Aryl Sulfonamides Induce Degradation of Aryl Hydrocarbon Receptor Nuclear Translocator through CRL4[DCAF15] E3 Ligase

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Aryl hydrocarbon receptor nuclear translocator (ARNT) plays an essential role in maintaining cellular homeostasis in response to environmental stress. Under conditions of hypoxia or xenobiotic exposure, ARNT regulates the subset of genes involved in adaptive responses, by forming heterodimers with hypoxia-inducible transcription factors (HIF1α and HIF2α) or aryl hydrocarbon receptor (AhR). Here, we have shown that ARNT interacts with DDB1 and CUL4-associated factor 15 (DCAF15), and the aryl sulfonamides, indisulam and E7820, induce its proteasomal degradation through Cullin-RING finger ligase 4 containing DCAF15 (CRL4[DCAF15]) E3 ligase. Moreover, the two known neo-substrates of aryl sulfonamide, RNA-binding motif protein 39 (RBM39) and RNA-binding motif protein 23 (RBM23), are not required for ARNT degradation. In line with this finding, aryl sulfonamides inhibited the transcriptional activities of HIFs and AhR associated with ARNT. Our results collectively support novel regulatory roles of aryl sulfonamides in both hypoxic and xenobiotic responses.

Keywords: aryl hydrocarbon receptor nuclear translocator, aryl sulfonamide, cullin ring ubiquitin ligase, DDB1 and CUL4 associated factor 15, E7820, indisulam

INTRODUCTION

Aryl hydrocarbon receptor nuclear translocator (ARNT), also known as HIF1β, is a transcription factor participating in the hypoxia-inducible factor (HIF) and xenobiotic metabolism pathways. ARNT plays a critical role in mediating signaling pathways and maintaining cellular homeostasis through regulating gene expression patterns in response to hypoxia and xenobiotic exposure (Gradin et al., 1996; McIntosh et al., 2010; Sogawa et al., 1995; Vorrink and Domann, 2014). Under normal physiological conditions, the aryl hydrocarbon receptor (AhR) in the cytoplasm is inactivated via interactions with HSP90. Once AhR recognizes xenobiotics, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), bound HSP90 dissociates and AhR forms a heterodimer with ARNT. The ARNT/AhR heterodimer translocates into the nucleus and occupies a specific enhancer element, xenobiotic response element (XRE), in the 5' upstream region of target genes involved in metabolism of xenobiotics, thereby regulating their

expression (Klinge et al., 1999; Kobayashi et al., 1997). On the other hand, HIF1α, an essential activator that functions in cellular adaptation to hypoxia, is degraded by the CRL2^{VHL} E3 ligase complex in normoxia. Under hypoxic stress conditions, HIF1α rapidly accumulates, leading to its stabilization, and translocates to the nucleus where it forms a heterodimer with ARNT. The HIF1α/ARNT heterodimer localizes to its consensus DNA element, the hypoxia response element (HRE), with the transcriptional co-activator, CBP/p300, to regulate numerous target genes involved in proliferation, metastasis, and metabolism (Weir et al., 2011; Wolff et al., 2013; Wood et al., 1996). As cancer cells rapidly proliferate and consume cellular oxygen, the oxygen concentration within tumors is significantly lower than that in normal tissue. Chronic hypoxia promotes uncontrolled cell growth and metabolic changes in cancer. Therefore, interventions targeting the HIF pathway should provide effective options for cancer therapy, in particular, hypoxia-addicted malignant tumors (Maltepe et al., 1997; Mandl and Depping, 2017; Semenza, 2003). However, small molecules that can efficiently target the HIF pathway are yet to be identified.

Cullin-RING finger ligases (CRL), the largest family of ubiquitin ligases, are composed of a cullin protein (CUL1, 2, 3, 4A, 4B, 5r, and 7), RING finger protein (Rbx1), a substrate recognition subunit, and generally an adaptor protein that links the substrate recognition subunit to the complex (Bosu and Kipreos, 2008). DDB1 and CUL4-associated factors (DCAFs) are a family of substrate recognition subunits forming the CRL4 complex with CUL4A/B, RBX1, and DDB1 (Hannah and Zhou, 2015). Several DCAFs possess the WD40 repeat domain crucial for binding to DDB1 and additional domain that determines the selectivity of substrates for the CRL4 complex (Lee and Zhou, 2007). DCAF15 has received considerable attention since elucidation of its relationship with aryl sulfonamide. Aryl sulfonamide is reported to exert anti-cancer activity by inducing G1 cell cycle arrest, leading to cell death (Assi et al., 2018; Owa et al., 1999; Van Kesteren et al., 2002). Several subsequent studies have demonstrated that aryl sulfonamide functions as molecular glue, facilitating ternary complex formation with DCAF15 and RNA-binding proteins (RBM39 and RBM23), thereby inducing their degradation (Han et al., 2017; Ting et al., 2019; Uehara et al., 2017). RBM39 not only regulates global alternative splicing but also acts as a transcriptional coactivator for ERα/β and AP-1 (Jung et al., 2002; Kang et al., 2015; Mai et al., 2016). RBM degradation by aryl sulfonamides induces anti-proliferative effects in various cancer types (Haddad et al., 2004; Han et al., 2017; Hsiehchen et al., 2020; Wang et al., 2019). Therefore, identification of novel DCAF15-interacting proteins that modulate its activity for therapeutic purposes is of significant research interest.

Based on the human interactome database, we identified ARNT as an interacting protein of DCAF15 in the present study. Furthermore, we showed that aryl sulfonamides promote proteasomal degradation of ARNT through CRL4^{DCAF15} and inhibit the transcriptional activities of HIF/ARNT and AhR/ANRT in response to hypoxia and xenobiotics, respectively. Our results highlight a novel link between ARNT and DCAF15, extending the potential therapeutic application of aryl sulfonamides.

MATERIALS AND METHODS

Antibodies and reagents
The following antibodies were used: anti-ARNT rabbit polyclonal (#5537; Cell Signaling Technology, USA), anti-RBM39 mouse monoclonal (sc-376531; Santa Cruz Biotechnology, USA), anti-RBM23 rabbit polyclonal (PA5-52060; Invitrogen, USA), anti-AhR mouse monoclonal (sc-133088; Santa Cruz Biotechnology), anti-DDB1 rabbit polyclonal (ab109027; Abcam, UK), anti-CUL4a rabbit polyclonal (ab72548; Abcam), anti-Myc mouse monoclonal (sc-40; Santa Cruz Biotechnology), and anti-HA rabbit polyclonal antibody (sc-805; Santa Cruz Biotechnology). Anti-DCAF15 antibody was developed using purified DCAF15 peptide (260-600 amino acids) of human DCAF15 as an immunogen in rabbits (AbClone, Korea).

The reagents used included MLN4924 (505477; Calbiochem, USA), Bortezomib (S1013; Selleckchem, USA), Indisulam (SML1225; Sigma-Aldrich, USA), and E7820 (25502; Cayman Chemical Company, USA).

Cell culture
HEK293T, HepG2, and HCT116 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml; Gibco) in a humidified atmosphere of 5% CO_2 in air at 37°C.

Plasmid constructs and transfection
The human gene encoding full-length DCAF15 was purchased from Novoprolabs in China (703683-1, NM_138353). Full-length and truncated DCAF15 were subcloned into pcDNA3.1 N-terminal FLAG-tagged or C-terminal HA-tagged vector. The plasmid encoding full-length ARNT was provided by Addgene in USA (87213, NM_001668). Full-length and truncated ARNTs were cloned into pCMV N-terminal MYC tagged vector.

Cells were transfected with various plasmids using X-treamGENE HP DNA Transfection Reagent (Roche, Switherzerland) according to the manufacturer's instructions.

RNA interference
Negative control siRNA (SN-1003) was obtained from Bioneer (Korea), and siRNAs against RBM39 (L-011965-00) and RBM23 (E-016689-00) from Dharmacon (USA). For RNA interference, cells were transfected with various siRNAs using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen), according to the manufacturer's instructions. After 48 to 72 h of incubation, cells were harvested via centrifugation at 5,000g for 10 min and analyzed.

Lentivirus production and transduction
For producing lentiviral particles, HEK293T cells were transfected with a lentiviral plasmid, pLKO.1, containing DCAF15 3'UTR-targeting shRNA (TRCN0000263998; Sigma-Aldrich) or non-targeted shRNA (SHC202; Sigma-Aldrich), psPAX2, and pMD2.G at a ratio of 1:0.75:0.25. After 72 h transfec-

tion, culture supernatants containing lentivirus were collected and filtered through a 0.45 μm sterile filter to remove cell debris. For generating HEK293T stably expressing DCAF15 or negative control shRNA, cells were treated with the viral supernatant and hexadimethrine bromide (8 μg/ml, H9268; Sigma-Aldrich) and incubated for 48 h. Following viral transduction, cells were selected with 2 μg/ml puromycin.

Immunoprecipitation and immunoblotting
Cells were lysed using lysis buffer (50 mM HEPES KOH pH7.4, 40 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM Na pyrophosphate, 10 mM Na β-glycerophosphate, 50 mM NaF, 1 mM NaVO$_4$, and 1% Triton X-100) containing protease inhibitor (05056489001; Roche). Lysates were clarified by centrifugation at 13,000g and 4°C for 10 min. The supernatant fraction was quantified and incubated with antibody and beads at 4°C for 3 h or overnight. Beads were washed twice in lysis buffer, twice in wash buffer (50 mM HEPES KOH pH7.4, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM Na pyrophosphate, 10 mM Na β-glycerophosphate, 50 mM NaF, 1 mM NaVO$_4$, 1% Triton X-100, and protease inhibitor) and once in lysis buffer. In co-IP experiments, beads were washed three times in lysis buffer. Elution was performed using 2X Laemmli buffer and samples separated via SDS-PAGE. After transfer to nitrocellulose membrane, immunoblotting was performed using the indicated antibodies.

Real-time polymerase chain reaction (PCR) analysis
Total RNA was extracted from siRNA- or chemical-treated cells using the RNeasy Plus Mini Kit (Qiagen, Germany) and 1 μg total RNA reverse-transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Real-time PCR was performed with Solg 2X Real-Time PCR Smart mix (SRH71-M40H; SolGent, Korea) and gene-specific primers using GAPDH for normalization. The following primers were employed for real-time PCR: DCAF15 (5'-GAGGTCTGCCCAGAAAC-CAA-3' and 5'-CTCAGTCAGGTCGCCTACA-C-3'), GAPDH (5'-GGTATCGTGGAAGGACTCATGA-C-3' and 5'-ATGCCAGT-GAGCTTCCCGTTCAGC-3'), GLUT1 (5'-AACTCTTCAGCC-AG-GGACCT-3' and 5'-CACAGTGAAGATGATGAAGA-3'), LDHA (5'-TTGGTCCAGC-GTAACGTGAA-3' and 5'-CCAGGATGTG-TAGCCTTTGA-3'), VEGF (5'-CTACCTCCA-CCATGCCAAGT-3' and 5'-GCAGTAGCTGCGCTCATAGA-3'), TGFα (5'-CTGC-CCG-CCCGCCCGTAAAA-3' and 5'-C-CGCATGCTCACAGC-GTGCA-3'), and β-actin (5'-C-ATGTACGTTGCTATCCAGGC-3' and 5'-CTCCTTAATGTCACGCACGAT-3').

Luciferase assay
HEK293 cells were co-transfected with HRE luciferase reporter plasmid and pRL-TK. After 24 h, cells were treated with indisulam (10 μM) and incubated under normoxia or hypoxia for an additional 12 h. HepG2 cells were co-transfected with XRE luciferase reporter plasmid and pRL-TK. After 24 h, cells were treated with indisulam (10 μM) and/or TCDD (10 nM) for 12 h. Luciferase activities were measured with Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions.

RESULTS

DCAF15 interacts with ARNT
Affinity purification-mass spectrometry studies on the human interactome have disclosed potential associations of DCAF15 with ARNT (Huttlin et al., 2015; 2017). To establish DCAF15-ARNT interactions, co-immunoprecipitation experiments were performed in the current study. Overexpressed HA-tagged DCAF15 (DCAF15-HA) and MYC-tagged ARNT (MYC-ARNT) reciprocally interacted with each other in HEK293T cells (Fig. 1A). It was of note that two bands (~70 and ~55 kDa) were detected for DCAF15-HA, suggesting potential cleavage of DCAF15. Since endogenous DCAF15 is not recognized by all commercially available antibodies, we failed to validate this interaction at endogenous levels. However, upon overexpression of DCAF15, endogenous ARNT clearly co-immunoprecipitated with DCAF15 (Fig. 1B). Next, we attempted to determine the specific regions of DCAF15 and ARNT involved in this interaction. For this purpose, MYC-ARNT was expressed with either an N-terminal domain (NTD; 1-300 aa) or C-terminal domain (CTD; 301-600 aa) fragment of DCAF15. Co-immunoprecipitation data showed binding of CTD of DCAF15 to ARNT (Fig. 1C). ARNT possesses four distinct domains (bHLH, PAS1, PAS2, and PAC). Subsequently, plasmids expressing serial deletion mutants of ARNT were generated and examined for interactions with DCAF15. Notably, all fragments incorporating the bHLH domain bound DCAF15 but not those without bHLH (Fig. 1D), clearly supporting the requirement of this region for ARNT-DCAF15 interactions. Notably, the bHLH domain is reported to be essential for heterodimerization with HIF1α or AhR (Reisz-Porszasz et al., 1994; Rezvani et al., 2011; Weir et al., 2011).

ARNT is not an endogenous substrate of CRL4^{DCAF15}
While DCAF15 has been characterized as a substrate receptor of Cullin-RING finger ligase 4 (CRL4) complex, possibly governing substrate specificity (Lee and Zhou, 2007), its endogenous substrates have not been identified to date. Based on the specific interactions between DCAF15 and ARNT, we examined the possibility that ARNT is a potential substrate of CRL4^{DCAF15}. Co-immunoprecipitation experiments revealed associations of ARNT with CUL4a and DDB1, common subunits of the CRL4 complex (Fig. 2A). To further establish whether DCAF15 regulates ARNT stability, we generated DCAF15-depleted cell lines with the aid of specific shRNAs (shDCAF15) and examined endogenous ARNT levels via western blot. Our data showed that endogenous ARNT levels were indistinguishable in both shControl and shDCAF15 cells (Fig. 2B). Given that ARNT regulates hypoxic responses via heterodimerization with HIF1α or HIF2α, we investigated the transcriptional activity of HIFs in both normoxia and hypoxia using the HRE reporter system. Overexpression of DCAF15 did not affect HIF transcriptional activity under both conditions (Fig. 2C) and levels of ARNT under normoxia condition (Fig. 2D). Moreover, DCAF15 knockdown had no influence on HIF transcriptional activity (Fig. 2E). Since ARNT is also involved in xenobiotic responses, we examined the transcriptional activity of AhR in TCDD-treated cells using the XRE reporter system. DCAF15 overexpression did not affect TCDD-induced
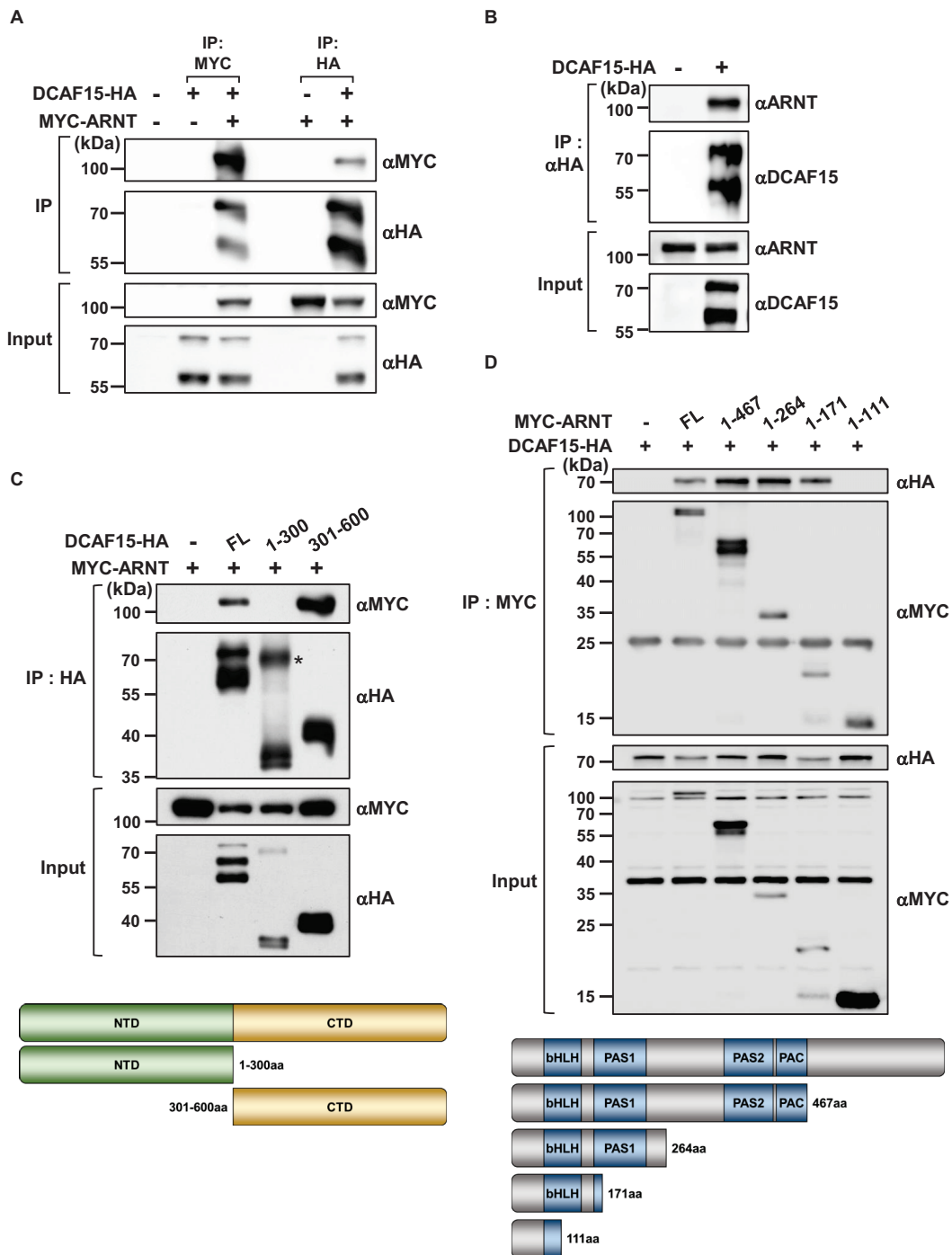
Fig. 1. ARNT interacts with DCAF15. (A) HEK293T cells were co-transfected with DCAF15-HA and MYC-ARNT. After 48 h, cell lysates were immunoprecipitated using MYC or HA antibody-conjugated magnetic beads. Immune complexes were analyzed by immunoblotting with the indicated antibodies. (B) HEK293T cells were transfected with DCAF15-HA. After 48 h, cell lysates were immunoprecipitated using HA antibody-conjugated magnetic beads. Immune complexes were analyzed by immunoblotting with the indicated antibodies. (C) HEK293T cells were transfected with MYC-ARNT and HA-full-length or truncated DCAF15 (1-300 aa: NTD or 301-600 aa: CTD). After 48 h, cell lysates were immunoprecipitated using HA antibody-conjugated magnetic beads. Immune complexes were analyzed by immunoblotting with the indicated antibodies (upper panel). Schematic diagram illustrating DCAF15 deletion mutants (lower panel). Asterisk (*) indicates a non-specific band. (D) HEK293T cells were co-transfected with DCAF15-HA and MYC-full length or truncated ARNT (1-467 aa, 1-264 aa, 1-171 aa, or 1-111 aa). After 48 h, cell lysates were immunoprecipitated using MYC antibody-conjugated magnetic beads. Immune complexes were analyzed by immunoblotting with the indicated antibodies (upper panel). Schematic diagram illustrating ARNT deletion mutants (lower panel).
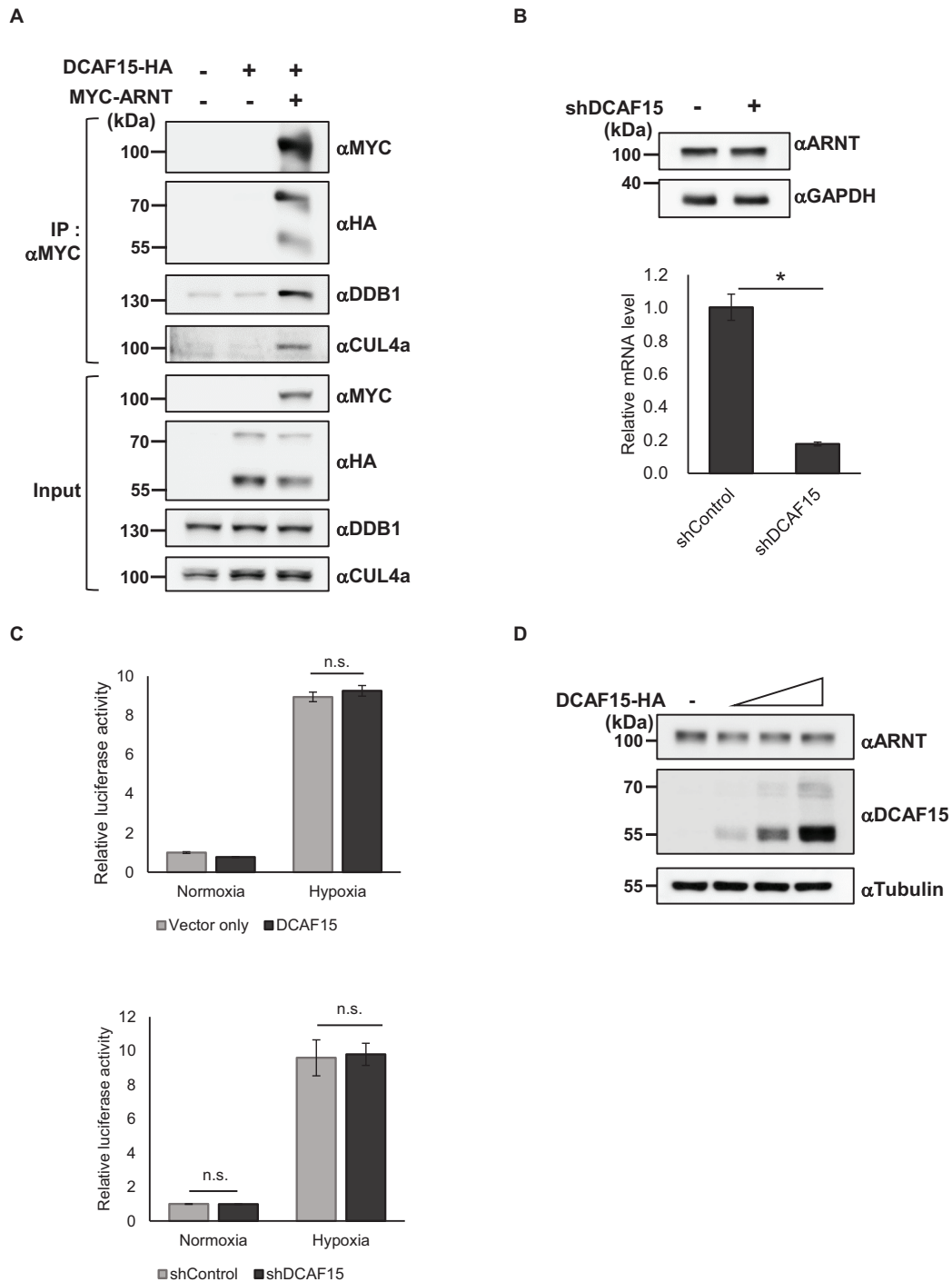
Fig. 2. ARNT is not an endogenous substrate of CRL4^{DCAF15}. (A) HEK293T cells were co-transfected with MYC-ARNT and DCAF15-HA. After 48 h, cell lysates were immunoprecipitated using MYC antibody-conjugated magnetic beads. Immune complexes were analyzed by immunoblotting with the indicated antibodies. (B) Lysates from shControl or shDCAF15 HEK293T cells were analyzed with the indicated antibodies (upper panel). qPCR validation of relative DCAF15 mRNA levels (lower panel). Data are presented as mean ± SD (n = 3). *$P < 0.001$. (C) HEK293T cells were transfected with pRL-TK, HRE luciferase reporter, and DCAF15 or vector control. After 24 h, transfected cells were additionally incubated under normoxia or hypoxia for 12 h. Luciferase activities were measured with Dual Luciferase Reporter Assay System. Data are presented as mean ± SD (n = 3). n.s., not significant ($P > 0.1$). (D) HEK293T cells were transfected with increasing amounts of DCAF15-HA. After 48 h, cell lysates were analyzed by immunoblotting with the indicated antibodies. (E) shControl or shDCAF15-containing HEK293T cells were transfected with pRL-TK and HRE luciferase reporter. After 24 h, transfected cells were incubated under normoxia or hypoxia for 12 h. Luciferase activities were measured with a Dual-Luciferase Reporter Assay System. Data are presented as mean ± SD (n = 3). n.s., not significant ($P > 0.1$).

transcriptional activity in our experiments (Supplementary Fig. S1). The collective data indicate that ARNT is not an endogenous substrate of CRL4[DCAF15].

Aryl sulfonamides induce degradation of ARNT through CRL4[DCAF15]

Aryl sulfonamides, including indisulam and E7820, promote ternary complex formation with DCAF15 and RBM proteins (RBM23 and RBM39), subsequently inducing degradation of RBM23 and RBM39 (Han et al., 2017; Ting et al., 2019; Uehara et al., 2017). The stable interactions observed between ARNT and subunits of CRL4[DCAF15] led us to investigate whether aryl sulfonamides affect the stability of ARNT. To this end, levels of ARNT and RBM39 were examined in the presence of indisulam and E7820 via western blot. Treatment with indisulam or E7820 suppressed ARNT levels in both 293T and HCT116 cells in a dose-dependent manner (Fig. 3A, Supplementary Fig. S2A). Following indisulam treatment, RBM39 levels were rapidly decreased while those of ARNT decreased relatively slowly (Fig. 3B). To ascertain whether the indisulam-induced decrease in ARNT was dependent on proteasome and Cullin-containing E3 ligases, we examined the effects of bortezomib, a proteasome inhibitor, and MNL4924, a Neddylation inhibitor, along with indisulam. Interestingly, co-treatment with indisulam and bortezomib or MNL4924 prevented degradation of ARNT and RBM39 (Fig. 3C, Supplementary Fig. S2B). The levels of poly-ubiquitinated ARNT were also augmented in the presence of indisulam (Fig. 3D), indicating that indisulam promotes poly-ubiquitination and subsequent degradation of ARNT. We further determined whether indisulam-mediated ARNT degradation is dependent on DCAF15. DCAF15 knockdown prevented indisulam-mediated degradation of both ARNT and BRM39, strongly supporting its requirement for these processes (Fig. 3E). Sulfonamides have been shown to promote ternary complex formation with DCAF15 and RBM39 or RBM23 (Bussiere et al., 2020; Du et al., 2019; Faust et al., 2020). Accordingly, we examined whether degradation of both RBM39 and RBM23 is a prerequisite for indisulam-mediated ARNT degradation. Notably, knockdown of both RBM39 and RBM23 using specific siRNAs did not influence ARNT degradation by indisulam (Fig. 3F), suggesting that the RBM proteins are dispensable for the degradation process. Notably, it also excluded the possibility that degradation of RBM proteins leads to ARNT degradation. Next, co-immunoprecipitation experiments were conducted to establish whether aryl sulfonamide enhances binding of DCAF15 and ARNT or DDB1. Levels of co-immunoprecipitated ARNT and DDB1 remained unchanged in the presence and absence of indisulam, suggesting no significant effects of indisulam on the binding affinity of DCAF15 with ARNT (Fig. 3G).

Aryl sulfonamide negatively regulates the transcriptional activity of ARNT

ARNT forms a heterodimer with HIF1/2α or AhR to regulate genes involved in adaptation to hypoxia and xenobiotics, respectively (Abel and Haarmann-Stemmann, 2010; Scheuermann et al., 2007; Vorrink and Domann, 2014; Wood et al., 1996). We thus examined whether the function of ARNT

is affected by indisulam. Transcriptional activity of AhR/ARNT was examined using the XRE reporter luciferase assay. Treatment with a known xenobiotic, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), induced XRE reporter activity > 100-fold in HepG2 cells, which was suppressed by indisulam (Fig. 4A). Aryl sulfonamides decreased the level of ARNT, but not AhR, both in the presence and absence of TCDD (Fig. 4B). We additionally examined the transcriptional activity of HIF in the presence of indisulam using the HRE reporter luciferase assay. Indisulam inhibited HIF transcriptional activity in both normoxia and hypoxia conditions (Fig. 4C). While ARNT degradation was promoted by indisulam and E7820 in both normoxia and hypoxia, stabilized HIF1α levels in hypoxia were not altered by the aryl sulfonamides (Fig. 4D). Moreover, the effects of indisulam on specific HIF target genes (glucose transporter 1 [GLUT1], lactate dehydrogenase A [LDHA], vascular endothelial growth factor [VEGF], and transforming growth factor α [TGFα]) were validated using quantitative reverse transcription-PCR (qRT-PCR). Consistent with the results obtained with the HRE reporter, indisulam significantly inhibited HIF target genes in both normoxia and hypoxia conditions (Figs. 4E-4H). These results suggest that degradation of ARNT by aryl sulfonamides reduces transcriptional activity in response to xenobiotics and hypoxia.

DISCUSSION

From a mechanistic viewpoint, molecular glue can mediate complex formation between two unrelated proteins (Hughes and Ciulli, 2017). For example, IMiDs bridge CRBN and Zn-finger proteins, inducing degradation of a subset of Zn-finger proteins (Kronke et al., 2014; Zhu et al., 2014), while aryl sulfonamides attach DCAF15 to RBM proteins and promote degradation of RBM proteins (Han et al., 2017; Uehara et al., 2017). Two consecutive studies using quantitative mass spectrometry have reported that aryl sulfonamide downregulates two specific RBM proteins (RBM23 and RBM39) possessing the conserved α-helical degron motif (Ting et al., 2019). ARNT was not identified as a degradation target of aryl sulfonamide in earlier studies (Han et al., 2017; Ting et al., 2019; Uehara et al., 2017), in contrast to our observation that ARNT levels were decreased in the presence of indisulam and E7820. This discrepancy may be attributed to the relatively slow degradation rate of ARNT. As shown in Fig. 3B, RBM39 was rapidly degraded and had almost disappeared at 6 h of treatment whereas ~50% ARNT was detected at this time-point. All proteomic analyses were performed over < 6 h that may have been insufficient for detection of reduced ARNT levels in these reports. In addition, interactions between DCAF15 and ARNT were not affected by aryl sulfonamides, suggesting a distinct mode of action from that of DCAF15 with RBM proteins. The mechanisms by which aryl sulfonamides induce ARNT degradation despite no significant augmentation effects on interactions between DCAF15 and ARNT are yet to be established. One potential explanation is that aryl sulfonamide-mediated complex formation of RBM proteins and DCAF15 facilitates ARNT degradation. However, knockdown of both RBM23 and RBM39 did not influence aryl sulfonamide-induced ARNT degradation, implying that
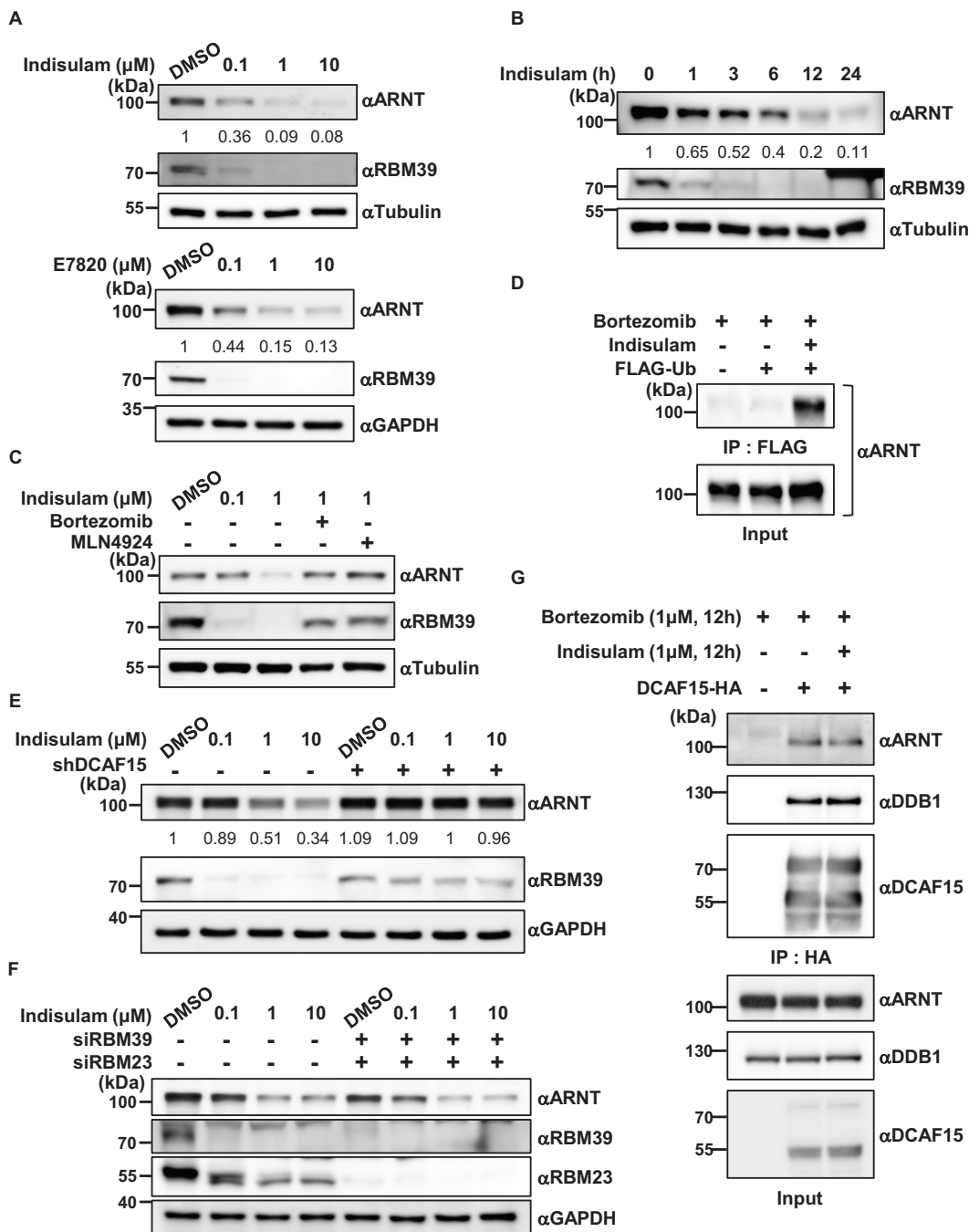
Fig. 3. Aryl sulfonamides induce ARNT degradation through CRL4^{DCAF15}. (A) HEK293T cells were treated with the indicated concentrations of indisulam or E7820 for 24 h. Cell lysates were analyzed via immunoblotting with indicated antibodies. (B) HEK293T cells were treated with indisulam (1 μM) for the indicated time-periods. Cell lysates were analyzed via immunoblotting with the indicated antibodies. (C) HEK293T cells were treated with indisulam (1 μM) along with bortezomib (1 μM) or MLN4924 (1 μM) for 12 h. Cell lysates were analyzed via immunoblotting with the indicated antibodies. (D) HEK293T cells were transfected with FLAG-ubiquitin. After 24 h, transfected cells were co-treated with DMSO or indisulam and bortezomib for 12 h. Whole-cell lysates were immunoprecipitated using FLAG antibody-conjugated magnetic beads and immune complexes analyzed by immunoblotting with anti-ARNT antibody. (E) shControl or shDCAF15 HEK293T cells were treated with the indicated concentrations of indisulam for 24 h. Cell lysates were analyzed via immunoblotting with the indicated antibodies. (F) HEK293T cells were transfected with siControl or siRNA against RBM39 and RBM23. After 48 h, transfected cells were treated with the indicated concentrations of indisulam for 24 h. Cell lysates were analyzed via immunoblotting with the indicated antibodies. (G) HEK293T cells were transfected with DCAF15-HA, followed by treatment with DMSO or indisulam (1 μM) and bortezomib (1 μM) for 12 h. Cell lysates were immunoprecipitated using HA antibody-conjugated magnetic beads and immune complexes analyzed via immunoblotting with the indicated antibodies.
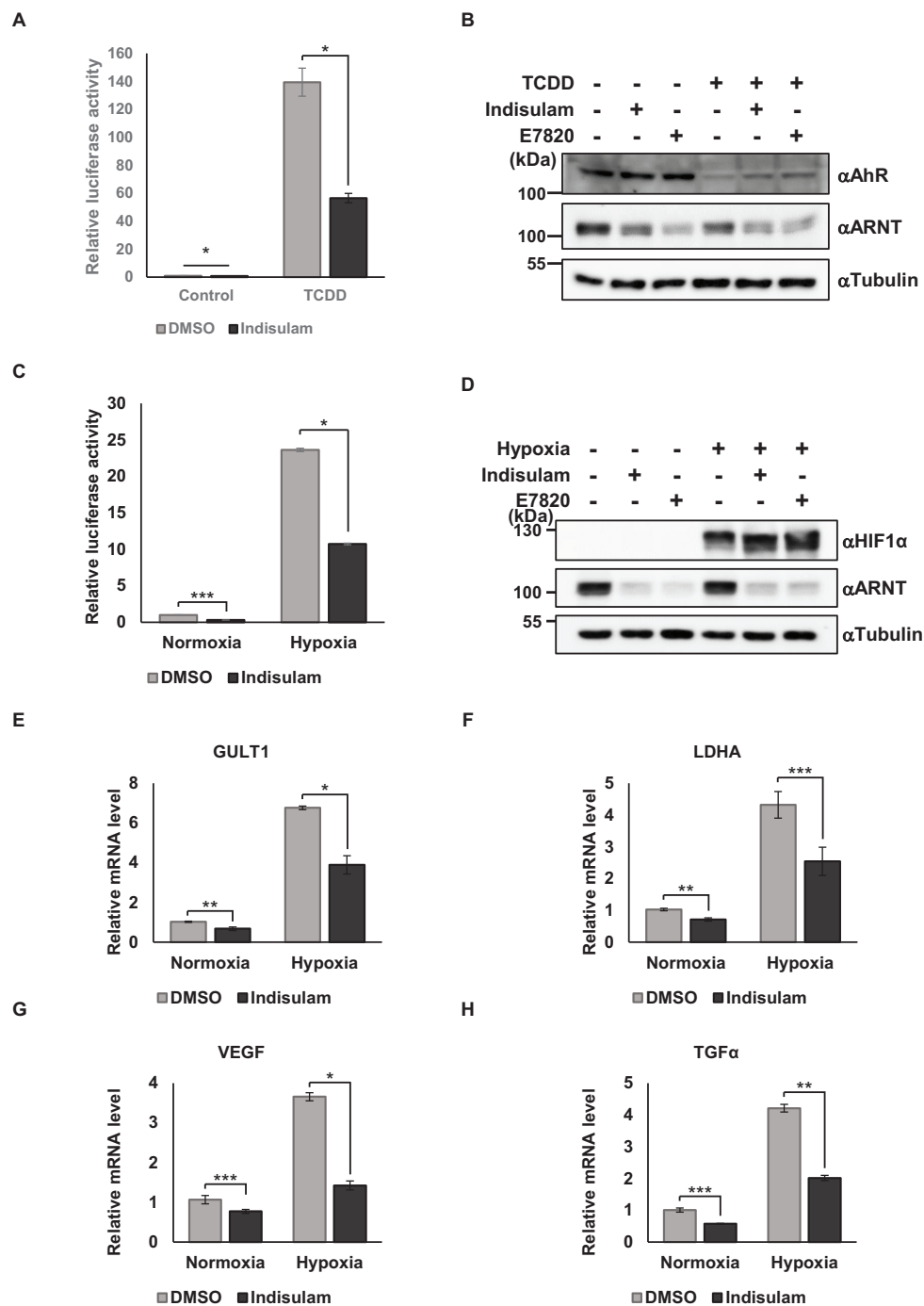
Fig. 4. Aryl sulfonamide negatively regulates transcriptional activity of ARNT. (A) HepG2 cells were transfected with pRL-TK, a XRE luciferase reporter. After 24 h, cells were treated with DMSO or indisulam (10 μM) along with TCDD (10 nM) for 12 h. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System. Data are presented as mean ± SD (n = 3). *$P < 0.001$. (B) HepG2 cells were treated with DMSO, indisulam (1 μM) or E7820 (1 μM) in the presence or absence of TCDD (10 nM) for 12 h. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (C) HEK293T cells were transfected with pRL-TK, a HRE luciferase reporter. After 24 h, cells were treated with indisulam (10 μM) under normoxia or hypoxia for 12 h and luciferase activities measured with a Dual-Luciferase Reporter Assay System. Data are presented as mean ± SD (n = 3). *$P < 0.001$, ***$P < 0.05$. (D) HEK293T cells were treated with DMSO, indisulam (1 μM) or E7820 (1 μM) under normoxia or hypoxia for 12 h. Cell lysates were analyzed via immunoblotting with the indicated antibodies. (E-H) Total mRNAs from HEK293T cells treated with DMSO or indisulam (1 μM) under normoxia or hypoxia for 12 h were subjected to RT-qPCR analysis for the indicated HIF target genes. GLUT1, glucose transporter 1; LDHA, lactate dehydrogenase A; VEGF, vascular endothelial growth factor; TGFα, transforming growth factor α; Data are presented as mean ± SD (n = 3). *$P < 0.001$, **$P < 0.01$, ***$P < 0.05$.

RBM proteins are not essential for this process. Another possibility is the conformational changes induced by engagement of aryl sulfonamides to DCAF15. Binding of aryl sulfonamide with DCAF15 could induce a tilt at the binding interface of ARNT and DCAF15, thereby repositioning ARNT for ubiquitination by the CRL4^{DCAF15} complex. Further structural analyses, such as crystallization or cryogenic electron microscopy (cryo-EM), of the ARNT-DCAF15-aryl sulfonamide complex are required to test this hypothesis.

What is the significance of the interaction between DCAF15 and ARNT in physiological conditions? This interaction may affect the substrate recognition of DCAF15, governing specificity for substrates. As ARNT is well-known as a nuclear translocator, this interaction may also facilitate nuclear translocation of DCAF15, and nuclear DCAF15 forms a CRL complex, inducing degradation of endogenous substrates. It is unknown so far, but if the endogenous substrate of DCAF15 was identified, these possibilities could be tested.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

AUTHOR CONTRIBUTIONS
S.A.K. designed and performed experiments, analyzed data, and wrote the manuscript. S.H.J., J.H.C., M.Y.Y., and H.C.S. performed experiments and analyzed data. J.A.K., S.G.P., and B.C.P. designed experiments and provided feedback. B.C.P., S.K., and J.H.K. supervised the research. J.H.K. wrote the manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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REFERENCES

Abel, J. and Haarmann-Stemmann, T. (2010). An introduction to the molecular basics of aryl hydrocarbon receptor biology. Biol. Chem. *391*, 1235-1248.

Assi, R., Kantarjian, H.M., Kadia, T.M., Pemmaraju, N., Jabbour, E., Jain, N., Daver, N., Estrov, Z., Uehara, T., Owa, T., et al. (2018). Final results of a phase 2, open-label study of indisulam, idarubicin, and cytarabine in patients with relapsed or refractory acute myeloid leukemia and high-risk myelodysplastic syndrome. Cancer *124*, 2758-2765.

Bosu, D.R. and Kipreos, E.T. (2008). Cullin-RING ubiquitin ligases: global regulation and activation cycles. Cell Div. *3*, 7.

Bussiere, D.E., Xie, L., Srinivas, H., Shu, W., Burke, A., Be, C., Zhao, J., Godbole, A., King, D., Karki, R.G., et al. (2020). Structural basis of indisulam-mediated RBM39 recruitment to DCAF15 E3 ligase complex. Nat. Chem. Biol. *16*, 15-23.

Du, X., Volkov, O.A., Czerwinski, R.M., Tan, H., Huerta, C., Morton, E.R., Rizzi, J.P., Wehn, P.M., Xu, R., Nijhawan, D., et al. (2019). Structural basis and kinetic pathway of RBM39 recruitment to DCAF15 by a sulfonamide molecular glue E7820. Structure *27*, 1625-1633.e3.

Faust, T.B., Yoon, H., Nowak, R.P., Donovan, K.A., Li, Z., Cai, Q., Eleuteri, N.A., Zhang, T., Gray, N.S., and Fischer, E.S. (2020). Structural complementarity facilitates E7820-mediated degradation of RBM39 by DCAF15. Nat. Chem. Biol. *16*, 7-14.

Gradin, K., McGuire, J., Wenger, R.H., Kvietikova, I., fhitelaw, M.L., Toftgård, R., Tora, L., Gassmann, M., and Poellinger, L. (1996). Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. Mol. Cell. Biol. *16*, 5221-5231.

Haddad, R.I., Weinstein, L.J., Wieczorek, T.J., Bhattacharya, N., Raftopoulos, H., Oster, M.W., Zhang, X., Latham, V.M., Jr., Costello, R., Faucher, J., et al. (2004). A phase II clinical and pharmacodynamic study of E7070 in patients with metastatic, recurrent, or refractory squamous cell carcinoma of the head and neck: modulation of retinoblastoma protein phosphorylation by a novel chloroindolyl sulfonamide cell cycle inhibitor. Clin. Cancer Res. *10*, 4680-4687.

Han, T., Goralski, M., Gaskill, N., Capota, E., Kim, J., Ting, T.C., Xie, Y., Williams, N.S., and Nijhawan, D. (2017). Anticancer sulfonamides target splicing by inducing RBM39 degradation via recruitment to DCAF15. Science *356*, eaal3755.

Hannah, J. and Zhou, P. (2015). Distinct and overlapping functions of the cullin E3 ligase scaffolding proteins CUL4A and CUL4B. Gene *573*, 33-45.

Hsiehchen, D., Goralski, M., Kim, J., Xie, Y., and Nijhawan, D. (2020). Biomarkers for RBM39 degradation in acute myeloid leukemia. Leukemia *34*, 1924-1928.

Hughes, S.J. and Ciulli, A. (2017). Molecular recognition of ternary complexes: a new dimension in the structure-guided design of chemical degraders. Essays Biochem. *61*, 505-516.

Huttlin, E.L., Bruckner, R.J., Paulo, J.A., Cannon, J.R., Ting, L., Baltier, K., Colby, G., Gebreab, F., Gygi, M.P., Parzen, H., et al. (2017). Architecture of the human interactome defines protein communities and disease networks. Nature *545*, 505-509.

Huttlin, E.L., Ting, L., Bruckner, R.J., Gebreab, F., Gygi, M.P., Szpyt, J., Tam, S., Zarraga, G., Colby, G., Baltier, K., et al. (2015). The BioPlex network: a systematic exploration of the human interactome. Cell *162*, 425-440.

Jung, D.J., Na, S.Y., Na, D.S., and Lee, J.W. (2002). Molecular cloning and characterization of CAPER, a novel coactivator of activating protein-1 and estrogen receptors. J. Biol. Chem. *277*, 1229-1234.

Kang, Y.K., Putluri, N., Maity, S., Tsimelzon, A., Ilkayeva, O., Mo, Q., Lonard, D., Michailidis, G., Sreekumar, A., Newgard, C.B., et al. (2015). CAPER is vital for energy and redox homeostasis by integrating glucose-induced mitochondrial functions via ERR-alpha-Gabpa and stress-induced adaptive responses via NF-kappaB-cMYC. PLoS Genet. *11*, e1005116.

Klinge, C.M., Bowers, J.L., Kulakosky, P.C., Kamboj, K.K., and Swanson, H.I. (1999). The aryl hydrocarbon receptor (AHR)/AHR nuclear translocator (ARNT) heterodimer interacts with naturally occurring estrogen response elements. Mol. Cell. Endocrinol. *157*, 105-119.

Kobayashi, A., Numayama-Tsuruta, K., Sogawa, K., and Fujii-Kuriyama, Y. (1997). CBP/p300 functions as a possible transcriptional coactivator of Ah receptor nuclear translocator (Arnt). J. Biochem. *122*, 703-710.

Kronke, J., Udeshi, N.D., Narla, A., Grauman, P., Hurst, S.N., McConkey, M., Svinkina, T., Heckl, D., Comer, E., Li, X., et al. (2014). Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. Science *343*, 301-305.

Lee, J. and Zhou, P. (2007). DCAFs, the missing link of the CUL4-DDB1 ubiquitin ligase. Mol. Cell *26*, 775-780.

Mai, S., Qu, X., Li, P., Ma, Q., Cao, C., and Liu, X. (2016). Global regulation of alternative RNA splicing by the SR-rich protein RBM39. Biochim. Biophys. Acta *1859*, 1014-1024.

Maltepe, E., Schmidt, J.V., Baunoch, D., Bradfield, C.A., and Simon, M.C. (1997). Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature *386*, 403-407.

Mandl, M. and Depping, R. (2017). ARNT is a potential direct HIF-1 target gene in human Hep3B hepatocellular carcinoma cells. Cancer Cell Int. *17*, 77.

McIntosh, B.E., Hogenesch, J.B., and Bradfield, C.A. (2010). Mammalian Per-Arnt-Sim proteins in environmental adaptation. Annu. Rev. Physiol. *72*, 625-645.

Owa, T., Yoshino, H., Okauchi, T., Yoshimatsu, K., Ozawa, Y., Sugi, N.H., Nagasu, T., Koyanagi, N., and Kitoh, K. (1999). Discovery of novel antitumor sulfonamides targeting G1 phase of the cell cycle. J. Med. Chem. *42*, 3789-3799.

Reisz-Porszasz, S., Probst, M.R., Fukunaga, B.N., and Hankinson, O. (1994). Identification of functional domains of the aryl hydrocarbon receptor nuclear translocator protein (ARNT). Mol. Cell. Biol. *14*, 6075-6086.

Rezvani, H.R., Ali, N., Nissen, L.J., Harfouche, G., de Verneuil, H., Taïeb, A., and Mazurier, F. (2011). HIF-1alpha in epidermis: oxygen sensing, cutaneous angiogenesis, cancer, and non-cancer disorders. J. Invest. Dermatol. *131*, 1793-1805.

Scheuermann, T.H., Yang, J., Zhang, L., Gardner, K.H., and Bruick, R.K. (2007). Hypoxia-inducible factors Per/ARNT/Sim domains: structure and function. Methods Enzymol. *435*, 3-24.

Semenza, G.L. (2003). Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer *3*, 721-732.

Sogawa, K., Nakano, R., Kobayashi, A., Kikuchi, Y., Ohe, N., Matsushita, N., and Fujii-Kuriyama, Y. (1995). Possible function of Ah receptor nuclear translocator (Arnt) homodimer in transcriptional regulation. Proc. Natl. Acad. Sci. U. S. A. *92*, 1936-1940.

Ting, T.C., Goralski, M., Klein, K., Wang, B., Kim, J., Xie, Y., and Nijhawan, D. (2019). Aryl sulfonamides degrade RBM39 and RBM23 by recruitment to CRL4-DCAF15. Cell Rep. *29*, 1499-1510.e6.

Uehara, T., Minoshima, Y., Sagane, K., Sugi, N.H., Mitsuhashi, K.O., Yamamoto, N., Kamiyama, H., Takahashi, K., Kotake, Y., Uesugi, M., et al. (2017). Selective degradation of splicing factor CAPERalpha by anticancer sulfonamides. Nat. Chem. Biol. *13*, 675-680.

Van Kesteren, C., Beijnen, J.H., and Schellens, J.H. (2002). E7070: a novel synthetic sulfonamide targeting the cell cycle progression for the treatment of cancer. Anticancer Drugs *13*, 989-997.

Vorrink, S.U. and Domann, F.E. (2014). Regulatory crosstalk and interference between the xenobiotic and hypoxia sensing pathways at the AhR-ARNT-HIF1alpha signaling node. Chem. Biol. Interact. *218*, 82-88.

Wang, E., Lu, S.X., Pastore, A., Chen, X., Imig, J., Chun-Wei Lee, S., Hockemeyer, K., Ghebrechristos, Y.E., Yoshimi, A., Inoue, D., et al. (2019). Targeting an RNA-binding protein network in acute myeloid leukemia. Cancer Cell *35*, 369-384.e7.

Weir, L., Robertson, D., Leigh, I.M., Vass, J.K., and Panteleyev, A.A. (2011). Hypoxia-mediated control of HIF/ARNT machinery in epidermal keratinocytes. Biochim. Biophys. Acta *1813*, 60-72.

Wolff, M., Jelkmann, W., Dunst, J., and Depping, R. (2013). The Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT/HIF-1beta) is influenced by hypoxia and hypoxia-mimetics. Cell. Physiol. Biochem. *32*, 849-858.

Wood, S.M., Gleadle, J.M., Pugh, C.W., Hankinson, O., and Ratcliffe, P.J. (1996). The role of the aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression. Studies in ARNT-deficient cells. J. Biol. Chem. *271*, 15117-15123.

Zhu, Y.X., Braggio, E., Shi, C.X., Kortuem, K.M., Bruins, L.A., Schmidt, J.E., Chang, X.B., Langlais, P., Luo, M., Jedlowski, P., et al. (2014). Identification of cereblon-binding proteins and relationship with response and survival after IMiDs in multiple myeloma. Blood *124*, 536-545.