J. Biochem. 2010;147(3):353–360 doi:10.1093/jb/mvp169

JB THE JOURNAL OF BIOCHEMISTRY

Suppression of AhR signaling pathway is associated with the down-regulation of UDP-glucuronosyltransferases during BBN-induced urinary bladder carcinogenesis in mice

Received August 18, 2009; accepted October 15, 2009; published online October 29, 2009

Katsuyuki Iida[1,2], Junsei Mimura[3], Ken Itoh[3,*], Chikara Ohyama[4], Yoshiaki Fujii-Kuriyama[5], Toru Shimazui[1], Hideyuki Akaza[1] and Masayuki Yamamoto[2,6,†]

[1]Department of Urology, Institute of Clinical Medicine; [2]Center for TARA and JST-ERATO Environmental Response Project, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577; [3]Department of Stress Response Science; [4]Department of Urology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562; [5]Center for TARA and JST-SORST, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, 305-8577; and [6]Departement of Medical Biochemistry, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan

*Ken Itoh, Department of Stress Response Science, Hirosaki University, Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan, Tel.: +81 172 39 5158, Fax: +81 172 39 5158, email: itohk@cc.hirosaki-u.ac.jp

†Masayuki Yamamoto, Department of Medical Biochemistry, Tohoku University, Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan, Tel.: +81 22 717 8088, Fax: +81 22 717 8090, email: masi@mail.tains.tohoku.ac.jp

Down-regulation of carcinogen detoxifying enzymes might be a critical factor in tumour formation by increasing the carcinogen concentration in the target organ. Previous reports revealed that the expression of *UGT1A* mRNA is either lost or decreased in certain human cancer tissues, including urinary bladder cancer. To elucidate this down-regulation mechanism, we used an *N*-nitrosobutyl (4-hydroxybutyl) amine (BBN)-induced mouse urinary bladder carcinogenesis model. Similar to human cancer, the expressions of *Ugt1a6*, *Ugt1a9* and total *Ugt1a* mRNA in the BBN-induced bladder cancer were markedly decreased compared with those of normal mice. BBN down-regulated the basal *Ugt1a* mRNA expression in a time-dependent manner and this was reversible in the first 2 weeks of BBN treatment. However, after 4 weeks of BBN treatment the repression became persistent after the cessation of BBN treatment. Aryl hydrocarbon receptor (AhR) regulates the constitutive and inducible expression of *Ugt1a* mRNA. We found that the constitutive *Ugt1a* mRNA expression is decreased in the bladder of AhR knockout (KO) mice. Furthermore, BBN-induced *Ugt1a* down-regulation was lost in AhR KO mice, and the canonical AhR target gene *Cyp1a1* was similarly down-regulated by BBN in the bladder. These results demonstrate that BBN repressed *Ugt1a* mRNA expression *via* suppression of AhR signaling pathway during BBN-induced carcinogenesis.

Keywords: UDP-glucuronosyltransferase/urinary bladder carcinogenesis/AhR.

Abbreviations: AhR, Aryl hydrocarbon receptor; BBN, *N*-nitrosobutyl (4-hydroxybutyl) amine; Cyp1a1, cytochrome P450, family 1, subfamily a, polypeptide 1; Gstp1, Glutathione *S*-transferase, placental isoform 1; 3-MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; UGT, UDP-glucuronosyltransferase.

Down-regulation of carcinogen detoxifying enzymes in target organs is likely to play a critical role in chemically induced carcinogenesis by leading to an increased local concentration of carcinogens (*1*–*5*). UDP-glucuronosyltransferases (UGTs) contribute to cellular detoxification through their glucuronidation of potentially toxic carcinogens and xenobiotics (*6*–*8*) and are thereby key players in the defense mechanism against chemical-induced carcinogenesis (*9*) and teratogenesis (*10*). Carcinogens, such as aromatic amines and polycyclic aromatic hydrocarbons (PAHs), are detoxified by conjugation with glutathione or UDP-glucuronic acid (*11*, *12*). The 19 human *UGT* cDNAs identified so far include nine *UGT1A* genes encoded by a single *UGT1A* locus on chromosome 2 and 10 individually encoded *UGT2* genes on chromosome 4 (*13*). *UGT1A* genes mainly catalyse the glucuronidation of aromatic amines and PAHs (*13*) and down-regulation of *UGT1A* gene expression has been associated with liver, digestive tract and urinary bladder tumors in human (*1*–*4*).

Each *Ugt1a* gene consists of a gene specific protein-encoding first exon and 2–5 common exons. Gene-specific promoters 5′ of the first exons control the specific expression of individual *Ugt1a* genes. Basal *Ugt1a* mRNA expression is regulated tissue-specifically by several transcription factors, such as hepatocyte nuclear factor 1 and CAAT-enhancer binding protein (*14*, *15*). On the other hand, the inducible expression of *Ugt1a* mRNA is regulated by several xenobiotic receptors including pregnenolone X receptor, constitutive androstane receptors, peroxisome proliferators-activated receptors, liver X receptor, aryl hydrocarbon receptor (AhR) and NF-E2-related factor 2 (Nrf2) (*16*–*20*).

The AhR regulates inducible expression of both phase 1 and phase 2 drug metabolizing enzyme genes (*21*, *22*). AhR is usually sequestered in the cytoplasm in association with Hsp90, p23 and XAP2/ARA9. Upon binding to halogenated or polycyclic aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene (3-MC),

respectively, AhR translocates into the nucleus, and dimerizes with its partner molecule, Arnt (AhR nuclear translocator), and then binds to its cognate enhancer sequences called XREs in the regulatory region of *Ugt1a*, *Cyp1a1* and glutathione S-transferase a1 (*Gsta*1) genes (*23–25*). Chen *et al.* (*26*) recently showed that the AhR controls *UGT1A* gene expression more profoundly than was previously anticipated from transgenic mouse studies with the human *UGT1A* locus. Although a distribution of XREs occurs immediately upstream of the *UGT1A1*, *UGT1A*6 and *UGT1A9* first exons, TCDD treatment activated the expression of all the human *UGT1A* genes in small and large intestines, suggesting that AhR regulates the transcriptional activity of the whole *UGT1A* locus (*26*). Nrf2 also plays important role in *Ugt1a* gene expression. Nrf2 is activated by electrophiles, such as oltipraz and sulforaphane, and coordinately regulates expression of phase 2 drug metabolizing enzymes including Ugt1a6 and Gsts (*27*, *28*).

Oral administration of *N*-nitrosobutyl(4-hydroxybutyl)amine (BBN) to rodents induced cancer specifically in urinary bladder (*29*). BBN itself is either metabolized by alcohol/aldehyde dehydrogenase-mediated oxidation to yield *N*-nitrosobutyl (3-carboxypropyl)amine (BCPN) or by UGT to form BBN-glucuronide conjugate which is easily excreted from bladder (*30*). If BBN or BCPN are metabolized through the α-hydroxylation pathway and chemically cleaved, their corresponding reactive species of alkylcarbonium ion are generated. Carbonium ion binds covalently to DNA and enhances carcinogenesis in uroepithelial cells (*31*, *32*). We previously demonstrated that *Ugt1a* mRNA expression is specifically down-regulated in the mouse urinary bladder after BBN exposure (*27*). This may reduce the local glucuronidation activity against carcinogens, allowing their accumulation and consequent promotion of DNA mutations. In this study, we used a BBN-induced urinary bladder carcinogenesis model to elucidate the mechanism of *Ugt1a* mRNA down-regulation during carcinogenesis.

Materials and methods

Animals and reagents

BBN was purchased from Tokyo Kasei (Tokyo, Japan) and 3-MC was bought from Sigma Chemical Co. (St Louis, MO, USA). Nrf2-deficient mice of an ICR/129SV background (*28*) were backcrossed for nine generations with C57BL/6J mice acquired from CLEA Japan (Tokyo, Japan). The $Ahr^{-/-}$ mice (*33*) have been backcrossed to C57BL/6J mice for seven generations. Mice were housed in stainless steel cages in an animal room maintained at 24 ± 2°C and with a 12 h light/dark cycle. Mice were fed a purified AIN-76A diet (Oriental MF; Oriental Yeast Co., Tokyo) and given water *ad libitum*. BBN was dissolved in tap water to the set concentrations and supplied *ad libitum* in dark bottles. 3-MC was dissolved in corn oil to a concentration of 4 mg/ml. Mice were treated with a single injection of 80 mg/kg of 3-MC intraperitoneally.

RNA blot analysis

After the experimental period, mice were analysed by autopsy. Total RNAs from whole urinary bladders or cancer lesions were extracted with Isogen (Nippon Gene, Toyama) according to the manufacturer's instructions. Total RNAs (10 μg) were separated by 1.5% agarose gel electrophoresis containing 2.2 M formaldehyde and transferred to a nylon membrane. Membranes were hybridized with the ^{32}P-labelled gene-specific cDNA probes and washed with the stringent washing conditions (final wash was done by 0.1% SSC, 0.1% SDS solution for 30 min at 55°C). cDNA probes for *Ugt1a6*, *Ugt1a9* and total *Ugt1a* have been described (*27*) and a cDNA probe for *Gstp1* was kindly provided by Dr Kimihiko Satoh.

Immunoblot analysis of mAhR

Total proteins from whole mouse bladders were homogenized on ice in 500 μl of RIPA lysis buffer [PBS (pH 7.4) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM sodium ortho-vanadate and 1 mM DTT]. After incubation for 30 min on ice, homogenates were centrifuged at maximum speed (15 000 r.p.m.) in a microcentrifuge for 30 min at 4°C. Protein concentrations of the supernatants were determined by Coomassie Plus Protein Assay Reagent (Pierce). Proteins were separated by 10% SDS–PAGE and electro-transferred onto an Immobile membrane (Millipore, Bedford, MA, USA). The membrane was incubated for 8 h at 4°C with anti-AhR antibody (SA-210; BIOMOL, PA, USA) diluted 1 : 200 in TBST (TBS plus 0.05% Tween-20). Immunoreactive proteins were detected using horseradish peroxidase-conjugated anti-IgG antibody and ECL (Amersham Biosciences).

Statistical analyses

Data were expressed as means ± SEM. The student's *t*-test was used to determine the statistical differences among groups. A $P < 0.05$ was interpreted as statistically significant.

Results

Reduced Ugt1a mRNA expression in BBN-induced mouse urinary bladder cancer

We previously found that BBN dose-dependently down-regulated *Ugt1a* mRNA expression after 2 weeks of BBN exposure in a manner independent of Nrf2 (*27*). In addition, decreased *UGT1A* mRNA expression has been reported in several human cancers. To examine whether *Ugt1a* mRNA expression is decreased in BBN-induced urinary bladder cancer, we examined the mRNA expressions of *total Ugt*1*a* and its representative isoforms *Ugt1a6* and *Ugt1a9* in BBN-induced urinary bladder carcinoma. For this purpose, mice were treated with 0.05% BBN for 12 weeks and *Ugt1a* mRNA expressions in the urinary bladder were analysed 10 weeks after ceasing treatment. Unlike untreated mice, those administered BBN suffered from apparent urinary bladder cancer, with bladder lesions that were nodular rather than papillary in shape. The expressions of *Ugt1a6*, *Ugt1a9* and *total Ugt*1*a* mRNAs in the urinary bladders of BBN-treated mice were significantly decreased by 87.7, 98.2 and 80.0%, respectively, compared to those of control mice (Fig. 1). On the other hand, *Gstp* mRNA expression was increased by 290.3%, indicating that the down-regulation of *Ugt1a* mRNA expression in the cancerous urinary bladder is specific amongst phase 2 genes.

Persistent down-regulation of *Ugt1a* mRNA after prolonged BBN exposure

In order to examine the mechanism of *Ugt1a* suppression in more detail, we analysed *Ugt1a* mRNA expression after exposure to 0.05% BBN for 0, 3, 7 and 14 days (Fig. 2A and B). The expressions of *Ugt1a6*, *Ugt1a9* and *total Ugt1a* mRNAs were down-regulated

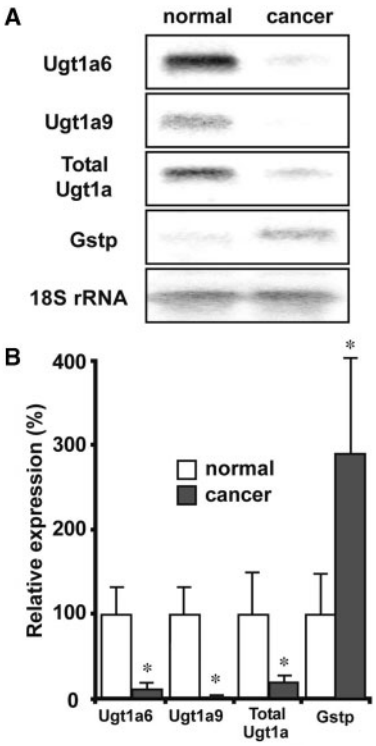

Fig. 1 The expressions of *Ugt1a6*, *Ugt1a9* and total *Ugt1a* mRNAs in BBN-induced urinary bladder cancer compared with those in normal urinary bladder. Mice were treated with vehicle (normal) or 0.05% BBN (cancer) for 12 weeks. Ten weeks after the cessation of BBN treatment, the expression of *Ugt1a* mRNA in the urinary bladder was analysed. The band intensities of the RNA blot (**A**) were quantified by densitometric analysis and the mRNA levels of phase 2 genes were normalized by *18S rRNA* levels. The expression level of each phase 2 gene in vehicle-treated mice was arbitrarily set to 100 and that of the BBN-treated mice is shown as a percentage of this control (**B**). The means from four mice are presented with the SEM ($n = 4$). *$P \leq 0.05$ compared with untreated wild-type mice.

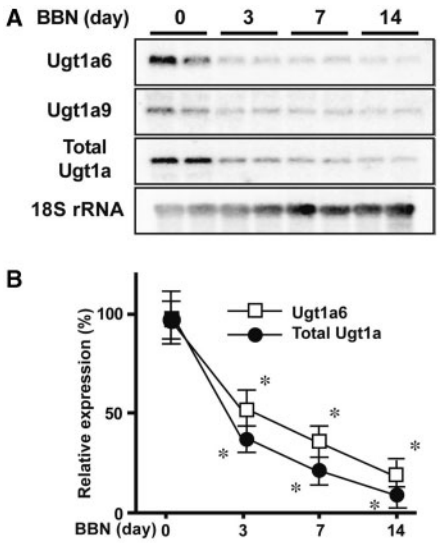

Fig. 2 Effect of BBN on *Ugt1a* mRNA expression in the urinary bladder. Mice were treated with 0.05% BBN or vehicle for 0, 3, 7 and 14 days, then urinary bladder RNA were extracted for RNA blot analysis. The band intensities of the RNA blot (**A**) were quantified by densitometric analysis and expression levels of phase 2 genes were normalized by 18*S rRNA* levels. The mRNA level of each *Ugt1a* gene in untreated mice was arbitrarily set to 100 and that of BBN-treated mouse is shown as a percentage of this control (**B**). The means from four mice are presented with the SEM ($n = 4$). *$P \leq 0.05$ compared with untreated wild-type mice.

by BBN treatment as early as 3 days and decreased in a time-dependent manner until 14 days.

It is known that gene expression is altered irreversibly after cancerous transformation. Therefore, we examined whether the down-regulation of *Ugt1a* mRNA expression is reversible after the cessation of BBN treatment. For this purpose, mice were treated with 0.05% BBN for 2 weeks and *Ugt1a* mRNA expression in the urinary bladder was examined 1, 2, 4, 7 and 14 days after the cessation of BBN treatment. Suppression of *Ugt1a6*, *Ugt1a9 and total Ugt1a* mRNA levels was maintained up to 4 days after the interruption of BBN treatment (Fig. 3A). However, 7 days after ceasing BBN administration, *Ugt1a* mRNA expression increased to a level exceeding those of untreated mice, demonstrating the reversibility of the process after a short period (*i.e.* 2 weeks) of carcinogen treatment.

But what of longer term BBN exposure? Mice were treated with 0.05% BBN for 2, 4, 6, 9 and 12 weeks and the expression of *Ugt1a6 and* total *Ugt1a* mRNAs was examined 7 days after discontinuing BBN treatment at each time point (Fig. 3B). We found that the expression of *Ugt1a6 and* total *Ugt1a* mRNAs did not fully recover after the mice were treated with BBN for 4 weeks or longer. Thus, chronic BBN exposure of greater than 2 weeks resulted in the persistent down-regulation of *Ugt1a* mRNA expression.

Ugt1a down-regulation by BBN treatment is not observed in the AhR KO mouse bladder

Ugt1a mRNA expression is regulated by multiple transcription factors, such as Nrf2 and the AhR (*16*, *17*). To elucidate if the Nrf2 or AhR pathway is responsible for the down-regulation of *Ugt1a* mRNA expression by BBN, we analyzed *Ugt1a* mRNA expression in the urinary bladders of $Nrf2^{-/-}$ and $Ahr^{-/-}$ mice after exposure to 0.05% BBN for 2 weeks (Fig. 4A and B). Importantly, the basal expressions of *Ugt1a6*, *Ugt1a9* and *total Ugt1a* mRNAs in $Ahr^{-/-}$ mice were significantly decreased by 80.0%, 92.8% and 83.8%, respectively, compared with those of wild-type mice. On the other hand, *Ugt1a6*, *Ugt1a9* and *total Ugt1a* mRNA expressions in $Nrf2^{-/-}$ mice were not significantly altered compared with those of wild-type mice. After BBN treatment, *Ugt1a* mRNA expression was significantly decreased by >80% in both $Nrf2^{-/-}$ and wild-type mice. However, in $Ahr^{-/-}$ mice, the constitutively low *Ugt1a* mRNA expression was not further reduced upon BBN exposure.

Further confirmation that the AhR indeed regulates *Ugt1a* mRNA expression in the urinary bladder was provided by treating both wild-type and $Ahr^{-/-}$ mice with the AhR activator 3-MC. The expressions of *Ugt1a* and *Cyp1a1* mRNAs were measured 48 h post-intraperitoneal injection of 3-MC (Fig. 4*C*). The expressions of *Ugt1a6*, *Ugt1a9*, *total Ugt1a* and *Cyp1a1* mRNAs in wild-type mice were significantly

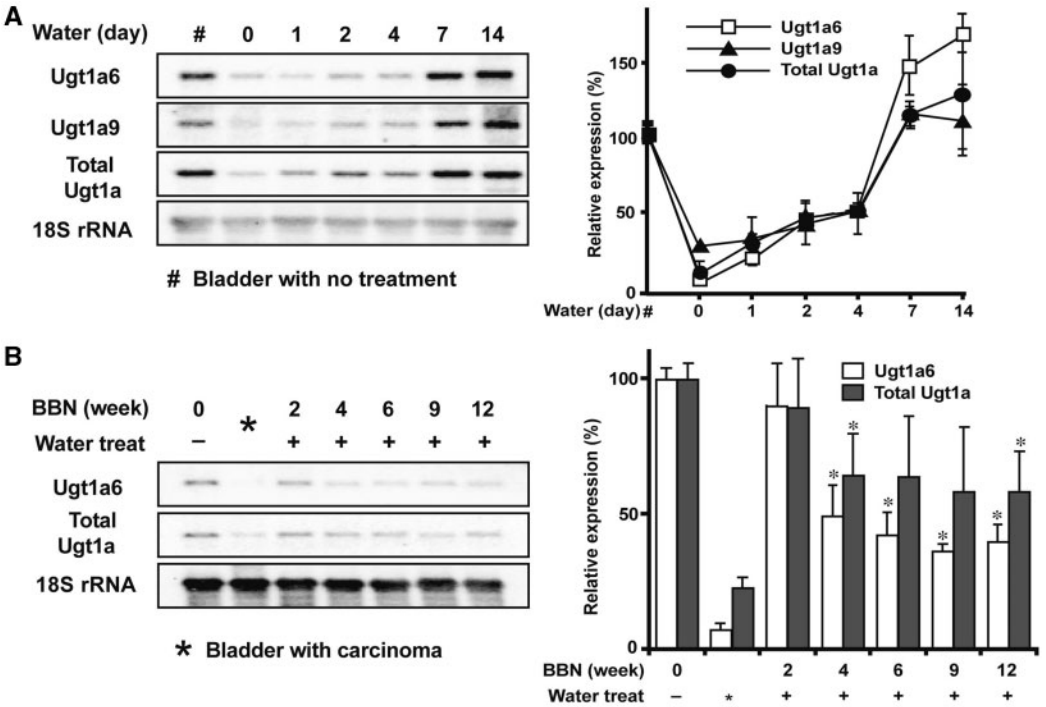

Fig. 3 Expression of *Ugt1a* mRNAs after the cessation of BBN treatment. (**A**) Mice were treated with 0.05% BBN for 2 weeks and total RNAs from the urinary bladders were examined for *Ugt1a* mRNA expression 1, 2, 4, 7 and 14 days after the cessation of BBN treatment. (**B**) Mice were treated with 0.05% BBN for 2, 4, 6, 9 and 12 weeks. Total RNAs from the urinary bladders were examined for *Ugt1a* mRNA expression 7 days after the cessation of BBN treatment at each time point. The band intensities of the RNA blots (left panels) were quantified by densitometry and the expression levels of *Ugt1a* mRNAs were normalized by *18S rRNA* levels. The expression level of *Ugt1a* mRNA in untreated mice was arbitrarily set to 100 and that of the BBN-treated mouse is shown as a percentage of this control (right panels). Values are presented as means ± SEM ($n=4$). *$P \leq 0.05$ compared with untreated wild-type mice.

increased by 157.7, 143.8, 194.0 and 1509.0%, respectively, after 3-MC treatment. However, the constitutive expression of these genes was decreased in $Ahr^{-/-}$ mice and induction by 3-MC was lost. These results clearly demonstrate a similar regulation of mRNA expression under the influence of the AhR between *Ugt1a* and *Cyp1a1* genes in the urinary bladder.

Expression of AhR protein and the activity of the AhR pathway after BBN treatment

To examine the effect of BBN on AhR activity, we measured the expression of AhR protein and the AhR target gene expression in the urinary bladder after 2 weeks of BBN treatment. Immunoblot analysis using whole urinary bladder extracts demonstrated that BBN treatment significantly increased AhR protein in a dose-dependent manner (Fig. 5A). Because RNA blot analysis showed that the *Ahr* mRNA level was not increased by BBN treatment (data not shown), AhR protein translation rate or stability may be increased by BBN treatment. We evaluated the AhR target gene expression after BBN treatment by measuring *Cyp1a1* mRNA expression by RNA blot analysis. Mice were treated with 0.01, 0.05 and 0.1% BBN for 2 weeks and the expression of *Cyp1a1* mRNA in urinary bladder was examined. The results revealed that BBN treatment decreased *Cyp1a1* mRNA expression. These results indicated that AhR signaling is suppressed by BBN treatment although AhR protein itself is increased by BBN (Fig. 5B).

AhR activity is repressed in BBN-induced cancerous tissue

Because AhR protein is increased but its activity may be suppressed by BBN, we next analyzed AhR protein level and *Cyp1a1* expression in cancerous tissue. Mice were administered BBN for 12 and then with water for 10 weeks following the stoppage of BBN treatment, *Cyp1a1* mRNA expression in the cancerous tissues was examined. RNA blot analysis demonstrated a decrease in *Cyp1a1* mRNA expression similar to the decrease in *Ugt1a* mRNA expression, indicating the persistent repression of AhR signaling in cancerous urinary bladder (Fig. 5C). Consistent with the results of Fig. 5A, the expression of AhR protein in whole bladder extract remained elevated even 10 weeks after ending BBN treatment (Fig. 5D). Thus, BBN suppresses AhR signaling pathway in cells, hence repressing *Ugt1a* mRNA expression during BBN-induced carcinogenesis.

Discussion

Our study established that *Ugt1a* mRNA expression is markedly decreased in BBN-induced urinary bladder cancer, with the decreased expression commencing during the early phase of continuous BBN administration. In *Ahr* KO mice, although the expression of

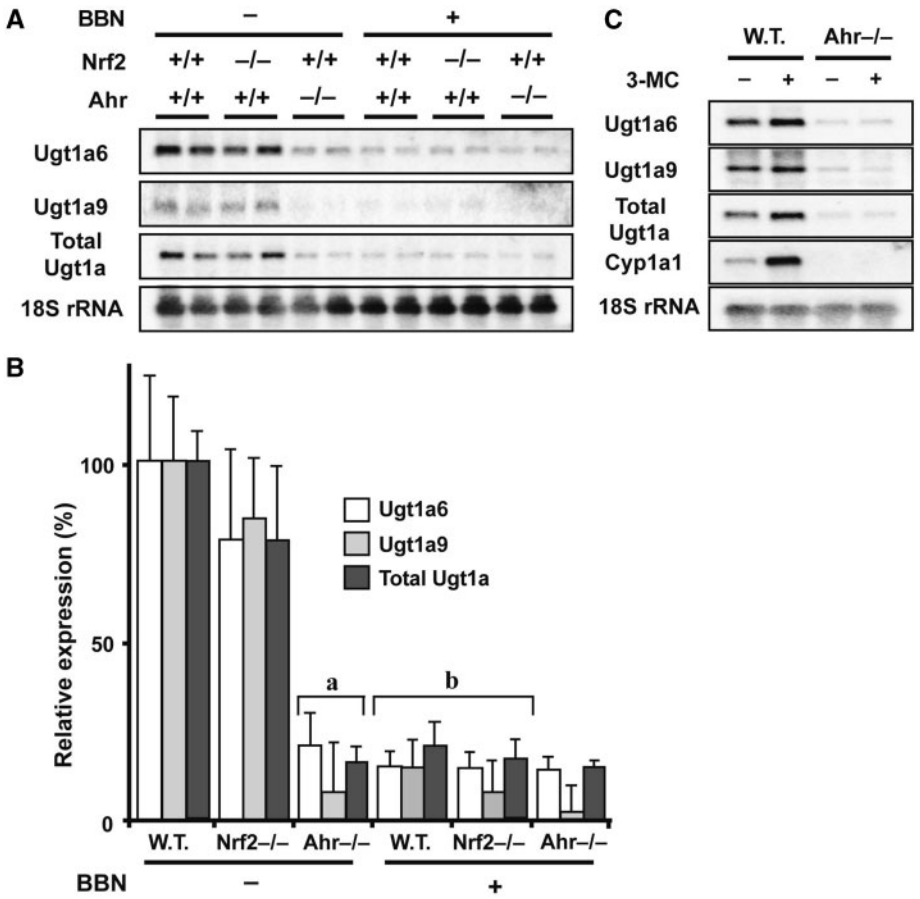

Fig. 4 Role of AhR on *Ugt1a* mRNA expression in the urinary bladder. (**A**) Effect of *Ahr* or *Nrf*2 gene deletion on *Ugt1a* mRNA expression. Wild-type, $Nrf2^{-/-}$ and $Ahr^{-/-}$ mice were treated with 0.05% BBN for 2 weeks and the expression levels of *Ugt1a* mRNAs in the urinary bladders were examined. (**B**) The expression level of *Ugt1a* mRNA in untreated wild-type mice was arbitrarily set to 100 and that of each mouse as indicated in the figure is shown as a percentage of this control. (**A**) $P \leq 0.05$ compared with untreated wild-type mice. (**B**) $P \leq 0.05$ compared with untreated mice of the same genotype. (**C**) Effect of 3-MC on *Ugt1a* mRNA expression in the urinary bladder. Wild-type and $Ahr^{-/-}$ mice were injected intraperitoneally with 80 mg/kg of 3-MC. After 48h, the expressions of *Ugt1a* mRNAs and the *Cyp1a1* mRNA were examined by RNA blot analysis.

Ugt1a mRNA was constitutively low, BBN treatment did not further suppress *Ugt1a* mRNA expression. BBN appears to down-regulate *Ugt1a* mRNA expression *via* the suppression of AhR-dependent signaling pathway. These results indicate that the potent carcinogen BBN facilitates carcinogenesis by repressing the expression of AhR-dependent detoxification genes.

Our current hypothesis of *Ugt1a* mRNA down-regulation during BBN-induced urinary bladder carcinogenesis is summarized in Fig. 6. Under normal conditions, when the urinary bladder is continually exposed to urine, the basal expression of *Ugt1a* mRNA is up-regulated by the AhR that is activated by urinary ligands such as indigos (*34*). BBN-treatment increases AhR protein level in the cells, but the *Ugt1a* mRNA expression is suppressed by BBN-treatment. After long BBN exposure, the down-regulation of *Ugt1a* mRNA becomes persistent and this may allow for the accumulation of carcinogen and consequently predispose the urinary bladder to carcinogenesis.

It is known that BBN is metabolized by the conjugation of glucuronic acid in rat (*30*). However, UGT isozymes responsible for BBN-glucuronidation are not clear at present. We previously demonstrated that Nrf2 activator oltipraz induce BBN-glucuronidation in an Nrf2-dependent manner in mouse liver (*27*). As Nrf2 regulates a battery of *Ugt1a* as well as *Ugt2b* mRNA expression, we surmise that either of these Ugt family members probably catalyse BBN glucuronidation in mice (*35*, *36*).

Several studies have clarified the mechanism of gene silencing in cancer. For example, methylation of the 5′ CpG island is thought to play an important role in the inactivation of tumor suppressor genes in cancer. The *GSTP1* gene is the major GST isoform expressed in normal human prostate and is silenced in the majority of prostate tumors by the hypermethylation of CpG islands in the 5′ regulatory region (*5*). Similarly, expression of *UGT1A* mRNA is also down-regulated in the early stages of human liver and biliary carcinogenesis, but the mechanism has not been elucidated (*1*). To determine the mechanism of down-regulation of *Ugt1a* mRNA, we evaluated methylation of the *Ugt1a6* gene promoter in mouse cancerous urinary bladder. However, we failed to detect methylation of CpG in the promoter between −1.2 kb and 0.1 kb (data not shown).

It is important to note that BBN does not repress *Ugt1a* mRNA expression in the liver. Since BBN is metabolized to its reactive species only in the urinary bladder, it might be the reactive metabolites of BBN that are repressing the AhR. The repression mechanism was examined using primary culture of urinary bladder epithelial cells, but we found that neither BBN nor BCPN down-regulated basal *Ugt1a* mRNA expression. This might reflect the fact that the AhR does not contribute to basal *Ugt1a* mRNA expression *in vitro*, because of the absence of its urinary ligands. In contrast to the urinary bladder, transcription factors other than the AhR may contribute to the basal expression of *Ugt1a* mRNA in the liver. If this is the case, even if BBN represses liver AhR, it would not repress the expression of *Ugt1a mRNA*. Clarification of these possibilities requires further study.

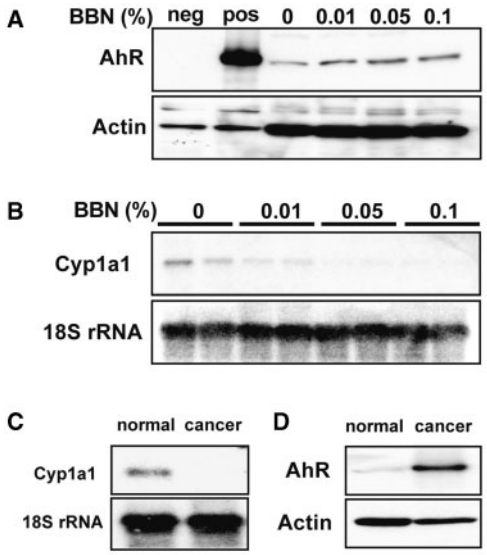

Fig. 5 The effect of BBN treatment on the AhR pathway. (**A**) Effect of BBN on the expression of the AhR in the urinary bladder. Mice were treated with vehicle, or 0.01%, 0.05%, 0.1% BBN for two weeks. Total cell extracts of the urinary bladders were examined by immunoblot analysis using anti-AhR antibody. neg, the total cell extract of COS-7 cells transfected with empty vector; posi, the total cell extract of COS-7 cells transfected with AhR expression vector. anti-β-actin antibody was used as a loading control. (**B**) Effect of BBN on *Cyp1a1* mRNA expression. Mice were treated with 0.01, 0.05 and 0.1% BBN or vehicle for two weeks and total RNAs from the urinary bladders were examined for the expression of *Cyp1a1* gene. (**C**) Down-regulation of *Cyp1a1* mRNA expression in urinary bladder cancer. Mice were treated with vehicle (normal) or 0.05% BBN (cancer) for 12 weeks and then with water for 10 weeks. *Cyp1a1* mRNA expression in the urinary bladder was examined by RNA blot analysis. (**D**) AhR protein expression in urinary bladder cancer. Mice were treated with vehicle (normal) or 0.05% BBN (cancer) for 12 weeks and then with water for 10 weeks. Total cell extracts of cancerous urinary bladders were examined by immunoblot analysis using anti-AhR antibody. Anti-β-actin antibody was used as a loading control.

It is known that ligand-coupled AhR is rapidly degraded by the ubiquitin–proteasome system (UPS) (*37*, *38*). Several studies reported that many transactivators possess rapid-turnover characteristics, indicating an association between transcriptional activation and protein degradation (*39*). Indeed, the proteolysis of some transcriptional activators by the UPS can stimulate transcription (*40*, *41*). However, since the proteasome inhibitors MG132 and lactacystin block AhR degradation by TCDD, but lead to an enhancement of AhR transcriptional activity, proteolysis *per se* may not be essential for the transactivation activity of the AhR (*37*, *38*). Recently, Chen *et al.* (*42*) demonstrated that MEK inhibitor U0126 stabilizes the AhR and increases its steady-state levels, but also diminishes the ability of the activated AhR to induce *Cyp1a1* in response to TCDD. In that paper, the authors speculated that Erk induces a conformational change that provokes both transcriptional activation and degradation of the AhR. Collectively, these studies argue that degradation of the cellular AhR is not necessarily a requirement for transcription, but a property of the ligand-activated form of the receptor. It is not clear whether the same phenomenon occurs in BBN-treated bladder, but an interesting possibility might be that BBN inhibits Erk in the urinary bladder.

The repression of *Ugt1a* mRNA expression becomes persistent after longer BBN exposure (i.e. after 4 weeks or longer exposure), and the inhibition of AhR signaling pathway and the repression of *Ugt1a* mRNA was also observed in the cancerous urinary bladder tissue, that is even 10 weeks after the cessation of BBN treatment (Fig. 5). Although it is not clear whether the inhibition mechanisms of AhR signaling pathway are identical in the non-cancerous and cancerous tissues, these results suggest that persistent

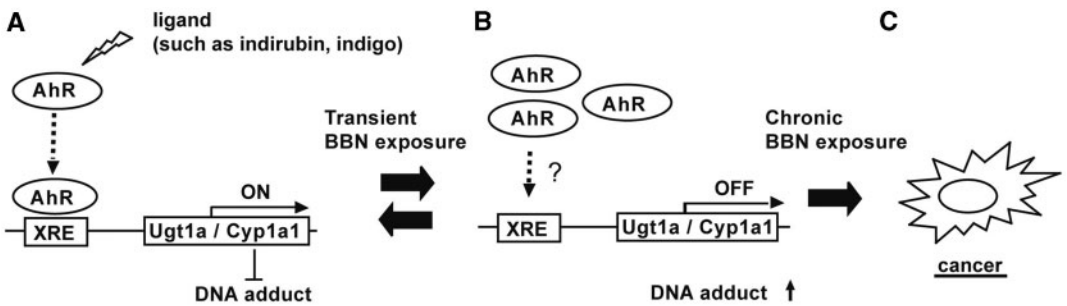

Fig. 6 The hypothetical mechanism of *Ugt1a* mRNA down-regulation during urinary bladder carcinogenesis. (**A**) Normally, the basal mRNA expressions of *Ugt1a* and *Cyp1a1* are mediated by the AhR that is constitutively activated by exposure to AhR ligands in the urine. (**B**) The AhR signaling pathway is repressed either by BBN or BBN metabolites by yet unidentified mechanisms, which down-regulates the mRNA expressions of *Ugt1a* and *Cyp1a1*. (**C**) If BBN exposure has been transient, the expression of *Ugt1a* mRNA swiftly recovers to normal levels. However, after a long exposure to BBN of >4 weeks, the down-regulation becomes persistent.

inhibition of *Ugt1a* expression *via* inhibition of AhR signaling pathway play an important role in carcinogenesis. Further studies will be required to find out how BBN or its metabolites inhibit AhR signaling pathway in the bladder.

This is the first report demonstrating that suppression of AhR signaling pathway is associated with the down-regulation of *Ugt1a* mRNA during urinary bladder carcinogenesis. Continuous exposure to carcinogen causes persistent repression of *Ugt1a* mRNA expression and may induce carcinogenesis. Conversely, we previously reported that Nrf2 activation antagonizes the BBN-induced repression of urinary *Ugt1a* mRNA expression (*27*). Thus, Nrf2 activation by dietary anticarcinogenic vegetables and fruits not only induces phase 2 expression, but also counteracts carcinogen-mediated repression of *Ugt1a* mRNA expression, thereby protecting the urinary bladder from carcinogenesis.

Acknowledgements

We thank Drs Atsushi Maruyama, Nobuhiko Harada and Tania O'Connor for their help and critical reading of the manuscript.

Funding

This work was supported in part by grants from JST-ERATO, JSPS, the Ministry of Education, Science, Sports and Technology, the Ministry of Health, Labor and Welfare, and the Naito Foundation.

Conflict of interest

None declared.

References

1. Strassburg, C.P., Manns, M.P., and Tukey, R.H. (1997) Differential down-regulation of the UDP-glucuronosyltransferase 1A locus is an early event in human liver and biliary cancer. *Cancer Res.* **57**, 2979–2985
2. Strassburg, C.P., Nguyen, N., Manns, M.P., and Tukey, R.H. (1998) Polymorphic expression of the UDP-glucuronosyltransferase UGT1A gene locus in human gastric epithelium. *Mol. Pharmacol.* **54**, 647–654
3. Giuliani, L., Gazzaniga, P., Caporuscio, F., Ciotti, M., Frati, L., and Agliano, A.M. (2001) Can down-regulation of UDP-glucuronosyltransferases in the urinary bladder tissue impact the risk of chemical carcinogenesis? *Int. J. Cancer* **91**, 141–143
4. Giuliani, L., Ciotti, M., Stoppacciaro, A., Pasquini, A., Silvestri, I., De Matteis, A., Frati, L., and Aglianò, A.M. (2005) UDP-glucuronosyltransferases 1A expression in human urinary bladder and colon cancer by immunohistochemistry. *Oncol. Rep.* **13**, 185–191
5. Nelson, C.P., Kidd, L.C., Sauvageot, J., Isaacs, W.B., De Marzo, A.M., Groopman, J.D., Nelson, W.G., and Kensler, T.W. (2001) Protection against 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]-pyridine cytotoxicity and DNA adduct formation in human prostate by glutathione S-transferase P1. *Cancer Res.* **61**, 103–109
6. Dutton, G.J. (1980) *Glucuronidation of Drugs and Other Compounds*. CRC Press, Boca Raton, FL
7. Burchell, B. and Coughtrie, M.W. (1989) UDP-glucuronosyltransferases. *Pharmacol. Ther.* **43**, 261–289
8. Burchell, B., Nebert, D.W., Nelson, D.R., Bock, K.W., Iyanagi, T., Jansen, P.L., Lancet, D., Mulder, G.J., Chowdhury, J.R., Siest, G., Tephly, T.R., and Mackenzie, P.I. (1991) The UDP glucuronosyltransferase gene superfamily: suggested nomenclature based on evolutionary divergence. *DNA Cell Biol.* **10**, 487–494
9. Vienneau, D.S., DeBoni, U., and Wells, P.G. (1995) Potential genoprotective role for UDP-glucuronosyltransferases in chemical carcinogenesis: initiation of micronuclei by benzo(a)pyrene and benzo(e)-pyrene in UDP-glucuronosyltransferase-deficient cultured rat skin fibroblasts. *Cancer Res.* **55**, 1045–1051
10. Kim, P.M., Winn, L.M., Parman, T., and Wells, P.G. (1997) UDP-glucuronosyltransferase-mediated protection against in vitro DNA oxidation and micronucleus formation initiated by phenytoin and its embryotoxic metabolite 5-(p-hydroxyphenyl)-5-phenylhydantoin. *J. Pharmacol. Exp. Ther.* **280**, 200–209
11. Ciotti, M., Lakshmi, V.M., Basu, N., Davis, B.B., Owens, I.S., and Zenser, T.V. (1999) Glucuronidation of benzidine and its metabolites by cDNA-expressed human UDP-glucuronosyltransferases and pH stability of glucuronides. *Carcinogenesis* **20**, 1963–1969
12. Filiadis, I. and Hrouda, D. (2000) Genetic factors in chemically-induced transitional cell bladder cancer. *BJU Int.* **86**, 794–801
13. Tukey, R.H. and Strassburg, C.P. (2000) Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu. Rev. Pharmacol. Toxicol.* **40**, 581–616
14. Auyeung, D.J., Kessler, F.K., and Ritter, J.K. (2003) Differential regulation of alternate UDP-glucuronosyltransferase 1A6 gene promoters by hepatic nuclear factor-1. *Toxicol. Appl. Pharmacol.* **191**, 156–166
15. Lee, Y.H., Sauer, B., Johnson, P.F., and Gonzalez, F.J. (1997) Disruption of the c/ebp alpha gene in adult mouse liver. *Mol. Cell. Biol.* **17**, 6014–6022
16. Chen, C., Staudinger, J.L., and Klaassen, C.D. (2003) Nuclear receptor, pregname X receptor, is required for induction of UDP-glucuronosyltranferases in mouse liver by pregnenolone-16 alpha-carbonitrile. *Drug Metab. Dispos.* **31**, 908–915
17. Xie, W., Yeuh, M.F., Radominska-Pandya, A., Saini, S.P., Negishi, Y., Bottroff, B.S., Cabrera, G.Y., Tukey, R.H., and Evans, R.M. (2003) Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. *Proc. Natl Acad. Sci. USA* **100**, 4150–4155
18. Verreault, M., Senekeo-Effenberger, K., Trottier, J., Bonzo, J.A., Bélanger, J., Kaeding, J., Staels, B., Caron, P., Tukey, R.H., and Barbier, O. (2006) The liver X-receptor alpha controls hepatic expression of the human bile acid-glucuronidating UGT1A3 enzyme in human cells and transgenic mice. *Hepatology* **44**, 368–378
19. Enomoto, A., Itoh, K., Nagayoshi, E., Haruta, J., Kimura, T., O'Connor, T., Harada, T., and Yamamoto, M. (2001) High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. *Toxicol. Sci.* **59**, 169–177
20. Auyeung, D.J., Kessler, F.K., and Ritter, J.K. (2003) Mechanism of rat UDP-glucuronosyltransferase 1A6 induction by oltipraz: evidence for a contribution of the Aryl hydrocarbon receptor pathway. *Mol. Pharmacol.* **63**, 119–127

21. Perdew, G.H. (1988) Association of the Ah receptor with the 90-kDa heat shock protein. *J. Biol. Chem.* **263**, 13802–13805
22. Pongratz, I., Mason, G.G., and Poellinger, L. (1992) Dual roles of the 90-kDa heat shock protein hsp90 in modulating functional activities of the dioxin receptor. Evidence that the dioxin receptor functionally belongs to a subclass of nuclear receptors which require hsp90 both for ligand binding activity and repression of intrinsic DNA binding activity. *J. Biol. Chem.* **267**, 13728–13734
23. Metz, R.P. and Ritter, J.K. (1998) Transcriptional activation of the UDP-glucuronosyltransferase 1A7 gene in rat liver by aryl hydrocarbon receptor ligands and oltipraz. *J. Biol. Chem.* **273**, 5607–5614
24. Fujisawa-Sehara, A., Sogawa, K., Yamane, M., and Fujii-Kuriyama, Y. (1987) Characterization of xenobiotic responsive elements upstream from the drug-metabolizing cytochrome P-450c gene: a similarity to glucocorticoid regulatory elements. *Nucleic Acids Res.* **15**, 4179–4191
25. Nebert, D.W., Roe, A.L., Dieter, M.Z., Solis, W.A., Yang, Y., and Dalton, T.P. (2000) Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochem. Pharmacol.* **59**, 65–85
26. Chen, S., Beaton, D., Nguyen, N., Senekeo-Effenberger, K., Brace-Sinnokrak, E., Argikar, U., Remmel, R.P., Trottier, J., Barbier, O., Ritter, J.K., and Tukey, R.H. (2005) Tissue-specific, inducible, and hormonal control of the human UDP-glucuronosyltransferase-1 (UGT1) locus. *J. Biol. Chem.* **280**, 37547–37557
27. Iida, K., Itoh, K., Kumagai, Y., Oyasu, R., Hattori, K., Kawai, K., Shimazui, T., Akaza, H., and Yamamoto, M. (2004) Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Res.* **64**, 6424–6431
28. Itoh, K., Chiba, T., Takahashi, S., ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* **236**, 313–322
29. Ito, N., Hiasa, Y., Tamai, A., Okajima, E., and Kitamura, H. (1969) Histogenesis of urinary bladder tumors induced by *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine in rats. *Gann* **60**, 401–410
30. Bonfanti, M., Magagnotti, C., Bonati, M., Fanelli, R., and Airoldi, L. (1988) Pharmacokinetic profile and metabolism of *N*-nitrosobutyl-(4-hydroxybutyl)amine in rats. *Cancer Res.* **48**, 3666–3669
31. Airoldi, L., Magagnotti, C., Bonfanti, M., and Fanelli, R. (1990) Alpha-oxidative metabolism of the bladder carcinogens N-nitrosobutyl(4-hydroxybutyl)-amine and N-nitrosobutyl(3-carboxypropyl)amine within the rat isolated bladder. *Carcinogenesis* **11**, 1437–1440
32. Hashimoto, Y. and Kitagawa, H.S. (1974) In vitro neoplastic transformation of epithelial cells of rat urinary bladder by nitrosamines. *Nature* **252**, 497–499
33. Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T.N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M., and Fujii-Kuriyama, Y. (1997) Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. *Genes Cells* **2**, 645–654
34. Adachi, J., Mori, Y., Matsui, S., Takigami, H., Fujino, J., Kitagawa, H., Miller, C.A. 3rd, Kato, T., Saeki, K., and Matsuda, T. (2001) Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. *J. Biol. Chem.* **276**, 31475–31478
35. Buckley, D.B. and Klaassen, C.D. (2009) Induction of mouse UDP-glucuronosyltransferase mRNA expression in liver and intestine by activators of aryl-hydrocarbon receptor, constitutive androstane receptor, pregnane X receptor, peroxisome proliferator-activated receptor alpha, and nuclear factor erythroid 2-related factor 2. *Drug Metab. Dispos.* **37**, 847–856
36. Reisman, S.A., Yeager, R.L., Yamamoto, M., and Klaassen, C.D. (2009) Increased Nrf2 activation in livers from Keap1-knockdown mice increases expression of cytoprotective genes that detoxify electrophiles more than those that detoxify reactive oxygen species. *Toxicol. Sci.* **108**, 35–47
37. Pollenz, R.S. (2002) The mechanism of AH receptor protein down-regulation (degradation) and its impact on receptor-mediated gene regulation. *Chemic-Biol. Interact.* **141**, 41–61
38. Pollenz, R.S. (2007) Specific blockage of ligand-induced degradation of the Ah receptor by proteasome but not calpain inhibitors in cell culture lines from different species. *Biochem. Pharmacol.* **74**, 131–143
39. Salghetti, S.E., Muratani, M., Wijnen, H., Futcher, B., and Tansey, W.P. (2000) Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. *Proc. Natl Acad. Sci. USA* **97**, 3118–3123
40. Muratani, M., Kung, C., Shokat, K.M., and Tansey, W.P. (2005) The F box protein Dsg1/Mdm30 is a transcriptional coactivator that stimulates Gal4 turnover and cotranscriptional mRNA processing. *Cell* **120**, 887–899
41. Lipford, J.R., Smith, G.T., Chi, Y., and Deshaies, R.J. (2005) A putative stimulatory role for activator turnover in gene expression. *Nature* **438**, 113–116
42. Chen, S., Operana, T., Bonzo, J., Nguyen, N., and Turkey, R.H. (2005) ERK kinase inhibition stabilizes the aryl hydrocarbon receptor. *J. Biol. Chem.* **280**, 4350–4359