Supplementary Information for

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

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Supplementary Materials and Methods

Cell Culture and Reagents
Karpas 299, Jurkat, and Peer cells were propagated in RPMI-1640 medium (Corning, 15-041-CV) complete with 10% FBS (Atlanta Biologicals, S11550) and 2 mM GlutaMAX (Gibco, 35050-061) at 37 °C, 5% CO2. Hepa-1c1c7 cells were cultured in MEM Alpha (1X) + 2 mM GlutaMAX (Gibco, 32561-037) with 10% FBS at 37 °C, 5% CO2. Stable BpRc1 cells, an Arnt-null variant of Hepa-1c1c7 cells, were cultured in DMEM (Corning, 15-018-CV) with 10% FBS, 2 mM GlutaMAX, and 2 µg/mL puromycin (Invivogen, ant-pr) at 37 °C, 5% CO2. For CK2 inhibitor experiments, 1 × 10^7 Karpas 299 or Peer cells were treated with 5 µM CX-4945 (Selleckchem, S2248) for one hour prior to AhR ligand exposure. AhR antagonist experiments were conducted by pretreating 1 × 10^7 Peer cells with 10 µM CH223191 (MilliporeSigma, C8125) for 2 hours prior to AhR ligand exposure. AhR ligands used in this study are as follows: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (Cerilliant, ED-901-C), 6-Formylindolocarbazole (FICZ) (MilliporeSigma, SML1489), b-Naphthoflavone (BNF) (MilliporeSigma, N3633), L-Kynurenine (KYN) (MilliporeSigma, K8625), Indolo[3,2-b]carbazole (ICZ) and Cinnabarinic Acid (CA) (obtained from Dr. Cornelis Elferink, The University of Texas Medical Branch, Galveston, TX).

RNA Interference
1 × 10^7 Karpas 299 or Peer cells were transfected with 4 µM of target siRNA duplexes using a Bio-Rad Gene Pulser Xcell electroporator set on infinite resistance, 300 V for Karpas 299 cells or 250 V for Peer cells, and 950 microfarads. Cells were suspended in RPMI-1640 medium containing 20% FBS and 2 mM GlutaMAX. Sixteen hours post-transfection, dead cells were removed by centrifugation at 400 × g for 20 minutes with Ficoll-Paque PLUS (GE Healthcare, 17-1440-02). The live cell layer was then transferred to RPMI-1640 medium complete with 10% FBS and 2 mM GlutaMAX at 0.5 × 10^6 cells/mL for 24 hours. The siRNA (MilliporeSigma) target sequences are as follows: siA-1 5’-UGC CAG GUC GGA UGA UGA GCA 3’, siA-3 5’-CGG UUU GCC AGG GAA AAG C-3’, siA-1/3 5’-GAC UCG UAC UUC CCA GGU U-3’ and 5’-CUU UGC UCG UCA GAC UGG A-3’, and AhR (MilliporeSigma, SASI_Hs01_00140202). The target sequence for siControl is a scrambled siA-1/3 sequence (1).

RT-qPCR
Total RNA was extracted using QIAshredder (Qiagen, 79656) and RNeasy Kits (Qiagen, 74104). 300 ng of RNA was converted to cDNA using iScript cDNA Synthesis Kits (Bio-Rad, 1706691). RT-qPCR was performed on the CFX96 Real Time System (Bio-Rad) using SsoAdvanced Universal Probes Supermix (Bio-Rad, 1725284) for Primer-Probe assays and SsaAdvanced Universal SYBR Green Supermix (Bio-Rad, 1725274) for primer only assays. GAPDH (qHsa CEP0041396) was the internal reference gene used in the Primer-Probe assays to determine fold changes with the ΔC_T method. The following primers were used for the SYBR Green assays: homo sapiens sample analysis: IL17C (qHsa CED0044039); CD93 5’-GGC AGA CAG TTA CTC CTG GGT T-3’ and 5’-GGG GTT CAA AGC TCT GAG GAT GG-3’; BAMBI 5’-TAC AGA GGG CGG CTG GAT GTT C-3’ and 5’-AAG TCA GCT CTC CCT GGA CCT TGG T-3’; CXCL10 5’-GGT GAG AAG AGA TGT CTG AAT CC-3’ and 5’-GTC CAG CAT CCT TGG AAG CAC TGC A-3’; CCR5 5’-TCT CTT CTG GGC TCC CTA CAA C-3’ and 5’-CCA AGA GTA GTC TCT GTC ACC TGC A-3’; TSC22D 5’-ATC TGC AAC CGC AAC ATC GAC C-3’ and 5’-GCA TAC ATC AGA TGA TGC TCC ACC-3’; NR3C1 5’-ATA GGT CTG TCC CAG ACT CAA CT-3’ and 5’-TCC TGA AAC CTC GTA TGG CCT C-3’; TNFRSF9 5’-TCT GCC TCA CGC TCC GTT TCT C-3’ and 5’-TGG AAA
Antibodies and Immunoblot Analysis
Whole cell lysates were extracted by incubating cells with radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling, 9806S) complete with phosphatase inhibitor cocktail 2 (MilliporeSigma, P5726), phosphatase inhibitor cocktail 3 (MilliporeSigma, P0044), complete mini protease inhibitor tablets (Roche, 11836170001), and 50 mM sodium fluoride (MilliporeSigma, S1504-100G) on ice for twenty minutes. Protein samples were normalized using the Bradford method, resolved on NuPAGE 4-12% Bis-Tris Gels (Invitrogen, NP0322BOX) and transferred onto nitrocellulose membranes (Bio-Rad, 1620215). Membranes were blocked with 1% Casein Blocker (Bio-Rad, 1610782) supplemented with 0.1% Tween 20. Membranes were incubated with the indicated antibodies, washed 4 times with 0.1% TBST, incubated with specific horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, NA931 (Mouse) or NA934 (Rabbit)) for one hour, and then washed 4 more times. Lastly, ECL substrate (Bio-Rad, 170-5060) was added to the membrane for five minutes and horseradish peroxidase activity was imaged on the ChemiDoc MP Imaging System (Bio-Rad). Antibodies used are as follows: ARNT-pS77 (ThermoFischer, custom antibody to sequence C-KERFARS*PDDEQSS-amide), ARNT (BD Transduction Laboratories, 611079), AhR (ENZO, BML-SA210), Lamin A/C (Santa Cruz, sc-20681), α-Tubulin (Cell Signaling, 2148S), β-actin (MilliporeSigma, A5316).

Co-immunoprecipitation
Protein extraction was performed on the indicated co-IP samples using complete RIPA lysis buffer, and then 2 µg of ARNT (Novus, NB100-124) or AhR (ENZO, BML-SA210-100) antibody was added to each sample and rotated at 4 °C overnight. The next morning, 30 µL of a 50% slurry of rProtein G Agarose beads (Invitrogen, 15920-010), prewashed three times with complete RIPA lysis buffer, was added to each sample and rotated at 4 °C for an additional hour. Samples were then washed four times with Triton-X 100 lysis buffer (25 mM Hepes pH 7.9, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton-X 100). Loading buffer containing sodium dodecyl sulfate and dithiothreitol was added to bead-bound protein complexes and subsequently boiled for 5 minutes prior to loading on reducing PAGE gels for immunoblot analysis.

Nuclear Translocation Assay
1 × 10^7 Karpas 299, Peer, or Hepa-BpRc1 cells were treated with vehicle control (DMSO), 10 nM TCDD, or 1 nM FICZ for the designated times as outlined in the figure legends. Cells were collected, washed with PBS (Corning, 21-040-CV), and cytoplasmic and nuclear protein fractions were collected with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, 78835) according to the manufacturer’s protocol.
Chromatin immunoprecipitation
40 hours after induction of RNA interference, 8 \times 10^6 Karpas 299 cells were exposed to DMSO or TCDD (10 nM) for 30 minutes. Protein-DNA complexes in Karpas 299 cells were then cross-linked with 1% formaldehyde (Polysciences Inc., 18814-10) for 10 minutes at room temperature, which was followed by quenching in 125 mM glycine for 5 minutes at room temperature. Cells were collected via centrifugation at 300 × g for 5 minutes at 4 °C, then washed in PBS and spun down again at the same speed at 4 °C. Cells were then lysed at a ratio of 100 µL per 2 million cells in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1), supplemented with phosphatase inhibitor cocktail 2 (MilliporeSigma, P5726), phosphatase inhibitor cocktail 3 (MilliporeSigma, P0044), complete mini protease inhibitor tablets (Roche, 11836170001), and 50 mM sodium fluoride, and a ChIP experiment was performed as previously described (2). Primer sequences used to amplify the AhRE cluster in CYP1A1 promoter are: 5’-TAA GAG CCC CGC CCC GAC TCC T-3’ and 5’-CTC CCG GGG TGG CTA GTG CTT TGA-3’.

Testing Specificity of ARNT-pS77 antibody
Human embryonic kidney (HEK)293T cells were transfected with an empty pEBB plasmid, a pEBB plasmid containing a cDNA encoding wildtype ARNT isoform 1, or the indicated individual ARNT isoform 1-point mutants using a standard calcium phosphate transfection protocol. Twenty-four hours post-transfection 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) or ARNT. Mutation of ARNT isoform 1 cDNA was performed using the Quickchange kit (Agilent) with primers: S77A sense 5’-GAG CGG TTT GCC AGG GCG GAT GAT GAG CAG-3’ and nonsense 5’-GAG CGG TTT GCC AGG GCG GAT GAT GAG CAG-3’; S83S84A sense 5’-GAG CGG TTT GCC AGG GCG GAT GAT GAG CAG-3’ and nonsense 5’-GAG CGG TTT GCC AGG GCG GAT GAT GAG CAG-3’. The S77S83S84A mutant was constructed using the ARNT isoform 1 S77A plasmid as a template in conjunction with the S83S84A primer pair.

ARNT phospho-peptide LC-MS/MS analysis
Karpas 299 cells were exposed to TCDD (10 nM) for 0, 0.5, 1, or 2 hours after which whole cell lysates were immunoprecipitated with an ARNT specific antibody. The immunoprecipitated ARNT samples were prepared for LC-MS/MS analysis as previously described (3). Samples were analyzed by nanoLC-MS/MS (nanoRSLC, ThermoFisher) using an Aurora series (Ion Opticks) reversed phase HPLC column (25 cm length x 75 µm inner diameter) directly injected to a ThermoScientific Orbitrap Eclipse using a 65 min method (mobile phase A = 0.1% formic acid (Thermo Fisher), mobile phase B = 99.9% acetonitrile with 0.1% formic acid (Thermo Fisher); hold 1% B for 5 min, 1-15% B in 40 min, 15-32% for 20 min) at a flow rate of 300 µL/min. Eluted peptide ions were analyzed using a data-dependent acquisition (DDA) method with resolution settings of 120,000 and 15,000 (at m/z 200) for MS1 and MS2 scans, respectively. DDA-selected peptides were fragmented using stepped high energy collisional dissociation (27, 32, 37%). Tandem mass spectra were analyzed according to a label-free proteomic strategy using Proteome Discoverer (version 2.5.0.400, ThermoFisher) with the Byonic (version 4.1.10, Protein Metrics) and Minora nodes, using a database consisting of human protein sequences (4, 5). Mass tolerances of 10 ppm and 20 ppm were used for matching parent and fragment masses, respectively. Mass spectra were searched with a fixed modification of carbamidomethyl (C), and up to 2 common variable modifications of deamination (N,Q), oxidation (M), and phosphorylation (S, T, Y). A trypsin-produced peptide with two missed
cleavages spanning residues F74-R88 was detected with zero and one phosphorylation; MS2 fragment data allowed determination of the phosphorylation site to be S77. A ratio of phosphorylated/unmodified F74-R88 peptide was calculated using the measured area under the curve peak areas (5 ppm extracted mass) of all the observed peptide ions corresponding to the tryptic peptide fragment (F74-R88) which was observed as a 3+ phosphorylated ion and 3+ and 4+ for the unmodified ion.

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). Lentiviral packaging in HEK 293 cells was performed as previously described (6). Lentivirus containing a puromycin resistance marker and engineered to express cDNA encoding ARNT isoform 1, or an ARNT isoform 1 S77A point mutant, were incubated with 5 × 10^4 BpRc1 cells for four hours at 37 °C, 5% CO_2. Media was then replaced with fresh DMEM and incubated forty-eight hours at 37 °C, 5% CO_2. Stable single cell clones were selected by limiting dilution in the presence of 2 μg/mL puromycin. Mutation of S77 to alanine in ARNT isoform 1 cDNA was performed using the Quickchange kit (Agilent) with primers: sense 5’-GAG CGG TTT GCC AGG GCG GAT GAT GAG CAG-3’ and nonsense 5’-CTG CTC ATC ATC CGC CCT GGC AAA CCG CTC-3’.

Statistical Analysis
All data was graphed as mean ± SEM of either two or three independent experiments performed in triplicate, as indicated in the figure legends. Graphing and analyses of statistics were performed using Prism (GraphPad). Statistical differences between two individual groups were assessed by performing a two-tailed unpaired Student’s t-test. Results from RT-qPCR were considered significant when a P value of less than 0.05 was achieved. Differential gene expression analysis utilized dispersion (biological coefficient of variation squared) to calculate the statistical significance of gene abundances between groups. In the absence of biological replicates (e.g., the TCDD treated samples where n=1), a common dispersion value was estimated and adjusted based on gene-wise dispersion, by taking the linear combination of the likelihood for the gene of interest and neighboring genes with similar expression values (i.e., the window of 1.5% of genes to the left and to the right when sorted by counts per million mapped reads), with a reported 92% positive predictive value on datasets consisting of single samples (7). Additionally, a modest increase in false positives can occur in the absence of biological replicates (~1%) (8). Thus, we subsequently validated the RNA-seq analysis by targeted RT-qPCR on 16 genes (with biological replicates of n=2-3 depending on the experiment, each performed in triplicate), all of which aligned with our RNA-seq data.
Fig. S1. ARNT isoform-specific regulation of AHR target gene expression. (A) Venn diagram showing relationship of all significant \((P<0.05)\) differentially expressed genes between each ARNT siRNA dataset exposed to DMSO or TCDD versus siControl samples exposed to DMSO or TCDD.
exposed to DMSO or TCDD. (B) Gene clustering analysis comparing gene expression changes of ± 1.5-fold (FDR<0.05) between all combinations of control and ARNT siRNA samples, untreated (n=2) or treated with TCDD (n=1). (C) Volcano plots of select genes showing the change in significance, and relationship in expression, between siA-1/3 (Top), siA-1 (Middle), and siA-3 (Bottom), as compared to siControl. The horizontal dashed grey line signifies $P=0.05$ and the vertical dashed grey lines mark ± 1 log₂ change on the x-axis. (D) IPA of the differentially expressed genes in siA-1/3 (Top), siA-1 (Middle), and siA-3 (Bottom), exposed to either DMSO or TCDD (10 nM) for 2 hours, showing the top ten most significant pathways unique to each siARNT condition. 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; pathways with undetermined z-score are shown in grey. The relative strength of the z-score is depicted by the intensity of the color. (E) Upstream regulators determined by IPA in siA-1 treated versus siA-3 treated Karpas 299 cells exposed to either DMSO or TCDD (10 nM) for 2 hours. Only those upstream regulators with a z-score of ± 2 within the DMSO and/or TCDD treated samples are included.
Fig. S2. Changes in the ARNT isoform 1:3 ratio modulates AhR activity. RNAi was performed using siControl, siA-1, or siA-3 in Karpas 299 cells for forty-eight hours. The cells were then treated with DMSO as a vehicle control or exposed to TCDD (10 nM) for 3 hours. (A) RT-qPCR analysis of inflammatory marker genes (IL17C, CD93, BAMBI, CXCL10, and CCR5), or of immune suppressive genes (TSC22D3 and NR3C1). (B) RT-qPCR analysis of Treg marker genes. 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<0.01, ***P<0.001, ****P<0.0001 (comparisons indicated by bars); †P<0.05, ††P<0.01, †††P<0.001, ††††P<0.0001 compared to the DMSO treated condition of the same RNAi sample.
Fig. S3. AhR activation induces ARNT isoform 1 phosphorylation. (A) HEK293T cells were transfected with a control empty plasmid or plasmids encoding the indicated ARNT isoform 1 wildtype or point mutants. Twenty-four hours post-transfection 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) or ARNT. (B) Karpas 299 cells were exposed to TCDD for 0, 0.5, 1, and 2 hours after which whole cell lysates were immunoprecipitated with an ARNT specific antibody and then analyzed by LC-MS/MS to quantitate the ratio of phosphorylated/unmodified ARNT isoform 1. The average phosphorylation/unmodified ratio is represented in bar graph form (Left) and a summary of the manually extracted ion areas is shown (Right).
Fig. S4. 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 hour. Cells were then exposed -/+ TCDD (10 nM) for two hours. (A) RT-qPCR analysis of inflammatory marker genes (IL17C, CD93, and CCR5), or of the inflammatory suppressive gene NR3C1. (B) RT-qPCR analysis of Treg marker genes. 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, n.s.=not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
**Dataset S1 (separate file).** List of global differentially expressed genes in Karpas 299 cells between samples where ARNT isoform 1 and 3 were simultaneously suppressed (siA-1/3), or each ARNT isoform was individually targeted by RNAi (siA-1 or siA-3), versus the control sample (siControl), that were exposed to vehicle control (DMSO) for 2 hours. The samples were subjected to RNA sequencing and the raw data was subsequently analyzed as described in Materials and Methods.

**Dataset S2 (separate file).** List of global differentially expressed genes in Karpas 299 cells between samples where ARNT isoform 1 and 3 were simultaneously suppressed (siA-1/3), or each ARNT isoform was individually targeted by RNAi (siA-1 or siA-3), or a control sample (siControl), after which each sample was exposed to TCDD (10 nM) for 2 hours. The samples were subjected to RNA sequencing and the raw data was subsequently analyzed as described in Materials and Methods.

**Dataset S3 (separate file).** Results of Ingenuity Pathway Analysis of data listed in Dataset S1. Performed as described in Materials and Methods.

**Dataset S4 (separate file).** Results of Ingenuity Pathway Analysis of data listed in Dataset S2. Performed as described in Materials and Methods.

**Dataset S5 (separate file).** Results and raw data of ARNT isoform 1 phosphorylation quantitation after TCDD exposure using LC-MS/MS analysis. The dataset includes (1) the LC-MS abundance raw data for the phosphorylated and unmodified S77 peptide ions, (2) MS/MS spectra for the identification of the phosphorylation and unmodified S77 peptide ions for the 0-hour timepoint data, and (3) the total list of ARNT peptides identified.

**Dataset S6 (separate file).** List of global differentially expressed genes in Hepa-1c1c7 cells, or in Hepa-BpRc1 cells stably expressing WT ARNT isoform 1 or ARNT isoform 1 S77A, exposed to DMSO or TCDD (10 nM) for 2 hours. The samples were subjected to RNA sequencing and the raw data was subsequently analyzed as described in Materials and Methods.

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