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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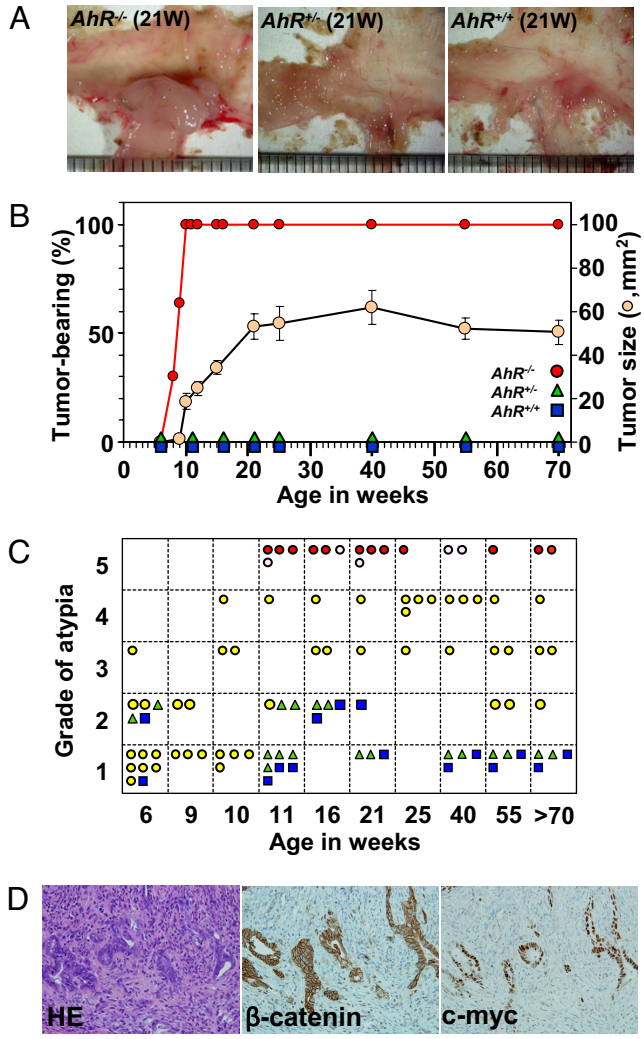

Fig. 1. Cecal tumor development in $AhR^{-/-}$ mice. (*A*) Representative profiles of colon tumors at the cecum in $AhR^{-/-}$ mice. (*B*) Relationship between the time course of macroscopic tumor incidence and tumor growth by age. Tumor size was estimated based on NIH images as shown by beige circles. 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.

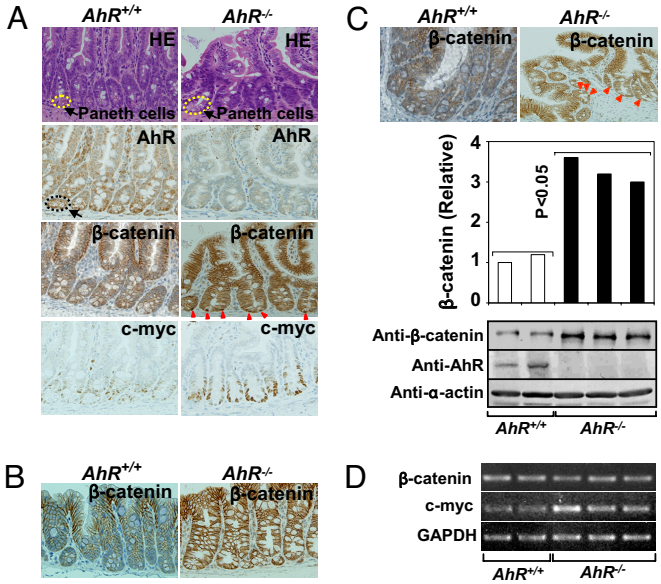

Fig. 2. Abnormal β-catenin accumulation in the intestines of $AhR^{-/-}$ mice. (*A*) H&E staining and immunohistochemical staining of mouse small intestines. Paneth cells were observed at the bottom of the crypts in the small intestine in both genotypes. Expression of AhR, β-catenin, and c-myc are shown. 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-

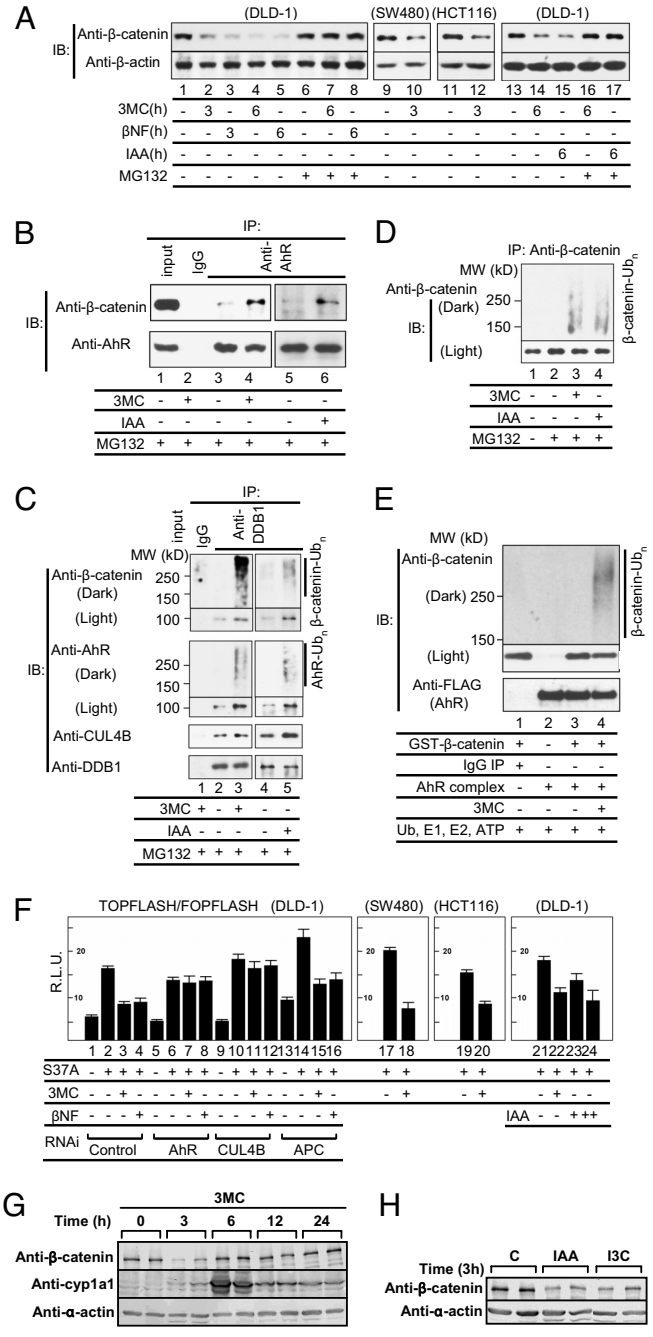

Fig. 3. Novel AhR ligand-dependent ubiquitylation and proteasomal degradation of β-catenin. (*A*) Activated AhR promotes proteasomal degradation of β-catenin. Cells were incubated as indicated with 3MC (1 μM), βNF (1 μM), or IAA (100 μM) in the presence or absence of the proteasome inhibitor MG132 (10 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

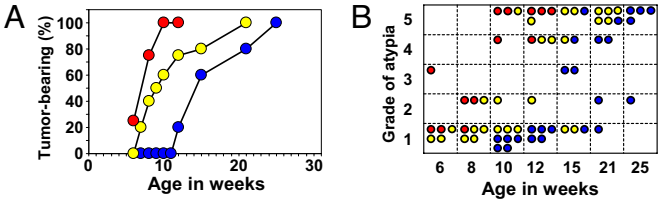

Fig. 4. Functional cooperation between Apc and AhR with regard to cecal tumor incidence. Macroscopic cecal tumor incidence by age in weeks (*A*) and summary of histological grades of atypia (*B*) that developed in $Apc^{Min/+}{\cdot}AhR^{+/+}$ (blue circles), $Apc^{Min/+}{\cdot}AhR^{+/-}$ (yellow circles),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

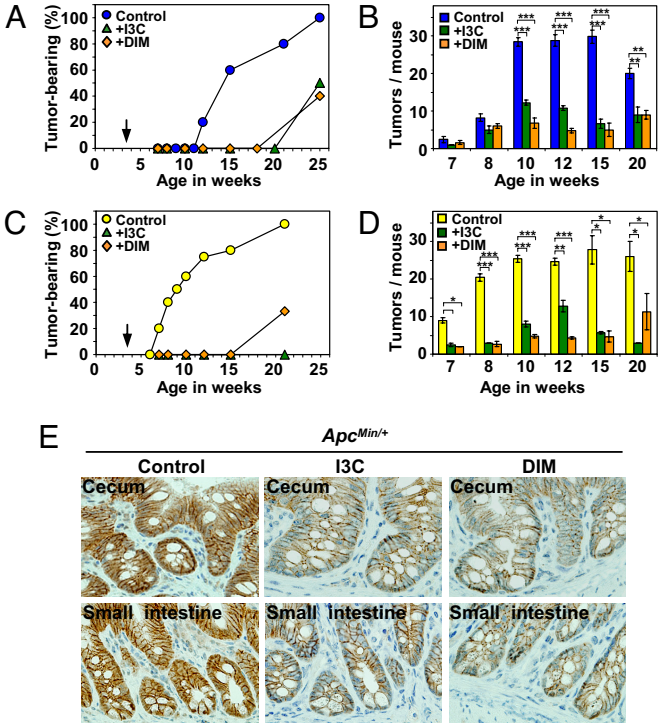

Fig. 5. Natural AhR ligands suppress intestinal carcinogenesis. Four to 5 mice were used in each group. Cecal carcinogenesis in the $Apc^{Min/+}$ (*A*) and $Apc^{Min/+}{\cdot}AhR^{+/-}$ (*C*) mice. Tumor development in mice fed a control diet (blue circles in *A* and yellow circles in *C*), 0.1% I3C-containing (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 $Apc^{Min/+}{\cdot}AhR^{+/-}$ (*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.

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 $Apc^{Min/+}{\cdot}AhR$-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 $Apc^{Min/+}{\cdot}AhR^{+/-}$ 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 $Apc^{Min/+}{\cdot}AhR^{-/-}$ 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 $Apc^{Min/+}{\cdot}AhR^{+/-}$ 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*).

To determine how compound $Apc^{Min/+}{\cdot}AhR$-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 $Apc^{Min/+}{\cdot}AhR^{-/-}$ and $Apc^{Min/+}{\cdot}AhR^{+/-}$ mice compared with $Apc^{Min/+}{\cdot}AhR^{+/+}$ 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 $Apc^{Min/+}{\cdot}AhR$-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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