoters was observed (Fig. 7D). These results suggest that the FSH-induced expression of GIOT-4 may potentiate ERβ function during an ovulatory cycle. This effect seems to result from the modulation of the chromatin structure by a recruited SWI/SNF-type chromatin-remodeling complex (Fig. 8).

DISCUSSION

Stimulation of ERβ transcriptional activity by FSH. ER KO mice exhibit different phenotypic abnormalities in the female reproductive organs depending on which ER subtype is deleted (23, 32, 34, 41). For example, the ovarian function of ERβ, but not ERα, appears to be controlled by gonadotropins (9). Distinct roles of LH and FSH in modifying ERβ function during follicle differentiation in granulosa cells have been proposed previously (9, 24, 41). The molecular mechanism behind the possible cross talk, however, has not yet been described. In the present study, the LH analogue (hCG) was not effective in coregulating the transcriptional activity of ERβ but FSH potently stimulated ERβ in KGN cells. Thus, the stimulation of ERβ activity by FSH may account for the FSH-specific biological actions at specific ovulatory stages in the granulosa cells of the ovary.

We used the promoters of two ERβ target genes, those for aromatase and the activin βA precursor, to model the potentiation of ERβ transcriptional activity by FSH (1, 49, 51, 58). These genes are responsible for regulating estrogen production by the granulosa cells via regulatory feedback by the pituitary. ERβ seems to participate in this regulatory axis through cross talk with the PKA-mediated gonadotropin cascade. The identification of other ERβ target genes will improve the understanding of the biological significance of this cross talk system (9).

The recruitment of an SWI/SNF-type chromatin-remodeling complex to ERβ through GIOT-4 is upregulated by gonadotropin at the gene expression level. Several ATP-dependent chromatin-remodeling complexes that coregulate the ligand-induced transactivation function of NRs exist (4, 37, 56). An SWI/SNF complex has already been reported to coactivate the function of ligand-bound ERα (3). However, a more recent

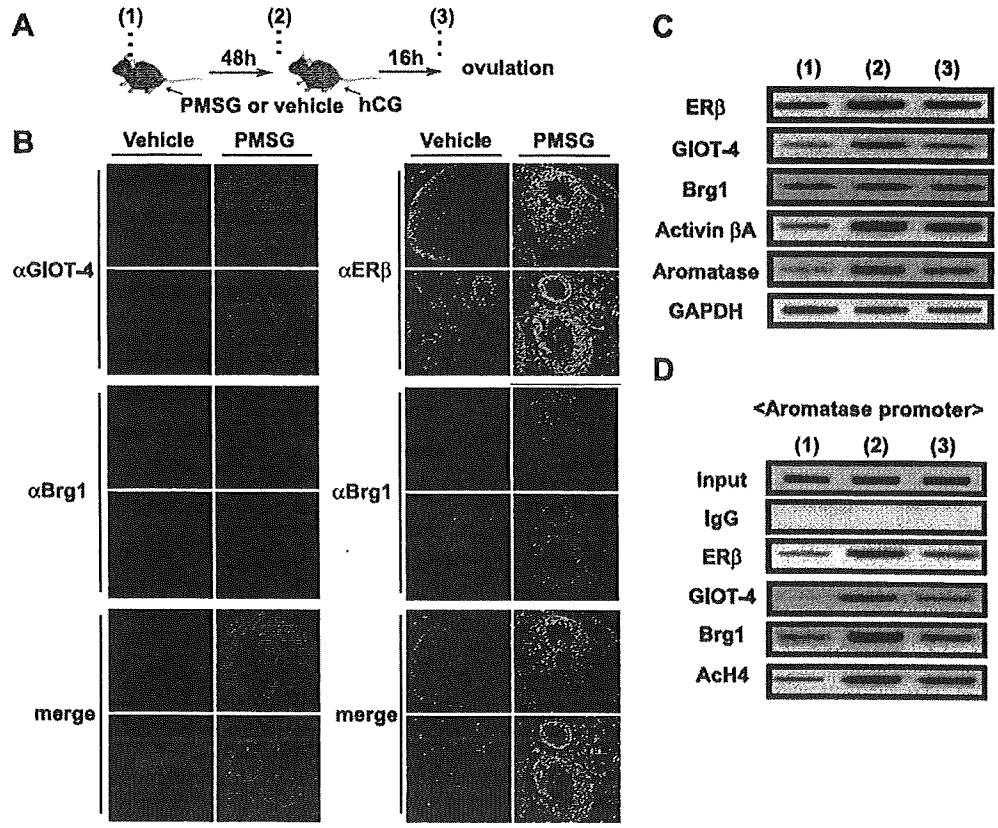

FIG. 7. GIOT-4 is expressed primarily in the growing follicles and functions with ERβ in a FSH-dependent manner in murine folliculogenesis. (A) Protocol for obtaining mouse ovary samples in the assays described herein. (1) Control sampling and pretreatment with an i.p. injection of 3.25 IU of PMSG. (2) Treatment with an i.p. injection of 3.25 IU of hCG 48 h after pretreatment. (3) Sampling 16 h after treatment. (B) Colocalization of ERβ (green) and GIOT-4 (blue) with Brg1 (red) in PMSG-treated mouse ovaries. Forty-eight hours after PMSG treatment (time point 2 as described in the legend to panel A), the expression of GIOT-4 was clearly induced in granulosa cells of various stages of follicles, including antral follicles (upper panel) and primary and secondary follicles (lower panel), but was undetectable in vehicle-treated ovaries. The colocalization of GIOT-4 and ERβ with Brg1 was detected in growing follicle granulosa cells at all developmental stages in PMSG-treated ovaries. αGIOT-4, αBrg1, and αERβ, anti-GIOT-4, anti-Brg1, and anti-ERβ antibodies. (C) Levels of expression of GIOT-4 and the ERβ target genes during the ovarian cycle. Semiquantitative RT-PCR was performed with follicles from mice at stages 1, 2, and 3, described in the legend to panel A. mRNA levels were the highest at a specific stage (preantral-antral follicle stage) during folliculogenesis and ovulation. (D) Recruitment of GIOT-4 and the SWI/SNF complex components to the aromatase promoter during an ovulatory cycle. An in vivo ChIP assay was performed as described in Materials and Methods. ERβ and BRG1 were recruited to the aromatase promoter at a specific stage (antral follicle formation) in folliculogenesis. IgG, immunoglobulin G; AcH4, acetylated H4.

report showed that ERβ does not interact with BAF57, a bridging component for ERα in the SWI/SNF complex (20). In the present study, GIOT-4 was a physical interactant with ERβ in vivo but not with ERα. Moreover, BAF57 was also dispensable for the targeting of ERβ or the subsequent histone modifications (Fig. 3A, 4C, and 6B). Thus, it is likely that the modes and/or mechanisms of recruiting ATP-dependent chromatin-remodeling complexes differ between ERα and ERβ subtypes.

In our ChIP assay, several histone modifications of adjacent chromatin areas in the ERβ target genes seem to have followed the recruitment of the SWI/SNF-type complex components (7, 37). These histone modifications occur through the anchoring of the other histone-modifying complexes, including a histone acetylase coactivator complex (19, 26, 59). In this respect, GIOT-4 may stabilize the assembly of coregulator complexes by inducing an association of an SWI/SNF-type complex with ERβ in the gene promoters. The targeting of ERβ by PMSG treatment in the absence of E2 (probably through GIOT-4 expression) may explain the E2-independent activation of ERβ transcriptional activity found in our luciferase assays (Fig. 1 and 5).

Functional regulation of GIOT family proteins as transcriptional cofactors. Certain GIOT family member proteins are induced by gonadotropins in both the male and female reproductive organs, but little is known about their functions (38, 52, 60). GIOT-1 serves as a corepressor for an orphan NR (SF-1) by recruiting histone deacetylase 2 through the GIOT-1 KRAB domain (52). Though GIOT-4 is a GIOT family protein, we have provided evidence that GIOT-4 is an ERβ coactivator in both cultured cells and the ovary. Nevertheless, we cannot exclude the possibility that GIOT-4 forms a distinct corepressor complex at another stage of folliculogenesis or in other tissues. It would be of interest to search for other transcription regulatory factors coregulated by GIOT-4. The identification

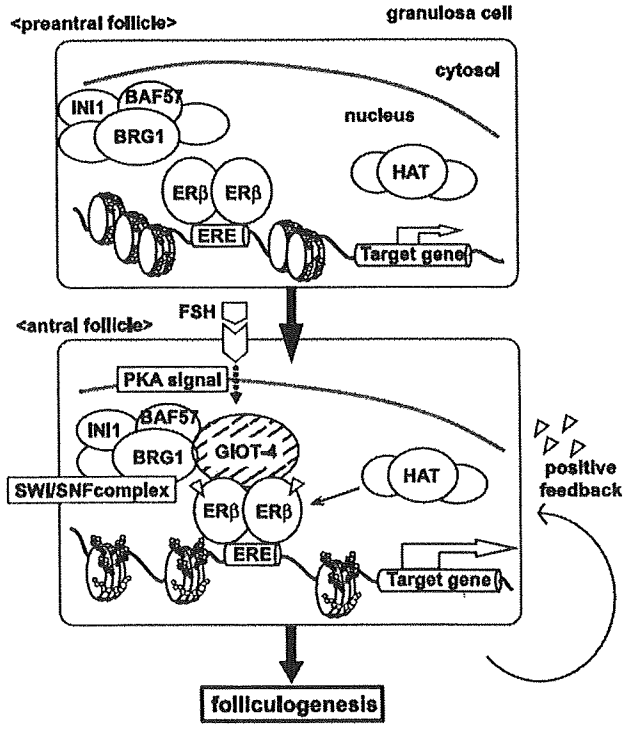

FIG. 8. Schematic representation of GIOT-4 function as a gonadotropin-induced cofactor of ERβ. At a specific antral stage during folliculogenesis, the expression of GIOT-4 in granulosa cells is induced by FSH stimulation. The association of GIOT-4 with ERβ recruits SWI/SNF-type complexes and other histone modifiers to ERβ on its target gene promoters and results in the potentiation of the genes. HAT, histone acetylase.

of other stage-specific participants in GIOT-4 complexes in folliculogenesis would improve the understanding of the molecular mechanisms of gonadotropin action at each ovulation stage (10, 25, 39).

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Review

AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions

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ABSTRACT

The arylhydrocarbon receptor (AhR) mediates the adverse effects of dioxins, including modulation of sex steroid hormone signaling. The role of AhR as a transcription factor is well described. AhR regulates the expression of target genes such as CYP1A1; however, the mechanisms of AhR function through other target-selective systems remain elusive. Accumulating evidence suggests that AhR modulates the functions of other transcription factors. The ligand-activated AhR directly associates with estrogen or androgen receptors (ERa or AR) and modulates their function both positively and negatively. This may, in part explain the sex steroid hormone-related adverse effects of dioxins. AhR has recently been shown to promote the proteolysis of ERa/AR through assembling a ubiquitin ligase complex, CUL4-B^{AhR}. In the $CUL4B^{AhR}$ complex, AhR acts as a substrate-recognition subunit to recruit ERa/AR. This action defines a novel role for AhR as a ligand-dependent E3 ubiquitin ligase. We propose that target-specific regulation of protein destruction, as well as gene expression, is modulated by environmental toxins through the E3 ubiquitin ligase activity of AhR.

Contents

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Abbreviations: AhR, arylhydrocarbon receptor; ERa, estrogen receptor; AR, androgen receptor; XRE, xenobiotic-responsive element; ERE, estrogen-responsive element; bHLH/PAS, basic helix-loop-helix/Per–Arnt–Sim; AF-1, autonomous activation function; E_2, 17b-estradiol; 3MC, 3-methylcholanthrene; bNF, b-naphthoflavone; CRL, cullin–RING ubiquitin ligase; SCF, Skp1–CUL1–F-box; CUL4B, cullin 4B; DDB1, damaged-DNA-binding protein 1.

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1. Introduction

Dioxin-type environmental contaminants, such as tetrachloro-dibenzo-*p*-dioxin (TCDD), exert toxic effects [1]. Some of these toxicities are estrogen- and androgen-related actions [2–7]. The arylhydrocarbon receptor (AhR) is a ligand-dependent transcription factor belonging to the basic helix-loop-helix/Per–Arnt–Sim (bHLH/PAS) family. AhR possesses a variety of biological and toxicological functions [8–11] (Figs. 1 and 2). AhR mediates the toxicological effects of dioxins. In addition, AhR plays a physiological role in various tissues such as the reproductive and immune systems. The transcriptional activity of AhR is regulated by direct binding of its ligands [12,13] (Figs. 1 and 2A). The unliganded AhR is sequestered in the cytosol by interacting with the Hsp90/XAP2 (also called as ARA9 or AIP) chaperon complex [8–11]. Ligand binding to the PAS-B region of AhR is thought to induce conformational changes and subsequent translocation of the AhR complex to the nucleus [8–10]. AhR then dimerizes with the AhR nuclear translocator (Arnt) in the nucleus after dissociating from the chaperon complex, recognizes the xenobiotic-responsive element (XRE), and recruits co-activators such as the histone acetyltransferase p300/CBP, chromatin remodeling factor Brg1, and the mediator (DRIP/TRAP) complex to activate transcription [8–10] (Fig. 1). The AhR/Arnt heterodimer induces the expression of target genes, such as CYP1A1, CYP1A2, and glutathione-S-transferase [1].

The actions of the direct target genes of AhR alone do not fully explain its toxicological and physiological effects. Accumulating evidence suggests that the AhR exhibits its regulatory functions by modulating the function of other transcription factors [2,11], including estrogen receptor (ERa and ERb) [14–19] and androgen receptor (AR) [18,19] (Fig. 1). These cross-talk pathways are important mediators of the functions of endogenous and exogenous AhR ligands. The liganded AhR recently has been shown to promote the ubiquitination and proteasomal degradation of ERs and AR by assembling a ubiquitin ligase complex, $CUL4B^{AhR}$ [18,19]. Thus, complexes of the AhR with ERs or AR appear to regulate transcription as functional units by multiple mechanisms. In this review, we will summarize a novel role for AhR as a component of an E3 ubiquitin ligase complex, which mediates cross-talk of AhR with sex steroid receptors through promotion of proteolysis.

2. Cross-talk of AhR with ERs or AR

2.1. *Transcriptional regulatory mechanism involving nuclear receptors*

ERs and AR belong to the nuclear receptor superfamily of transcription factors [20–22] (Fig. 2). Nuclear receptors, by acting as ligand-dependent transcription factors serve as

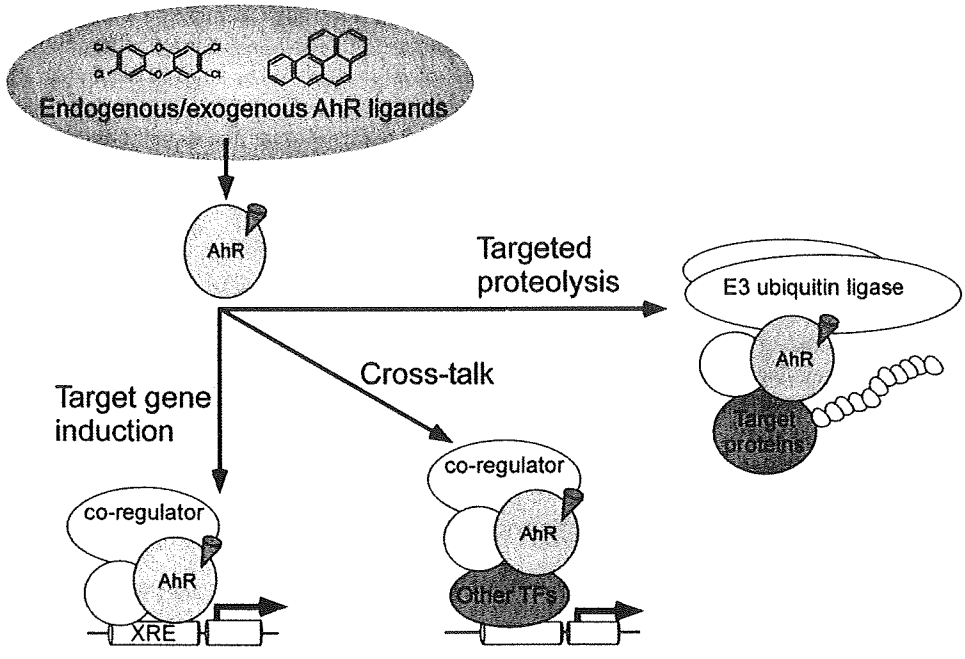

Fig. 1 – Different modes of the AhR signaling pathways. Molecular pathways for AhR-mediated biological actions. AhR may exhibit its biological actions through different modes of pathways as illustrated. Typically, AhR directly binds to its target gene promoters and induces expression of these genes. In addition, cross-talk of AhR with other transcription factors, as well as the function of AhR as an E3 ubiquitin ligase, is considered important for AhR biology. XRE, xenobiotic-response element; TF, transcription factor.

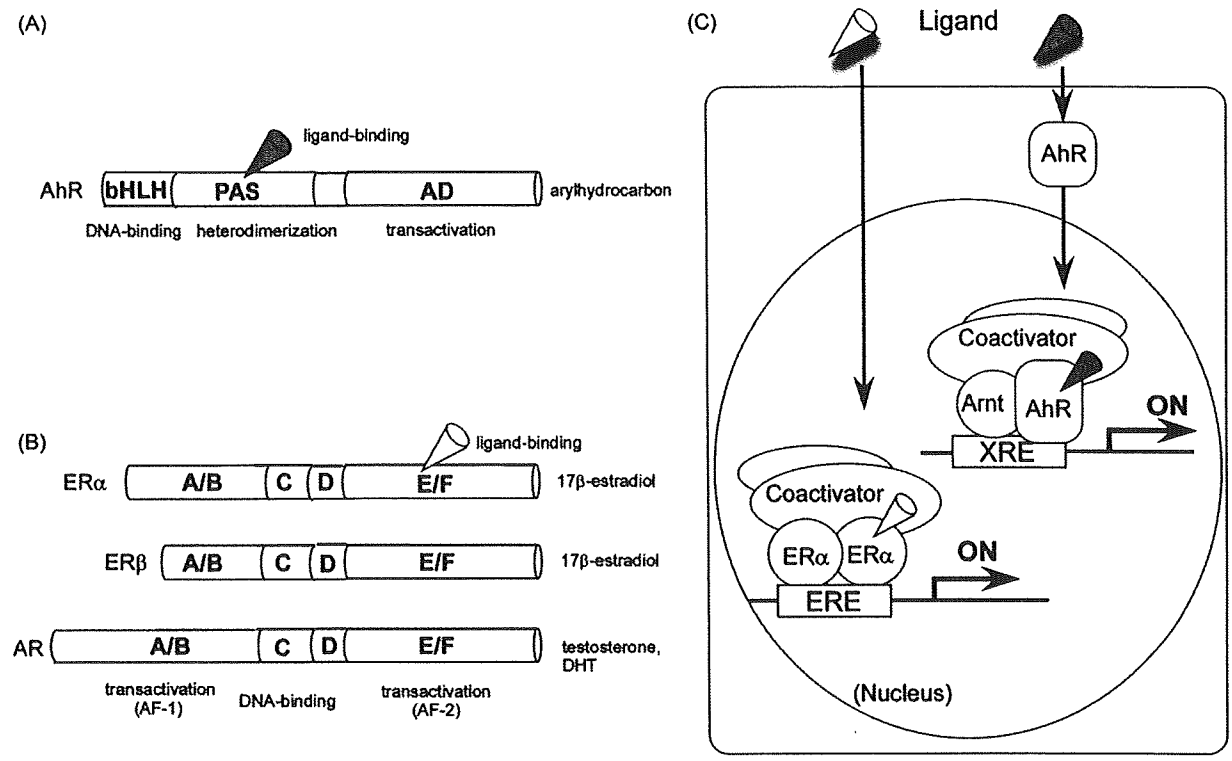

Fig. 2 – Structure and molecular mechanism of AhR and nuclear receptors. A and B domain structures of AhR (A) and nuclear receptors (B). Domain architectures and cognate ligands for these receptors are illustrated. bHLH, basic helix-loop-helix; PAS, Per–Arnt–Sim domain; AD, activation domain; AF, activation function; DHT, dihydrotestosterone. (C) Mechanisms of gene regulation mediated by AhR and nuclear receptors. ERE, estrogen-response element.

sensors for low molecular weight, fat-soluble ligands such as steroids/thyroid hormones, and vitamins A and D [20,21]. Members of the nuclear receptor gene superfamily share a common domain structure with distinct functional domains, designated A–E [21] (Fig. 2B). The ligand-binding domain is located in the C-terminal E domain. The most conserved C domain, located in the middle of the receptor, serves as the zinc finger-type DNA-binding domain. This domain specifically recognizes its cognate response elements in the target gene promoters. The N-terminal A/B domain and the C-terminal E domain are required for ligand-induced nuclear receptor transactivation functions. The autonomous activation function (AF-1) in the A/B domain is constitutively active but is presumably masked in the absence of ligand. The autonomous activation function (AF-2) in the ligand-binding E domain is, in contrast, dependent on ligand binding through the ligand-dependent conformational change of helix 12 and subsequent formation of a hydrophobic surface for the interaction with co-regulators [20] (Fig. 2).

Ligand-bound nuclear receptors recruit a number of transcriptional co-regulators and co-regulator complexes to the target gene promoters to mediate ligand-dependent transcriptional control [21,22] (Fig. 2). These complexes can be classified into three categories according to their functions. The first class of co-regulator complexes modifies histone tails covalently [23]. The amino-terminal tails of histones are subjected to various covalent modifications such as acetylation, methylation, phosphorylation, and ubiquitination by specific histone-modifying enzymes. These post-translational histone modifications are thought to serve as a 'histone code' that fine-tunes the transcriptional state through chromatin structure rearrangement [23]. The second class of complex mediates ATP-dependent dynamic remodeling of chromatin structure [22]. Chromatin remodeling complexes use ATP hydrolysis to rearrange nucleosomal arrays in a non-covalent manner. These chromatin remodeling complexes support the accessibility of co-regulator complexes and transcription factors to specific promoter regions. The last co-regulator complex class, the mediator complex, directly regulates transcriptional control by physically interacting with general transcription factors and RNA polymerase II. Recent evidence suggests that numerous co-regulators and nuclear receptors are recruited onto the promoters in an ordered manner, associating and dissociating transiently [24,25]. Nuclear receptors, as well as other transcription factors, serve as specific adaptors that connect co-regulator complexes and specific promoter regions.

The ligand-dependent nuclear receptor function is also regulated by other classes of signal transduction pathways. Such cross-talk pathways include at least two mechanisms: functional modulation through post-translational modification, and the association with other classes of transcription factors. MAPK, activated by EGF, phosphorylates ERa at serine 118 [26]. This in turn potentiates the ligand-dependent transactivation function of ERa [26] as well as its rapid turn-over. Phosphorylation-mediated functional modulation has been reported for a number of nuclear receptors to date.

Complex formation-based cross-talk mechanisms are also seen in several nuclear receptors including the glucocorticoid receptor (GR) [27]. GR ligands have an anti-inflammatory action, which is mediated through ligand-dependent repression of AP-1 activity through direct association. More recently, the exchange of different classes of co-regulator complexes has been reported to underlie the signal cross-talk pathway. Ligand-activated PPARg typically assembles co-activator complexes on its cognate promoters. In the repression of NF-kB activity, PPARg forms a complex with NF-kB, and this complex stably associates with an NCoR co-repressor complex by inhibiting the degradation of NcoR [28]. A current view of signal cross-talk at the transcription levels is that signal/ligand-dependent transcription factors associate with each other to assemble diverse types of co-regulator complexes. These exchange dynamically and regulate transcription in a manner specific for each cross-talk pathway [22].

2.2. *Molecular mechanisms of cross-talk of AhR with estrogen or androgen receptors*

Signal cross-talk pathways are important mediators of the functions of AhR ligands in various tissues. Dioxin-type environmental contaminants exert both estrogen- and androgen-related effects [1–3,5–7,29–32] (Fig. 3). Dioxins have well-described anti-estrogenic effects, such as the inhibition of estrogen-induced uterine enlargement, MCF-7 cell growth, and target gene induction [3,7]. However, there is also evidence to the contrary as dioxins have also been shown to have estrogenic effects including the stimulation of uterine enlargement [29], induction of estrogen-responsive genes such as *VEGF*, *c-fos*, and *TERT*, and a similar pattern to estrogen of transcriptional regulation in a genome-wide study [6]. In addition, AhR-deficient mice exhibit impaired ovarian follicle maturation [33]. Using AhR-deficient cells, the importance of AhR in the proliferation of mammary cells has been confirmed [34]. These findings suggest that AhR, activated by its endogenous ligand, may modulate the estrogen signaling pathway. Similarly, dioxins exert both androgenic and anti-androgenic effects on prostate development in an age-specific manner [5]. As is true for other cross-talk pathways [22], the AhR appears to modulate estrogen/androgen signaling both positively and negatively depending on cellular context.

The molecular mechanisms of AhR modulation of ERa have been extensively studied, and both direct and indirect regulatory mechanisms have been proposed. First, TCDD/AhR either increases or decreases estrogen levels through an indirect mechanism [2,35]. TCDD promotes the clearance of estrogen, thereby repressing ER transcriptional activity [35]. AhR-deficient mice have decreased estrogen production due to impaired induction of aromatase (CYP19) gene expression [33]. Another indirect mechanism involves competitive DNA binding of AhR and ER on the responsive promoters [2]. AhR and ER, each bound to its own target promoter recruits transcriptional co-regulators

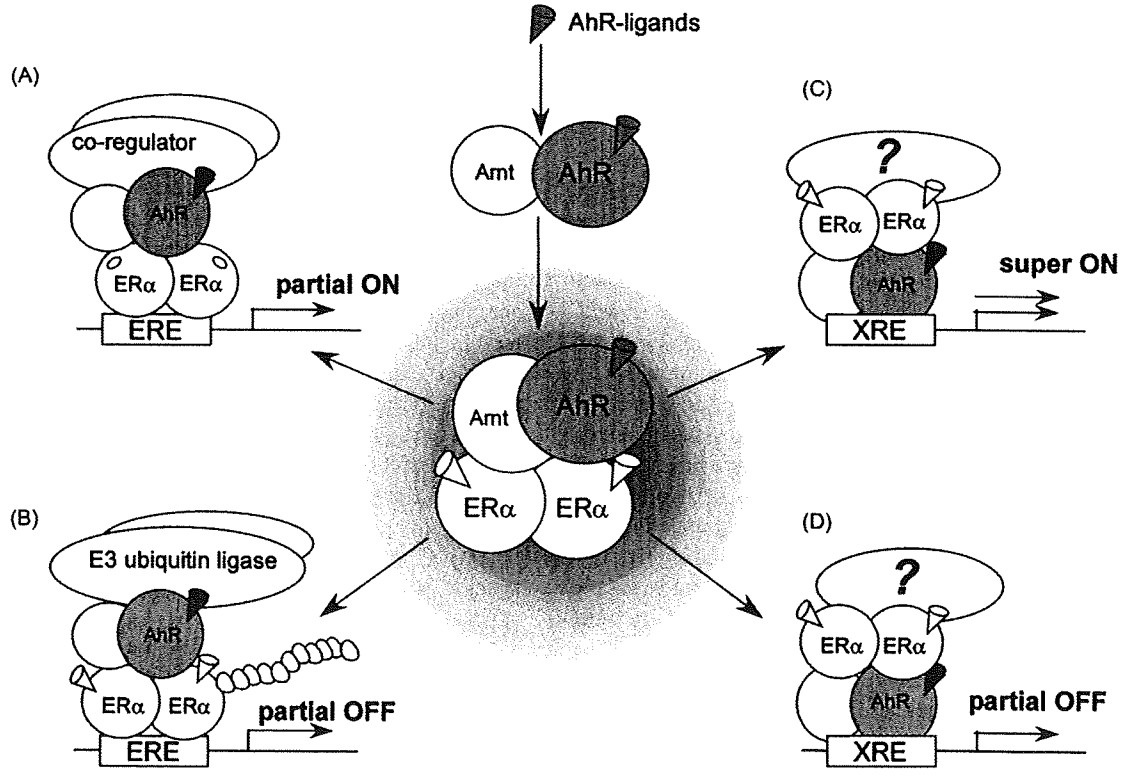

Fig. 3 – Cross-talk of AhR with ERα through direct association. Ligand-bound AhR directly associates with estrogen or androgen receptors (ERα, ERβ, or AR) in the nucleus. This association leads to different types of cross-talk between AhR and ERs/AR, as illustrated (see text for details). (A) Ligand-bound AhR associates with unliganded ERs upon ERE and recruits transcriptional co-activators. (B) Ligand-bound AhR forms E3 ubiquitin ligase complex and recognizes ERs for proteolysis. (C) Ligand-bound ERα associates with AhR and activates transcription through XRE. (D) Association of ERα with AhR results in repression through XRE.

in a competitive manner. This mechanism may be limited to specific genes and conditions since not all of the estrogen-responsive promoters contain XRE.

More recently, direct association of AhR with ERs has been independently reported. Ligand-activated AhR/Arnt associates with ERa and ERb through the N-terminal A/B region within ERs [14–18] (Fig. 3). By means of this association, the liganded AhR potentiates the transactivation function of 17b-estradiol (E_2)-unbound ERa (Fig. 3A), while it represses E_2-bound ERa-mediated transcription upon the estrogen-responsive element (ERE) [14] (Fig. 3B). The interaction of AhR/ER is induced by different AhR ligands, such as TCDD, 3-methylcholanthrene (3MC), and b-naphthoflavone (bNF). The activation of AhR is thought to be sufficient for the interaction with ERa, as a constitutively active form of AhR [12] modulates ERa function in the absence of AhR ligand [19]. These results suggest that the cross-talk of AhR with ER is initiated primarily through stimulation of AhR. Supporting this, ERa is predominantly located in the nucleus, whereas AhR translocates to the nucleus upon ligand stimulation. The association of AhR/ERa has been shown by several independent approaches, including *in vitro* [36], *in vivo*, and biochemical methods [18]. Moreover, AhR/ERa cross-talk in the transcriptional regulation of ERa-responsive genes is abolished in AhR-deficient mice [10,33], confirming the specificity of the molecular pathway *in vivo* [14]. Reciprocally, E_2-bound ERa associates with XRE-bound AhR to either potentiate [15] (Fig. 3C) or repress [16] (Fig. 3D) AhR-mediated transcription. Considered together, the AhR/ERa complex may be able to bind to either XRE or ERE through the attachment functions of AhR or ERa, respectively. Alternatively, different complex subtypes that contain AhR/ERa may control promoter selectivity (Fig. 3). Reflecting this functional cross-talk, Arnt also acts as a co-regulator for both ERa and ERb [37].

The proposed mechanism of AhR/ER association is a reasonable explanation for dioxin/estrogen cross-talk. First, this mechanism explains the functional AhR/ER cross-talk irrespective of differences in target gene promoters. Second, ligand-dependent AhR/ER association may result in a rapid cellular response to dioxins in terms of ER activity. The responses of ER transcriptional activity to AhR ligands are observed within a few hours in cultured cells as well as in mice, which supports the existence of direct cross-talk mechanisms. Third, variations in the AhR/ER containing co-regulator complexes may result in the complex, bi-phasic consequences of AhR/ER cross-talk. Given that complexes containing different classes of transcription factors can recruit co-regulator complexes distinct from their cognate associating complexes [22], it is possible that the AhR/ER complex, acting as a functional unit, may recruit different types of complexes depending on the cellular context. A current area of interest is the identification of the molecular determinants by which the activity of the AhR/ER complex is controlled.

3. Ubiquitin ligase activity of AhR

3.1. *The ubiquitin–proteasome system*

The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels in response to various cellular contexts in metazoans (Fig. 4A). Whereas the transcriptional regulatory system is targeted by environmental fat-soluble ligands, the involvement of the ubiquitin–proteasome system in the adverse effects of these environmental toxins remains largely unknown. The target selectivity of these systems depends on the recognition of specific DNA elements by sequence-specific transcription factors [20–22] and recognition of degradation substrates by E3 ubiquitin ligases [38–41] (Fig. 4B). These transcription factors and E3 ubiquitin ligases primarily serve as specific adapters to subsequently recruit enzymes such as transcriptional co-

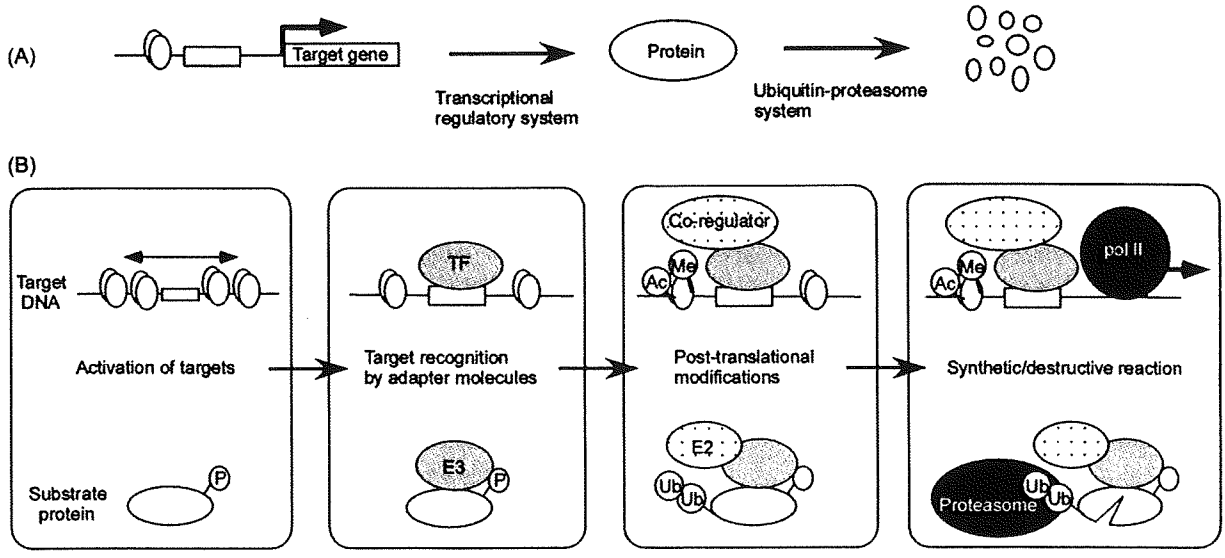

Fig. 4 – The ubiquitin–proteasome system. (A) The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels. (B) The transcription factors and E3 ubiquitin ligases primarily serve as target-specifying adapters in these systems. Ub, ubiquitin; P, phosphorylated serine/threonine; Ac, acetylated lysine; Me, methylated lysine; Pol-II, RNA polymerase II.

regulators and E2 ubiquitin-conjugating enzymes, respectively, to appropriate targets. Considering the functional analogy of E3 ubiquitin ligase and transcription factors, it is possible that E3 ubiquitin ligase also serves as a target of environmental toxins.

The ubiquitin–proteasome system, which regulates cellular protein degradation, plays a pivotal role in cellular homeostasis [38–41]. Ubiquitin is a 76 amino acid polypeptide that is highly conserved among eukaryotes. Ubiquitin is covalently attached to lysine (Lys) residues of substrate proteins. Ubiquitination of proteins is catalyzed by sequential reactions involving ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3). Ubiquitin is conjugated either as one molecule (mono-ubiquitination) or as a tandem polymer (poly-ubiquitination). Poly-ubiquitination can occur at any of seven lysine residues in the ubiquitin molecule. The Lys48-linked poly-ubiquitin chain is then recognized by the 26S proteasome for subsequent proteolysis (Fig. 4B).

Among E1, E2, and E3 enzymes, the E3 ubiquitin ligases are most diverse and therefore possess substrate specificity. E3 acts as a bridge between E2 and the substrate, maintaining the appropriate distance. E2 then conjugates ubiquitin to the substrate [38–41]. Of the RING-type E3s, the largest class is comprised of the cullin–RING ubiquitin ligases (CRLs) [40–44]. CRLs are multisubunit complexes that include a cullin (CUL1, 2, 3, 4A, 4B, or 5) subunit, a RING finger protein Rbx1/Roc1 or Rbx2/Roc2, and a substrate-recognition subunit. Cullin serves as a scaffold protein, binding to the substrate-recognition subunit or adapter protein at its N-terminus while binding to Rbx1 at its C-terminus [41]. Rbx1 binds to E2 enzymes through RING finger to support efficient conjugation of ubiquitin to the substrates. Their diverse substrate-recognition subunits enable CRLs to target numerous substrates. The best characterized CRLs are the SCF (Skp1–CUL1–F-box) complexes. In SCF complexes, F-box proteins function as a substrate-recognition subunit by binding to Skp1, which is bound to the N-terminal region of CUL1. F-box proteins and other types of substrate-recognition subunits serve as adapters for target-specific substrates. Therefore, any protein binding to E3 core components can potentially act in a manner similar to substrate-recognition subunits. More interestingly, F-box proteins and other types of substrate-recognition subunits are rapidly degraded through an auto-catalytic mechanism once they are integrated into the CRL core complexes [42]. In this way, CRLs can efficiently ubiquitinate different substrates by associating with different substrate-recognition subunits. This raises the possibility that F-box and F-box 'equivalent' proteins act either as substrates or as adapter components, as in the case of DDB2 in the CUL4-based CRL complex [45–50].

3.2. *AhR is an E3 ubiquitin ligase*

As discussed above, dioxins, through activating the AhR, have well-described effects on the transcriptional regulatory system. TCDD is also reported to decrease the uterine ERa protein level in the rat [51], suggesting that AhR may also be involved in the control of protein stability. Somewhat unexpectedly, our own study has shown that in a ChIP analysis, the ligand-bound AhR does not block co-activator recruitment of liganded ERa. In addition, repression of ERa transcriptional activity by AhR is not observed when ERa is over-expressed in transient reporter assays (Ohtake et al., unpublished data). These observations imply that the ligand-activated AhR has an additional molecular role beyond transcriptional regulation, at least in the modulation of sex hormone signaling.

Exploring the functions of AhR in sex hormone signaling, we found that upon activation of AhR by binding of AhR ligands such as 3MC and bNF, as well as by expression of constitutively active AhR, protein levels of endogenous ERa, ERb, and AR, were drastically decreased without alteration in mRNA levels [19] (Fig. 5). Since ligand-bound AhR and ERa proteins are ubiquitinated for proteasome-mediated degradation [52–57], we tested whether the functional modulation of ERs and AR by activated AhR is related to this degradation system. 3MC-enhanced degradation of sex steroid receptors is attenuated in the presence of a proteasome inhibitor MG132, and 3MC-enhanced poly-ubiquitination of ERa is consistently observed irrespective of E2 binding. MG132 treatment abrogates the transcriptional modulation of liganded sex steroid receptor function by activated AhR. This indicates that the ubiquitin–proteasome system mediates the repressive AhR–ER cross-talk pathway.

These experiments provide evidence that AhR acts as an E3 ubiquitin ligase component. First, FLAG–AhR immunoprecipitated complexes exert a self-ubiquitination activity in an E1/E2 enzyme-dependent manner *in vitro*. Second, 3MC-dependent recognition of ER and AR by AhR [14] appears to induce ubiquitination of ER/AR. Third, degradation of AhR itself is accelerated upon activation of degradation of sex steroid receptors, which is a typical sign of self-ubiquitination of the E3 component [42]. Taken together, these properties of AhR resemble that of classical adapter components of the E3 ubiquitin ligase complex such as F-box proteins in the SCF complex [39,42], DDB2/CSA in the CUL4A complex [45–49], and VHL in the CUL2 complex [58]. Therefore, we reasoned that activated AhR might serve as an E3 ubiquitin ligase component.

Supporting this idea, an AhR associating ubiquitin ligase complex has been biochemically purified [59] from HeLa cells. This complex includes cullin 4B (CUL4B) [39,60], damaged-DNA-binding protein 1 (DDB1) [61,62], and Rbx1 [39] together with subunits of the 19S regulatory particle (19S RP) of 26S proteasome as well as Arnt and transducin-beta-like 3 (TBL3) (Fig. 5). The core complex appears to constitute a CRL-type E3 ligase, and therefore is referred to as $CUL4B^{AhR}$. Although the typical CUL4B-type CRL complex contains substrate-recognition components having a WDXR/DWD motif [45–49], no such component has been identified in this complex. AhR directly interacts with the N-terminal region of CUL4B in GST pull-down assays. Together with the direct interaction of AhR with ER, it appears that AhR may act as a substrate-recognition component in the $CUL4B^{AhR}$ complex. Using an *in vitro* reconstituted ubiquitination assay, the E3 ubiquitin ligase activity of $CUL4B^{AhR}$ for ERa is dependent only on 3MC, and not on E_2. This suggests that $CUL4B^{AhR}$ has the unique property of being able to respond to ligand signals by complex assembly and ubiquitin ligase activity (Fig. 5). The importance of the $CUL4B^{AhR}$ components for the promotion of ERa ubiquitina-

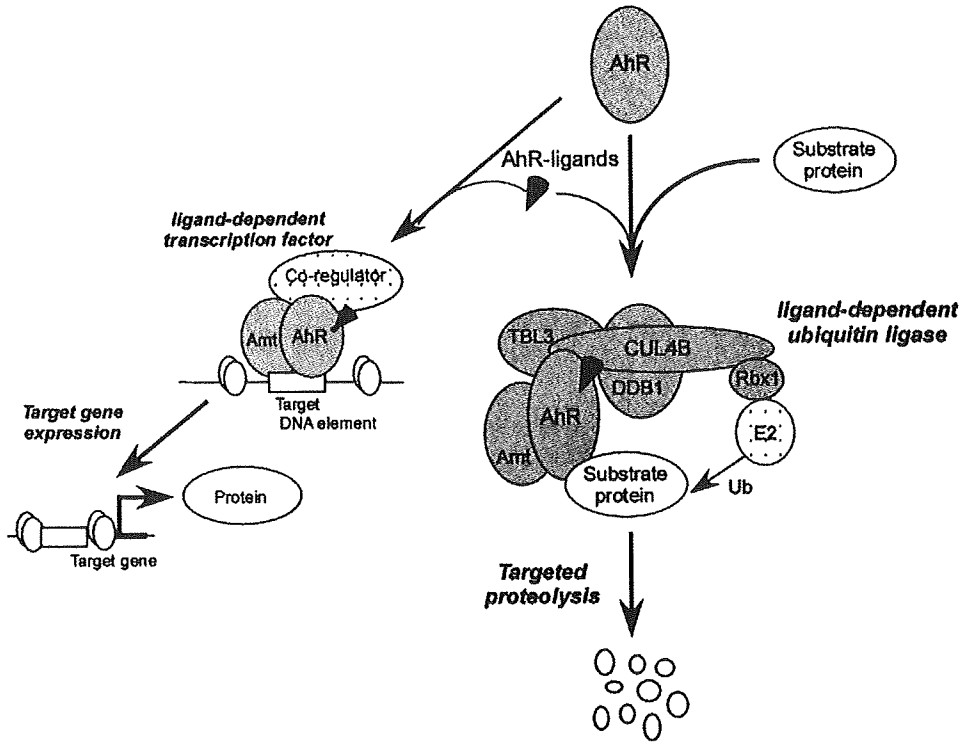

Fig. 5 – An E3 ubiquitin ligase activity of AhR. Ligand-bound AhR assembles a CUL4B-based atypical E3 ubiquitin ligase complex, $CUL4B^{AhR}$, to mediate a non-genomic signaling pathway of fat-soluble ligands. AhR serves as a ligand-dependent ubiquitin ligase, as well as a transcription factor (see text for details). DDB1, damaged-DNA-binding protein 1; TBL3, transducin-beta-like 3.

tion and degradation has been demonstrated in knock-down experiments. Degradation of ERa or AR in the uterus and prostate is inducible by treatment with AhR ligands. Such degradation of ERa or AR is not seen in AhR-deficient mice [10,33]. This confirms that the AhR has E3 ubiquitin ligase activity *in vivo*. The anti-estrogenic effects of AhR ligands on estrogen-dependent uterine cell proliferation [14] appear to be mediated by the E3 ubiquitin ligase activity of AhR.

3.3. *Perspectives on the E3 ubiquitin ligase activity of AhR in cross-talk pathways*

Although it is well established that AhR is a key factor in mediating the adverse effects of dioxin-type compounds [8–10], the underlying mechanisms for this remain elusive. The putative functions of the previously identified target genes for AhR appear unlikely to fully explain the diverse range of biological actions of AhR ligands [11] (Fig. 1). The discovery of $CUL4B^{AhR}$ suggests that the adverse effects of AhR ligands in sex hormone signaling are, at least in part, attributable to the enhanced degradation of sex steroid receptors through E3 ubiquitin ligase activity of AhR [18,19] (Fig. 5). Target selectivity of the transcriptional regulatory system and the ubiquitin–proteasome system depends on specificity conferred by sequence-specific transcription factors and E3 ubiquitin ligases. To date, however, no single factor has been shown to function as a specificity factor in both target selection systems. Therefore, AhR is the first sequence-specific transcription factor identified that acts as an E3 ubiquitin ligase that also targets substrates for accelerated protein degradation. It is possible that other transcription factors, such as nuclear receptors, also function as E3 ubiquitin ligase components in some cellular contexts. Fat-soluble ligands for nuclear receptors are reported to have 'non-genomic' actions independent of transcriptional regulation-mediated effects. Considered together, ubiquitin ligase-based signaling mechanisms may possibly be involved in these non-genomic actions of various fat-soluble ligands.

From a mechanical point of view, AhR appears to be a unique and atypical type of substrate-specific component in cullin-based E3 complexes. AhR does not bear the reported signature motifs such as F-box [39], but directly associates with CUL4B. Substrate recognition by the other substrate-specific components in ubiquitin ligase complexes is usually evoked by substrate modifications such as phosphorylation [38–41] and hydroxylation [43,44,58]. However, recognition and subsequent ubiquitination of sex steroid receptors by AhR requires dioxin-type ligands, and does not occur following normal modifications of sex steroid receptors. Thus, it is plausible that activation of atypical E3 complexes may be a strategy of sensors for environmental stresses to respond to these stresses (Fig. 6). Supporting this, Hsp70 acts as an atypical substrate-specific adapter within the CHIP E3 complex in response to heat shock stress [63]. Hsp70 interacts with misfolded proteins and promotes their degradation. It later undergoes auto-catalytic degradation through CHIP [63]. In response to DNA damage, an atypical E3 complex alters the stability of TIP60, which in turn regulates ataxia-telangiectasia

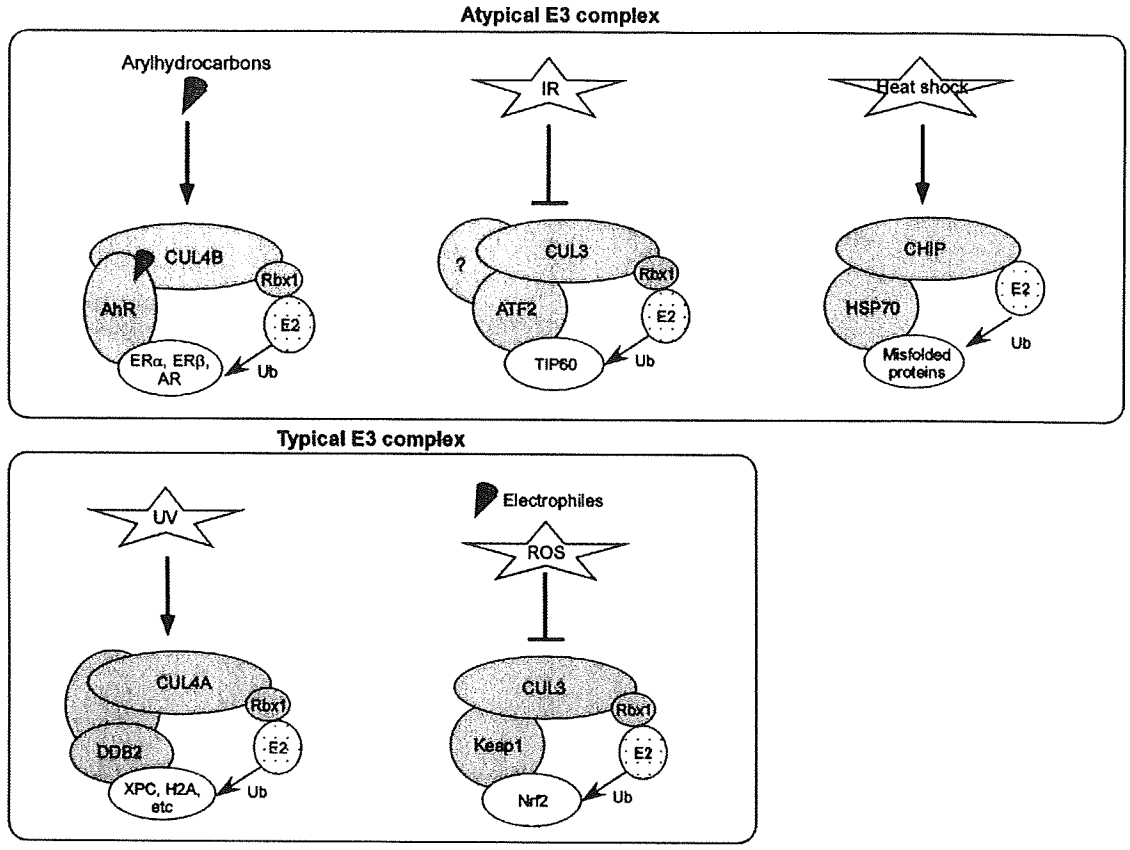

Fig. 6 – Atypical E3 complexes as sensors for environmental stresses. Several examples of E3 ubiquitin ligase-based perception of environmental stresses are illustrated. In the top panel, signal-responsive factors serve as atypical components of E3 complexes. In the bottom panel, canonical E3 components with conserved signature motif act as signal-responsive factors. ATF2, activating transcription factor-2; TIP60, tat interactive protein 60; CHIP, C-terminus of Hsp70 interacting protein; Hsp70, heat shock protein 70; XPC, xeroderma pigmentosum group C; H2A, histone H2A; Keap1, Kelch-like ECH-associated protein 1; Nrf2, NF-E2-related factor 2; IR, ionizing radiation; ROS, reactive oxygen species.

mutated (ATM) activation in DNA repair [64]. Activating transcription factor-2 (ATF2) promotes the degradation of TIP60 by assembling a CUL3-based complex under non-stressed conditions. ATF2 dissociates from TIP60 in response to ionizing radiation (IR), resulting in enhanced TIP60 stability and activity [64]. Functional regulation of E3 components is also seen with the CUL3-based component Keap1 in the oxidative stress response [65], and CUL4A-based components DDB2 and CSA in the DNA damage response [61]. Considered together, E3 components that respond to environmental stress may be more diverse than initially believed (Fig. 6). It is possible that $CUL4B^{AhR}$ may cross-talk with these stress-responsive E3 ligases to modulate their functions. As WDXR/DWD motif containing components, including DDB2 and CSA, also bind to CUL4B [46], it is possible that AhR may associate or interfere with these CRL subunits.

The E3 ubiquitin ligase activity of AhR and the transcriptional activity of AhR appear to be responsible for a distinct set of biological events induced by AhR ligands (Fig. 5). As substrate-specific adapters of ubiquitin ligase complexes are capable of recognizing a number of proteins, identification of other $CUL4B^{AhR}$ substrate proteins may reveal new molecular links between AhR-mediated signaling and other signaling pathways and cellular events. In this regard, it is of interest that AhR interacts with various transcription factors [11], such as Rb/E2F1 [66], SF1/Ad4BP [33], and NF-kB [67], to modulate their functions. AhR has recently been shown to regulate the differentiation of Th17 and T_{reg} cells [68–70]. This may be mediated by a functional interaction with STAT1 [70]. In addition, although the underlying mechanisms remain unknown, AhR also modulates the function of transcription factors [71] such as GR and RAR [72,73]. Considering the evolutionary conservation of AhR, it is likely that the intrinsic function of AhR is to mediate the signal transduction of endogenous ligands in cross-talk pathways. A current area of interest is the identification of candidate degradation substrates for AhR which are abnormally stabilized in AhR-deficient mice. In summary, several lines of recent evidence define a novel role for AhR as a ligand-dependent E3 ubiquitin ligase to regulate target-specific protein destruction. The ubiquitin ligase activity of AhR, together with the cross-talk of AhR with nuclear receptors through direct association, provides an additional layer of complexity for AhR biology. Characterization of these new molecular aspects of AhR function may lead to a greater understanding of the diverse biological actions induced by endogenous and exogenous AhR ligands.

Conflict of interest

The authors declare no competing financial interests.

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Aryl hydrocarbon receptor suppresses intestinal carcinogenesis in $Apc^{Min/+}$ mice with natural ligands

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Intestinal cancer is one of the most common human cancers. Aberrant activation of the canonical Wnt signaling cascade, for example, caused by adenomatous polyposis coli (APC) gene mutations, leads to increased stabilization and accumulation of β-catenin, resulting in initiation of intestinal carcinogenesis. The aryl hydrocarbon receptor (AhR) has dual roles in regulating intracellular protein levels both as a ligand-activated transcription factor and as a ligand-dependent E3 ubiquitin ligase. Here, we show that the AhR E3 ubiquitin ligase has a role in suppression of intestinal carcinogenesis by a previously undescribed ligand-dependent β-catenin degradation pathway that is independent of and parallel to the APC system. This function of AhR is activated by both xenobiotics and natural AhR ligands, such as indole derivatives that are converted from dietary tryptophan and glucosinolates by intestinal microbes, and suppresses intestinal tumor development in $Apc^{Min/+}$ mice. These findings suggest that chemoprevention with naturally-occurring and chemically-designed AhR ligands can be used to successfully prevent intestinal cancers.

cecal cancer | ubiquitin ligase | β-catenin | tumor chemoprevention

The aryl hydrocarbon receptor (AhR, also known as dioxin receptor) is a member of a transcription factor superfamily that is characterized by structural motifs of basic helix–loop–helix (bHLH)/Per-AhR nuclear translocator (Arnt)-Sim (PAS) domains, and also includes hypoxia-inducible factors (HIFs). Over the past decade, many studies have been focused on elucidating the functions of AhR as a mediator of multiple pharmacological and toxicological effects such as the induction of drug-metabolizing enzymes, teratogenesis, tumor promotion, and immunosuppression caused by environmental contaminants such as 3-methylcholanthrene (MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (1, 2). On ligand binding, AhR translocates from the cytoplasm into the nucleus where it heterodimerizes with the Arnt and activates the transcription of target genes such as *Cyp1a1*. Induction of the *Cyp1a1* gene leads to the biotransformation of polycyclic aromatic hydrocarbons into active genotoxic metabolites, resulting in the initiation of chemical carcinogenesis (3). AhR-deficient ($AhR^{-/-}$) mice are resistant to most, if not all, of these toxicological adverse effects, indicating that AhR is a key factor in the development of these chemical-induced diseases (4, 5). Also, we recently found that AhR functions as a ligand-dependent E3 ubiquitin ligase of certain nuclear receptors (6), such as the estrogen (ER) and androgen receptors (AR). Most recently, AhR has been reported to have a crucial role in the differentiation of regulatory T cells (7–9).

AhR is a nucleocytoplasmic shuttling protein, the intracellular localization of which is changed depending on cell density in the absence of exogenous ligands (10). Such cell density-dependent movements between the cytoplasm and nucleus have also been reported for some tumor suppressor gene products, such as VHL (11) and adenomatous polyposis coli (APC) (12). Also, the natural AhR ligands of indole derivatives (13, 14), such as indole-3-acetic acid (IAA, so-called plant auxin), indole-3-carbinol (I3C) and 3,3′-diindolylmethane (DIM), are natural AhR ligands and generated through conversion from dietary tryptophan (Trp) and glucosinolates, respectively, by commensal intestinal microbes (15). Notably, glucosinolates have been reported to exert the chemopreventive effects on colorectal cancers in humans by cruciferous vegetables (16–18). Together, these lines of evidence suggest that AhR has some functional association with intestinal carcinogenesis.

Results

Cecal Tumor Development in $AhR^{-/-}$ Mice. After thoroughly examining the digestive tracts of $AhR^{-/-}$ mice, we found that $AhR^{-/-}$ mice, but not heterozygous $AhR^{+/-}$ or wild-type $AhR^{+/+}$ mice, frequently developed colonic tumors, mostly in the cecum near the ileocecal junction (Fig. 1 *A* and *B*). $AhR^{-/-}$ mice bred at 2 independent animal houses showed a similar time course of macroscopic tumor incidence (Fig. S1*B*), and the tumor size increased gradually by age, reached a plateau at ≈30 to 40 weeks (Fig. 1*B*). To date, 3 independent $AhR^{-/-}$ mice lines have been reported (4, 19, 20). Although one report described frequent rectal prolapse (Fig. S1*A*) and marked colonic hyperplasia with severe inflammation in $AhR^{-/-}$ mice (19), there have been no systematic studies on intestinal carcinogenesis, which may explain why the tumor suppressor function of AhR has been unreported to date. Colorectal cancer is one of the most common human cancers, 5–10% of which originates in the cecum. Therefore, we were interested in investigating how $AhR^{-/-}$ mice develop spontaneous cecal tumors.

Randomly selected mice were examined histologically for atypia classified according to the standards as shown in Fig. S2. Although $AhR^{+/+}$ and $AhR^{+/-}$ mice of all ages had normal (Grade 1) to mild hyperplasia (Grade 2) at worst, $AhR^{-/-}$ mice older than 11 weeks had abnormal histology with atypia ranging from mild malignancy of polyps to severe carcinomas that were exacerbated with age (Fig. 1*C*). Close microscopic examination revealed that the $AhR^{-/-}$ mice bore cecal lesions with a mod-

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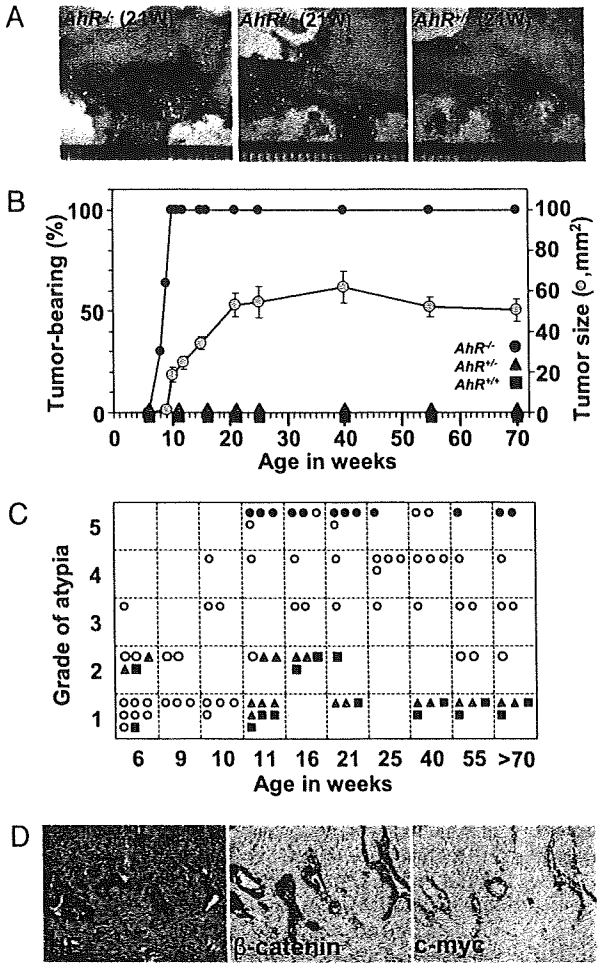

Fig. 1. Cecal tumor development in $AhR^{-/-}$ mice. (*A*) Representative profiles of colon tumors at the cecum in $AhR^{-/-}$ mice. (*B*) Relationship between the time course of macroscopic tumor incidence and tumor growth by age. Tumor size was estimated based on NIH images as shown by beige circles. Error bars, means ± SD. (*C*) Summary of histological atypia grades of tumors in $AhR^{-/-}$ mice by age. $AhR^{+/+}$ (blue squares), $AhR^{+/-}$ (green triangles), and $AhR^{-/-}$ (yellow circles) are shown. $AhR^{-/-}$ mice with adenocarcinomas (Grade 5) that had invaded the submucosal region or beyond (red circles) and within the intramucosal region (pink circles) are shown separately. (*D*) Representative H&E staining profile of a moderately differentiated adenocarcinoma and immunohistochemical staining with an antibody against β-catenin or c-myc.

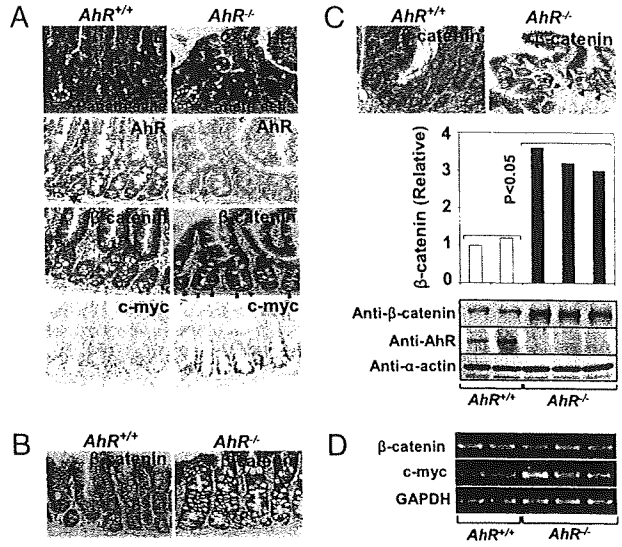

Fig. 2. Abnormal β-catenin accumulation in the intestines of $AhR^{-/-}$ mice. (*A*) H&E staining and immunohistochemical staining of mouse small intestines. Paneth cells were observed at the bottom of the crypts in the small intestine in both genotypes. Expression of AhR, β-catenin, and c-myc are shown. Nuclear accumulation of β-catenin in Paneth cells of the small intestine and cecum is noted by red arrowheads. Immunohistochemical staining of β-catenin in the colons (*B*) or cecum (*C*) of $AhR^{+/+}$ or $AhR^{-/-}$ mice. (*C*) Levels of β-catenin, AhR and α-actin in the cecum were detected by Western blotting. The amount of β-catenin was quantified using the ImageJ software (NIH). ($P < 0.05$; $AhR^{+/+}$ versus $AhR^{-/-}$ group). (*D*) RT-PCR was performed to detect mRNA levels for β-catenin, c-myc ($P < 0.05$; $AhR^{+/+}$ versus $AhR^{-/-}$ group), and GAPDH in the cecal epithelium of $AhR^{+/+}$ or $AhR^{-/-}$ mice. Data are representative of 3 independent experiments.

erate (Grade 3: 9/42) or a high grade of atypia, adenoma (Grade 4: 12/42), and adenocarcinoma (Grade 5: 17/42). Among the 17 diagnosed adenocarcinomas, 12 tumors (71%) invaded the submucosal region or beyond, and the remainder were located within the intramucosal region. Overall survival rates estimated by the Kaplan-Meyer method (Fig. S1*C*) revealed that $AhR^{-/-}$ mice had a significantly shorter lifespan than wild-type or heterozygous mice (log-rank test; $P = 4.4 \times 10^{-9}$), although this shorter longevity might not be only due to cecal tumors in the $AhR^{-/-}$ mice (19).

The detected cecal cancers were predominantly tubular adenocarcinomas with various degrees of malignancy (Fig. S3). A representative profile of moderately differentiated adenocarcinomas with irregularly shaped and fused tubular structures that sometimes invaded the submucosal regions is presented in Fig. 1*D*. In these cells, immunohistochemical staining showed concomitant overexpression of β-catenin and c-myc, a target gene of β-catenin/TCF4 (21). It remains uninvestigated whether there should occur any further genetic alterations in $AhR^{-/-}$ mice leading to carcinogenesis. In human cecal cancers, markedly reduced expression of AhR was also found concomitantly with an abnormal accumulation of β-catenin in all of 12 cancer specimens from our hospital (Fig. S4).

The β-Catenin Accumulation in $AhR^{-/-}$ Mice. To examine the molecular mechanism underlying tumor development in $AhR^{-/-}$ mice, we analyzed the expression of both AhR and β-catenin in the intestines of 6-week-old $AhR^{+/+}$ and $AhR^{-/-}$ mice, which had a morphologically normal epithelium. AhR expression was relatively abundant in Paneth cells (22), which have a host-defensive role against microbes in the small intestine and the cecum in $AhR^{+/+}$ mice, but was undetectable in $AhR^{-/-}$ mice (Fig. 2*A*). Significant AhR expression was also observed in Paneth cells of the small intestine and the cecum in humans (Fig. S5). Notably, β-catenin expression was abnormally high in epithelial cells of the ileum (Fig. 2*A*), colon (Fig. 2*B*), and cecum (Fig. 2*C*) in $AhR^{-/-}$ mice, suggesting that the intestines of $AhR^{-/-}$ mice may be in a "cancer-prone" or "precancerous" state (23). In particular, these elevated levels of β-catenin were observed in the nuclei of Paneth cells compared with the corresponding regions in wild-type mice (Fig. 2*A*).

Using Western blotting (Fig. 2*C*), we confirmed that $AhR^{-/-}$ mice had significantly higher levels of β-catenin in the cecum than wild-type mice ($P < 0.05$), whereas β-catenin mRNA expression levels were unchanged (Fig. 2*D*), suggesting that the stabilization, but not enhanced synthesis of the β-catenin protein in the $AhR^{-/-}$ intestine leads to β-catenin accumulation. Con-

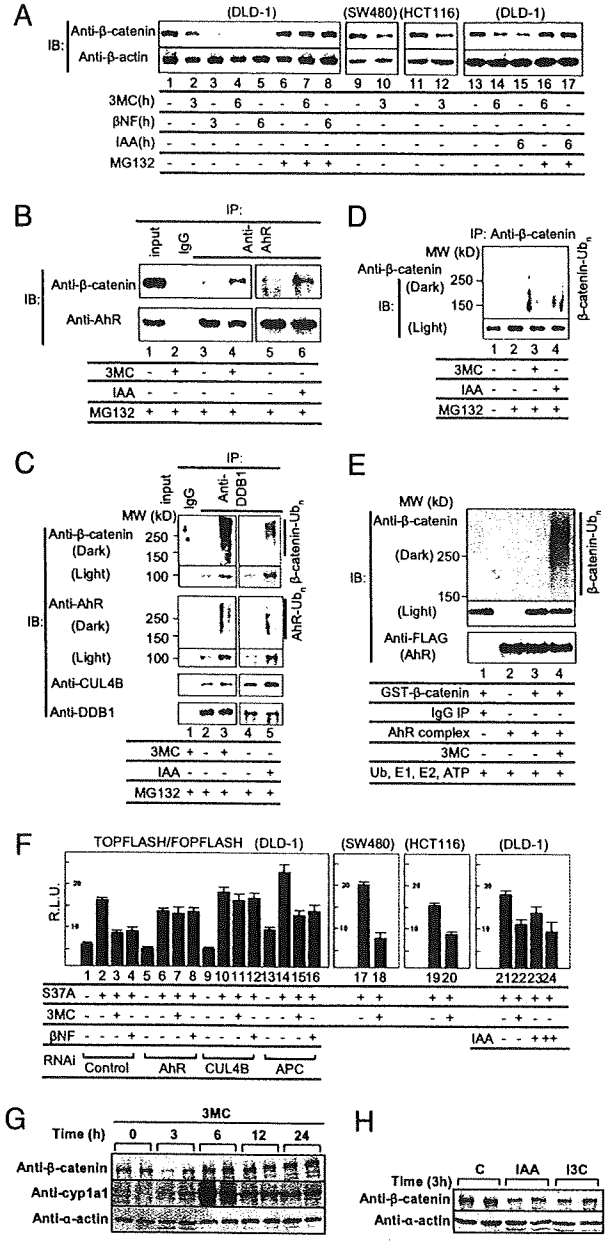

Fig. 3. Novel AhR ligand-dependent ubiquitylation and proteasomal degradation of β-catenin. (*A*) Activated AhR promotes proteasomal degradation of β-catenin. Cells were incubated as indicated with 3MC (1 μM), βNF (1 μM), or IAA (100 μM) in the presence or absence of the proteasome inhibitor MG132 (10 μM) for 3 or 6 h. Cell lysates were subjected to Western blotting with antibodies indicated. (*B*) Ligand-dependent recognition of β-catenin by AhR. DLD-1 cells were incubated with 3MC or IAA and MG132 for 2 h. Then, the extracts were prepared and immunoprecipitated. (*C*) Ligand-dependent complex assembly of CUL4B^{AhR} E3 ligase with β-catenin. DLD-1 cells were incubated with 3MC or IAA and MG132 for 2 h, after which the cell extracts were prepared and immunoprecipitated with an anti-DDB1 antibody to detect CUL4B^{AhR} complexes with β-catenin. Western blottings were subjected to a long exposure (Dark) to detect polyubiquitylated forms of the proteins. (*D*) AhR ligand-induced ubiquitylation of β-catenin. DLD-1 cells were incubated with the indicated ligands and MG-132 for 6 h. (*E*) The AhR complex directly ubiquitylates β-catenin in vitro. The FLAG-HA-AhR-associated immunocomplex in the presence of CUL4B^{AhR} components was mixed with recombinant GST-β-catenin (Fig. S6*D*) and His-ubiquitin, and an in vitro ubiquitylation assay was performed. (*F*) CUL4B^{AhR} components are essential for AhR ligand-dependent repression of hyperactive β-catenin (S37A) transactivation. Cells were incubated as indicated with 3MC (1 μM), βNF (1 μM), or IAA (+, 10 μM; ++, 100 μM). All values are means ± SD for at least 3 independent experiments. (*G*) AhR ligand-dependent β-catenin degradation in vivo. AhR$^{+/+}$ mice received a single i.p. injection of 3MC (4 mg/kg). The levels of proteins in the cecal epithelium were determined. (*H*) *AhR*$^{+/+}$ mice received a single i.p. injection of IAA or I3C (25 mg/kg).

sistent with the abnormal accumulation of β-catenin, expression of the downstream target, c-myc, showed ≈2-fold induction (Fig. 2 *A* and *D*).

Ligand-Dependent Degradation of β-Catenin. Next, we examined whether the AhR E3 ubiquitin ligase participates in the degradation of β-catenin (Fig. 3) as reported (6) for the degradation of ER and AR. On activation of AhR by exogenous ligands, 3MC or β-naphthoflavone (βNF), endogenous β-catenin protein levels markedly decreased in DLD-1 cells derived from a colon cancer and in other colon cancer-derived cells, SW480 and HCT116 (Fig. 3*A*). These results clearly show that β-catenin is degraded in an AhR ligand-dependent manner even in colon cancer-derived cells harboring mutations (24) in *APC* or *β-catenin* that stabilize β-catenin protein against APC-dependent degradation. These findings suggest that AhR participates in a previously undescribed mechanism of β-catenin degradation that is independent of the APC pathway. Also, after the addition of IAA, which is produced in the intestine from Trp by intestinal microbes (15), and was detected in the cecal contents by HPLC (Fig. S6*H*), AhR-dependent degradation of β-catenin was also observed (Fig. 3*A*; Fig. S6*A*). Degradation of β-catenin induced by xenobiotics or natural AhR ligands was abrogated in the presence of either the proteasome inhibitor MG132 (Fig. 3*A*) or AhR siRNA (Fig. S6*A*). We observed that the AhR ligands promoted selective degradation of β-catenin in the soluble fractions, but not in the membrane fraction of cells (Fig. S6*B*), suggesting that β-catenin involved in the Wnt signaling pathway is selectively degraded. Recognition of endogenous β-catenin by AhR was clearly ligand-dependent, as shown by coimmunoprecipitation assays (Fig. 3*B*). Also, AhR ligand-dependent assembly of the Cullin (CUL)4B^{AhR} E3 ligase complex with β-catenin (Fig. 3*C*) was detected by immunoprecipitation assays using an antibody to DDB1 (6), a component of the E3 ubiquitin ligase complex of AhR, together with ligand-induced polyubiquitylation of β-catenin (Fig. 3 *C* and *D*) and self-ubiquitylation of AhR (Fig. 3*C*). AhR-mediated degradation of β-catenin was reconstituted in an in vitro ubiquitylation assay. In this assay, immunopurified CUL4B^{AhR} complexes showed, as expected, E3 ubiquitin ligase activity toward ER (Fig. S6*C*) and purified GST-β-catenin (Fig. 3*E*; Fig. S6*D*). In both these cases, the E3 ubiquitin ligase activity was increased by addition of the ligand, 3MC (Fig. 3*E*; Fig. S6*C*). These data strongly suggest that the ligand-dependent E3 ubiquitin ligase activity of AhR participates in β-catenin degradation, and is consistent with the repression of the transcriptional activity of endogenous β-catenin by 3MC (Fig. S6*E*).

To substantiate AhR-dependent degradation of β-catenin in terms of its transcriptional activity and its relationship with the canonical APC-dependent degradation system, we performed reporter assays with TOPFLASH/FOPFLASH mediated by a hyperactive β-catenin (S37A) mutant (Fig. 3*F*) (25). The reporter activity was enhanced by the addition of β-catenin, and the enhanced reporter expression was repressed by the AhR ligands, 3MC, βNF, and IAA ($P < 0.05$). Repression of the transcriptional activity of β-catenin by AhR ligands was reversed by AhR or CUL4B siRNA, but not by APC siRNA, confirming that AhR is involved in a previously undescribed ligand-dependent mechanism of proteasomal degradation of β-catenin

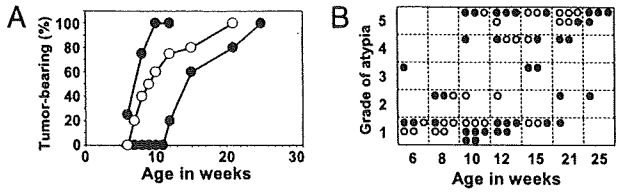

Fig. 4. Functional cooperation between Apc and AhR with regard to cecal tumor incidence. Macroscopic cecal tumor incidence by age in weeks (*A*) and summary of histological grades of atypia (*B*) that developed in $Apc^{Min/+}$·$AhR^{+/+}$ (blue circles), $Apc^{Min/+}$·$AhR^{+/-}$ (yellow circles), and $Apc^{Min/+}$·$AhR^{-/-}$ (red circles) mice. Four to 5 mice were used in each group.

that is distinct from the canonical APC-dependent pathway (Fig. 3*F*; Fig. S6*F*).

We were interested to investigate whether β-catenin protein is reduced in vivo in the intestines of mice after AhR ligand treatment. AhR ligand-dependent degradation of the β-catenin protein was clearly observed in vivo in the intestines of mice with a peak at 3 h after i.p. injection of 3MC, whereas cyp1a1 expression was markedly enhanced as expected (Fig. 3*G*). This transient degradation of β-catenin is likely due to the rapid down-regulation of AhR after ligand activation (6). Also, this in vivo degradation of β-catenin by 3MC was AhR-dependent, because accumulated β-catenin levels in the cecal epithelia of $AhR^{-/-}$ mice were not altered by 3MC treatment (Fig. S6*G*). Also, in vivo degradation of β-catenin was observed after i.p. injection of the natural AhR ligands, IAA and I3C (Fig. 3*H*). HPLC analysis of cecal materials demonstrated that the production of natural AhR ligands [IAA (≈1.2 μM), TA (tryptamin) (≈7.2 μM), and indole (≈43 μM)] depended on the presence of intestinal microbes (Fig. S6*H*), and the concentrations of these ligands were in a range that effectively activates AhR. During 3MC treatment, β-catenin mRNA levels remained unchanged with a slight, but reproducible decrease in c-myc mRNA expression, whereas cyp1a1 mRNA levels were markedly enhanced (Fig. S6*I*). These in vivo observations are highly consistent with the in vitro experiments, and provide a basis for possible chemoprevention against intestinal carcinogenesis by using natural AhR ligands.

Cooperative Function Between Apc and AhR Pathways. The tumor suppressor *APC* gene was originally discovered as a gene responsible for a hereditary cancer syndrome termed familial adenomatous polyposis (FAP) (26, 27). *APC* mutations are also found in most sporadic colorectal cancers (28) with an abnormal accumulation of β-catenin. The murine model of FAP, $Apc^{Min/+}$ (multiple intestinal neoplasia/+), carries an *Apc* mutation (29). However, in contrast to FAP patients who develop tumors in the colon (28), these mice develop numerous adenomatous polyps mostly in the small intestine, although the reasons for this difference remain unknown.

To investigate a functional association between the Apc- and AhR-mediated pathways of β-catenin degradation with regard to intestinal tumor development, we generated mice with compound mutations in both the *Apc* and *AhR* genes with the same genetic background. We observed no effect of AhR mutation on the expression of Apc, and vice versa (Fig. S7*A*). The tumor incidence in compound $Apc^{Min/+}$·*AhR* disrupted mutant mice was compared with that of single gene mutant $Apc^{Min/+}$ mice. In the cecum (Fig. 4*A*), $Apc^{Min/+}$ mice showed a tumor incidence of ≈50% of the total at 14 weeks of age that reached 100% at 25 weeks of age, whereas no tumors were found in $AhR^{+/-}$ mice (Fig. 1*B*). Remarkably, the compound $Apc^{Min/+}$·$AhR^{+/-}$ mutant mice had a tumor incidence of 50% at 9–10 weeks of age, and were much more susceptible to cecal tumorigenesis than $Apc^{Min/+}$ mice, which supports a cooperative tumor suppression function between the 2 genes. Compound $Apc^{Min/+}$·$AhR^{-/-}$ mutant mice displayed this tendency more prominently, although in limited numbers because of difficulty in breeding. A similarly accelerated carcinogenesis in the small intestine at 7 and 8 weeks was observed in $Apc^{Min/+}$·$AhR^{+/-}$ mice (Fig. 5*D*) compared with $Apc^{Min/+}$ mice (Fig. 5*B*) ($P < 0.001$). In the compound mutant mice, the grade of atypia of cecal tumors progressed with age in a cooperative manner, reflecting a cooperative interaction between the AhR and Apc pathways (Fig. 4*B*).

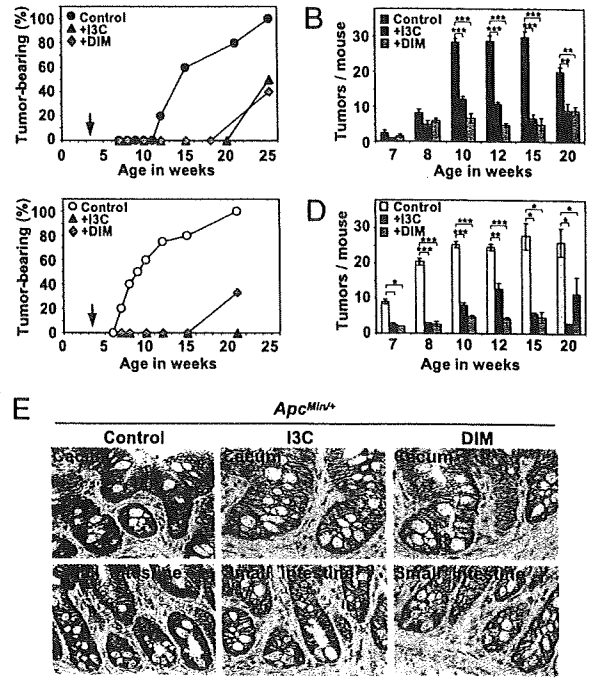

Fig. 5. Natural AhR ligands suppress intestinal carcinogenesis. Four to 5 mice were used in each group. Cecal carcinogenesis in the $Apc^{Min/+}$ (*A*) and $Apc^{Min/+}$·$AhR^{+/-}$ (*C*) mice. Tumor development in mice fed a control diet (blue circles in *A* and yellow circles in *C*), 0.1% I3C-containing (green triangles) or 0.01% DIM-containing (beige diamondes) diet just after weaning of 3–4 weeks of age as noted by the arrows. Number of small intestinal polyps in $Apc^{Min/+}$ (*B*, blue squares) or $Apc^{Min/+}$·$AhR^{+/-}$ (*D*, yellow squares) mice fed a control diet. Number of polyps in the small intestines of mice fed an I3C-containing (green squares) or DIM-containing (beige squares) diet. Data are presented as means ± SD. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$. (*E*) Representative profile of immunohistochemical staining with an antibody against β-catenin in the intestines from 15-week-old $Apc^{Min/+}$ mice fed a control or ligand-containing diet.

To determine how compound $Apc^{Min/+}$·*AhR*-disrupted mutant mice were more susceptible to cecal tumorigenesis than $Apc^{Min/+}$ mice, β-catenin levels were monitored in the cecum by Western blotting (Fig. S7*B*) and immunohistochemistry (Fig. S7*C*) at 6 to 8 weeks of age, when a morphologically normal epithelium was observed (Fig. 4*B*). And we found elevated levels of β-catenin in the cecum of both $Apc^{Min/+}$·$AhR^{-/-}$ and $Apc^{Min/+}$·$AhR^{+/-}$ mice compared with $Apc^{Min/+}$·$AhR^{+/+}$ mice, suggesting an association between the levels of β-catenin and tumor susceptibility. Expression levels of the β-catenin/TCF4 target genes, c-myc and cyclin D1, were concomitantly enhanced in $Apc^{Min/+}$·*AhR*-disrupted mice, suggesting that AhR-mediated β-catenin degradation has a suppressive role in intestinal carcinogenesis in parallel to the Apc system.

Tumor Suppression by AhR Natural Ligands. As described in Fig. 3, IAA and I3C accelerated β-catenin degradation in the intestine.

We were interested to study whether natural AhR ligands actually suppress carcinogenesis in the cecum or small intestine in $Apc^{Min/+}$ mice (Fig. 5). The chemoprevention (30) study was designed so that $Apc^{Min/+}$ or $Apc^{Min/+}$·$AhR^{+/-}$ mice were fed natural AhR ligand-containing diets, such as I3C (31) and DIM (32), immediately after weaning at 3–4 weeks of age. When fed the control diet, $Apc^{Min/+}$ mice started to develop small intestinal polyps at 7 weeks of age with the number of tumors containing polyps plateauing (≈30 tumors per mouse) at ≈10 to 15 weeks (Fig. 5*B*), whereas the cecal tumor incidence was as described (Figs. 4*A* and 5*A*). However, when fed an I3C (0.1%)- or DIM (0.01%)-containing diet, $Apc^{Min/+}$ mice showed a cecal tumor incidence of ≈50% of the total at 25 weeks of age (Fig. 5*A*) and a markedly reduced number of tumors in the small intestine (Fig. 5*B*). Similar chemopreventive effects were also clearly observed with the compound $Apc^{Min/+}$·$AhR^{+/-}$ mutant mice (Fig. 5*C* and *D*). However, no suppressive effect was observed in $AhR^{-/-}$ mice (Fig. S7*D*), suggesting that AhR ligand-dependent chemoprevention requires the presence of AhR.

Using immunohistochemical analysis, we showed a marked reduction of β-catenin except for the molecules associated with adherence junctions in the intestines of $Apc^{Min/+}$ (Fig. 5*E*; Fig. S7*F*) and $Apc^{Min/+}$·$AhR^{+/-}$ mice (Fig. S7 *E* and *F*) fed AhR ligand-containing diets compared with those fed a control diet. These results clearly demonstrate that chemoprevention of intestinal carcinogenesis by AhR ligands in $Apc^{Min/+}$ and $Apc^{Min/+}$·$AhR^{+/-}$ mice is due to β-catenin degradation mediated by the natural ligand-activated AhR E3 ubiquitin ligase.

Discussion

In this study, we provide both loss-of-function and gain-of-function data to show that the AhR mediates ligand-dependent degradation of β-catenin, leading to suppression of intestinal carcinogenesis. The AhR-mediated pathway of β-catenin degradation is independent of the canonical APC-mediated pathway, but functions cooperatively with it, because (*i*) $AhR^{-/-}$ mice develop colonic tumors mostly in the cecum, whereas numerous polyps develop mostly in the small intestine of $Apc^{Min/+}$ mice; (*ii*) even in cells containing mutations in APC or β-catenin gene, β-catenin is clearly degraded in an AhR ligand-dependent manner; and (*iii*) experiments using siRNAs against AhR, its E3 ubiquitin ligase cofactor CUL4B, and APC clearly indicate the independency between the 2 pathways. The cooperative function is strongly confirmed by additional experiments, in which (*i*) accelerated carcinogenesis was observed in the compound $Apc^{Min/+}$·*AhR*-disrupted mutant mice compared with $Apc^{Min/+}$ mice, and (*ii*) AhR natural ligands suppress intestinal carcinogenesis in $Apc^{Min/+}$ mice. These distinct roles are most likely because the AhR- and APC-dependent β-catenin degradation pathways are considered to be in different subcellular compartments (Fig. S8*A*); ligand-activated AhR translocates to the nucleus where it forms an ubiquitylation complex containing CUL4B (7) and the constitutively nuclear protein Arnt, whereas the APC-dependent pathway functions in the cytoplasm (33–35).

It is noteworthy that $AhR^{-/-}$ mice mainly develop tumors in the cecum, but not in the small intestine, whereas numerous polyps develop mostly in the small intestine of $Apc^{Min/+}$ mutant mice (29). Our findings that AhR is abundantly expressed in Paneth cells of the small intestine, as well as the cecum near the ileocecal junction, and that abnormal β-catenin accumulation is observed in the intestines of $AhR^{-/-}$ mice, suggest that intestines of $AhR^{-/-}$ mice may be in a cancer-prone or precancerous state (23). Although it is still unknown why $AhR^{-/-}$ mice specifically develop cecal cancers, the host genetic predisposition to these cancers may be potentiated by stimuli from bacteria colonized in the cecum (36). Abnormal β-catenin accumulation, together with microbial interaction or subsequent inflammation, may promote cecal carcinogenesis in $AhR^{-/-}$ mice. In conjunction with the involvement of intestinal microbes, different structural and functional properties of intestinal epithelial cells (34) may also be associated with the specific development of cecal tumor in $AhR^{-/-}$ mice.

We show evidence that natural AhR ligands converted from dietary Trp and glucosinolates in the intestine are as efficient as exogenous AhR ligands in promoting degradation of endogenous β-catenin. These results provide a molecular basis for chemopreventive mechanisms against intestinal carcinogenesis that were observed in $Apc^{Min/+}$ and $Apc^{Min/+}$·$AhR^{+/-}$ mice fed diets containing the AhR ligands I3C and DIM. Also, our findings lend credence to previous reports on the chemopreventive effects on colorectal cancers in humans by cruciferous vegetables that contain a high content of glucosinolates (16–18), and suggest that AhR ligands define a potent strategy for dietary chemoprevention of intestinal cancer.

In conclusion, this study shows that AhR has a critical role in suppression of intestinal carcinogenesis by a previously undescribed ligand-dependent mechanism of proteasomal degradation of β-catenin, which functions independently of and cooperatively with the canonical APC-dependent pathway. $AhR^{-/-}$ mice provide a murine model for spontaneously developing tubular adenocarcinomas, which have the most common histologic characteristics of sporadic colorectal cancers in humans. Although the reasons remain to be established, reduced AhR expression was observed in 12 specimen of human cecal cancers and their surrounding tissues (Fig. S4). Together, we conclude that $AhR^{-/-}$ mice are a useful model to study human intestinal cancer, and will help us to investigate the molecular mechanisms of pathogenesis and chemoprevention of intestinal cancer.

Materials and Methods

Animal Experiments. C57BL/6 wild-type and *AhR*-deficient ($AhR^{-/-}$) (4) mice on the C57BL/6 background were obtained from CLEA Japan. $Apc^{Min/+}$ mice (29) on a C57BL/6 genetic background were purchased from The Jackson Laboratory. Generation of germ-free (GF) mice or compound $Apc^{Min/+}$·*AhR*-disrupted mutant mice, carcinogenesis, and chemoprevention studies were performed as described in the *SI Materials and Methods*. All animal experiments were approved by the Saitama Cancer Center Animal Care and Use Committee.

Biochemical Analyses. Immunohistochemistry was performed on 4–5 μm sequential paraffin sections using the antibodies described. Total RNA was extracted from the intestines of $AhR^{+/+}$ or $AhR^{-/-}$ mice using an Isogen kit (Nippon Gene), and RT-PCR was performed using TaKaRa RNA PCR kits (Takara Shuzo). Cell culture and transfection assays were performed using standard methods. Protein stability analysis and in vitro ubiquitylation assay were performed as previously reported (6). Sequences of the siRNAs used in this study and HPLC analysis are described in *SI Materials and Methods*.

Statistical Analyses. Differences in survival in the mouse genotypes were analyzed using the Kaplan-Meyer method, and statistical analyses were performed with the log-rank test. We analyzed numeric data for statistical significance using the Student's *t* test. We considered $P < 0.05$ as significant.

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