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Knockdown of a Zebrafish Aryl Hydrocarbon Receptor Repressor (AHRRa) Affects Expression of Genes Related to Photoreceptor Development and Hematopoiesis

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The aryl hydrocarbon receptor repressor (AHRR) is a transcriptional repressor of aryl hydrocarbon receptor (AHR) and hypoxia-inducible factor (HIF) and is regulated by an AHR-dependent mechanism. Zebrafish (*Danio rerio*) possess two AHRR paralogs; AHRRa regulates constitutive AHR signaling during development, whereas AHRRb regulates polyaromatic hydrocarbon-induced gene expression. However, little is known about the endogenous roles and targets of AHRRs. The objective of this study was to elucidate the role of AHRRs during zebrafish development using a loss-of-function approach followed by gene expression analysis. Zebrafish embryos were microinjected with morpholino oligonucleotides against AHRRa or AHRRb to knockdown AHRR protein expression. At 72 h postfertilization (hpf), microarray analysis revealed that the expression of 279 and 116 genes was altered by knockdown of AHRRa and AHRRb, respectively. In AHRRa-morphant embryos, 97 genes were up-regulated and 182 genes were down-regulated. Among the down-regulated genes were several related to photoreceptor function, including cone-specific genes such as several opsins (*opn1sw1*, *opn1sw2*, *opn1mw1*, and *opn1lw2*), phosphodiesterases (*pde6H* and *pde6C*), retinol binding protein (rbp4l), phosducin, and arrestins. Down-regulation was confirmed by RT-PCR and with samples from an independent experiment. The four genes tested (*opn1sw1*, *pde6H*, *pde6C*, and *arr3b*) were not inducible by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. AHRRa knockdown also caused up-regulation of embryonic hemoglobin (*hbbe3*), suggesting a role for AHRR in regulating hematopoiesis. Knockdown of AHRRb caused up-regulation of 31 genes and down-regulation of 85 genes, without enrichment for any specific biological process. Overall, these results suggest that AHRRs may have important roles in development, in addition to their roles in regulating xenobiotic signaling.

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Key words: **microarrays; morpholino oligonucleotides; opsins; cones; development; repressor; zebrafish; aryl hydrocarbon receptor; dioxin; TCDD; AHR; AHRR.**

The aryl hydrocarbon receptor repressor (AHRR) is a member of the basic helix-loop-helix/Per-AHR nuclear translocator (ARNT)-Sim (bHLH-PAS) protein family and is well-established as a transcriptional repressor of aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor well known for its roles in toxicology, physiology, and development (Haarmann-Stemmann and Abel, 2006; Hahn *et al.*, 2009). AHRR shares a high degree of sequence similarity with AHR in the amino terminus that contains bHLH and PAS-A domains. However, AHRR lacks the PAS-B domain that acts as the ligand-binding region in AHR (Mimura *et al.*, 1999); thus, AHRR cannot bind ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Karchner *et al.*, 2002) and acts in a ligand-independent fashion. The presence of bHLH and PAS-A domains enables AHRR to heterodimerize with ARNT and bind to AHR response elements (AHREs) in the promoter regions of target genes. AHR regulates AHRR transcription by the binding of AHR-ARNT heterocomplex to AHREs in the promoter regions of the AHRR gene (Mimura *et al.*, 1999) and putative AHREs have been identified in the AHRR promoter regions in a number of species (Haarmann-Stemmann *et al.*, 2007; Karchner *et al.*, 2002).

Competition between AHR and AHRR for binding to AHREs is one mechanism of repression by AHRR (Hahn *et al.*, 2009; Mimura *et al.*, 1999). Recently, other mechanisms of repression by AHRR have been proposed, including small ubiquitin modification (SUMOylation) of AHRR and ARNT (Oshima *et al.*, 2009) and a transrepression mechanism involving protein-protein interactions (Evans *et al.*, 2008). The presence of multiple mechanisms of repression by AHRR suggests that there may be a broader role for this protein in addition to the regulation of AHR signaling.

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The specificity of AHRR in suppressing the function of other transcription factors is not fully understood, but recent studies have suggested that transcription factors other than AHR may also be targets of repression by AHRR. Kanno *et al.* (2008) reported that the repression of estrogen receptor alpha (ERα)-mediated activation of target genes *in vitro* occurs by direct interaction between AHRR and ERα protein. Similarly, Karchner *et al.* (2009) demonstrated that AHRR could repress hypoxia-inducible factor 1α (HIF-1α)-mediated transactivation *in vitro* in cells transfected with a hypoxia response element (HRE)-coupled luciferase construct. In addition, AHRR has been shown to act as a tumor suppressor gene in several types of cancer, in which knockdown of AHRR expression enhances the angiogenic potential of tumors (Zudaire *et al.*, 2008). Furthermore, Mimura *et al.* (1999) demonstrated that a NF-κB activator, 12-*O*-tetradecanoylphorbol-13-acetate, induces AHRR transcription. A NF-κB binding site is present in the murine AHRR regulatory region, suggesting the potential for activation of AHRR by other transcription factors. There are also reports linking polymorphisms in AHRR to reproductive abnormalities (Watanabe *et al.*, 2004). AHRR mRNA expression profiles also suggest that AHRR might be involved in physiological processes other than AHR signaling. AHRR mRNA is constitutively expressed in most adult tissues and the fetus in mammals, suggesting multiple biological roles in both adults and developing animals (Bernshausen *et al.*, 2006; Nishihashi *et al.*, 2006; Thackaberry *et al.*, 2005; Yamamoto *et al.*, 2004).

We are using zebrafish embryos as a model system to investigate AHRR function and its possible role in embryonic development and developmental toxicity. Zebrafish possess two AHRR paralogs (AHRRa and AHRRb), thought to be the result of a teleost-specific genome-duplication; phylogenetic analysis and comparative genomics suggests that they are co-orthologs of mammalian AHRR (Evans *et al.*, 2005). We recently demonstrated that knockdown of AHRRa in zebrafish embryos using morpholino oligonucleotides (MOs) causes developmental abnormalities similar to those observed in TCDD-exposed embryos (Jenny *et al.*, 2009). These phenotypes were not observed in embryos injected with AHRRb-MO. Knockdown of AHRRb, but not AHRRa, enhanced the induction of *cyp1a*, *cyp1b*, and *cyp1c* by TCDD, suggesting distinct roles for AHRRa and AHRRb in regulating developmental processes and TCDD-induced embryotoxicity. Thus, the zebrafish embryo model provides a unique opportunity to investigate the functions of these duplicated genes and gain insights into the various roles played by their single mammalian ortholog (Jenny *et al.*, 2009). Based on the above evidence, we hypothesized that AHRRa and AHRRb may regulate distinct sets of genes during embryonic development. Here, we tested this hypothesis by knocking down the individual AHRRs using antisense MOs and profiling the transcriptional changes using microarrays. Our results demonstrate that AHRRa knockdown alters expression profiles of several genes involved in photoreceptor signaling and hematopoiesis.

MATERIALS AND METHODS

Experimental animals. All the experiments were conducted using the Tupfel/Long fin (TL) wild-type strain of zebrafish. The experimental procedures used were approved by the Animal Care and Use Committee of the Woods Hole Oceanographic Institution. Animal maintenance, breeding, and rearing of embryos were carried out as described previously (Jenny *et al.*, 2009; Jonsson *et al.*, 2007).

Morpholino oligonucleotide microinjections. All the MOs used in this study were previously described (Jenny *et al.*, 2009). Fluorescein tagged MOs against AHRRa and AHRRb as well as control MO were obtained from Gene tools, LLC (Philomath, OR). The efficacy of AHRRa and AHRRb MOs in blocking translation was verified *in vitro* as described earlier (Jenny *et al.*, 2009).

MOs were diluted to 0.18mM in deionized water and approximately 2.1 nl (3.3–3.4 ng) of each MO was injected into the yolk of 1–2 cell embryos using a Narishige IM-300 microinjector (Jenny *et al.*, 2009). Embryos were screened for fluorescence at 3 h postfertilization (hpf) for successful MO incorporation; any embryos that did not have fluorescence were discarded. Three biological replicates of 20 pooled embryos per replicate were set up for each treatment (Ctrl-MO, AHRRa-MO, and AHRRb-MO). Embryos were maintained in 0.3× Danieau's solution at 28.5°C in 14:10 light-dark cycle. At 72 hpf, all replicate samples were collected for each treatment and stored in −80°C for RNA isolation from each set of 20 pooled embryos followed by gene expression analysis using microarrays (two of the replicates) and, subsequently, quantitative, real-time RT-PCR (all three replicates). The microarray analyses were performed using only two replicates because of limitations on the number of arrays available for hybridization at the time these analyses were performed.

TCDD exposure. Two additional experiments were used to obtain an independent assessment of the effects of knocking down AHRRa and AHRRb, as well as to determine the effect of TCDD exposure in MO-injected embryos. (These experiments were part of an earlier study (Jenny *et al.*, 2009) that focused on CYP1 expression after knockdown of AHRRs.) For those experiments, MOs were injected into the zebrafish embryos as described in the above section. At 6 hpf, MO-injected (AHRRa, AHRRb, and Ctrl) or noninjected (control) embryos were divided into three biological replicates of 20 pooled embryos for each treatment (0.1% dimethyl sulfoxide (DMSO) or 2nM TCDD) and exposed for 1 h in glass Petri dishes, as described earlier (Jenny *et al.*, 2009). At the end of the exposure period, embryos were rinsed thoroughly with fresh Danieau solution and maintained at 28.5°C in 14:10 light-dark cycle until sampling at 72 hpf. All treatments were collected at 72 hpf and stored in −80°C for later analysis of gene expression us-

ing quantitative, real-time RT-PCR. CYP1 expression data from these experiments was reported previously (Jenny *et al.*, 2009).

RNA isolation. Total RNA was prepared using the RNA STAT60 protocol (Tel-Test Inc., TX). RNA was quantified using a Nanodrop spectrophotometer and assessed for quality using an Agilent 2100 Bioanalyzer Lab-on-chip system. Only samples with RNA integrity number (RIN) between 9.8 and 10 were used for microarray analysis.

Microarray analysis. Whole-genome mRNA expression analysis was performed using Agilent 4×44 K zebrafish microarrays (Agilent Technologies Inc.). Microarray experiments were done in a two-color experimental design by comparing individual samples to a universal reference (UR) pool prepared from pooling equal amounts of total RNA from all the experimental samples.

RNA labeling was done using Agilent Low Input Quick Amp Labeling kit for two-color microarray-based gene expression analysis. Five hundred nanograms of total RNA were reverse-transcribed during which a T7 sequence was introduced into cDNA. T7 RNA polymerase-driven RNA synthesis was used for the preparation and labeling of RNA with fluorescent dyes. UR RNA was labeled with Cy3 and individual sample RNA was labeled with Cy5. The fluorescent cRNA probes were purified using Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA) to remove any excess dye. Equal amount of Cy3 (UR) and Cy5 (samples) labeled cRNA probes were mixed and hybridized to the array. Hybridization was done at 55°C for 20 h in a rotating hybridization oven (20 rpm). The hybridized slides were washed using Agilent microarray wash buffers and scanned using Genepix 4100A scanner (Molecular Devices Corporation, Sunnyvale, CA).

The resulting images were analyzed using Agilent Feature Extraction (AFE) software version 9.5.3. Image analyses included signal and spatial detrending and applying a universal error model. Quality control (QC) reports generated by AFE software were used to assess data quality for each microarray and to identify outliers. The raw data were used in statistical analysis for determining differential gene expression patterns.

Statistical analysis was performed using the Bioconductor package, linear microarray analysis (LIMMA) (Wettenhall and Smyth, 2004). Background-corrected mean signal intensities were used in the analysis. Within-array and between-array normalization was done using intensity-dependent global loess normalization. For statistical analysis, a linear model was fitted and differential gene expression was determined using the empirical Bayes method. This method moderates the standard errors of the log-fold changes and provides statistical power in situations where the sample size is small (Smyth, 2005). Differentially expressed genes were identified using *p*-values adjusted for multiple testing. Benjamini and Hochberg's method was used to control False Discovery Rate (adjusted *p*-value < 0.05). In addition, *B*-statistic – log-odds that the gene is differentially expressed was also taken into consideration for determining differentially expressed genes. All microarray data presented in this manuscript are in accordance with Minimum Information About a Microarray Experiment (MIAME) guidelines and are deposited in the NCBI GEO database (GEO accession number: GSE52229).

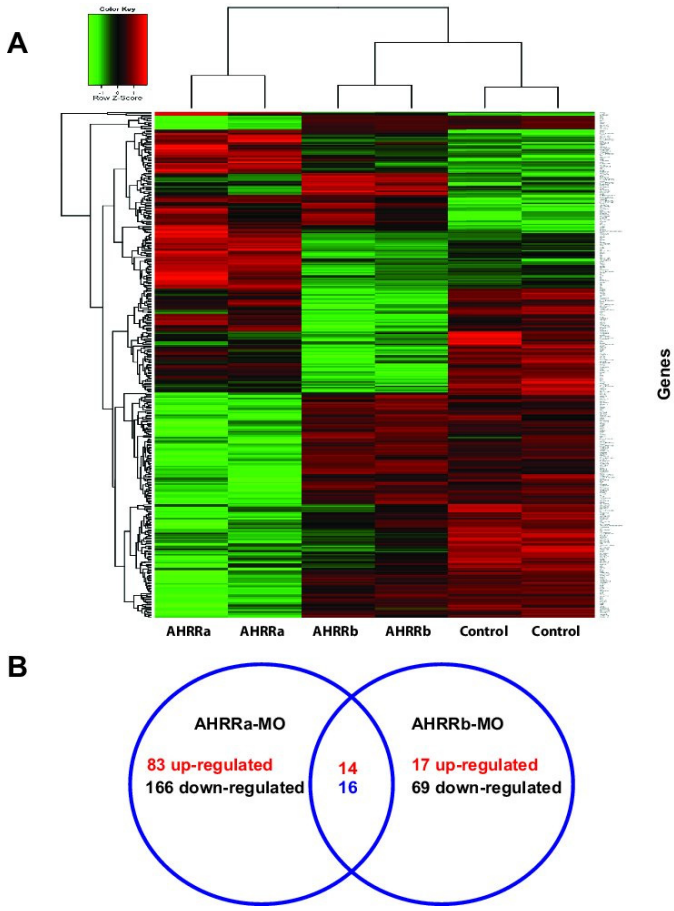

FIG. 1. Altered gene expression in response to MO-induced knockdown of AHRR in zebrafish embryos. (A) Heat map representation of gene expression patterns significantly altered by AHRRa and AHRRb knockdown at 72 hpf, in comparison to a control morpholino-injected group. Hierarchial clustering of significant genes was performed. Adjusted *p*-value less than 0.01 is considered to be statistically significant. (B) Venn diagram showing the total number of up- and down-regulated genes that are uniquely altered by AHRRa and AHRRb MOs at 72 hpf. The set of genes that are differentially expressed by both MOs are shown in the intersection.

Gene Ontology term enrichment and biological network analysis. Differentially expressed genes were functionally annotated with Gene Ontology (GO) terms using DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resource 6.7 (http://david.abcc.ncifcrf.gov/) (Huang da *et al.*, 2009). GO term enrichment for biological process was determined by comparing the list of differentially expressed genes against all the probes on the microarray.

Biological network analysis was conducted to determine the functional association between the proteins encoded by the differentially expressed genes. This was conducted using the String database (www.string-db.org) (Szklarczyk *et al.*, 2011). Default prediction methods were used except text mining and the required confidence score was set as high (0.7). The network obtained was imported into Cytoscape for identification of subnetworks with differentially expressed genes using jActiveModules version 2.23 (Cline *et al.*, 2007; Yeung *et al.*, 2008).

Quantitative real-time PCR. Complementary DNA was synthesized from 2 μg total RNA using random hexamers and the Omniscript cDNA Synthesis Kit (Qiagen). Quantitative PCR was carried out using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). The PCR conditions used were 95°C for 3 min and 95°C for 15 s/T_m°C for 1 min (40 cycles). The primers used to amplify different transcripts and their annealing temperatures (T_m, °C) are given in Table 1. Each PCR reaction was run in triplicate and a no-template control reaction was run on each PCR plate. Melt curve analysis was performed at the end of each PCR run to ensure that no nonspecific products are amplified. Relative levels of transcript abundance were calculated using the $2^{-\Delta\Delta Ct}$ method (where $\Delta\Delta Ct = [Ct_{(GOI)} - Ct_{(\beta\text{-actin})}]_{TCDD} - [Ct_{(GOI)} - Ct_{(\beta\text{-actin})}]_{DMSO}$ (GOI, gene of interest; Livak and Schmittgen, 2001). Statistical analysis of qPCR data was conducted using the Prism 4 software package (GraphPad Software Inc., San Diego, CA). Logarithmic transformed relative expression values were used in analysis of variance (ANOVA). One-way ANOVA was used to determine the effect of MOs on gene expression (confirmation of microarray results). Two-way ANOVA was used to determine the effect of MO and TCDD. Bonferroni's *post hoc* test was used to determine the statistical significance. A p-value of ≤ 0.05 is considered statistically significant.

RESULTS

Altered Gene Expression Profiles After AHRR Knockdown

To test the hypothesis that AHRRa and AHRRb have distinct roles in regulating gene expression during development, we knocked down each one separately and measured changes in global gene expression by microarray. Altogether, 279 and 116 genes showed significant differential expression in embryos injected with AHRRa-MO or AHRRb-MO, respectively, as compared with embryos injected with the control-MO. Knockdown of AHRRa caused up-regulation of 97 genes and down-regulation of 182 genes, whereas knockdown of AHRRb caused up-regulation of 31 genes and down-regulation of 85 genes in comparison to the control MO-injected group. All differentially expressed genes were hierarchically clustered and the results are shown as a heat map in Figure 1A.

The genes with twofold or higher induction with AHRRa knockdown are shown in Table 2 and the list of ≥2-fold down-regulated genes is in Table 3. Genes with a ≥2-fold change in expression after AHRRb knockdown are listed in Table 4 (up-regulated) and Table 5 (down-regulated). Among the genes that were differentially expressed in AHRRa and AHRRb morphants, 30 genes were common to both treatment groups (Fig. 1B; Table 6). Of these, 14 genes were up-regulated and 16 were down-regulated. All but three genes (fibrinogen gamma polypeptide (*fgg*), cathepsin 1La (*ctsl1a*), and type IV antifreeze protein precursor (zgc:161979)) were altered in the same direction with both AHRRa and AHRRb knockdown. There was no effect of AHRR knockdowns on mRNA expression of AHR1a, AHR2, HIF-1αb, HIF-2α, HIF-3α, or ARNT2 (other members of these gene subfamilies were not on the array; Supplementary table 1). The full list of all the significantly altered genes is provided in Supplementary table 2 (AHRR-MO differential gene expression.xls).

Functional Annotation of Differentially Expressed Genes

Functional annotation of genes differentially expressed in AHRRa morphants revealed enrichment of genes related to photoreceptor function. Based on GO term analysis (biological process), the majority of the genes are grouped under GO term neurological system process (GO:0050877) and response to stimulus (GO:0009416). The list of genes grouped under each GO term, their enrichment scores, and adjusted p-values are shown in Table 7. Biological network analysis also revealed a network of 17 genes related to cone photoreceptor signaling (Fig. 2). The entire network of all the differentially expressed genes is provided in the supplementary information (Supplementary fig. 1). Similar functional annotation and network analysis for the AHRRb-MO group did not reveal enrichment of genes for any specific biological process.

Confirmation of Microarray Results

We selected six genes (*opn1sw1*, *pde6c*, *pde6h*, *arr3b*, *hbbe3*, and *mmp9*) that were differentially expressed in AHRRa morphants for confirmation by qRT-PCR (Fig. 3). All the genes except *mmp9* showed a change similar to that observed with microarrays. *Opn1sw1*, *pde6c*, *pde6h,* and *arr3b* showed significant down-regulation after AHRRa knockdown compared with the control-MO and AHRRb-MO groups (Figs. 3A–D). *Hbbe3* showed significant up-regulation with AHRRa-MO treatment (Fig. 3E). *Mmp9* showed an increasing trend with AHRRa-MO treatment, but it was not significantly different compared with the control group (Fig. 3F). However, *mmp9* was significantly up-regulated with AHRRb-MO treatment, as observed with microarrays (Fig. 3F).

Effect of TCDD on Gene Expression Patterns in AHRR Knockdown Embryos

To further examine the effect of AHRR knockdown and to determine whether genes responsive to loss of AHRR are also inducible by the AHR ligand TCDD, we performed qRT-PCR on an independent set of samples from an earlier study that in-

TABLE 1
List of qPCR Primers Used in the Study

Gene	Accession number	Primers	T_m (°C)	Product size (bp)
pde6h	NM_200785.1	Forward 5′-CAG AAG CTC CAG CAC AGC AC-3′ Reverse 5′-GAT GTC TGT GCC GAG ACC CTC-3′	65	179
pde6c	NM_200871.1	Forward 5′-GGA CAT GAC CAA AGA GAA GGA G-3′ Reverse 5′-GCA GGA GGT TGG GTA TAA CTT TG-3′	60	170
arrestin3b	BC076177.1	Forward 5′-CTC TGA CAC AGA AAC GTT AGC AG-3′ Reverse 5′-CAC CAT CAA CTG AAT CAA CGC-3′	65	145
mmp9	NM_213123.1	Forward 5′-CAC ATA CAG GAT TTT GAA CTA TTC G-3′ Reverse 5′-GAT CAC CGT GAT CTG CTT TCC-3′	59	170
opn1sw1	NM_131319.1	Forward 5′-CGA TTG CAG GTC TTG TGA CG-3′ Reverse 5′-GAC CCT CGG GAA TGT ATC TGC-3′	60	196
β-actin	AF057040.1	Forward 5′-CAA CAG AGA GAA GAT GAC ACA GAT CA-3′ Reverse 5′-GTC ACA CCA TCA CCA GAG TCC ATC AC-3′	65	140
hbbe3	NM_001015058.1	Forward 5′-CGA GAC CTT GAC AAG ATG CTT GGT-3′ Reverse 5′-CCT TTT CAA GTC CCT TGA GGA CC-3′	59	175

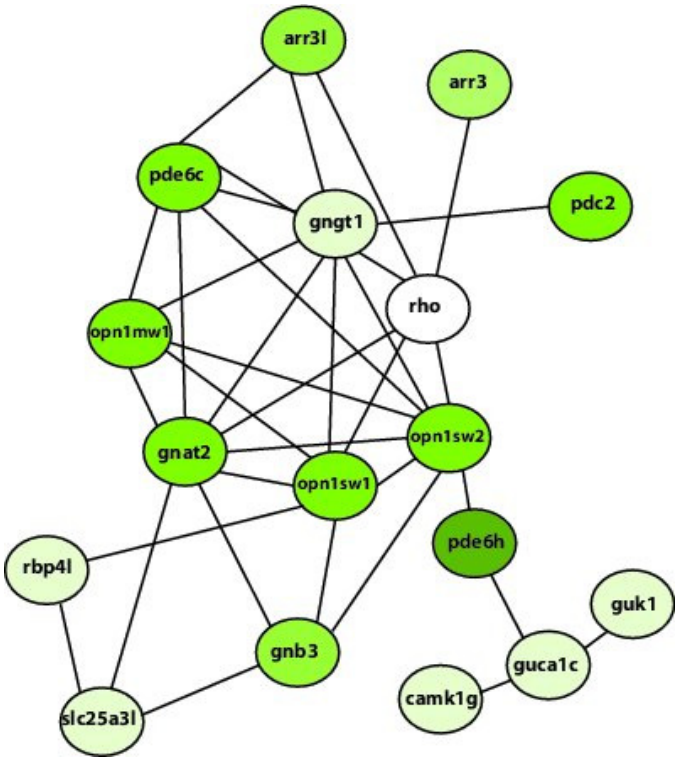

FIG. 2. Biological network of genes involved in cone photoreceptor signaling. All the differentially expressed genes from the AHRRa-MO group were analyzed for functional association using the STRING database and statistically significant networks were identified using jActiveModules version 2.23. Only the network of genes involved in cone photoreceptor signaling is shown. The color of each node represents the fold change in gene expression. Different shades of green (light green to dark green) denote the magnitude of down-regulation. Unchanged nodes are colored white.

cluded combinations of AHRR knockdown and TCDD treatment (Jenny *et al.*, 2009). Knockdown of AHRRa caused significant down-regulation of *opn1sw1*, *pde6c*, *pde6h*, and *arr3* genes, as observed in the experiment described in the previous section. However, there was no effect of TCDD treatment on the expression of these four genes as compared with vehicle controls, in any of the groups (Figs. 4A–D). The up-regulation of *hbbe3* in AHRRa-morphants but not AHRRb-morphants occurred as before. Interestingly, TCDD exposure strongly induced the expression of *hbbe3* in control-MO, AHRRa-MO-injected, and AHRRb-MO-injected embryos in comparison to MO-injected, DMSO-exposed embryos (Fig. 4E). In a separate, replicate experiment that also involved injection of AHRR-MOs and exposure to TCDD, the TCDD induction of *hbbe3* in control-MO-injected embryos did not occur, but the induction of *hbbe3* by TCDD in AHRRa-MO-injected and AHRRb-MO-injected embryos was confirmed (Supplementary fig. 2). TCDD also induced *mmp9* gene expression, but only in AHRRb-MO injected embryos (Fig. 4F) and these results were confirmed in the replicate experiment (Supplementary fig. 2).

DISCUSSION

We have previously demonstrated that the knockdown of AHRRa, but not AHRRb, induces developmental phenotypes similar to those seen with TCDD exposure (Jenny *et al.*, 2009). In order to understand the molecular basis of these phenotypes, transcriptional profiling was carried out using microarrays after knocking down each of the two AHRRs. Comparing the two paralogs, knockdown of AHRRa down-regulated a larger set of genes than did knockdown of AHRRb and there was very little overlap between the two groups, suggesting that they play distinct physiological roles during development.

AHRRa Regulates Genes Involved in Photoreceptor Signaling

Functional annotation of all the differentially expressed genes, especially the large number of down-regulated genes involved in cone photoreceptor signaling, suggests a unique role

TABLE 2
List of Genes Up-regulated in AHRRa-MO-injected Zebrafish Embryos

Probe name	Description	Accession number	Fold change	Adjusted *p*-value	*B*
A_15_P106583	Hemoglobin beta embryonic-3 (hbbe3)	NM_001015058	16.28	0.000	14.22
A_15_P107501	Fibrinogen, gamma polypeptide (fgg)	NM_213054	2.43	0.000	8.07
A_15_P114154	Matrix metalloproteinase 9 (mmp9)	NM_213123	9.59	0.000	7.09
A_15_P100851	Cyclin G1 (ccng1)	NM_199481	2.27	0.000	6.41
A_15_P114425	Uncharacterized protein LOC494049 (zgc:101853)	NM_001008592	2.43	0.000	6.37
A_15_P117758	Jun B proto-oncogene (junb)	NM_213556	2.51	0.001	5.65
A_15_P106305	Type IV antifreeze protein precursor (zgc:161979)	NM_001045488	2.17	0.001	5.63
A_15_P102660	Tumor protein p53 (tp53)	NM_131327	2.44	0.002	4.93
A_15_P111458	Hemoglobin alpha embryonic-1 (hbae1)	NM_182940	2.05	0.002	4.94
A_15_P118692	Hypothetical protein LOC550501 (zgc:111983)	NM_001017803	2.28	0.002	4.65
A_15_P103191	Matrix metalloproteinase 9 (mmp9)	NM_213123	3.70	0.002	4.56
A_15_P120536	Tumor protein p53 (tp53)	NM_131327	2.05	0.002	4.47
A_15_P111302	APOEB_BRARE (O42364) apolipoprotein Eb precursor (Apo-Eb), partial (62%)(TC312510)	NM_131098.1	2.03	0.003	4.10
A_15_P118068	*Danio rerio* cDNA clone IMAGE: 6965466.	BC160607	14.24	0.004	3.80
A_15_P113920	CCAAT/enhancer binding protein (C/EBP), beta (cebpb)	NM_131884	2.47	0.004	3.57
A_15_P107566	Apolipoprotein Eb (apoeb)	NM_131098	2.13	0.004	3.56
A_15_P120997	Stanniocalcin 1 (stc1)	NM_200539	2.33	0.006	3.15
A_15_P117178	Fibrinogen, gamma polypeptide (fgg)	NM_213054	2.09	0.006	3.08
A_15_P101751	Glycine N-methyltransferase (gnmt)	NM_212816	2.19	0.006	3.04
A_15_P102187	Unknown		2.04	0.008	2.64
A_15_P112685	Lectin, galactoside-binding, soluble, 1 (galectin 1)-like 1 (lgals1l1)	NM_001005958	2.12	0.009	2.41
A_15_P120578	Beaded filament structural protein 2, phakinin (bfsp2) (zgc:103750)	NM_001008633	2.25	0.017	1.63
A_15_P116825	Beaded filament structural protein 2, phakinin (bfsp2) (zgc:103750)	NM_001008633	2.31	0.022	1.15
A_15_P109667	Crystallin, gamma M2c (crygm2c)	NM_001007783	2.18	0.023	1.12
A_15_P103210	N-myc downstream regulated gene 1, like (ndrg1l)	NM_200692	2.43	0.025	0.97
A_15_P120206	Arrestin domain containing 3b (arrdc3b) (zgc:92034)	NM_001004605	2.32	0.025	0.96
A_15_P101920	Novel protein similar to vertebrate PX domain containing serine/threonine kinase (PXK)	CAK11481.1	2.35	0.027	0.80
A_15_P110005	Histone H1-like (zgc:110380)	NM_001017660	2.15	0.041	0.07

Note. Probe name corresponds to the Agilent probe ID. Fold change values, adjusted *p*-value and *B*-statistic are based on the statistical analysis. Only genes that showed ≥2-fold change are shown in the table. The full list of differentially expressed genes is given in Supplementary table 2.

for AHRRa in eye development. In vertebrates, cone photoreceptors are primarily responsible for color vision and responses to bright light. The cone photoreceptor-specific genes that were down-regulated in AHRRa morphants include those encoding short wave opsins (*opn1sw1* and *opn1sw2*), medium wave opsins (*opn1mw1*), long wave sensitive opsins (*opn1lw2*), phosphodiesterase subunits (*pde6c* and *pde6h*), cone arrestin (*arr3*), cone-specific G-protein coupled receptor kinase (*grk7a*) and transducin α-subunit (*gnat2*). The down-regulation of several of these (*opn1sw1*, *pde6c, pde6h,* and *arr3b*) was confirmed by qRT-PCR. These genes are critical for vision in zebrafish (Leung and Dowling, 2005; Weger *et al.*, 2011). Opsin is the photopigment found in cone photoreceptors, and based on the sensitivity to different wavelengths of light these receptors can be classified as red (∼565 nm), green (∼520 nm), blue (∼430 nm), and ultraviolet (UV, ∼365 nm). In the zebrafish genome, there are two red (*opn1lw1* and *opn1lw2*), four green (*opn1mw1–4*), and single blue (*opn1sw2*) and ultraviolet (*opn1sw1*) opsin genes (Robinson *et al.*, 1993; Vihtelic *et al.*, 1999). Opsins are membrane-bound G-protein-coupled receptors (GPCR). Photoreceptor signaling begins with the absorption of a photon of light by a GPCR together with 11-*cis*-retinal, a chromophore. This activates the regulatory G-protein, transducin, and downstream second messenger cGMP by activating cGMP phosphodiesterases (PDE) (Collery and Kennedy, 2009). PDE breaks down cGMP to 5′-GMP, lowering intracellular cGMP concentration and subsequently hyperpolarizing the plasma membrane by closing of the cGMP-gated cationic channels. PDE6 is specific to photoreceptor signaling and *pde6c* and *pde6h* are the subunits of PDE specific to cone photoreceptors. Mutations in *pde6c* cause degeneration of cones in zebrafish (Stearns *et al.*, 2007) and also are responsible for the inherited degenerative disease *retinitis pigmentosa* in humans (Fadool and Dowling, 2008; Gestri *et al.*, 2012).

Recovery from the photoreceptor response involves phosphorylation of opsin by rhodopsin kinase (*grk7a*), which prepares the receptor for binding to arrestin (*arr3*). This prevents transducin-mediated signaling and targets the GPCR for internalization. Recoverin (*rcv1*), a calcium binding protein, regulates the rate of phosphorylation. Transcript levels of *grk7a*, *arr3*, and *rcv1* were down-regulated in AHRRa-morphant embryos. Knockdown of *grk7a* delays the recovery of

TABLE 3
List of Genes Down-regulated in AHRRa-MO-injected Zebrafish Embryos

Probe name	Description	Accession number	Fold change	Adjusted *p*-value	*B*
A_15_P107533	Phosphodiesterase 6C, cGMP-specific, cone, alpha prime (pde6c)	NM_200871	−8.09	0.000	13.30
A_15_P115980	Opsin 1 (cone pigments), short-wave-sensitive 1 (opn1sw1)	NM_131319	−7.24	0.000	13.53
A_15_P114133	Phosphodiesterase 6H, cGMP-specific, cone, gamma (pde6h)	NM_200785	−10.09	0.000	12.54
A_15_P117046	Opsin 1 (cone pigments), medium-wave-sensitive, 1 (opn1mw1)	NM_131253	−7.35	0.000	12.35
A_15_P106879	Arrestin 3, retinal (X-arrestin) (arr3)	NM_200792	−4.40	0.000	11.94
A_15_P120294	Retinal degradation slow 2 (rds2)	NM_131566	−5.22	0.000	11.37
A_15_P111532	Guanine nucleotide binding protein (G protein), beta polypeptide 3 (gnb3)	NM_213202	−5.58	0.000	11.16
A_15_P121399	Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2 (gnat2)	NM_131869	−7.08	0.000	10.98
A_15_P107383	Phosducin 2 (pdc2)	NM_001025464	−3.61	0.000	10.50
A_15_P102875	Retinal degradation slow 4 (rds4)	NM_131567	−5.60	0.000	10.19
A_15_P110693	Retinal degradation slow 2 (rds2)	NM_131566	−3.57	0.000	10.05
A_15_P104722	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3, like (slc25a3l), nuclear gene encoding mitochondrial protein	NM_200715	−3.25	0.000	10.05
A_15_P109108	Villin 1 like (vil1l)	NM_200238	−3.95	0.000	9.43
A_15_P119413	Opsin 1 (cone pigments), short-wave-sensitive 2 (opn1sw2)	NM_131192	−5.59	0.000	9.17
A_15_P119429	Opsin 1 (cone pigments), long-wave-sensitive, 2 (opn1lw2)	NM_001002443	−2.98	0.000	8.72
A_15_P111012	Opsin 1 (cone pigments), long-wave-sensitive, 2 (opn1lw2)	NM_001002443	−3.37	0.000	8.54
A_15_P104674	Retinol binding protein 4, like (rbp4l)	NM_199965	−3.26	0.000	8.48
A_15_P104664	Arrestin 3, retinal (X-arrestin), like (arr3l)	NM_001002405	−5.21	0.000	8.32
A_15_P103714	Guanylate cyclase activator 1C (guca1c)	NM_194393	−2.79	0.000	7.59
A_15_P106371	XM_678537 carboxypeptidase B1 (tissue) isoform 1 (*Danio rerio*) (exp = −1; wgp = 0; cg = 0), complete	TC311008	−2.81	0.000	7.47
A_15_P115516	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3, like (slc25a3l), nuclear gene encoding mitochondrial protein	NM_200715	−2.82	0.000	7.11
A_15_P108311	Chymotrypsinogen B2 precursor (zgc:112160)	NM_001017724	−4.05	0.000	6.98
A_15_P116791	Calcium/calmodulin-dependent protein kinase IG (camk1g)	NM_199966	−2.26	0.000	6.56
A_15_P109678	Calcium binding protein 5b (cabp5b) (zgc:109977)	NM_001020732	−2.64	0.000	6.47
A_15_P119059	Guanylate kinase 1 (guk1)	NM_200724	−2.52	0.000	6.42
A_15_P108795	Retinaldehyde binding protein 1b (rlbp1b)	NM_205690	−2.99	0.000	6.34
A_15_P102721	Chitinase, acidic.2 precursor (zgc:55941)	NM_213249	−2.53	0.000	6.21
A_15_P100788	SH3-domain GRB2-like 2 (sh3gl2)	NM_201116	−2.08	0.001	5.83
A_15_P117814	Trypsin (try)	NM_131708	−3.13	0.001	5.30
A_15_P113799	Chymotrypsinogen B1 (ctrb1)	NM_212618	−3.05	0.002	5.05
A_15_P115606	Q6PBU3_BRARE (Q6PBU3) Stathmin-like 4, complete	NM_213401	−2.07	0.002	4.86
A_15_P112264	Group-specific component (vitamin D binding protein) (zgc:92753)	NM_001002568	−2.86	0.002	4.70
A_15_P113364	Glycogenin, like (gygl)	NM_001002062	−2.08	0.002	4.57
A_15_P113940	Uncharacterized protein LOC445282 precursor (zgc:92041)	NM_001003737	−4.09	0.002	4.53
A_15_P107695	Fatty acid binding protein 10, liver basic (fabp10)	NM_152960	−2.58	0.003	4.15
A_15_P110245	Unknown		−2.64	0.004	3.81
A_15_P110192	Pyrophosphatase (inorganic) (pp)	NM_200733	−2.00	0.004	3.83
A_15_P110812	Trypsin-1-like (zgc:66382) NM_199605	NM_199605	−2.98	0.004	3.78
A_15_P115802	Cathepsin L, 1 b (ctsl1b)	NM_131198	−2.79	0.004	3.69
A_15_P118837	3-Oxoacid CoA transferase 1a (oxct1a)	NM_001007291	−2.28	0.004	3.66
A_15_P115815	G-Protein-coupled receptor kinase 7a (grk7a)	NM_001031841	−2.18	0.004	3.70
A_15_P112986	Solute carrier family 31 (copper transporters), member 1 (slc31a1)	NM_205717	−2.05	0.004	3.67
A_15_P114820	Recoverin (rcv1)	NM_199964	−2.12	0.004	3.60
A_15_P113750	Serum/glucocorticoid regulated kinase 1-like (si:ch211–195b13.1)	NM_001077302	−2.21	0.004	3.53
A_15_P100752	Alanine-glyoxylate aminotransferase, like (agxtl)	NM_001002331	−3.16	0.005	3.35
A_15_P120962	ATPase, Na^+/K^+ transporting, beta 2b polypeptide (atp1b2b)	NM_131838	−2.07	0.005	3.36
A_15_P112975	Transmembrane 4 L six family member 4 (tm4sf4) (zgc:92479)	NM_001003489	−2.21	0.005	3.27
A_15_P103143	Uncharacterized protein LOC327506 (zgc:56085)	NM_199816	−2.75	0.006	3.15
A_15_P112053	Retinal outer segment membrane protein 1b (zgc:56548)	NM_201014	−2.73	0.006	3.17
A_15_P120424	Lysozyme [source: RefSeq_peptide; Acc:NP_631919]	ENSDART00000080549	−2.04	0.006	3.15
A_15_P105587	Prominin 1 b (prom1b)	NM_198071	−2.28	0.006	2.98
A_15_P108560	Internexin neuronal intermediate filament protein, alpha a (inaa)	NM_001144784	−2.28	0.007	2.89
A_15_P116896	Guanine nucleotide binding protein (G protein), beta 5 (gnb5)	NM_200746	−2.07	0.007	2.85
A_15_P102378	Glutamate decarboxylase 1 (gad1)	NM_194419	−2.50	0.007	2.80
A_15_P104848	Tubulin polymerization-promoting protein family member 3 (tppp3)	NM_201335	−2.35	0.007	2.80
A_15_P118058	Unknown		−3.22	0.008	2.67
A_15_P103108	Trypsin-1-like (zgc:66382) NM_199605	CF595078	−2.10	0.008	2.69
A_15_P120202	SH3-domain GRB2-like 2 (sh3gl2)	NM_201116	−2.18	0.008	2.65
A_15_P112659	Transferrin-a (tfa)	NM_001015057	−2.17	0.009	2.45
A_15_P118533	*D. rerio* cDNA clone IMAGE: 7395709	BC092811	−2.37	0.010	2.25
A_15_P120388	Chymotrypsinogen B1 (ctrb1)	NM_212618	−2.07	0.015	1.83
A_15_P121355	Uncharacterized protein LOC393832 (zgc:77748)	NM_200858	−2.21	0.016	1.73
A_15_P113063	Similar to cathepsin L (MGC174155)	NM_001103118	−2.15	0.017	1.59
A_15_P114615	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2 (ENSDART00000024136) (gngt2a)	XM_001336994	−2.13	0.017	1.57
A_15_P103612	Family with sequence similarity 3, member C (fam3c)	NM_212725	−2.02	0.017	1.56
A_15_P118740	Oxysterol binding protein-like 7 (osbpl7)	NM_001005927	−2.02	0.019	1.37
A_15_P101585	Heat shock protein, alpha-crystallin-related, b11 (hspb11)	NM_001099427	−2.28	0.023	1.10
A_15_P110869	Urate oxidase (uox)	NM_001002332	−2.19	0.026	0.87
A_15_P110951	PREDICTED: *D. rerio* similar to myosin heavy chain fast skeletal type 2 (LOC100002040)	XM_001339170	−2.14	0.028	0.73
A_15_P121427	Amylase, alpha 2A; pancreatic (amy2a)	NM_213011	−2.37	0.032	0.54
A_15_P112046	Probable protein BRICK1 (zgc:86903)	NP_001002097	−2.04	0.034	0.40
A_15_P111425	Amylase, alpha 2A; pancreatic (amy2a)	NM_213011	−2.22	0.038	0.25
A_15_P114488	Q4SAD3_TETNG (Q4SAD3) chromosome 19 SCAF14691, whole genome shotgun sequence, partial (43%)	TC324157	−2.00	0.039	0.17
A_15_P106129	Ependymin (epd)	NM_131005	−2.01	0.041	0.07
A_15_P119722	Trypsin-1-like (zgc:66382) NM_199605	CF595078	−2.23	0.048	−0.15

Note. Probe name corresponds to the Agilent probe ID. Fold change values, adjusted *p*-value and *B*-statistic are based on the statistical analysis. Only genes that showed ≥2-fold change are shown in the table. The full list of differentially expressed genes is given Supplementary table 2.

TABLE 4
List of Genes Up-regulated in AHRRb-MO-injected Zebrafish Embryos

Probe name	Description	Accession number	Fold change	Adjusted *p*-value	*B*
A_15_P114154	Matrix metalloproteinase 9 (mmp9)	NM_213123	3.46	0.040	1.183
A_15_P103210	N-myc downstream regulated gene 1, like (ndrg1l)	NM_200692	3.08	0.017	3.093
A_15_P110005	Histone H1-like (zgc:110380)	NM_001017660	2.85	0.019	2.810
A_15_P110431	CRCB2_HALSA (Q9HNW1) protein crcB homolog 2, partial (12%)	TC354377	2.74	0.012	5.303
A_15_P120578	Beaded filament structural protein 2, phakinin (bfsp2) (zgc:103750)	NM_001008633	2.30	0.027	1.883
A_15_P111127	*Danio rerio* thioredoxin interacting protein, like (cDNA clone IMAGE:6034364).	BC055213	2.28	0.016	3.342
A_15_P108784	Protein phosphatase 1, regulatory (inhibitor) subunit 3Ca (ppp1r3ca) (zgc:92069)	NM_001002376	2.23	0.015	3.784
A_15_P109667	Crystallin, gamma M2c (crygm2c)	NM_001007783	2.13	0.043	0.911
A_15_P113062	Crystallin, beta A4 (cryba4)	NM_001018125	2.10	0.032	1.656
A_15_P115533	fw75d04.x1 Gong zebrafish testis *D. rerio* cDNA clone IMAGE:5616055 3′ sequence	BM571211	2.07	0.041	1.132
A_15_P119407	Uncharacterized protein LOC561929 (zgc:165347)	NM_001099243	2.06	0.032	1.601

TABLE 5
List of Genes Down-regulated in AHRRb-MO-injected Zebrafish Embryos

Probe name	Description	Accession number	Fold change	Adjusted *p*-value	*B*
A_15_P113328	ATP-binding cassette, subfamily F (GCN20), member 2 (abcf2), nuclear gene encoding mitochondrial protein	NM_201315	−3.54	0.015	4.422
A_15_P108311	Chymotrypsinogen B2 precursor (zgc:112160)	NM_001017724	−2.95	0.015	4.151
A_15_P120388	Unknown		−2.86	0.012	5.176
A_15_P104868	Predicted: *Danio rerio* hypothetical LOC100003999 (LOC100003999)	XM_001343386	−2.85	0.015	3.804
A_15_P111425	Amylase, alpha 2A; pancreatic (amy2a)	NM_213011	−2.79	0.019	2.490
A_15_P117308	Uncharacterized protein LOC692313 precursor (zgc:136461)	NM_001045282	−2.75	0.043	0.915
A_15_P121427	Amylase, alpha 2A; pancreatic (amy2a)	NM_213011	−2.64	0.032	1.612
A_15_P110812	Trypsin-1-like (zgc:66382) NM_199605	NM_199605	−2.64	0.019	2.555
A_15_P100902	FK506 binding protein 5 (fkbp5)	NM_213149	−2.54	0.004	6.970
A_15_P120582	Purine nucleoside phosphorylase 4b (pnp4b) (zgc:77825)	NM_205643	−2.43	0.045	0.818
A_15_P110245	Unknown		−2.43	0.019	2.866
A_15_P110547	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (serpina1)	NM_001077758	−2.38	0.019	2.665
A_15_P120732	Q5ERC9_CARAU (Q5ERC9) 14 kDa apolipoprotein, partial (72%)	TC361450	−2.33	0.014	4.810
A_15_P106533	Transferrin-a (tfa)	NM_001015057	−2.28	0.019	2.724
A_15_P105980	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 {*D. rerio*} (exp = −1; wgp = 0; cg = 0), partial (81%)	NM_001013259	−2.26	0.015	4.470
A_15_P106963	Carboxylesterase 2 (intestine, liver) (ces2) (zgc:153863)	NM_001077252	−2.24	0.019	2.634
A_15_P110799	Apolipoprotein A-II (apoa2) zgc:193613	NM_001130586	−2.20	0.016	3.485
A_15_P103108	Trypsin-1-like (zgc:66382) NM_199605	CF595078	−2.20	0.017	3.198
A_15_P112659	Transferrin-a (tfa)	NM_001015057	−2.20	0.019	2.560
A_15_P116208	Carboxypeptidase A5 (cpa5)	NM_199271	−2.19	0.022	2.201
A_15_P110520	Growth hormone receptor b (ghrb)	NM_001111081	−2.18	0.043	0.998
A_15_P100289	RRS1 ribosome biogenesis regulator homolog (*Saccharomycescerevisiae*) (rrs1)	NM_200062	−2.12	0.037	1.339
A_15_P113799	Chymotrypsinogen B1 (ctrb1)	NM_212618	−2.12	0.038	1.271
A_15_P107483	DnaJ (Hsp40) homolog, subfamily C, member 21 (dnajc21)	NM_200044	−2.11	0.015	3.981
A_15_P108821	Eukaryotic translation initiation factor 4A, isoform 1A (eif4a1a)	NM_198366	−2.10	0.015	3.864
A_15_P102590	Heat shock protein 90-alpha 1 (hsp90a.1)	NM_131328	−2.09	0.019	2.504
A_15_P117814	Trypsin (try)	NM_131708	−2.08	0.043	1.029
A_15_P100880	Heat shock protein 47 (hsp47)	NM_131204	−2.08	0.021	2.296
A_15_P100921	Collagen, type X, alpha 1 (col10a1)	NM_001083827	−2.07	0.031	1.707
A_15_P118831	Collagen, type X, alpha 1 (col10a1)	NM_001083827	−2.06	0.016	3.451
A_15_P103885	Methionine adenosyltransferase I, alpha (mat1a)	NM_199871	−2.06	0.019	2.820
A_15_P121273	RRS1 ribosome biogenesis regulator homolog (*S. cerevisiae*) (rrs1)	NM_200062	−2.02	0.037	1.408

Note. Probe name corresponds to the Agilent probe ID. Fold change values, adjusted *p*-value and *B*-statistic are based on the statistical analysis. Only genes that showed ≥2-fold change are shown in the table. The full list of differentially expressed genes is given in Supplementary table 2.

cone cells from the photoreceptor response in zebrafish (Rinner *et al.*, 2005). In addition to these cone photoreceptor-specific genes, several other eye-related genes were down-regulated with AHRRa-knockdown (Supplementary table 3).

These results suggest that AHRRa has a specific role in cone photoreceptor development. However, it is unclear if all the genes are direct targets of AHRRa or indirectly targeted by yet to be identified mechanisms. Based on the time at which MOs were injected into the embryos (1–2 cell stages) and the time at which the gene expression profiling was done (72 hpf), it is likely that at least some of these changes, and perhaps many, are secondary effects. Measuring the effect of AHRRa knockdown

TABLE 6
List of Genes Differentially Expressed with both AHRRa-MO and AHRRb-MO

Agilent probe ID	Gene	AHRRa-MO	AHRRb-MO
A_15_P107501	**Fibrinogen, gamma polypeptide (fgg)**	**2.431**	**– 1.574**
A_15_P114154	Matrix metalloproteinase 9 (mmp9)	9.588	3.458
A_15_P108311	Chymotrypsinogen B2 precursor (zgc:112160)	– 4.052	– 2.946
A_15_P116791	Calcium/calmodulin-dependent protein kinase IG (camk1g)	– 2.262	– 1.800
A_15_P102721	Chitinase, acidic.2 precursor (zgc:55941)	– 2.528	– 1.907
A_15_P117758	jun B proto-oncogene (junb)	2.510	1.786
A_15_P106305	**Type IV antifreeze protein precursor (zgc:161979)**	**2.174**	**– 1.927**
A_15_P117814	Trypsin (try)	– 3.135	– 2.080
A_15_P113799	Chymotrypsinogen B1 (ctrb1)	– 3.050	– 2.124
A_15_P110245	Unknown	– 2.639	– 2.431
A_15_P110812	Trypsin-1-like (zgc:66382)	– 2.981	– 2.636
A_15_P100902	FK506 binding protein 5 (fkbp5)	– 1.892	– 2.542
A_15_P103108	cDNA clone IMAGE:7015062	– 2.099	– 2.201
A_15_P112659	Transferrin-a (tfa)	– 2.169	– 2.198
A_15_P107950	si:dkey-42i9.4 (si:dkey-42i9.4)	1.914	1.862
A_15_P108038	si:dkeyp-113d7.7 (si:dkeyp-113d7.7)	1.804	1.845
A_15_P120388	Unknown	– 2.070	– 2.858
A_15_P120578	Beaded filament structural protein 2, phakinin (bfsp2) (zgc:103750)	2.246	2.300
A_15_P109667	Crystallin, gamma M2c (crygm2c)	2.177	2.131
A_15_P103210	N-myc downstream regulated gene 1, like (ndrg1l)	2.427	3.080
A_15_P110837	Transmembrane protein 41ab (tmem41ab)	– 1.832	– 1.844
A_15_P116208	Carboxypeptidase A5 (cpa5)	– 1.945	– 2.186
A_15_P103675	**Cathepsin L1, a (ctsl1a)**	**1.848**	**– 1.981**
A_15_P111425	Amylase, alpha 2A; pancreatic (amy2a)	– 2.216	– 2.793
A_15_P109854	IMAGE:7278570 5′	1.780	1.841
A_15_P117166	Nucleolar complex associated 3 homolog (*Saccharomyces cerevisiae*) (noc3l)	– 1.649	– 1.842
A_15_P110005	Histone H1-like (zgc:110380)	2.150	2.847
A_15_P116605	Suppressor of cytokine signaling 3b (socs3b)	1.706	1.967
A_15_P119407	Uncharacterized protein LOC561929 (zgc:165347)	1.807	2.056
A_15_P121427	Amylase, alpha 2A; pancreatic (amy2a)	– 2.366	– 2.644

Note. Fold change values for both the AHRRa and AHRRb MOs are shown. Rows in bold show the genes with opposite responses to AHRRa and AHRRb knockdown.

at additional time points during development will help to clarify this. The inability of TCDD to alter the expression of *opn1sw1*, *pde6c*, *pde6h*, or *arr3b* (Figs. 4A–D) suggests that at least these four genes are not directly regulated by AHR.

One possible explanation for the down-regulation of so many eye-related genes is that AHRRa knockdown impacts early cellular fate specification of progenitor cells by interfering with an essential signaling pathway. These changes could lead to differences in terminal differentiation and altered gene expression patterns in the photoreceptors. In the fruit fly, *Drosophila melanogaster*, loss of the AHR ortholog *spineless* disrupts color vision by altering the transcriptional regulation that distinguishes different classes of photoreceptors (Wernet *et al.*, 2006). Even though invertebrate and vertebrate eyes differ morphologically, recent evidence suggests that underlying molecular mechanisms in photoreceptor development are highly conserved (Charlton-Perkins and Cook, 2010). Although there is no direct evidence for a role of AHRR in retinal function in zebrafish, it is possible that AHRRa is expressed in the developing eye, where it could regulate AHR, HIF, or other transcription factors. AHR expression in mammalian eye has been reported (Chevallier *et al.*, 2013; Dwyer *et al.*, 2011; Liu and Piatigorsky, 2011) and we have recently detected expression of AHRRa mRNA in the eyes of adult zebrafish (unpublished results). Future studies will focus on identifying the expression patterns of AHRR and its possible targets in the developing eye.

The toxicological implications of the altered gene expression patterns that we observed in AHRRa-morphant embryos are not clear, but a possible role of AHRRa in the development of the eye suggests a mechanism that could help explain the reported effects of TCDD on visual function in fish (Carvalho and Tillitt, 2004) or ocular defects that appear in AHR-deficient mice (Chevallier *et al.*, 2013; Hu *et al.*, 2013). Expression of AHRR is regulated by AHR and induced by AHR agonists in mammals and fish (Karchner *et al.*, 2002; Mimura *et al.*, 1999). Moreover, AHRR regulates the responses to AHR agonists in adults and embryos (Hosoya *et al.*, 2008; Jenny *et al.*, 2009; Korkalainen *et al.*, 2004, 2005). Thus, induction of AHRR expression by AHR agonists during development or in adult animals could interfere with physiological roles of AHR or other transcription factors involved in the differentiation or function of cells in the eye. In light of our results, a detailed examination

TABLE 7
Gene Ontology Enrichment Analysis of Genes Differentially Expressed in AHRRa-MO-treated Embryos

Gene Ontology term (biological process)	Benjamini Hochberg adjusted *p*-value
NEUROLOGICAL SYSTEM PROCESS (GO:0050877)	2.1E−4 (Enrichment score: 5.67)
ATPase, Na^+/K^+ transporting, beta 2b polypeptide	
Arrestin 3, retinal (X-arrestin)	
Arrestin 3, retinal (X-arrestin), like	
Glutamate decarboxylase 1	
Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2	
Opsin 1 (cone pigments), long-wave-sensitive, 2	
Opsin 1 (cone pigments), medium-wave-sensitive, 1	
Opsin 1 (cone pigments), short-wave-sensitive 1	
Opsin 1 (cone pigments), short-wave-sensitive 2	
Phosphodiesterase 6C, cGMP-specific, cone, alpha prime	
zgc:56548	
zgc:73336 phosphodiesterase 6H, cGMP-specific, cone	
RESPONSE TO LIGHT STIMULUS (GO:0009416)	6.7E−3 (Enrichment score: 3.83)
Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2	
Opsin 1 (cone pigments), long-wave-sensitive, 2	
Opsin 1 (cone pigments), medium-wave-sensitive, 1	
Opsin 1 (cone pigments), short-wave-sensitive 1	
Opsin 1 (cone pigments), short-wave-sensitive 2	
Retinaldehyde binding protein 1b	
Solute carrier family 31 (copper transporters), member 1	
Tumor protein p53	

of eye development and adult visual function in TCDD-treated animals is warranted.

AHRR Knockdown Induces Stress Signaling

In contrast to the down-regulation of critical players in phototransduction, knockdown of both AHRRa and AHRRb up-regulated two genes that act as chaperone proteins in the eye. Crystallin (*crygm2c*), a small heat shock protein found exclusively in the lens and cornea of the eye, was up-regulated in AHRRa and AHRRb morphants. Beaded filament structural protein 2 (*bfsp2*), a lens-specific intermediary filament, was also up-regulated in the morphants. Intermediary filaments act as a scaffold for chaperone proteins and stress-activated kinases (Song *et al.*, 2009). These results suggest that knockdown of AHRRa and AHRRb activated stress signaling in the eye. This is perhaps not surprising considering that AHRR knockdown affected several genes involved in photoreceptor signaling and the induction of chaperone proteins may help to repair any misfolded or damaged proteins. The regulation of crystallin by AHRRa could be associated with AHR signaling, as suggested by the recent finding that AHR regulates crystallin (αb) in mice under normal and TCDD-induced states (Liu and Piatigorsky, 2011).

Role of AHRRa in Erythropoiesis and Hemostasis

We also observed that AHRRa morphants exhibited up-regulation of genes associated with erythropoiesis and hemostasis. The up-regulated genes included embryonic globins involved in oxygen transport (embryonic hemoglobin beta 3 subunit (*hbbe3*) and alpha subunit (*hbae1*)) and three genes encoding fibrinogen polypeptides (*fga*, *fgb,* and *fgg*), key players in blood coagulation.

Globins are an important group of proteins involved in oxygen transport. Most vertebrates possess multiple globin genes that vary in their affinity for oxygen and are differentially expressed during development (Brownlie *et al.*, 2003). In zebrafish, hematopoiesis occurs in two distinct waves. The first wave (primitive hematopoiesis) occurs in the first 12–48 hpf, during which progenitor hematopoietic cells with embryonic globin proteins are involved in oxygen transport (Ellett and Lieschke, 2010). In the second wave (definitive erythropoiesis), pluripotent hematopoietic stem cells are formed and adult globin proteins are involved in oxygen transport (Ellett and Lieschke, 2010). This phase extends from 4 to 5 dpf to the adult life.

It remains to be determined whether AHRRa knockdown directly or indirectly derepresses embryonic hemoglobins. We examined that possibility that globins may be AHR-regulated genes by measuring the expression of *hbbe3* after exposure of embryos to TCDD. The results provided evidence for induction of this gene by TCDD (Fig. 4E; Supplementary fig. 2), consistent with the possibility that AHRRa may negatively regulate AHR-dependent *hbbe3* expression during development. Several transcription factors are involved in the regulation of primitive and definitive phases of erythropoiesis (Paik and Zon, 2010). In light of the emerging understanding of an important

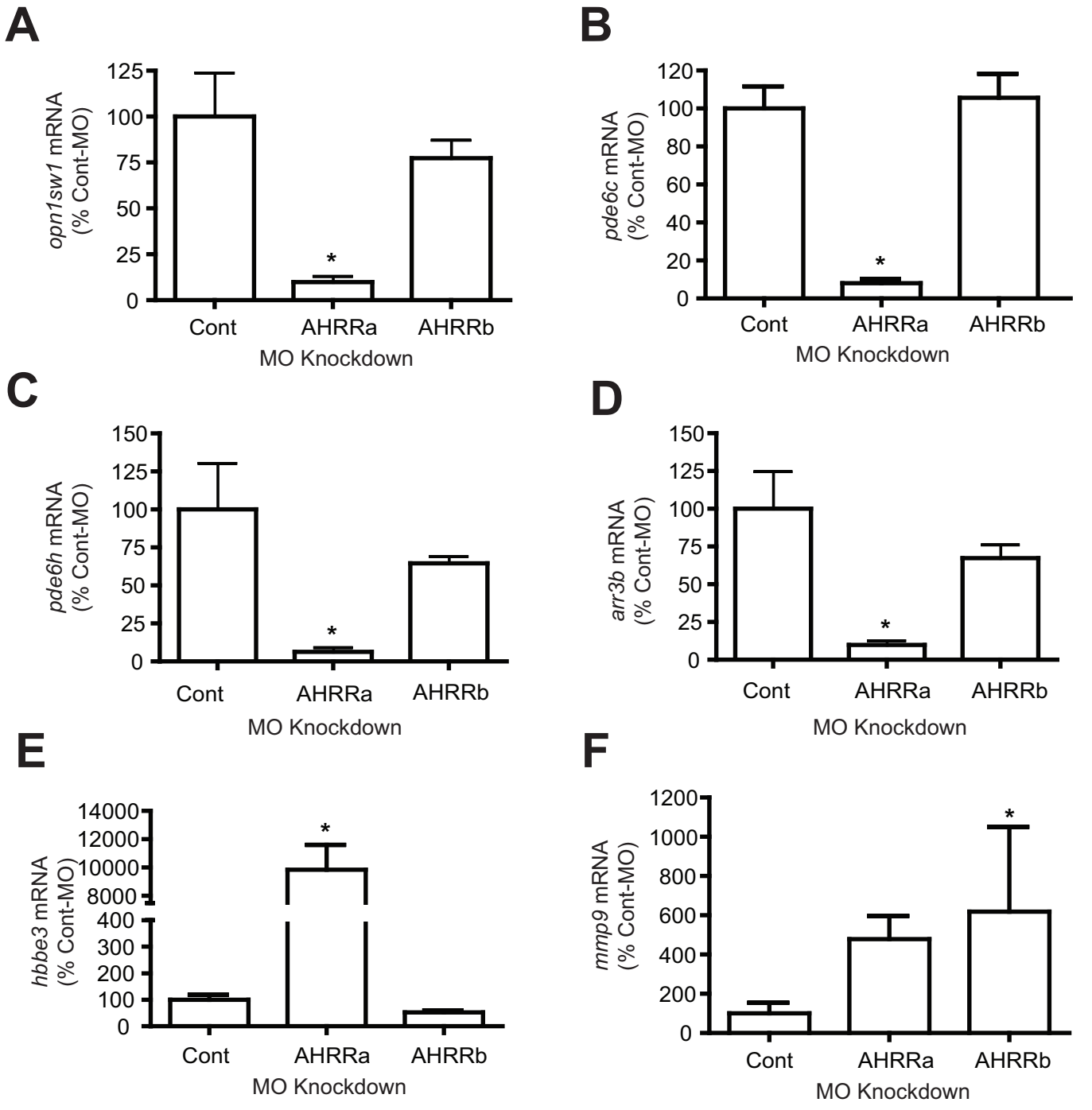

FIG. 3. qRT-PCR confirmation of microarray results. Six genes that were significantly altered by AHRR knockdown as suggested by microarray analysis were selected for qRT-PCR. β-Actin was used as an internal standard. The ΔΔCt method was used to determine the fold change in gene expression. RNA was from larvae sampled at 72 hpf. Values represent mean + SD (one-way ANOVA; $n = 3$), $*p \leq 0.05$.

role for AHR in hematopoiesis (Boitano *et al.*, 2010; Casado *et al.*, 2010; Lindsey and Papoutsakis, 2012; Singh *et al.*, 2011; Smith *et al.*, 2013), it is conceivable that AHRRa could participate in regulatory interactions involving this function of AHR. One possibility is that knockdown of AHRRa leads to an increase in endogenous AHR activity, disrupting the switch from primitive to definitive erythropoiesis and thereby delaying the appearance of erythrocytes expressing adult globins. Such a dis-

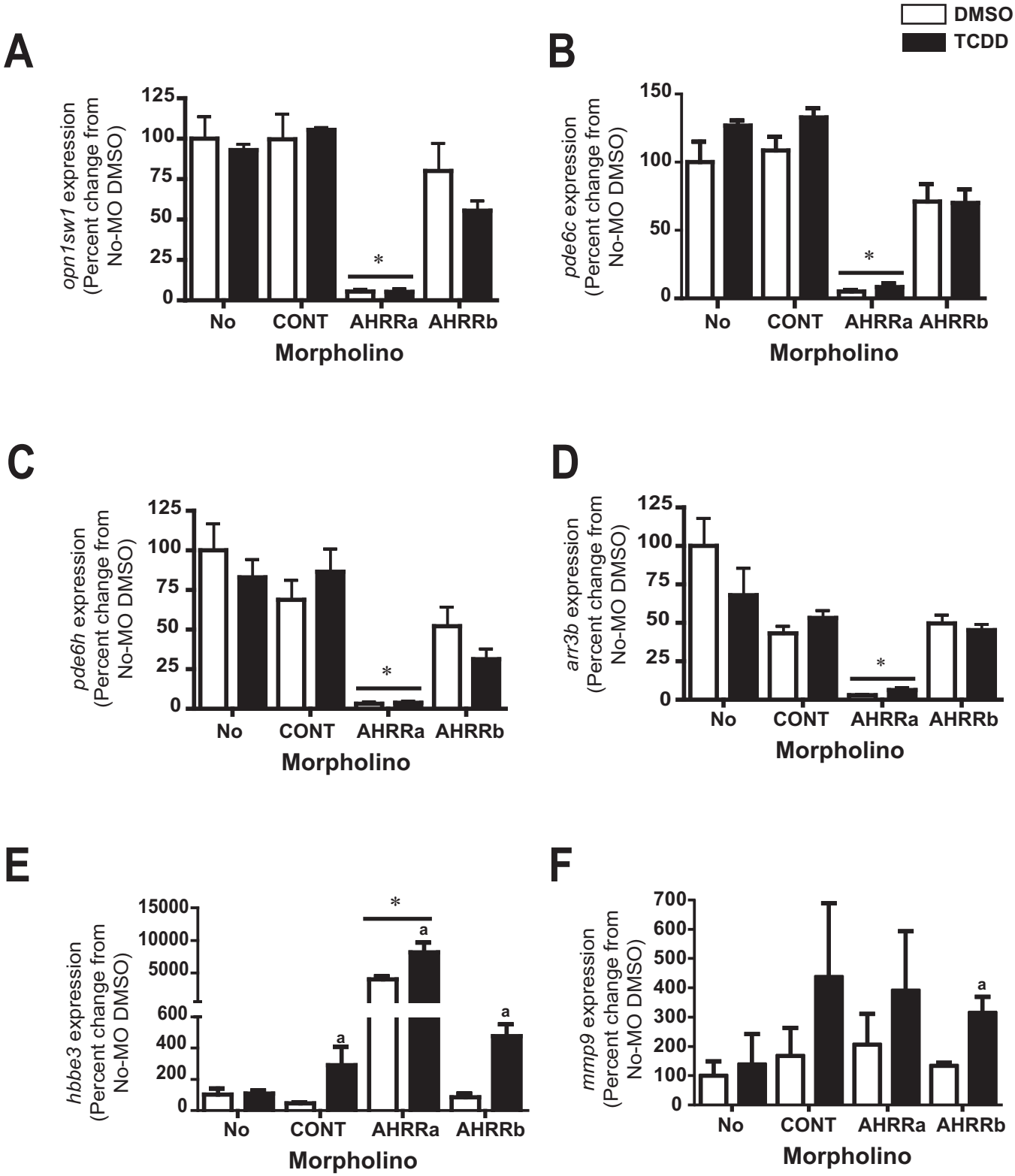

FIG. 4. Effect of TCDD on AHRR-MO induced gene expression changes. Injection of AHRR MOs and exposure to TCDD was carried out as described in Materials and Methods section. RNA was from larvae sampled at 72 hpf. β-Actin was used as an internal standard. The ΔΔCt method was used to determine the fold change in gene expression. Values represent mean + SD (two-way ANOVA; $n = 3$). Bonferroni's *post hoc* test was used for determining statistical significance. [a]$p \leq 0.05$ TCDD versus DMSO; *$p \leq 0.05$ AHRRa-MO group versus no-MO treatment. CYP1 expression data from these experiments was reported previously (Jenny *et al.*, 2009).

ruption of definitive erythropoiesis has been observed in zebrafish embryos in which AHR has been activated exogenously, by exposure to the AHR agonist TCDD (Belair *et al.*, 2001). Similarly, chronic TCDD exposure caused down-regulation of adult hemoglobins in juvenile rainbow trout (Liu *et al.*, 2013). Consistent with the idea that knockdown of AHRRa may lead to enhanced endogenous activity of AHR, the phenotypes observed with AHRRa knockdown are similar to those observed following TCDD treatment (Jenny *et al.*, 2009). Alternatively, or in addition, loss of AHRRa could affect hematopoiesis via altered function of HIF-1α or ARNT (Adelman *et al.*, 1999; Karchner *et al.*, 2009).

Similar to what was seen for embryonic globins, AHRRa knockdown up-regulated fibrinogen polypeptide subunit mRNAs (*fga*, *fgb*, and *fgg*). Compared with hematopoiesis, very little is known about developmental regulation of hemostasis in zebrafish. Fibrinogen is a hexamer consisting of two sets of three polypeptide subunits (alpha, beta, and gamma). Thrombin converts fibrinogen to fibrin during blood coagulation (Fish *et al.*, 2012). High levels of fibrinogen are associated with cardiovascular disease involving the accumulation of fibrinogen, which initiates platelet aggregation and atherogenesis (Tousoulis *et al.*, 2011). Whether the increase in fibrinogen expression causes defects in blood clotting in AHRRa morphant embryos is not yet known. Such an effect could also be associated with increased AHR signaling, as exposure of medaka embryos to TCDD leads to an apparent increase in the formation of blood clots (Kawamura and Yamashita, 2002).

AHRRa May Act as a Tumor Suppressor Gene

Some of our results appear related to recent observations that AHRR acts as a tumor suppressor gene (Zudaire *et al.*, 2008). Knockdown of AHRRa up-regulated two genes (*tp53* and *ccng1*) involved in cell cycle regulation. Tumor protein p53 (*tp53*) is an important regulator of cell cycle progression and cyclin G1 (*ccng1*) is involved in the regulation of transition from G1 to S-stage of the cell cycle (Zhao *et al.*, 2003). Previous studies have demonstrated that AHRR acts as a tumor suppressor in several cancer cell lines (Zudaire *et al.*, 2008). Stable transfection of AHRR into ER alpha-positive MCF-7 cells decreased the expression of E2F and cyclin genes important for tumor cell growth when induced with DMBA. In the same study, siRNA-induced down-regulation of AHRR increased the growth of lung carcinoma cell lines and caused them to become more invasive (Kanno *et al.*, 2006). In addition, the region of the chromosome containing the AHRR gene is deleted in several human cancer tissues, suggesting that AHRR plays an important role in tumor suppression by repressing the AHR activity in tumors (Zudaire *et al.*, 2008). Additional studies examining the roles of AHRR in regulation of cell growth are warranted.

One of the off-target effects of MO administration is the activation of *tp53* expression (Robu *et al.*, 2007). However, off-target effects involve up-regulation of protein expression without concomitant increase in the mRNA expression (Robu *et al.*, 2007). Our results involving increased *tp53* RNA are thus distinct from those associated with off-target effects. In addition, we only observed the induction of *tp53* with AHRRa-MO injected embryos and not with AHRRb-MO- and Control-MO-injected embryos, suggesting that the response is specific to loss of AHRRa.

Role of AHR Activation

It is tempting to conclude that the effects occurring upon knockdown of AHRRs result from derepression of AHR signaling. However, because AHRR is known to affect other signaling pathways, such as estrogen and hypoxia signaling (Kanno *et al.*, 2006, 2008; Karchner *et al.*, 2009), it is conceivable that the effects of AHRR knockdown reported here could involve these or other signaling pathways. A limitation of the present study is that MO-based knockdown does not completely eliminate expression of the targeted gene, so there could be functions that are maintained even at the reduced levels of protein expression. Experiments to generate targeted null-mutants of AHRRs are in progress and should provide further insight into AHRR functions and the role of AHR versus other signaling pathways.

CONCLUSIONS

Our results lend further support to our previous suggestion (Jenny *et al.*, 2009) that duplicated AHRR genes (AHRRa and AHRRb) have distinct physiological roles during development in zebrafish. We have identified several genes related to photoreceptor signaling, erythropoiesis, and hemostasis to be differentially expressed in AHRRa knockdown embryos. These results suggest that AHRRa has a role in normal embryonic development. In contrast, AHRRb knockdown affected fewer genes and there was little overlap in the genes differentially expressed in the two AHRR morphants, suggesting that they regulate different processes during development. The results here further demonstrate the value of the zebrafish model and its duplicated AHRRs for obtaining insight into the various functions of AHRR.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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