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AhR promotes phosphorylation of ARNT isoform 1 in human T cell malignancies as a switch for optimal AhR activity

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The aryl hydrocarbon receptor nuclear translocator (ARNT) is a transcription factor present in immune cells as a long and short isoform, referred to as isoforms 1 and 3, respectively. However, investigation into potential ARNT isoform–specific immune functions is lacking despite the well-established heterodimerization requirement of ARNT with, and for the activity of, the aryl hydrocarbon receptor (AhR), a critical mediator of immune homeostasis. Here, using global and targeted transcriptomics analyses, we show that the relative ARNT isoform 1:3 ratio in human T cell lymphoma cells dictates the amplitude and direction of AhR target gene regulation. Specifically, shifting the ARNT isoform 1:3 ratio lower by suppressing isoform 1 enhances, or higher by suppressing isoform 3 abrogates, AhR responsiveness to ligand activation through preprograming a cellular genetic background that directs explicit gene expression patterns. Moreover, the fluctuations in gene expression patterns that accompany a decrease or increase in the ARNT isoform 1:3 ratio are associated with inflammation or immunosuppression, respectively. Molecular studies identified the unique casein kinase 2 (CK2) phosphorylation site within isoform 1 as an essential parameter to the mechanism of ARNT isoform–specific regulation of AhR signaling. Notably, CK2-mediated phosphorylation of ARNT isoform 1 is dependent on ligand-induced AhR nuclear translocation and is required for optimal AhR target gene regulation. These observations reveal ARNT as a central modulator of AhR activity predicated on the status of the ARNT isoform ratio and suggest that ARNT-based therapies are a viable option for tuning the immune system to target immune disorders.

ARNT | AhR | isoform | immunomodulation

Significance

Nontoxic agonists and antagonists of the aryl hydrocarbon receptor (AhR) hold high therapeutic potential for treating autoimmune disease and cancer. However, AhR activation by different ligands can lead to opposing phenotypical outcomes in a cell- and tissue-specific manner. In this study, we demonstrate that proportional flux in the levels of aryl hydrocarbon receptor nuclear translocator (ARNT) isoforms 1 and 3 modulates AhR signaling in terms of amplitude and expression of distinct gene programs. These results delineate a molecular mechanism of ARNT isoform–mediated AhR regulation, simplify our understanding of a complex AhR signaling pathway, and provide feasibility for ARNT-targeted therapies that could be used in conjunction with nontoxic AhR ligands for the purpose of immunomodulation.

Environmental exposures, and certain lifestyle choices such as diet and cigarette smoking, can skew normal cell physiology. These fluctuations in homeostasis are detected, in part, by members of the basic-helix–loop–helix-PER/ARNT/SIM (bHLH-PAS) superfamily of transcription factors, which activate metabolic gene programs aimed at reestablishing cell and tissue homeostasis (1). The aryl hydrocarbon receptor nuclear translocator (ARNT), also known as hypoxia-inducible factor (HIF)-1β, is a class II bHLH-PAS family member that heterodimerizes with class I members such as the aryl hydrocarbon receptor (AhR) or HIF-1/2α as a necessary step for transcriptional regulation (2). Furthermore, we and others have characterized a key role for ARNT in regulating the nuclear factor-kappaB inflammatory response (3–5).

Given that ARNT serves as an important link between transcription factor pathways that are instrumental in regulating immune cell function, it is not surprising that ARNT has been implicated in maintaining immune system homeostasis. For instance, studies examining *Arnt* conditional deletion within myeloid cells revealed dysregulated immune responses that enhanced or reduced inflammation in a tissue-specific fashion (6–8). Additionally, ARNT is required for long-term HIF-dependent hematopoietic stem cell homeostasis (9, 10), and mice null for *Arnt* in fetal liver or bone marrow hematopoietic stem cells displayed abnormal numbers of B and T cells in the bone marrow, spleen, and thymus (11). Moreover, the presence of altered T cell subsets in mice with *Arnt*-deficient $CD4^+$ T cells predicts a role for ARNT in T cell differentiation (12).

Importantly, loss of AhR-mediated target gene regulation in *Arnt*-deficient $CD4^+$ T cells is a major factor in the resultant diminished T cell differentiation (12). The AhR is a soluble receptor that binds a wide range of ligands, including the xenobiotic compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and diet- or microbiota-derived metabolites, with tryptophan catabolites being the most notable (13–15). Like ARNT, and in addition to orchestrating xenobiotic metabolism, the AhR is critically involved in diverse physiological processes, including development, immunity, cell cycle,

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hematopoiesis, differentiation, and polyunsaturated fatty acid metabolism, and aberrant AhR activity is associated with disease propagation, including autoimmunity and cancer (2, 16, 17). Restricted to the cytoplasm by chaperone proteins (18–21), AhR is poised in a ligand-binding conformation and upon agonist binding translocates to the nucleus, where it sheds the chaperone proteins and dimerizes with ARNT (22). Subsequently, the AhR-ARNT heterodimer binds to genomic AhR-responsive elements (AhREs) for the regulation of target gene expression (23, 24). Notably, certain AhR ligands have been reported to modulate T cell polarization. Specifically, TCDD triggers immunosuppression in part by promoting increased regulatory T cell (Treg) differentiation (25–28), whereas the tryptophan photoproduct 6-formylindolo[3,2-b]carbazole (FICZ) induces the differentiation of autoimmune-promoting inflammatory T helper17 (TH17) cells (27, 28).

While AhR activity appears to be critical for determining T cell fate, it is unknown whether ARNT has a role in this process outside of serving as an obligate binding partner for AhR. In consideration of a more complex regulatory role for ARNT in AhR signaling, we noted that ARNT was initially identified as a long and a short transcript (now referred to as ARNT isoform 1 and ARNT isoform 3, respectively), which is a consequence of exon 5 alternative splicing (29). Interestingly, exon 5 within ARNT isoform 1 encodes an additional 15 amino acids that encompass a unique casein kinase 2 (CK2) phosphorylation site, which is not present in ARNT isoform 3 (30). Notably, CK2 phosphorylation of recombinant ARNT isoform 1 was shown to control promoter affinity (30). This observation predicts specific ARNT isoform 1 activities, which is supported by our previous findings that high levels of isoform 1 relative to isoform 3 (i.e., a high isoform 1:3 ratio) is necessary for propagation and survival of diverse lymphoid malignancies (31). Despite these observations, putative ARNT isoform–specific effects on the activity of bHLH-PAS family members, like AhR, have been largely overlooked. Thus, we hypothesized that modulation of the ARNT isoform 1:3 ratio directs discreet AhR target gene expression patterns.

As an initial study into the mechanisms by which the ARNT isoforms regulate AhR activity, we suppressed individual ARNT isoforms in human T cell lymphoma lines and treated them with the AhR agonists TCDD or FICZ. Accordingly, we found that a high ARNT isoform 1:3 ratio dampens, whereas a low ARNT isoform 1:3 ratio enhances, the regulation of specific AhR target genes. Moreover, we observed that CK2-mediated phosphorylation of ARNT isoform 1 is dependent on AhR activation and functions as a rheostat for AhR target gene transcription, with phosphorylation corresponding to higher expression levels of certain target genes. Collectively, these results increase our understanding of a complex regulatory mechanism by which the ARNT isoforms specifically regulate AhR signaling, further aiding in the comprehension of their roles in immunity and supporting the potential of targeting ARNT alternative splicing as a means of therapeutic intervention in hematological diseases.

Results

ARNT Isoforms Differentially Regulate AhR Activity. To test whether modulation of the ARNT isoform ratio distinctly impacts global AhR target gene expression, we utilized small interfering RNA (siRNA) to simultaneously suppress both ARNT isoforms (siA-1/3) or to suppress each isoform individually (siA-1 or siA-3) in Karpas 299 cells, an anaplastic large cell lymphoma cell line with Treg characteristics (32). Each ARNT suppression condition was incubated with vehicle (dimethylsulfoxide [DMSO]) as a negative control or with TCDD for 2 h to activate AhR signaling; RNA sequencing (RNA-seq) was subsequently performed. Manipulation of the ARNT isoform levels prior to addition of the exogenous AhR ligand leads to thousands of significant distinct and shared differential gene expression patterns between the ARNT siRNA conditions (Fig. 1*A*; *SI Appendix*, Fig. S1*A*; and Dataset S1). Notably, TCDD exposure promotes a modest shift in the variance of each unique genetic background, including the scrambled siRNA control (siControl) sample (Fig. 1*A*). Thus, to obtain the set of AhR-responsive genes in Karpas 299 cells, we compared transcription profiles of TCDD- versus DMSO-treated siControl cells. Out of the 186 significant ($P < 0.05$) gene expression changes that occur in the control cells after TCDD exposure, the expression of 164 of these genes was significantly affected by modulating the ARNT isoform ratio (Fig. 1*B*). Interestingly, an unbiased gene clustering analysis comparing gene expression changes of ±1.5-fold between all combinations of control and ARNT siRNA samples, untreated or treated with TCDD, suggests that ARNT regulates AhR activity in an isoform-specific manner (*SI Appendix*, Fig. S1*B*).

Further examination into the set of AhR target genes derived from siControl cells shows that TCDD-induced AhR activity is abrogated at approximately half of the identified AhR-responsive genes after individual or combined suppression of the ARNT isoforms (Fig. 1 *C*, *Left*). Interestingly, in the other half of TCDD-regulated genes, AhR activity is enhanced or stunted after manipulation of the ARNT isoform 1:3 ratio both in DMSO control– and in TCDD-treated samples (Fig. 1 *C*, *Right*, and *D*; *SI Appendix*, Fig. S1*C*; and Dataset S2). Specifically, suppression of ARNT isoform 1 significantly enhances basal and TCDD-induced expression of canonical AhR targets like *CYP1A1* and *AHRR* (33, 34) and of apparent AhR target genes such as *BLK*, *GLCCI1*, *TNFRSF19*, *TSC22D3*, *CARD10*, *NFATC2*, and *ULBP3*, whereas suppression of both isoforms or suppression of isoform 3 significantly abrogates basal and TCDD-induced expression (Fig. 1 *C*, *Right*, and *D*; *SI Appendix*, Fig. S1*C*; and Dataset S2). Similarly, for genes that are repressed after TCDD treatment of siControl Karpas 299 cells, such as *CD274*, *PDCD1LG2*, *IL1R1*, *GAS1*, *ANKRD22*, *SOX2*, *TRIB1*, *PCDH18*, *CHST2*, and *CEBPB*, suppression of ARNT isoform 1 further down-regulates the expression of these same genes, but suppression of both isoforms or suppression of isoform 3 significantly enhances expression (Fig. 1 *C*, *Right*, and *D*; *SI Appendix*, Fig. S1*C*; and Dataset S2). In fact, the genes that are differentially expressed upon suppression of both or individual ARNT isoforms, prior to TCDD exposure, include many of the same genes that are further augmented or abrogated after TCDD treatment.

Conversely, a proportion of established AhR target genes, e.g., *IL22*, *IL10*, *SOCS2*, *STAT3*, *SERPINE1*, *IKZF3*, *TGFB1*, *SPP1*, and *GZMB* (27, 35–41), are significantly differentially expressed, often exhibiting the opposite expression pattern to that of *CYP1A1*, in response to modulation of the ARNT isoform ratio but do not change expression to a significant degree in siControl Karpas 299 cells after TCDD exposure, according to the RNA-seq dataset (Fig. 1*C* and *SI Appendix*, Fig. S1*C*). Remarkably, suppression of ARNT isoform 1 correlates with increased expression of inflammatory and cytotoxic lymphoid cell markers such as *IL17C*, *IL17D*, *CD93*, *BAMBI*, *CXCL10*, *GZMA*, *CCR1*, and *CCR5* (42, 43), whereas suppression of ARNT isoform 3 correlates with decreased expression of some of these same genes (*SI Appendix*, Fig. S1*C*). Yet, the

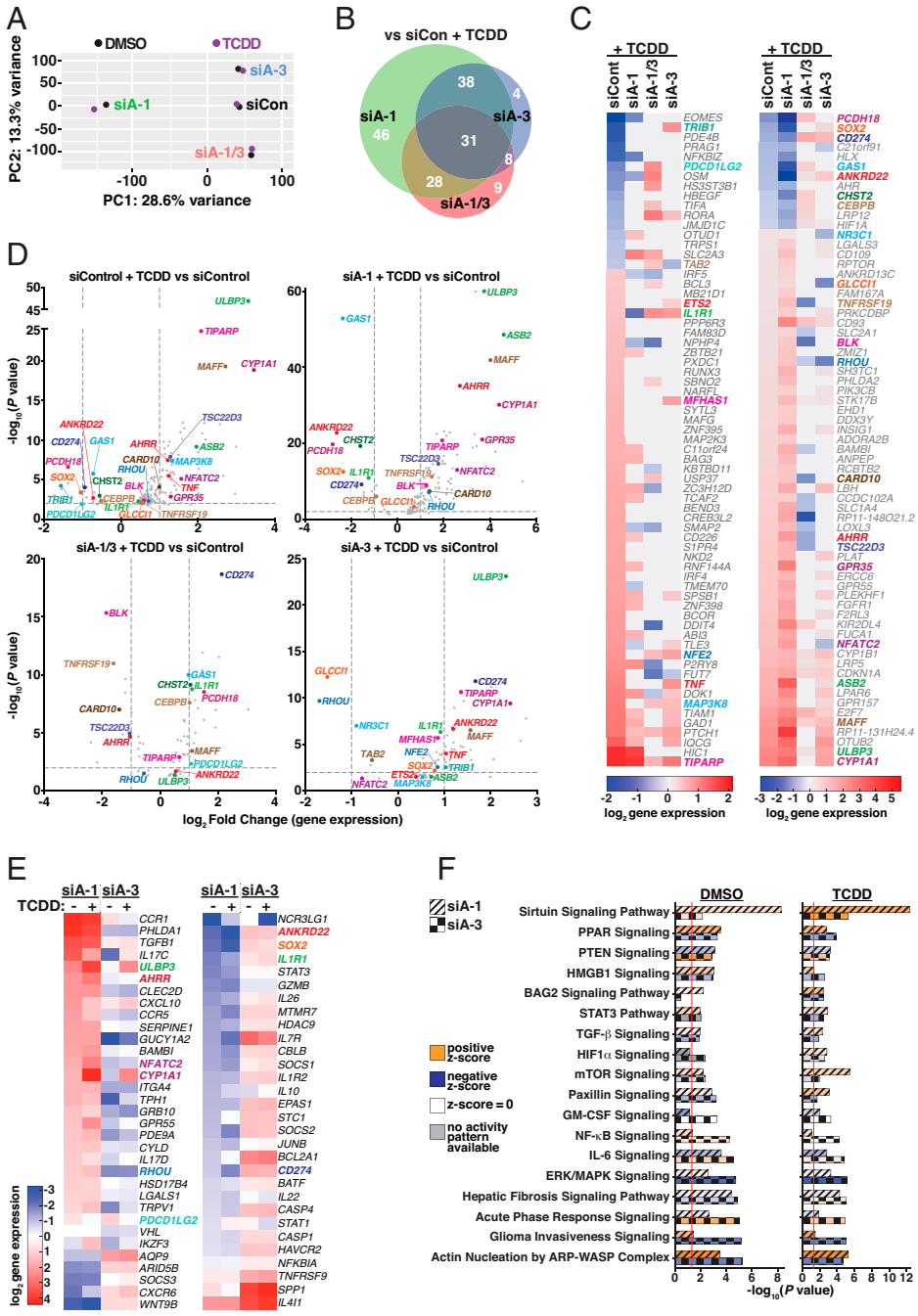

Fig. 1. ARNT isoform–specific regulation of AhR target gene expression. (*A*) Principal component analysis of differential gene expression between the indicated siRNA-treated Karpas 299 cells exposed to DMSO or TCDD (10 nM) for 2 h. (*B*) Venn diagram showing the relationship of only the significant ($P < 0.05$) TCDD-induced, differentially expressed genes between each ARNT siRNA dataset, as determined by the set of differentially expressed genes in the TCDD versus DMSO siControl samples. (*C*) Heatmaps representing the $\pm\log_2$ fold change ($P < 0.05$) in expression between the ARNT siRNA + TCDD samples and the siControl + TCDD sample (using the gene set described in *B*). (*D*) Volcano plots of select genes from *C* showing the change in significance, and relationship in expression, of siControl (*Top Left*), siA-1 (*Top Right*), siA-1/3 (*Bottom Left*), and siA-3 (*Bottom Right*). The horizontal dashed gray line signifies $P = 0.05$, and the vertical dashed gray lines mark ±1 $\log_2$ change on the *x* axis. (*E*) Heatmaps representing the $\pm\log_2$ fold change in gene expression profiles of representative genes with inverse expression patterns between the siA-1 and siA-3 ± TCDD samples as compared to the corresponding siControl ± TCDD samples. (*F*) IPA of the differentially expressed genes in siA-1–treated (striped bars) versus siA-3–treated (checkered bars) Karpas 299 cells exposed to either DMSO or TCDD (10 nM) for 2 h. The vertical red line (i.e., threshold) denotes $P = 0.05$. Pathways with positive z-scores are shown in orange, pathways with negative z-scores are shown in blue, pathways that have a zero z-score are shown in white, and pathways with an undetermined z-score are shown in gray. The relative strength of the z-score is depicted by the intensity of the color.

opposite is observed for expression of Treg and other immune suppressive markers like *TNFRSF9*, *IL1R2*, *HAVCR2*, *CD274*, *PDCD1LG2*, *SOCS2*, *STC1*, *SPP1*, *MTMR7*, *ARID5B*, *EPAS1*, and *BATF* (44–49), which exhibit repressed expression after ARNT isoform 1 suppression but enhanced expression after suppression of ARNT isoform 3 (*SI Appendix*, Fig. S1*C*). While many of these genes were not observed in the RNA-seq dataset as significantly induced by TCDD in siControl cells, they are modestly affected by TCDD treatment after modulation of the ARNT isoform ratio. Specifically, the enhanced expression of *IL17C*, *IL17D*, *CXCL10*, *TPH1*, *SOCS1*, and *CASP4*, among others, observed after suppression of ARNT

isoform 1 is reduced after TCDD treatment, but their repressed expression as observed upon suppression of ARNT isoform 3 is enhanced after TCDD exposure, as compared to their respective DMSO controls (Fig. 1*E*). Together, these observations suggest that suppression of individual ARNT isoforms sets up distinct gene expression patterns that correlate with the functional status of AhR.

Ingenuity Pathway Analysis (IPA) of all significant differentially expressed genes after targeted suppression of ARNT isoform 1 or isoform 3 revealed 18 common transcriptional modules that were significantly affected, the majority of which exhibited an inverse activity pattern between siA-1 and siA-3 conditions (Fig. 1*F* and Dataset S3). Exposure to TCDD changed the relative strength of the activity pattern, in addition to the overall significance, of the same transcriptional modules (Fig. 1*F* and Dataset S4). Similarly, inverse activation patterns in certain pathways that are unique to each specific ARNT isoform background were also observed between DMSO- and TCDD-treated cells (*SI Appendix*, Fig. S1*D* and Datasets S3 and S4). Moreover, the inverse activity pattern between siA-1 and siA-3 conditions is further exemplified in the common upstream regulators, with TCDD exposure enhancing the activation differences (*SI Appendix*, Fig. S1*E*). Notably, the AhR pathway was not shared between siA-1 and siA-3 conditions but was only identified by IPA after suppression of ARNT isoform 1, both in the DMSO control– and in the TCDD-treated samples (*SI Appendix*, Fig. S1*D*). Together, these analyses of global differential gene expression suggest that ARNT isoform 1 acts to limit AhR activity both before and after TCDD exposure, while ARNT isoform 3 augments AhR signaling.

Consistent with the findings of the transcriptomics analyses, and with the requirement of ARNT for AhR activity, siA-1/3 treatment decreases basal AhR activity and prevents TCDD- and FICZ-induced AhR signaling in Karpas 299 cells as measured by expression of the AhR target genes *CYP1A1* and *AHRR* (Fig. 2 *A* and *C*). Moreover, siA-1 treatment augments, while siA-3 treatment abrogates, basal and ligand-induced AhR activity (Fig. 2 *A* and *C*), suggesting that isoform-specific influences on AhR activity are ligand independent. Fractionation experiments further revealed differences in nuclear retention of ligand-induced AhR, suggesting that the ARNT isoforms possibly affect subcellular localization of AhR, thereby affecting transcriptional activity (Fig. 2 *B* and *D*). To further test this possibility, we utilized chromatin immunoprecipitation (ChIP) to examine the binding of AhR and ARNT to the *CYP1A1* promoter after manipulation of the ARNT isoform ratio. Introduction of siA-1/3 results in ablation of AhR DNA binding, as expected (Fig. 2*E*). Conversely, siA-1 enhances AhR and ARNT DNA binding, whereas siA-3 treatment shows AhR and ARNT DNA binding equivalent to that of siControl (Fig. 2*E*), suggesting a mechanism of ARNT isoform–specific AhR regulation that is more complex than retaining AhR in the nucleus.

Intriguingly, after siA-1 treatment, RT-qPCR analysis revealed significant augmentation of basal and TCDD-induced gene expression in the inflammatory lymphoid cell markers *IL17C*, *CD93*, *BAMBI*, *CXCL10*, and *CCR5*, whereas suppression of ARNT isoform 3 generally abrogated expression (*SI Appendix*, Fig. S2*A*). Conversely, the immune suppressive markers *TNFRSF9*, *IL1R2*, *CD274*, *SOCS2*, *STC1*, and *EPAS1* exhibit repressed expression after ARNT isoform 1 suppression but enhanced expression after suppression of ARNT isoform 3 (*SI Appendix*, Fig. S2*B*). These observations suggest that the ARNT isoforms significantly influence the expression of classical and immune-related AhR target genes in a similar fashion.

Next, we employed Peer cells, human leukemic T cells that possess effector-like properties (50), to test ARNT isoform–specific regulation of AhR signaling in an alternative T cell line. Interestingly, we found that ARNT isoforms 1 and 3 control AhR activity similarly in Peer cells as we observed for Karpas 299 cells, as monitored by *CYP1A1* expression levels (Fig. 2*F*), and analysis of AhR localization (Fig. 2*G*). Given that Peer and Karpas 299 cells are disparate T cell lines, these observations indicate that the ARNT isoforms might engage a general mechanism to regulate AhR signaling in lymphoid T cells.

ARNT Isoform 1 Is Phosphorylated during AhR Activation. To further explore the mechanism of ARNT isoform–specific regulation of AhR, we interrogated their amino acid sequence differences. The only divergence between ARNT isoforms 1 and 3 lies within the extra 15 amino acids encoded by alternative exon 5 that is included in isoform 1, which encompasses a canonical CK2 phosphorylation site at serine 77 (S77) (30). We surmised that CK2-mediated phosphorylation of S77 in ARNT isoform 1 is an important regulatory component of AhR signaling and set out to assess whether AhR activation has any bearing on S77 phosphorylation in both Karpas 299 and Peer cells. Immunoblot analysis reveals a dramatic increase in ARNT isoform 1 phosphorylation 30 min after TCDD or FICZ exposure (Fig. 3 *A* and *B*), suggesting that AhR activation is necessary for ARNT isoform 1 S77 phosphorylation. Importantly, mutational analysis of ARNT isoform 1 shows that immunoblot detection of ARNT isoform 1 phosphorylation is specific for posttranslational modification at S77 (*SI Appendix*, Fig. S3*A*). Moreover, in response to TCDD-induced AhR activation, phosphorylation increases from a basal state of 0.9:1 phosphorylated-to-unmodified ARNT isoform 1 to greater than a 10:1 phosphorylated-to-unmodified ARNT isoform 1 at 30 min post-TCDD exposure (*SI Appendix*, Fig. S3*B* and Dataset S5), as quantitated by liquid chromatography with tandem mass spectrometry.

Next, we sought to determine whether other AhR ligands elicit S77 phosphorylation. Markedly, AhR activation by each tested ligand promoted the phosphorylation of ARNT isoform 1 to varying degrees, which correlated with expression of *CYP1A1* and *AHRR* (Fig. 3*C*). These data demonstrate that S77 of ARNT isoform 1 is specifically and rapidly phosphorylated following AhR activation, obtaining a 10:1 ratio of phosphorylated-to-unmodified ARNT isoform 1 at 30 min post-TCDD exposure.

Phosphorylation of ARNT Isoform 1 Is Necessary for Optimal AhR Activity. To elucidate the role of ARNT isoform 1 phosphorylation in AhR signaling, we first sought to validate CK2 as the kinase responsible for ARNT isoform 1 phosphorylation at S77 within intact cells, given that the previous study used an in vitro CK2 kinase assay with recombinant proteins (30). To test this, we assayed for S77 phosphorylation upon AhR activation after first inhibiting CK2. As expected, immunoblot analysis showed that ARNT isoform 1 phosphorylation was abolished following inhibition of CK2 with the high-affinity CK2 inhibitor CX-4945 (51) (Fig. 4 *A* and *B*, *Left*). Intriguingly, RT-qPCR analysis shows that blocking TCDD- or FICZ-induced ARNT isoform 1 phosphorylation by CK2 inhibition significantly decreases expression of the AhR target genes *CYP1A1* and *AHRR* (Fig. 4 *A* and *B*, *Right*). Moreover, expression of immune-associated genes, which exhibit up-regulated expression after ARNT isoform 1 suppression (e.g., *IL17C*, *CD93*, *CCR5*, and *NR3C1*), is similarly significantly decreased upon CK2 inhibition (*SI Appendix*, Fig. S4*A*). In contrast,

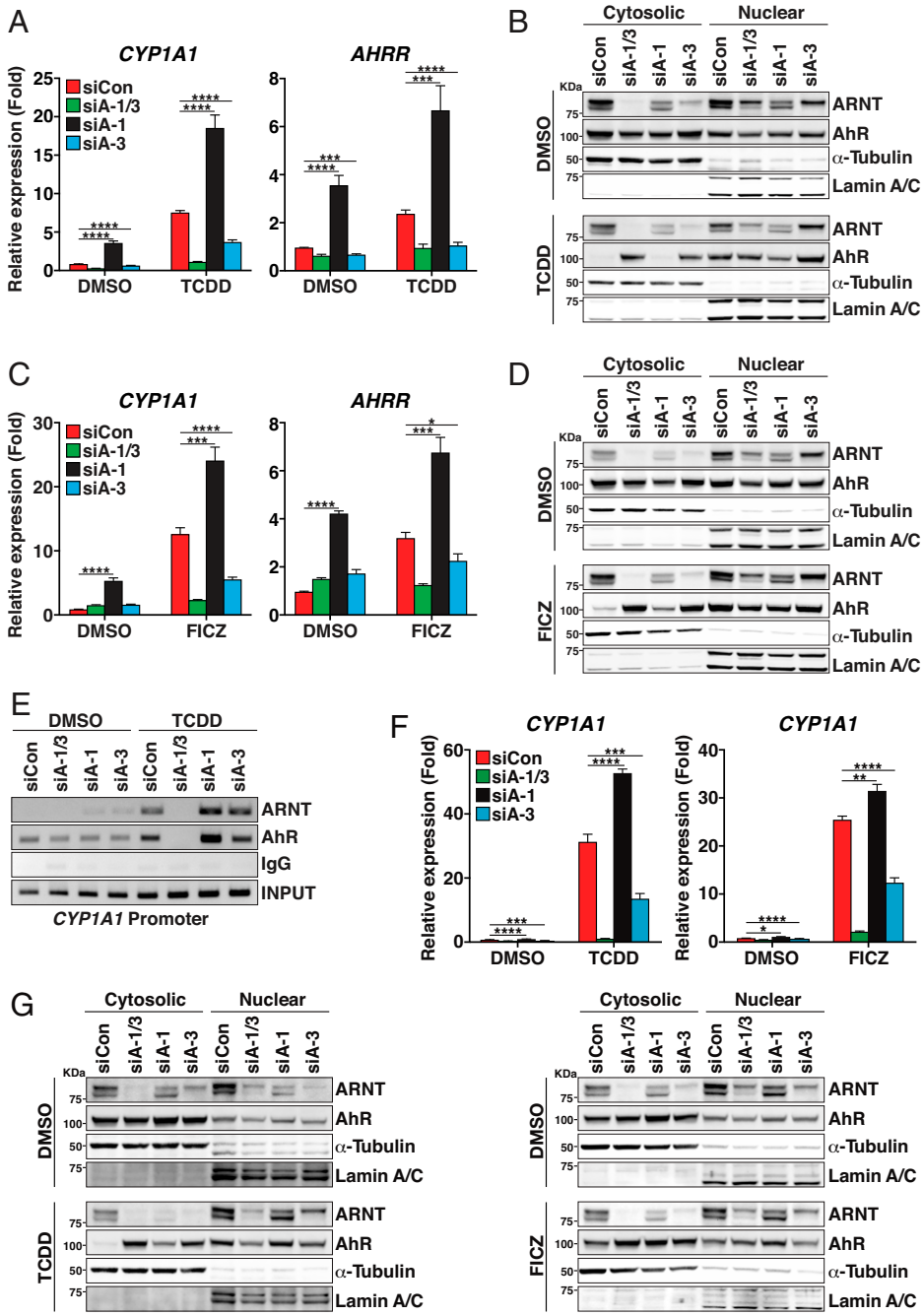

Fig. 2. Changes in the ARNT isoform 1:3 ratio modulate AhR activity. RNA interference was performed using siControl, siA-1/3, siA-1, or siA-3 in Karpas 299 cells for 48 h. The cells were then treated with DMSO as a vehicle control or exposed to TCDD (10 nM) or FICZ (1 nM) for 3 h. (*A* and *C*) RT-qPCR analysis of *CYP1A1* and *AHRR* gene expression. (*B* and *D*) Cytoplasmic and nuclear fractions were collected and analyzed by immunoblotting for ARNT, AhR, α-tubulin, and lamin A/C. (*E*) ChIP was performed with Karpas 299 cells after treatment with the indicated siRNA and exposed to TCDD (10 nM) for 30 min. Lysates were immunoprecipitated with antibodies specific to AhR and ARNT, or a negative control IgG antibody, and analysis was performed with primers specific for the AhRE cluster on the *CYP1A1* promoter. (*F* and *G*) RNAi was performed in Peer cells that were then exposed and analyzed by RT-qPCR or immunoblotting as described for *A*–*D*. RT-qPCR data are means ± SEM of three independent experiments performed in triplicate. *P* values are derived using a two-tailed unpaired Student's *t* test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. Western blots and ChIP images are representative of one experiment and were repeated at least three times.

immune genes whose expression is up-regulated after ARNT isoform 3 suppression are unaffected (*TNFRSF9*), modestly but significantly refractory to TCDD-mediated effects (*CD274* and *EPAS1*), or significantly up-regulated (*IL4I1*) after CK2 inhibition (*SI Appendix*, Fig. S4*B*). Importantly, AhR translocation and interaction with the ARNT isoforms is unaffected by CK2 inhibition, revealing that the observed decrease in gene expression is not a consequence of antagonistic effects by CX-4945 on AhR activation (Fig. 4*C*). Furthermore, like our observations in Karpas 299 cells, inhibition of CK2 in Peer cells also blocks TCDD- and FICZ-induced ARNT isoform 1 phosphorylation (Fig. 4 *D* and *E*, *Left*) and hinders expression of *CYP1A1* (Fig. 4 *D* and *E*, *Right*). These observations suggest that ARNT isoform 1 phosphorylation is a critical modification for optimal AhR target gene regulation.

AhR Is Required for ARNT Isoform 1 Phosphorylation. To further investigate the role of AhR in mediating ARNT isoform 1

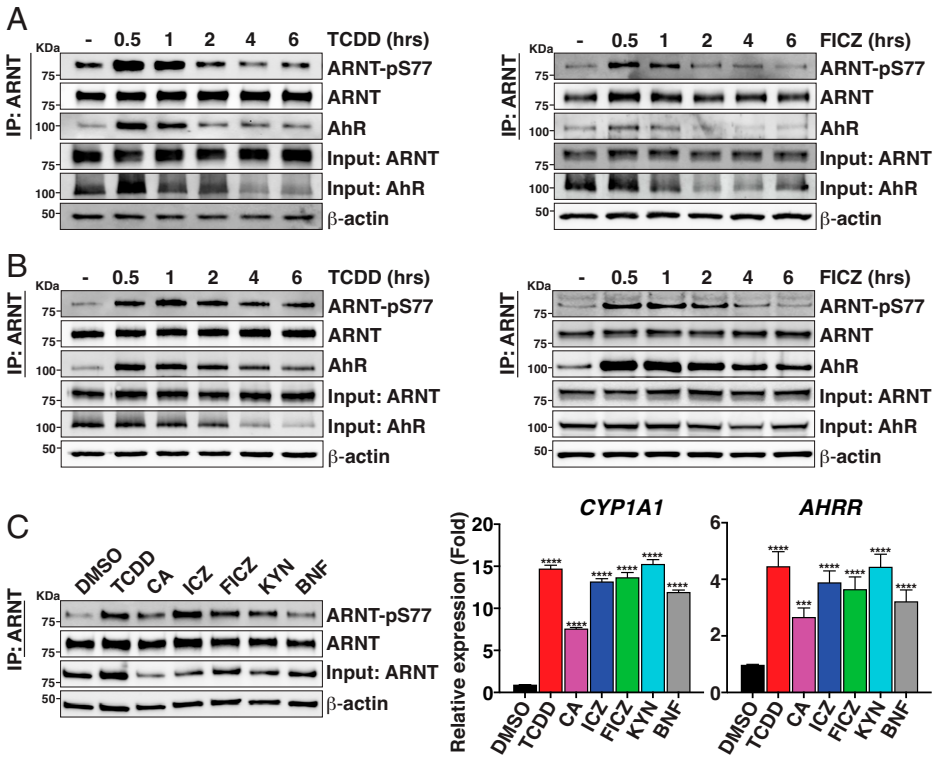

Fig. 3. AhR activation induces ARNT isoform 1 phosphorylation. (*A*) Karpas 299 cells were exposed to TCDD (10 nM, *Left*) or FICZ (1 nM, *Right*) for the indicated times, and then whole-cell lysates were immunoprecipitated with an ARNT-specific antibody and immunoblot analysis was performed with antibodies specific to phosphorylated ARNT isoform 1 (ARNT-pS77), ARNT, AhR, and β-actin. (*B*) Peer cells analyzed as described in *A*. (*C*) Karpas 299 cells were exposed to the AhR ligands TCDD (10 nM), cinnabarinic acid (CA, 30 μM), indolo[3,2-b]carbazole (ICZ, 20 nM), FICZ (1 nM), L-kynurenine (KYN, 50 μM), and β-naphthoflavone (BNF, 1 μM) for 30 min. Cells were then lysed and analyzed as in *A* (*Left*) or exposed to the same ligands for 2 h and analyzed by RT-qPCR to monitor *CYP1A1* and *AHRR* expression (*Right*). RT-qPCR data are means ± SEM of three independent experiments performed in triplicate. *P* values are derived using a two-tailed unpaired Student's *t* test: ***$P < 0.001$, ****$P < 0.0001$. Western blot images are representative of one experiment and were repeated at least three times.

phosphorylation, we examined ARNT isoform 1 phosphorylation after suppression of AhR. Strikingly, cells with reduced AhR levels are refractory to TCDD- or FICZ-induced ARNT isoform 1 phosphorylation (Fig. 5*A*). To further support the requirement of AhR for ARNT isoform 1 phosphorylation, we utilized Jurkat T cells, which are an acute T cell leukemia cell line devoid of AhR (52). Accordingly, Jurkat cells exposed to TCDD do not exhibit phosphorylation of ARNT isoform 1 (Fig. 5 *B*, *Left*). A lack of AhR signaling in Jurkat cells was validated by RT-qPCR analysis (Fig. 5 *B*, *Right*).

Considering our observation that ARNT isoform 1 phosphorylation is prevented upon loss of AhR, we utilized the AhR antagonist CH223191 to determine if AhR nuclear translocation is required for the phosphorylation of ARNT isoform 1. Immunoblot analysis reveals that ARNT isoform 1 phosphorylation at S77 is dramatically reduced in cells with antagonized AhR after exposure to TCDD or FICZ (Fig. 5 *C* and *D*, *Left*), with validation of AhR antagonism by RT-qPCR analysis of *CYP1A1* expression (Fig. 5 *C* and *D*, *Right*). Together, these results indicate that AhR ligation and nuclear translocation are necessary for ARNT isoform 1 phosphorylation.

ARNT Isoform 1 Phosphorylation Functions as a Rheostat for AhR Target Gene Expression. To test if ARNT isoform 1 phosphorylation is required for global AhR target gene regulation, we mutated S77 to an alanine (S77A) and transduced Hepa-BpRc1 cells, an *Arnt*-null cell line (53), with a lentivirus for the stable expression of wild-type (WT) or S77A ARNT isoform 1. Validation studies revealed that TCDD-induced phosphorylation at S77 could only occur in the WT ARNT isoform 1 cell line (Fig. 6*A*). Next, RNA-seq analysis was performed on these stable cell lines after treatment with DMSO or TCDD for 2 h. Interestingly, the stable S77A mutant cell line exhibits significant deficiencies in the regulation of TCDD-responsive genes, including a number of genes associated with immune function, and similar genes that were observed in the Karpas 299 RNA-seq dataset, such as *Socs2*, *Mafb*, *Tdo2*, *Alox5ap*, *Vegfd*, *Slc2a3*, *Myc*, *Ahr*, *Sod2*, *F5*, *Acer2*, *Tnfrsf19*, *Atg9b*, *Serpine1*, *Bmpr2*, *Nr3c1*, *Gdf15*, *Cdkn1b*, *Ido2*, *Glcci1*, *Aldh3a1*, *Tiparp*, *Gadd45b*, and *Cyp1a1* (Fig. 6*B* and Dataset S6). To confirm the transcriptomics analysis, we measured *Tiparp* (54) and *Cyp1a1* expression levels by RT-qPCR between the WT and the S77A cell line and observed a significant reduction in TCDD-induced expression in the S77A mutant cell line (Fig. 6*C*). Together, these data demonstrate that ligand-induced AhR-mediated ARNT isoform 1 phosphorylation at S77 is a necessary modification for controlled and optimal AhR target gene transcriptional regulation.

Discussion

While AhR-ARNT heterodimers are well-established regulators of AhRE gene transcription (55), whether the ARNT isoforms individually control different aspects of AhR signaling was unclear. Thus, given our previously observed differences in the ARNT isoform 1:3 ratio between normal human T cells and human T cell neoplasms (31), as well as the critical nature of AhR signaling in T cell function (56), we turned our attention to assessing whether the ARNT isoforms might distinctively regulate AhR activity. Interestingly, three regulatory patterns

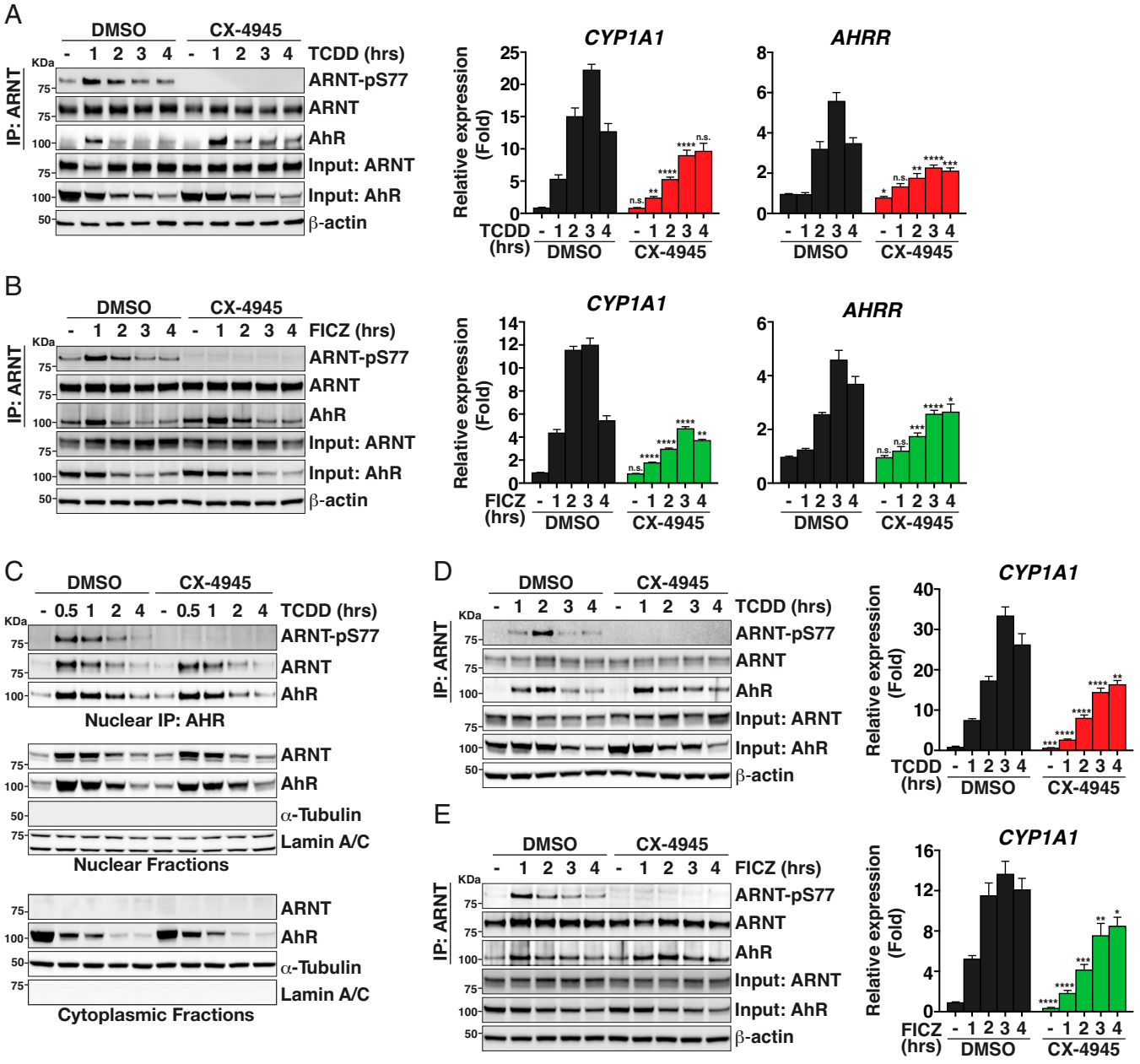

Fig. 4. Phosphorylation of ARNT isoform 1 is necessary for optimal AhR activity. Karpas 299 cells were treated with DMSO or the CK2 inhibitor CX-4945 (5 μM) for 1 h. Cells were then exposed to (*A*) TCDD (10 nM) or (*B*) FICZ (1 nM) for the indicated times, and whole-cell lysates were immunoprecipitated with anti-ARNT and analyzed via immunoblot, with antibodies directed to ARNT-pS77, ARNT, AhR, and β-actin (*Left*), or analyzed by RT-qPCR to monitor *CYP1A1* and *AHRR* expression (*Right*). (*C*) Karpas 299 cells were pretreated with DMSO or CX-4945 (5 μM) for 1 h and then exposed to DMSO or TCDD (10 nM) for the indicated times. Cytosolic and nuclear fractions were collected, and AhR was immunoprecipitated in the nuclear fraction. Protein fractions were analyzed by immunoblot with antibodies to ARNT-pS77, ARNT, AhR, α-tubulin, and lamin A/C. (*D* and *E*) Peer cells were analyzed as described in *A* and *B*. RT-qPCR data are means ± SEM of three independent experiments in *A* and *B*, or two independent experiments in *D* and *E*, performed in triplicate. *P* values are derived using a two-tailed unpaired Student's *t* test: n.s. = not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Western blot images are representative of one experiment that was repeated three times (*A*–*C*) or two times (*D* and *E*).

emerged from our transcriptional analyses in unstimulated and TCDD-treated Karpas 299 cells. First, AhR target genes exhibited abrogated or augmented regulation after siRNA-mediated suppression of ARNT isoform 3 or isoform 1, respectively (i.e., a high or a low isoform 1:3 ratio), which mirrored that of *CYP1A1* and *AHRR* expression. These results suggest that a high or a low ARNT isoform 1:3 ratio corresponds to stunted or enhanced AhR activity. However, certain AhR target genes exhibited an opposite regulatory pattern to that of *CYP1A1* and *AHRR* after modulation of the ARNT isoform ratio, suggesting that the ARNT isoforms function differently at specific loci. This second pattern of gene regulation includes *NR3C1* (encoding glucocorticoid receptor) and its target genes *TSC22D3* and *GLCCI1*. Interestingly, we observed that the regulatory pattern of *AHR* expression is inversely proportional to *NR3C1* expression, corresponding to long-held observations regarding the cross-regulation of these two receptors (57–60), and implies that changes in the ARNT isoform ratio might regulate the inverse relationship between their expression. Moreover, the differential regulation of *NR3C1* by the ARNT isoforms is bound to have important implications in T cell biology, given the role of glucocorticoids in shaping T cell

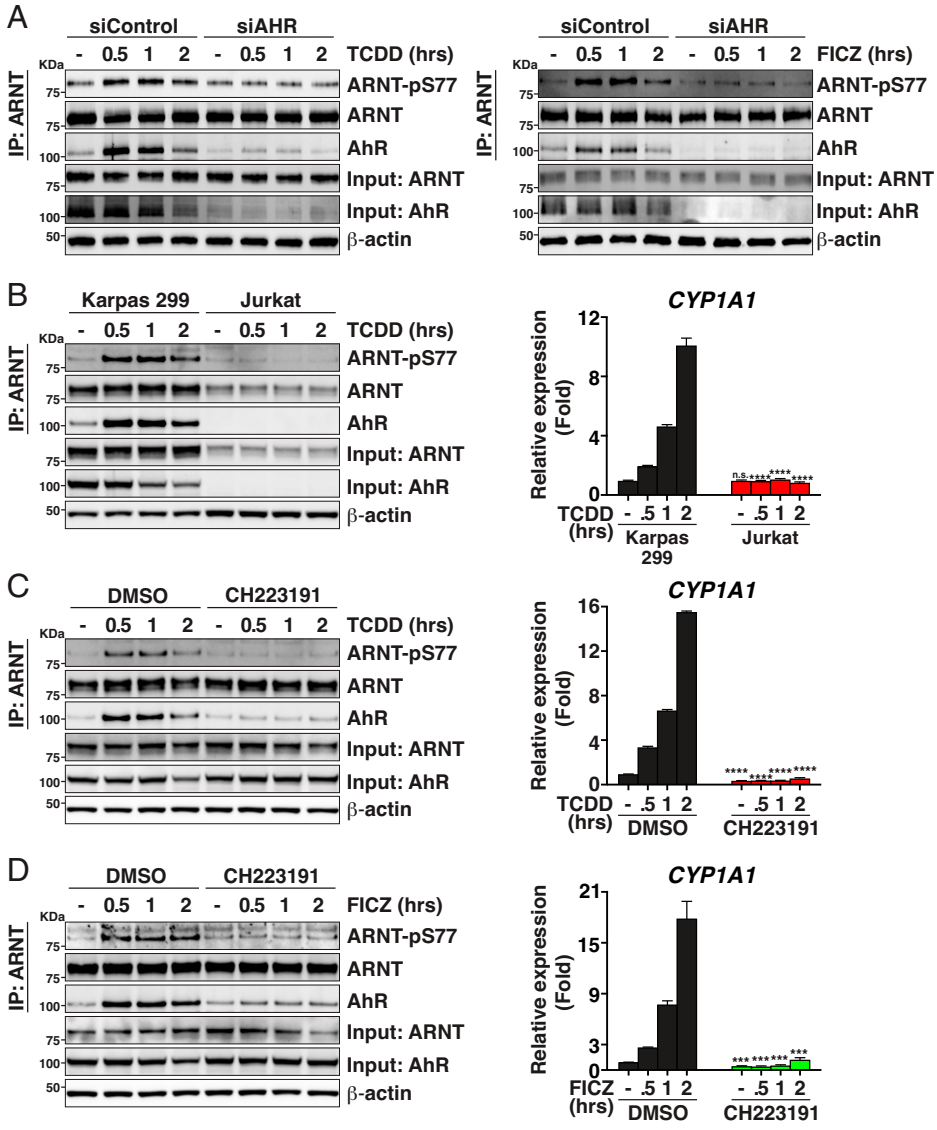

Fig. 5. AhR is required for ARNT isoform 1 phosphorylation. (*A*) Control or AhR-targeted siRNAs were introduced into Karpas 299 cells for 48 h, and then the cells were exposed to TCDD (10 nM, *Left*) or FICZ (1 nM, *Right*) for the indicated times, lysed, and immunoprecipitated with an antibody specific to ARNT, followed by immunoblotting with antibodies to ARNT-pS77, ARNT, AhR, and β-actin. (*B*) Karpas 299 and Jurkat cells were exposed to TCDD (10 nM) for the indicated times, and whole-cell lysates were immunoprecipitated with anti-ARNT and analyzed by immunoblot, with antibodies directed to ARNT-pS77, ARNT, AhR, and β-actin (*Left*), or analyzed by RT-qPCR to monitor *CYP1A1* expression (*Right*). (*C* and *D*) Peer cells were pretreated for 2 h with DMSO or the AhR antagonist CH223191 (10 μM) and then exposed to (*C*) TCDD (10 nM) or (*D*) FICZ (1 nM) for the indicated times. Whole-cell lysates were immunoprecipitated with anti-ARNT and analyzed via immunoblot, with antibodies directed to ARNT-pS77, ARNT, AhR, and β-actin (*Left*), or analyzed by RT-qPCR to monitor *CYP1A1* expression (*Right*). RT-qPCR data are means ± SEM of three independent experiments in *B*, or two independent experiments in *C* and *D*, performed in triplicate. *P* values are derived using a two-tailed unpaired Student's *t* test: n.s. = not significant, ***$P < 0.001$, ****$P < 0.0001$. Western blot images are representative of one experiment that was repeated three times (*A* and B) or two times (*C* and *D*).

responses (61). Lastly, several gene expression changes move in the same direction between all ARNT siRNA samples, regardless of combined or individual ARNT isoform suppression. This third pattern is exemplified by the expression of *IL4I1*, the product of which is an enzyme that catabolizes tryptophan into indole metabolites and kynurenic acid to serve as endogenous AhR ligands (62). Given that *IL4I1* is highly expressed in hematological malignancies and promotes an AhR-dependent immunosuppressive state for cancer immune evasion, the up-regulation of *IL4I1* after ARNT isoform suppression in Karpas 299 cells is possibly a compensatory measure for the perturbations in AhR signaling that accompany modulations in the ARNT isoform ratio. Combined, these observed expression patterns that emerge after manipulation of the ARNT isoform levels, in the absence and presence of TCDD, point to a paradigm whereby the duration and amplitude of AhR activity at specific gene loci are ARNT isoform dependent. Additionally, in the absence of a particular ARNT isoform, AhR might preferentially dimerize with a different binding partner (e.g., KLF6 or RelB), leading to divergent gene expression patterns (63, 64).

Confirmation of the global transcriptional analyses by targeted quantitation of *CYP1A1* and *AHRR* expression revealed similar augmentation or abrogation in Karpas 299 and Peer T cells after targeted depletion of isoform 1 or isoform 3, respectively. Furthermore, targeted RT-qPCR analysis of genes whose products regulate immune function also aligned with the observed differential gene expression in our transcriptomics datasets. Interestingly, this significant change in AhR target

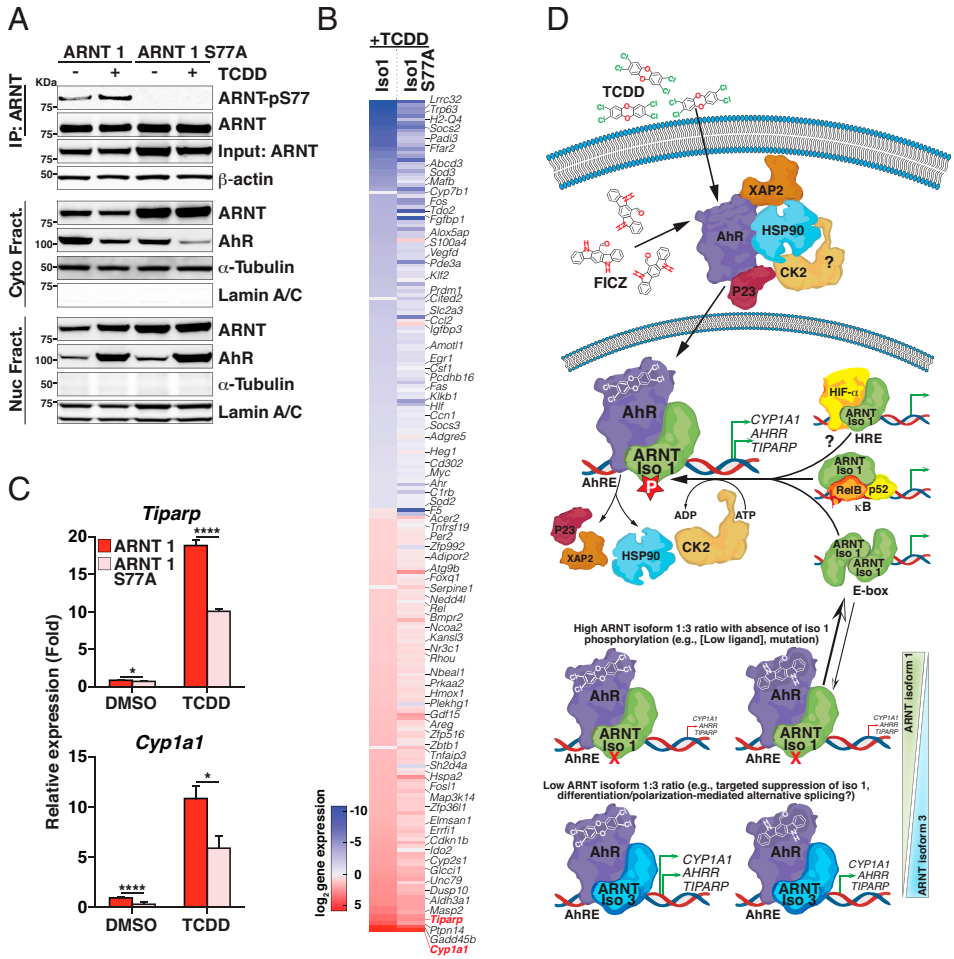

Fig. 6. ARNT isoform 1 phosphorylation functions as a rheostat for AhR target gene expression. (*A*) Hepa-BpRc1 cells stably expressing WT ARNT isoform 1 or ARNT isoform 1 S77A were exposed to TCDD (10 nM) for 30 min. Whole-, cytoplasmic, and nuclear cell lysates were extracted. The whole-cell lysate was immunoprecipitated with an antibody to ARNT. The eluates and lysates were analyzed by immunoblotting for ARNT-pS77, ARNT, AhR, α-tubulin, lamin A/C, and β-actin. (*B*) Heatmap of gene expression profiles ($P < 0.05$; $\geq \pm 1$ $\log_2$ fold change) between Hepa-BpRc1 cells stably expressing WT ARNT 1 or ARNT 1 S77A that were exposed to TCDD (10 nM) for 2 h and subjected to RNA-seq analysis. Significant differentially expressed genes, after TCDD exposure within the ARNT 1 or ARNT 1 S77A RNA-seq dataset, were identified by matching to RNA-seq differential gene analysis ($P < 0.05$) of the parental Hepa-1c1c7 cell line (expresses endogenous functional *Arnt*) exposed to TCDD for 2 h. The change in $\log_2$ fold gene expression depicted in the heatmap is versus the DMSO control–treated parental Hepa-1c1c7 cells, and genes with similar TCDD-induced expression profiles between the ARNT 1 or ARNT 1 S77A stable cells ($\leq \pm 0.5$ $\log_2$ fold change) were removed to highlight those genes whose expression is affected by the S77A mutation. (*C*) Cell lines stably expressing ARNT isoform 1 or ARNT isoform 1 S77A were exposed to TCDD (10 nM) for 2 h and then monitored for *Cyp1a1* and *Tiparp* gene expression using RT-qPCR. RT-qPCR data are means ± SEM of two independent experiments performed in triplicate. *P* values are derived using a two-tailed unpaired Student's *t* test: $*P < 0.05$, $****P < 0.0001$. Western blot images are representative of one experiment that were performed two times. (*D*) A working hypothetical model for ARNT isoform-mediated regulation of AhR signaling. Upon ligand binding, AhR translocates to the nucleus, where it sheds cochaperone proteins and binds to ARNT. Data presented support an AhR-mediated CK2 phosphorylation of ARNT isoform 1, possibly by bringing CK2 into proximity with ARNT as a component of the cochaperone complex, but the exact mechanism is yet to be elucidated. We further speculate that unphosphorylated ARNT isoform 1 prefers other transcription factors and/or DNA enhancer sequences, as supported by previous reports, with an overall effect of minimal AhR activity in the presence of a high ARNT isoform 1:3 ratio. Subsequent phosphorylation of ARNT isoform 1 precludes binding to E-box sequences and possibly other DNA recognition sequences, without affecting AhRE binding, thereby allowing for a tunable AhR response depending on the relative pool of phosphorylated ARNT isoform 1. Conversely, a low ARNT isoform 1:3 ratio would not be governed by this mechanism and would result in robust AhR activity. At this point, a low isoform 1:3 ratio has been achieved only by experimental means, but we predict that T cell activation/differentiation might result in alternative splicing of ARNT, producing modulations within the ARNT isoform ratio, given the variations observed between normal versus malignant human T cells (31).

gene expression occurred in the absence and presence of TCDD or FICZ, albeit at a reduced level without the addition of exogenous ligand, most likely a consequence of AhR ligands present in the cells and/or serum. As an initial step into delineating the mechanism of ARNT isoform–specific regulation of AhR signaling, we focused on the unique CK2 phosphorylation site at S77 present only in isoform 1, as this is the most obvious distinction that might dictate functional variances between the ARNT isoforms with respect to modulating AhR responses. Intriguingly, we found that exposure of cells to all AhR ligands tested promoted rapid and dynamic phosphorylation of ARNT isoform 1 S77 to varying degrees. Moreover, our study revealed that phosphorylation of ARNT isoform 1 is dependent on ligand-activated AhR and likely requires AhR nuclear translocation. We further found that inhibition of ARNT isoform 1 phosphorylation via CK2 inhibition, or mutation of S77, significantly reduces the expression of AhR ligand-induced target genes in diverse cell types, revealing the importance of ARNT isoform 1 phosphorylation for driving AhR activity. Regarding our reconstitution experiments, the results are even more striking when considering that cells reconstituted with the S77A mutant harbor relatively higher levels of ARNT and nuclear AhR versus cells reconstituted with WT ARNT, indicating that relative levels of phosphorylated isoform 1 serve as a rheostat for AhR signaling. We are currently focused on understanding the complete molecular mechanism of how AhR directs the

CK2-mediated phosphorylation of ARNT isoform 1 at S77. Our working hypothesis is that heat shock protein 90 (HSP90), a cochaperone of AhR and well-defined target of CK2 (65), recruits CK2 and brings it into proximity with ARNT isoform 1 upon AhR-ARNT dimerization (Fig. 6*D*). This proximity hypothesis is further supported by the fact that dimerization of ARNT with AhR is necessary for HSP90 displacement (22). We further speculate that the rapid and relatively high CK2-mediated phosphorylation of ARNT isoform 1 (>10:1 phosphorylated to unmodified) inhibits the binding of ARNT to other transcriptional response elements, as has been reported for E-box sequences (30), thereby directing ARNT to AhRE sites where S77 phosphorylation has been shown not to impede binding (30). This hypothetical scenario, combined with our observations reported here showing abrogated or enhanced AhR binding to the *CYP1A1* promoter that correlates with differential expression of *CYP1A1* depending on the ARNT isoform 1:3 ratio, suggests that the ARNT isoforms may confer different DNA binding affinities to AhR or possibly recruit specific transcriptional coregulators, some of which could be dependent on isoform 1 phosphorylation.

As a practical application of our hypothetical model, let us consider the role of AhR in T cell polarity. Early studies into AhR biology identified that TCDD triggers immunosuppression (26, 66–68), which was later partially attributed to TCDD-induced Treg differentiation (25, 27, 38). Conversely, FICZ promotes the differentiation of inflammatory TH17 cells in certain contexts (28, 69). AhR ligand-dependent conformational states leading to the recruitment of specific transcriptional coregulators and chromatin remodelers, or tissue-specific expression of coregulators, have been reported as mechanisms of AhR ligand-mediated immune cell polarization (70–75). Additionally, the concentration of AhR ligands within the microenvironment, and the subsequent duration of AhR activation, have been shown to elicit opposing T cell subsets (76). Given our observations reported in this study, it is tempting to speculate that the relative levels of the ARNT isoforms, and isoform 1 phosphorylation, also contribute to divergent T cell differentiation. For instance, CK2 activity regulates the TH17/Treg balance, and targeting CK2 activity with CX-4945 was shown to inhibit TH17 differentiation but promote Treg generation (77). Indeed, suppression of ARNT isoform 3 in DMSO-treated Karpas 299 cells resulted in the down-regulation of inflammatory markers including *IL17C*, whereas subsequent TCDD exposure led to derepression of the same inflammatory markers correlating with CK2 activity in determining T cell fate. Accordingly, unphosphorylated ARNT isoform 1 might reprogram the AhR target gene battery or have higher affinity toward other binding partners such as HIF-α (Fig. 6*D*).

It is feasible that a high ARNT isoform 1:3 ratio, which corresponds to a general reduction in AhR activity, might instead promote ARNT-HIF-α activity (78). Moreover, HIF-2α (encoded by *EPAS1*) is an essential mediator of Treg suppressor cell function, and deletion of *EPAS1* in Treg cells drives reprogramming toward interleukin-17–secreting cells (46). Notably, Karpas 299 cells harbor modest levels of normoxic HIF-α protein (79), and our transcriptomics dataset from DMSO-treated cells revealed up-regulated or repressed *EPAS1* expression in cells with a high or a low ARNT isoform 1:3 ratio, respectively, which corresponds to the expression pattern of immunosuppressive versus inflammatory markers observed between the siA-3 and siA-1 backgrounds. Specifically, the expression of *CD274* (encoding PD-L1), which is a common target of AhR and HIF-α (80–82), was down-regulated in cells after suppression of ARNT isoform 1 but enhanced after suppression of ARNT isoform 3. These observations support the notion that ARNT isoform ratio modulation might contribute to variations in AhR activity through the inverse regulation of AhR and HIF-α activity, with isoform 1 phosphorylation possibly serving to switch ARNT to AhR signaling (Fig. 6*D*). Thus, further investigation is needed to test whether differential ARNT isoform utilization, possibly as a function of T cell activation–induced alternative splicing of ARNT (31), or ARNT isoform 1 phosphorylation, contributes to the reported differences in AhR ligand-mediated T cell polarity. While beyond the scope of the current study, we are intrigued with these possibilities and more work is required for delineation of the molecular mechanism used by the ARNT isoforms to regulate AhR target gene expression in normal versus neoplastic T cells. Nevertheless, our results demonstrate that the ARNT isoforms have specific, and in many instances opposite, influences on AhR activity that together ultimately shape the outcome of AhR signaling.

In summary, our findings greatly enhance the understanding of AhR signaling by revealing an additional layer of AhR regulation by the ARNT isoforms that should be considered when investigating AhR signaling, especially in immune cells where ARNT isoforms 1 and 3 are robustly expressed. Unequivocally, our data demonstrate that a high ARNT isoform 1:3 ratio impedes low basal levels of AhR signaling until after robust AhR activation. In turn, AhR activation promotes ARNT isoform 1 phosphorylation to allow for optimal AhR target gene transcription. Conversely, a low ARNT isoform 1:3 ratio appears to readily promote AhR activity in an augmented fashion. Together, our results indicate that manipulation of the ARNT isoform ratio, or targeting ARNT isoform 1 phosphorylation, offers potential therapeutic options to inhibit or enhance AhR activity for treating hematological malignancies and other immune disorders.

Materials and Methods

Cell Culture and Reagents. Karpas 299, Jurkat, and Peer cells were propagated in Roswell Park Memorial Institute (RPMI) 1640 Medium, Hepa-1c1c7 cells were cultured in Minimum Essential Medium (MEM) Alpha, and stable BpRc1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and 2 μg/mL puromycin (InvivoGen). For CK2 inhibitor experiments, cells were treated with 5 μM CX-4945 (Selleckchem) for 1 h prior to AhR ligand exposure. AhR antagonist experiments were conducted by pretreating cells with 10 μM CH223191 (MilliporeSigma) for 2 h prior to AhR ligand exposure. Culture and reagent details are described in *SI Appendix*.

RNA Interference. Cells were transfected with 4 μM of target siRNA duplexes ~44 h prior to analysis. The siRNA (MilliporeSigma) target sequences are siA-1 5′-UGC CAG GUC GGA UGA UGA GCA-3′, siA-3 5′-CGG UUU GCC AGG GAA AAU C-3′, siA-1/3 5′-GAC UCG UAC UUC CCA GUU U-3′, 5′-CUU UGC UCC UGA GAC UGG A-3′, and AhR (MilliporeSigma, SASI_Hs01_00140202). The target sequence for siControl is a scrambled siA-1/3 sequence (31). See *SI Appendix* for details.

RNA-Seq and Transcriptomics Analysis. Total extracted RNA was subjected to library preparation and sequencing. The sequenced reads were trimmed and filtered based on adapter content and quality by a modified-Mott trimming algorithm (CLC Genomics Workbench 21, Qiagen). Filtered sequencing reads were locally aligned against the *Homo sapiens* (hg38; Fig. 1; *SI Appendix*, Fig. S1; and Datasets S1–S4) or *Mus musculus* (GRCm38; Fig. 6 and Dataset S6) reference genomes with annotated genes and transcripts (83). The resulting gene counts were normalized, and differential expression analysis on the complete list of genes and transcripts was used to evaluate the level and significance of

mRNA expression changes (84). All genes with a fold change ≥ 1.5 were included in the pathway analysis using IPA to highlight molecular function, biological process, and cellular component.

RT-qPCR. The *H. sapiens* primer-probe assays (Bio-Rad) used were *CYP1A1* (qHsa CEP0058439), *AHRR* (qHsa CEP0057513), *IL1R2* (qHsa CIP0039159), *CD274* (qHsa CIP0039192), *SOCS2* (qHsa CEP0024541), and *GAPDH* (qHsa CEP0041396). SYBR Green assays were used for all other *H. sapiens* and *M. musculus* sample analyses. See *SI Appendix* for primer sequences and details.

Antibodies, Immunoblot Analysis, Nuclear Translocation Assay, and Coimmunoprecipitation. See *SI Appendix* for details.

ChIP. The ChIP experiment was performed as previously described (4) with some minor modifications. Details and primer sequences used to amplify the AhRE cluster in the *CYP1A1* promoter can be found in *SI Appendix*.

Stable Cell Line Generation. Hepa-1c1c7 cells and the variant BpRc1 (*Arnt*-null) cells were kindly provided by Dr. Cornelis Elferink (The University of Texas Medical Branch, Galveston, TX), transduced with a lentivirus containing a puromycin resistance marker, and engineered to express complementary DNA encoding ARNT isoform 1 or an ARNT isoform 1 S77A point mutant. Stable single-cell clones were selected by limiting dilution in the presence of 2 μg/mL puromycin. See *SI Appendix* for details.

Statistical Analysis. Statistical differences between two individual groups were assessed by performing a two-tailed unpaired Student's *t* test. Graphing and analyses of statistics were performed using Prism (GraphPad). Further details on statistical analysis of RNA-seq and RT-qPCR data are provided in *SI Appendix*.

Data Availability. All study data are included in the article and/or *SI Appendix*. Additionally, raw data files for the expression profiling by RNA-seq have been deposited in Gene Expression Omnibus (accession no. GSE184151) (85).

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