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Research Article

Developmental Regulation of Nuclear Factor Erythroid-2 Related Factors (*nrfs*) by AHR1b in Zebrafish (*Danio rerio*)

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ABSTRACT

Interactions between regulatory pathways allow organisms to adapt to their environment and respond to stress. One interaction that has been recently identified occurs between the aryl hydrocarbon receptor (AHR) and the nuclear factor erythroid-2 related factor (NRF) family. Each transcription factor regulates numerous downstream genes involved in the cellular response to toxicants and oxidative stress; they are also implicated in normal developmental pathways. The zebrafish model was used to explore the role of AHR regulation of *nrf* genes during development and in response to toxicant exposure. To determine if AHR1b is responsible for transcriptional regulation of 6 *nrf* genes during development, a loss-of-function experiment using morpholino-modified oligonucleotides was conducted followed by a chromatin immunoprecipitation study at the beginning of the pharyngula period (24 h postfertilization). The expression of *nrf1a* was AHR1b dependent and its expression was directly regulated through specific XREs in its *cis*-promoter. However, *nrf1a* expression was not altered by exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), a toxicant and prototypic AHR agonist. The expression of *nrf1b*, *nrf2a*, and *nfe2* was induced by TCDD, and AHR1b directly regulated their expression by binding to *cis*-XRE promoter elements. Last, *nrf2b* and *nrf3* were neither induced by TCDD nor regulated by AHR1b. These results show that AHR1b transcriptionally regulates *nrf* genes under toxicant modulation via binding to specific XREs. These data provide a better understanding of how combinatorial molecular signaling potentially protects embryos from embryotoxic events following toxicant exposure.

Key words: AHR; ChIP; development; nrf; TCDD; zebrafish.

The expression of most genes is carefully regulated by multiple transcription factors. The crosstalk between these transcription factors allows biological systems to respond to endogenous and exogenous signals, and adapt to stress. Recently, the aryl hydrocarbon receptor (AHR) and the nuclear factor erythroid-2 related factor-2 (NRF2) were discovered to engage in crosstalk in multiple biological systems (Kalthoff *et al.*, 2010; Ma *et al.*, 2004; Miao *et al.*, 2005; Shin *et al.*, 2007; Timme-Laragy *et al.*, 2012; Yeager *et al.*, 2009). Because AHR and NRF2 are already known to each regulate a large and diverse set of target genes (Boutros *et al.*, 2009; Ma, 2013), crosstalk could also play a significant role.

AHR is a highly conserved ligand-activated transcription factor (Denison and Nagy, 2003) that binds to dioxin response elements (DREs)/xenobiotic response elements (XREs) (Denison *et al.*, 1988) on the promoter of target genes (Beischlag *et al.*, 2008). The AHR transcriptional program is extensively implicated in toxicology, carcinogenesis, physiology, and development, which highlights the importance of understanding how it interacts with other signaling pathways, including those controlled by NRF2 and related proteins.

In mammals, 4 Cap'n'collar (CNC) basic leucine zipper (bZIP) transcription factors have been characterized: nuclear factor erythroid-2 (NF-E2), NF-E2-related factor-1 (NRF1; also called

NFE2L1), NF-E2-related factor- 2 (NRF2 or NFE2L2), and NF-E2-related factor- 3 (NRF3 or NFE2L3). CNC-bZIP transcription factors are responsible for the cellular transcriptional response to oxidative stress (Ma and He, 2012; Sykiotis and Bohmann, 2010). Although the expression patterns and cellular functions of various NRF proteins vary widely, most have been found to play a role in basal cellular functions and response to imbalances in cellular redox conditions (Chevillard and Blank, 2011; Gasiorek and Blank, 2015; Hahn *et al.*, 2015; Ma, 2013; Zhang and Xiang, 2016). The many and diverse cellular roles make understanding NRF regulation and intrapathway interactions essential.

Genes that are transcriptionally activated through either xenobiotic or antioxidant response elements (XRE or ARE), and thus by AHR or NRF proteins, respectively, were originally thought to be independently regulated. The first gene found to be dependent on both proteins was NAD(P)H: quinone oxidoreductase 1 (NQO1). Induction of NQO1 by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) requires XRE (Favreau and Pickett, 1991), whereas induction by the prototypical oxidant *tert*-butylhydroquinone (tBHQ) proceeds through protein binding to AREs (Radjendirane and Jaiswal, 1999). A subsequent study showed that basal and inducible expression of NQO1 by either TCDD or tBHQ required the interaction of AHR and NRF2 (Ma *et al.*, 2004). The promoter region of NQO1 as well as GSTA1 and UGT1A10 contain XRE and ARE in close proximity to each other, suggesting that AHR and NRF2 may physically interact to drive transcription of the genes (Kalthoff *et al.*, 2010; Vasiliou *et al.*, 1995). In addition, the induction by TCDD of AHR-regulated genes, including UGT1A6, GSTA1, and other UDP glucuronosyltransferases and glutathione S-transferase isoforms was then shown to require NRF2 (Yeager *et al.*, 2009). These studies presented a new paradigm in gene regulation, that of the "TCDD-inducible AHR-NRF2 gene battery" (Yeager *et al.*, 2009), raising important questions about which other genes may be regulated through this mechanism, especially during the most sensitive and consequential life stage, the embryo.

Zebrafish have emerged as a powerful model for studying molecular mechanisms of vertebrate development and developmental toxicology (Horzmann and Freeman, 2018; Shin and Fishman, 2002; Tanguay, 2018; Ward and Lieschke, 2002). In zebrafish, 3 *ahr* genes (Hahn *et al.*, 2017) and 6 *nrf* genes (Timme-Laragy *et al.*, 2012) have been identified, arising from a whole genome duplication event (Amores *et al.*, 1998; Taylor *et al.*, 2001). Subsequently, the genes (single copies of *nfe2* and *nrf3* as well paralogs *nrf1a*, *nrf1b*, *nrf2a*, and *nrf2b*) and their protein products have subfunctionalized in their temporal and spatial expression patterns as well as in their transcriptional roles (Kobayashi *et al.*, 2009; Pratt *et al.*, 2002; Timme-Laragy *et al.*, 2012; Williams *et al.*, 2013). All *nrf* genes have putative XREs in their *cis*-promoters (Williams *et al.*, 2013), making them potential targets of Ahr regulation.

Of particular interest to this study was *ahr1b* due to its expression throughout development (Karchner *et al.*, 2005) and spatial expression in the developing eye (Karchner *et al.*, 2017; Sugden *et al.*, 2017), an organ that at 24 h postfertilization (hpf) makes up a large percentage of the larval body mass and is critical to later life behavior such as feeding and predator evasion (Glass and Dahm, 2004). Although *ahr1b*'s transcription is unaffected by chemical exposure to TCDD (Karchner *et al.*, 2005) or β-naphthoflavone (Sugden *et al.*, 2017), AHR1b has the ability to bind TCDD and is able to activate transcription of a reporter gene under the control of XREs (Karchner *et al.*, 2005). The goal of this study was to assess whether AHR1b transcriptionally regulates the expression of *nrf* gene family members through binding to XREs.

MATERIALS AND METHODS

Chemicals. TCDD was purchased from Ultra Scientific (N. Kingston, Rhode Island) and dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St Louis, Missouri).

Fish husbandry and strains. For all experiments, the Tupfel/Longfin mutation wild-type strain was used. Adults and embryos were maintained and used as previously described in Jönsson *et al.* (2007). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Woods Hole Oceanographic Institution Animal Care and Use Committee (Animal Welfare Assurance Number A3630-01) and the Bates College Animal Care and Use Committee (Animal Welfare Assurance Number A3320-01).

Morpholino oligonucleotides. A morpholino antisense oligonucleotide (MO), designed to block translation of *ahr1b* (AGGCACCCAAAATCTCAATATCACA) by binding upstream of the translational start site, was obtained from Gene Tools, LLC (Philomath, Oregon). The standard control-MO from Gene Tools (CCTCTTACCTCAGTTACAATTTATA) was used as an injection and MO control.

The efficacy of the translational knockdown of AHR1b was assessed via the TNT T7 Quick Coupled Reticulocyte Lysate system (Promega, Madison, Wisconsin). [^{35}S]methionine-labeled AHR1b was synthesized per manufacturer's instructions in the presence of *ahr1b*-MO or control-MO (0.05 μM final concentration) and synthesis of AHR1b was analyzed by gel electrophoresis and fluorography as previously described (Jenny *et al.*, 2009). Efficacy of knockdown was quantified for a single experiment by comparing the densitometric signals of the *ahr1b*- and control-MO samples using ImageJ (Schneider *et al.*, 2012).

Microinjection of zebrafish embryos with morpholinos and chemical exposure. Embryos between the 2- and 4-cell stage were injected with 2–2.5 nL of a 0.1 mM solution of *ahr1b* MO as previously described in Jenny *et al.* (2009) and Williams *et al.* (2013).

At 6 hpf, control MO and *ahr1b* injected embryos (3 pools of 30 embryos) were placed in glass scintillation vials containing no more than 3 embryos per milliliter of 0.3× Danieau's and then exposed to either 0.1% DMSO (vehicle control) or 2 nM TCDD (dissolved in DMSO) for 1 h. The timing, duration, and TCDD concentration were chosen because they did not cause severe toxicity but did elicit strong induction of *cyp1a* (Jenny *et al.*, 2009), a prototypic AHR-regulated gene (Whitlock, 1999). After the exposure, embryos were washed in 0.3× Danieau's and then placed in petri dishes containing fresh 0.3× Danieau's and held in an incubator with a 14/10-light/dark cycle until 24 hpf (Jenny *et al.*, 2009).

RNA extraction, cDNA synthesis, and real-time quantitative RT-PCR. Using RNA STAT-60 (AMS Biotechnology, Abingdon, UK), total RNA was isolated from pooled embryos following manufacturer's instructions. Following analysis of the isolated RNA with a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts), 1 μg of total RNA was used to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, California).

Quantitative RT-PCR (qPCR) on all nrf transcripts (nfe2, nrf1a, nrf1b, nrf2a, nrf2b, and nrf3) and a housekeeping gene (β-actin) in control MO and ahr1b MO samples was conducted

using the MyiQ Single-Color Real-Time PCR Detection system (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad) with the protocol and primers described previously (Evans et al., 2005; Timme-Laragy et al., 2012; Williams et al., 2013). Relative expression of each gene was analyzed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Statistical differences in expression were determined using a 2-way ANOVA followed by a Sidak's multiple comparisons test in Graphpad Prism 7 software (La Jolla, California).

In silico *promoter analysis of XREs*. An *in silico* promoter analysis was carried out on 10 000 bp upstream of the transcriptional start site to identify potential XREs [KNGCGTG] (Lusska *et al.*, 1993; ZeRuth and Pollenz, 2007) in the *cis*-promoter of *nrf* genes where AHR1b could potentially bind. Both strands of the DNA were searched using a fuzzy search algorithm, fuzznuc (Rice *et al.*, 2000), as previously described in Williams *et al.* (2013).

Antibody against AHR1b and confirmation of antibody specificity. A rabbit polyclonal antibody targeting AHR1b protein was raised against the peptide LENQTEDPAESQKPSTA (amino acids 592-608 of AHR1b) by 21st Century Biochemicals (Marlboro, Massachusetts) and affinity-purified. Antibody specificity was assessed by Western blotting of COS-7 lysates expressing AHR1b from transfected cDNA (data not shown) and zebrafish embryo homogenates. In 3 independent experiments, 200 embryos were exposed to DMSO or 2 nM TCDD starting at 6 hpf for 1 hour and prepared for Western blotting at 24 hpf using methods that are previously described in Westerfield (2007). Following electrophoresis of 10 μL of homogenate from each sample on a 10% Mini-PROTEAN TGX Stain-Free gel (Bio-Rad), total protein was quantified with a Biorad Chemidoc imaging system by measuring the total density of protein in each lane using Biorad ImageLab 6.0 (Gilda and Gomes, 2013). This step ensured equal loading of total protein in each well. Protein was then transferred to an Immuno-Blot LF PVDF membrane (Bio-Rad) and preblocked in 5% milk in 1× Tris-buffered saline Tween (TBST) for 1 hour at room temperature. After blocking, the membrane was incubated with AHR1b (1 μg/ml) antibody in Tris-buffered saline for 1 h at room temperature. Following 3 washes with 1× TBST, a goat antirabbit IgG (H + L)-HRP conjugate secondary antibody (Bio-Rad) was used at a dilution of 1:3000 and incubated for 1 h. Following three 1× TBST washes, Clarity Max Western ECL substrate (Bio-Rad) was added to the blot and the blot was exposed for 60 s on a Biorad Chemidoc imaging system. ImageJ (Schneider *et al.*, 2012) was used to quantify and compare the AHR1b protein between DMSO and TCDD samples. A 1-way ANOVA was used to determine statistical differences in the relative density of the AHR1b specific band between DMSO and TCDD treated embryos in GraphPad Prism 7 software, with the DMSO-treated samples serving as the control in the density calculations (Taylor *et al.*, 2013).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was carried out in triplicate on 24 hpf embryos as described previously in Aday *et al.* (2011) with slight modifications. For each independent sample, 300 embryos were used. Embryos were manually dechorionated. Samples were sonicated with a Diagnode Bioruptor Pico with a water cooler set to 4°C (Denville, New Jersey) for 13 cycles of 30 s on and 30 s off. Shearing was verified with gel electrophoresis to ensure a fragment size between 200 and 300 bp. Samples were not precleared or preblocked. A total of 8 μg of AHR1b antibody was used in the immunoprecipitation.

Immunoprecipitated and input samples were amplified for quantification with qPCR using a Stratagene Mx3000P qPCR Machine and Agilent Brilliant II SYBR Green dye. Primers for each XRE in the *cis*-promoter of all *nrf* genes were designed (Table 1) and used. For the positive control, primers were designed to amplify XRE 4 from *cyp1a1* (ZeRuth *et al.*, 2007), and the negative control primers amplified a segment of *nrf1a cis*-promoter that was at least 5800 bp upstream from any of the 2 identified XREs. Promoter content was calculated as previously described in Hestermann and Brown (2003).

RESULTS

Given that there was at least 1 well-conserved putative XRE in the *cis*-promoter of each *nrf* gene (Figure 1, Table 1) (Williams *et al.*, 2013), we hypothesized that the temporary suppression of AHR1b by a MO could affect the transcription of these genes during the pharyngula period. *In vitro*, the MO knocked down the expression of AHR1b by 66% (Figure 2) and thus was considered to be effective at reducing AHR1b *in vivo*. It is advisable that future studies test the knockdown of AHR1b via MO in embryos using the AHR1b antibody.

Upon exposure to TCDD, *nrf1b*, *nrf2a*, and *nfe2* transcripts were induced and the relative fold change values of *nrf1b* and *nrf2a* were significantly decreased upon knockdown of AHR1b (Figs. 3B and 3C). Interestingly, there was little difference in the TCDD-induced fold induction ratio of *nrf1b* in the control morphants (2.8) as compared with *ahr1b* morphants (3.7). The same was true for *nrf2a*, which had a ratio of 2.7 for control morphants and 3.5 for *ahr1b* morphants. For *nfe2*, the knockdown of AHR1b did not block the induction by TCDD (Figure 3E); moreover, the fold induction ratio caused by TCDD in the *ahr1b* morphants (12.1) was much greater than that in the control morphants (3.3). The constitutive expression of *nrf1b*, *nrf2a*, and *nfe2* (in DMSO-treated samples) was not significantly affected by knockdown of AHR1b. *nrf1a* was down-regulated upon knockdown of AHR1b in the vehicle controls, but its expression was not affected by exposure to TCDD (Figure 3A). Although the relative fold change values of this gene were not significant, there was an induction difference due to TCDD treatment in control morphant (1.3) versus *ahr1b* morphant samples (3.4). The expression of *nrf2b* was not affected by TCDD in the embryos injected with the control MO, but in the AHR1b morphants *nrf2b* was induced by TCDD (Figure 3D). TCDD had an effect on the fold induction of this gene in *ahr1b* morphants (4.3) as compared with control morphants (1.4). Under both vehicle control and TCDD conditions, the expression of *nrf3* remained the same and the loss of AHR1b had no effect (Figure 3F). There was no difference in TCDD induction ratios between control morphants (0.9) and *ahr1b* morphants (1.7).

Prior to using the AHR1b antibody in the ChIP assay, its efficacy in recognizing AHR1b in 24 hpf embryos was verified using a Western blot. In both the DMSO and TCDD-treated embryos, the antibody recognized a major band of approximately 105 kDa, very close to the predicted size of 104.8 kDa (Karchner *et al.*, 2005) (Figure 4A). There were a few nonspecific bands seen in the blots, some of which were of high molecular weight and only found in the DMSO sample. Total immunodetectable AHR1b protein was quantified by densitometry across 3 blots from independent samples and the average relative density of the 105-kDa band did not differ between DMSO and TCDD 24 hpf samples as determined by a 1-way ANOVA (Figure 4B).

The antibody was subsequently used to immunoprecipitate the AHR1b-bound regions of the chromatin. PCR amplification

Table 1. Quantitative Real-Time PCR Primers Used to Amplify XRE Containing Regions of *nrf* cis-Promoters Following ChIP in 24 hpf zebrafish

Gene and XRE	Forward Primer	Reverse Primer	XRE Sequence	Amplicon Length
nrf1a XRE1	TGTTGTTTA TATAGGTCA ACTCATAAG	TTGGAGGAG ATCAGGGTT ACT	CACGCGC	76
nrf1a XRE2	GGCACACAT AACCTACCA GAG	CGCTACGGT GAGGTTGAG CC	GGGCGTG	166
nrf1a XRE3	ACACTGATG AACGGAGTG AG	GAGTCG GTG AGTGCTGGA AAGC	CACGCGC	104
nrf1a Negative Control	CTCATCTGC ATACAGGAC TCAC	GTCTACAAG AGCAGCATA GACC	—	82
nrf1b XRE1	GACGCAGTG CTCTAATAT GGC	CTTGTCCTG AAAGAACTC GG	TAGCGTG	73
nrf2a XRE1	CTGCTCTCC GCCTGTTTA C	AGTGCTCTG CTCCACATT TG	GCGCGTG	108
nrf2a XRE2	CATGCACGC ACAGTCTAA TC	CAGTGTTCC CAGTGCTTT AC	CACGCAC	84
nrf2a XRE3	AGCTTCAGC TGGAGAATG TC	GACTAGGAG AACAGTGAA GTGC	CACGCACGCGC	115
nrf2a XRE4	GCACTTCAC TGTTCTCCT AGTC	CAGACACAC ATCTCCAGC AC	GTGCGTG	147
nrf2b XRE1	GTCCAGCAG TTCTTATCA GC	GACCATCTG TGTAGTCTT CTGC	CACGCCA	115
nrf2b XRE2	GTGACGCAG TGCTCTAAT ATG	TTTCTTGTC CTGAAAGAA CTCG	CACGCGA	89
nfe2 XRE1	CCTCTTTGG ACGGTGTCA GCC	CGCTCCATC AAGCCTCAT GCC	CACGCCA	146
nfe2 XRE2	CAAAGCCTC CACTGGTCA TGG	GCCACATTG TTCCTGTAT CTGC	CACGCTC	79
nrf3 XRE1	AACCTTATT GTAAAGTGT GACCATC	ATAGCTCCA GTCCCCCTA GC	CACGCCA	125
nrf3 XRE2	CTCGGACAG CAATATCTC CTTG	CGACCTTGC CATTCCTAT AAC	CACGCAA	89
cyp1a1 XRE4 Positive Control	CATTCCGCC AGCTCTTCC TG	GCCTGCATG TTTGAGTCT CTGC	CGCGTG	114

Primers are written 5' to 3' and amplicon length is given. Putative XRE sequence locations are mapped in Figure 1.

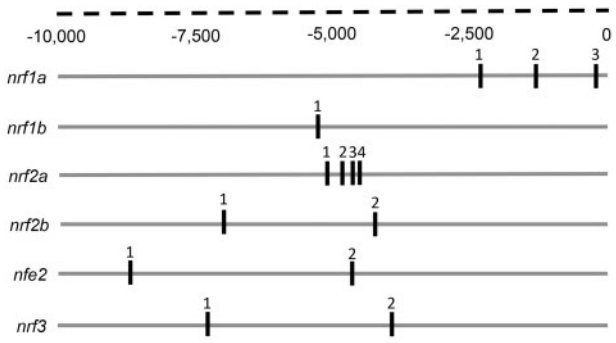

Figure 1. Putative XREs in the proximal regulatory region of zebrafish *nrf* genes. Specific XREs were identified using a fuzzy search for canonical zebrafish XREs within each *cis-nrf* promoter 10, 000 bp upstream of the transcriptional start site. Exon positions were exported from the Ensembl database (Zv9).

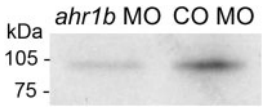

Figure 2. Morpholino efficacy as assessed by *in vitro* translation. The *in vitro* translation of AHR1b was measured using incorporation of [^{35}S]methionine in the presence of *ahr1b* MO or control MO. The *ahr1b* MO decreased the expression of AHR1b by 66%.

of the *cyp1a* *cis*-promoter, containing XRE4 (TGGCGTGCAAAG), which has been shown to drive expression of luciferase upon exposure to TCDD (ZeRuth *et al.*, 2007), was used as a positive control. Indeed, AHR1b directly bound to XRE4 under conditions of TCDD exposure (Figure 5A), serving as a positive control for the assay. For our negative control, we tested the promoter content of a region of the *nrf1a* *cis*-promoter that was located at least 5800 bp upstream from any of the 3 identified XREs. This negative control showed very little promoter content after immunoprecipitation (Figure 5A). For *nrf1b*, *nrf2a*, and *nfe2*, which showed transcriptional activation via TCDD exposure, the ChIP assay verified that there was direct binding of AHR1b in a XRE-specific manner (Figs. 5B, C, and E). Despite the presence of only a single XRE (TAGCGTG) in the *nrf1b* proximal promoter (Figure 1), this putative element was able to bind AHR1b (Figure 5B). Although the 4 XREs for *nrf2a* are all clustered together (within 1000 bp), only 2 seem to play a major role in binding AHR1b. XRE1 (GCGCGTG) and XRE4 (GTGCGTG) were both bound by AHR1b after treatment with TCDD, and XRE4 also bound AHR1b under vehicle control conditions. XRE3 (CACGCACGCGC) also bound some AHR1b weakly under control and TCDD treatments. There are 2 XREs in the proximal promoter for *nfe2*; however, only XRE1 (CACGCCA) bound AHR1b under TCDD conditions; in the control samples, the binding of AHR1b was weak for both XRE1 and XRE2 (CACGCTC). For *nrf1a*, XRE2 (GGGCGTG) bound AHR1b under control and TCDD conditions, whereas XRE3 (CACGCGC) participated in binding AHR1b following TCDD treatment (Figure 5A). Both of these XREs were located more proximal to the transcriptional start site (Figure 1) as compared with XRE1, which was more distal and did not show binding of AHR1b. AHR1b did not bind to the XREs in either of the *cis*-promoters of *nrf2b* or *nrf3* (Figs. 5D and 5F).

DISCUSSION

Since its discovery in the 1970s (Okey *et al.*, 1979; Poland *et al.*, 1976), the direct transcriptional role of AHR in cellular signaling has been well studied in the context of both its toxicological and physiological functions (Hahn *et al.*, 2017; Jan *et al.*, 2011; Lindsey and Papoutsakis, 2012; Mulero-Navarro and Fernandez-Salguero, 2016; Okey, 2007; Zhang, 2011). What has been less studied is its effects on cell signaling through crosstalk with other transcription factors (Puga *et al.*, 2009). Due to genome duplication in the teleost lineage (Amores *et al.*, 1998; Taylor *et al.*, 2001), there are multiple Ahr and Nrf proteins for which this crosstalk has not been evaluated. This study is the first to demonstrate direct regulation of *nrf* genes by 1 AHR paralog, AHR1b, during early embryonic development.

At the beginning of the pharyngula period (24 hpf), a phylotypic stage, all of the *nrf* genes and *ahr1b* are expressed (Karchner *et al.*, 2005; Mukaigasa *et al.*, 2012; Pratt *et al.*, 2002; Timme-Laragy *et al.*, 2012; Williams *et al.*, 2013). At 24 hpf, embryos are differentiating, utilizing endogenous ROS for cellular signaling (Thannickal and Fanburg, 2000). Yet, these embryos have low concentrations of total glutathione and oxidizing

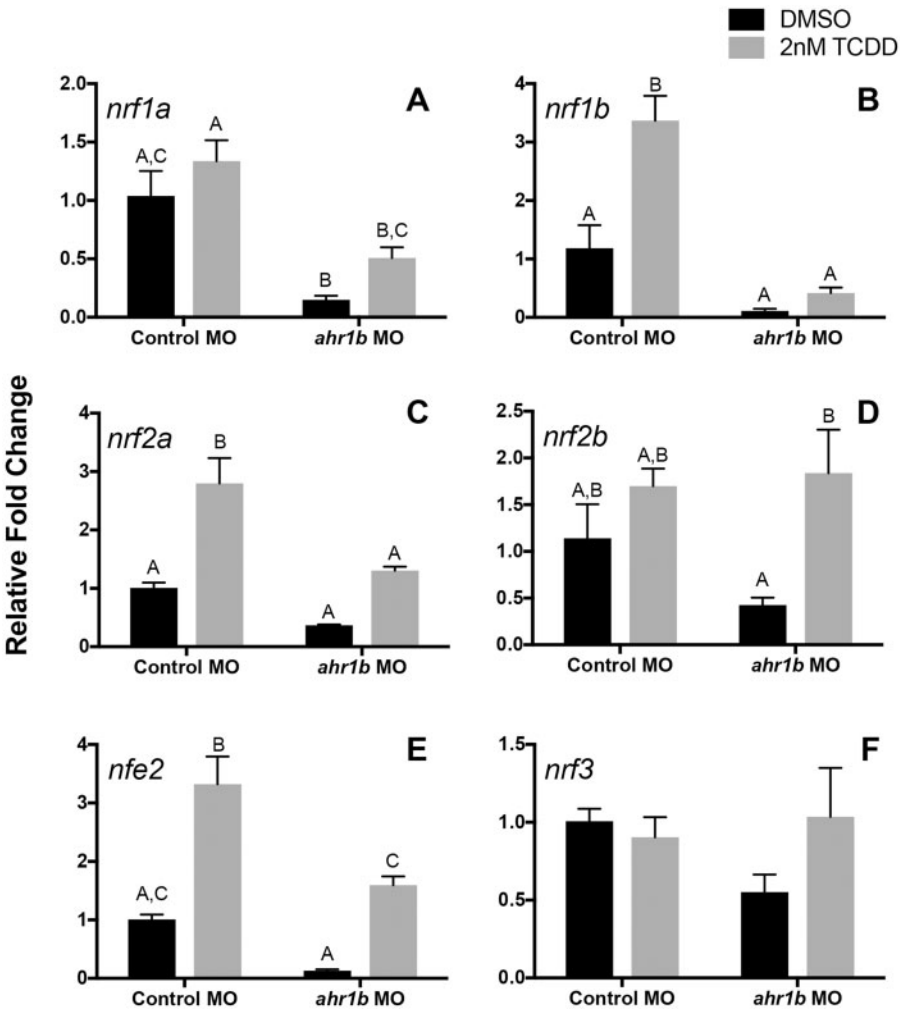

Figure 3. AHR1b knockdown resulted in decreased expression of *nrf* genes in zebrafish embryos. Relative fold change of nuclear factor erythroid 2 (*nfe2*)-related factors (*nrf*) genes in 24 hpf zebrafish embryos is shown for control and *ahr1b* morphants. Zebrafish were dosed with DMSO or 2 nM TCDD for 1 h starting at 6hpf. Data are presented as mean + standard deviation, where different letters over bars indicate significant difference (2-way ANOVA with Sidak's multiple comparisons test, $p = .05$). β-actin, whose expression was consistent across treatments, was used as a housekeeping gene and relative expression was calculated using the $\Delta\Delta C_T$ method.

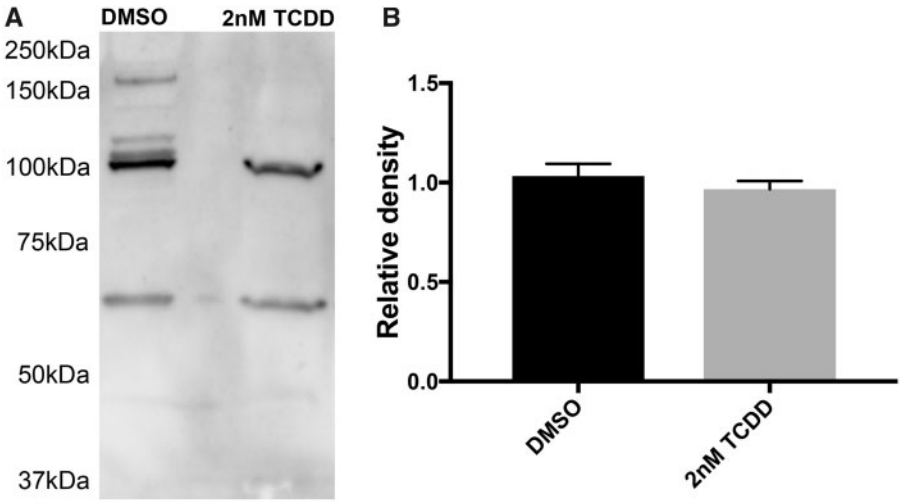

Figure 4. Western blot analysis of 24 hpf embryos with AHR1b antibody. A, Total protein from 24 hpf embryos was electrophoresed and blotted with a custom AHR1b antibody in 3 independent experiments and a representative image from 1 experiment is shown. B, Relative density of AHR1b concentration (band at approximately 105 kDa) was determined across 3 independent experiments by ImageJ and mean + SD is shown. No statistical difference was found between treatments (1-way ANOVA, $p = .05$).

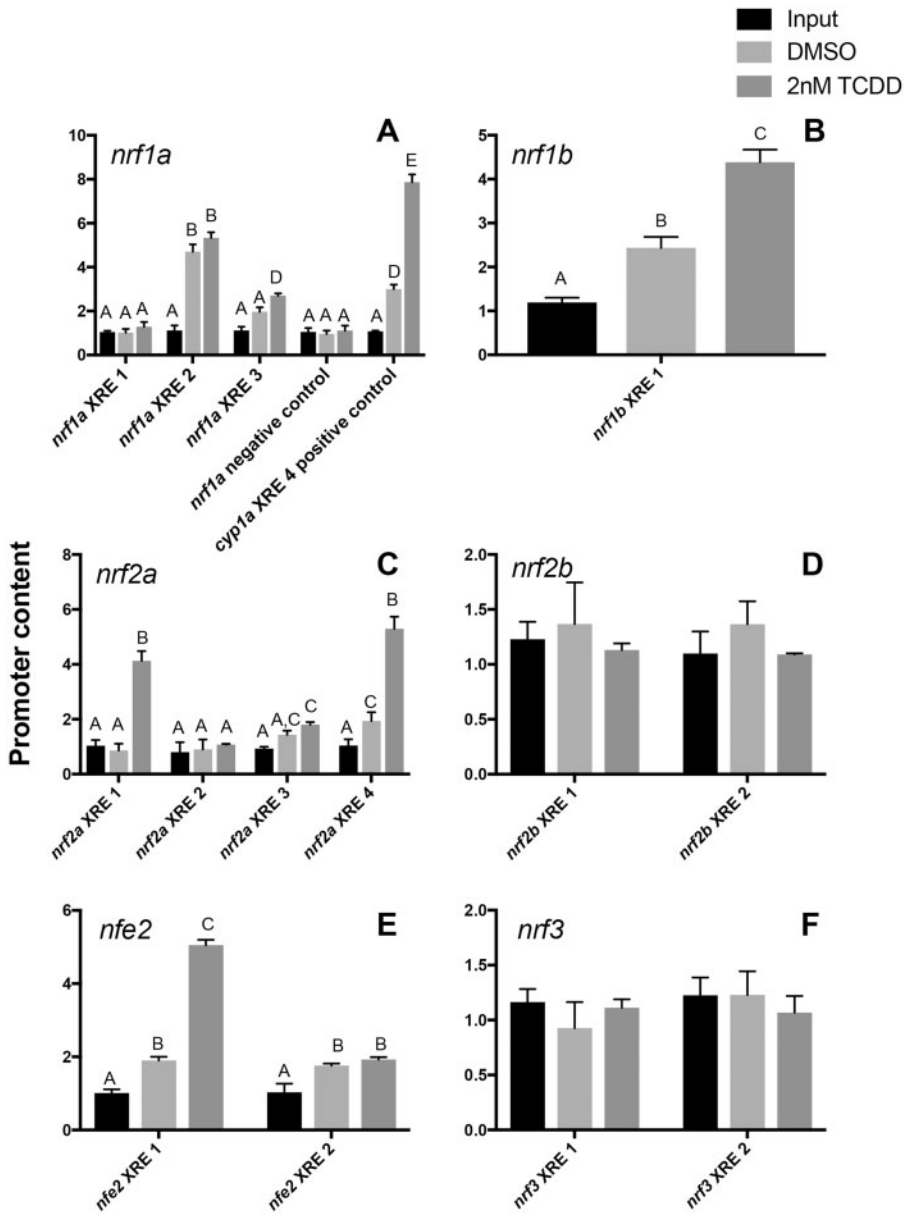

Figure 5. AHR is bound specifically to *cis*-XREs in the promoters of *nrf* genes in zebrafish embryos. ChIP assays were performed on 24 hpf zebrafish treated with either DMSO or 2 nM TCDD using an antibody specific for AHR1b followed by qPCR of individual XREs. qPCR of DNA from input fraction and AHR1b immunoprecipitation fraction are shown for samples treated with the control DMSO vehicle or TCDD. Shown in the *nrf1a* panel is the negative control which was an AHR1b IP followed by an amplification of a *cis* region of the *nrf1a* promoter lacking an XRE, and the positive control involved an AHR1b IP followed by an amplification of a *cis* region of the *cyp1a* promoter with XRE4 that has been shown to bind AHR2 (ZeRuth and Pollenz, 2007). Data are pooled from 3 experiments and presented as mean + SD where different letters over bars indicate significant difference (2-way ANOVA with Sidak's multiple comparisons test, $p = .05$).

redox potential (Timme-Laragy *et al.*, 2013), leaving them highly susceptible to imbalances in redox status that can be brought about by chemical exposure to compounds such as TCDD (Lin *et al.*, 2007).

Exposure to TCDD can cause oxidative stress (Reichard *et al.*, 2006). The AHR1b-dependent up-regulation of *nrf* genes, namely *nrf1b*, *nrf2a*, and *nfe2* (Figs. 3 and 5), which would likely enhance the transcription of antioxidant defenses, may help the embryos to maintain redox balance upon exposure to TCDD. The up-regulation of *nrf2a* is similar to results obtained in mammalian systems, in which AHR regulated NRF2 expression in mouse cells (Miao *et al.*, 2005). However, Hahn *et al.* (2015) did not find acute induction of *nrf2a* following a 6-h TCDD exposure in 24 hpf zebrafish with immediate sampling nor did a study that exposed 4 hpf embryos for 1-h to 1 nM TCDD with sampling at 24 hpf (Alexeyenko *et al.*, 2010). Disparities in *nrf2a* gene expression patterns between the studies are likely due to differences in exposure (time and concentration) and collection regimes. Induction results may also vary by embryonic stage. Timme-Laragy *et al.* (2012) found that at 48 hpf *nrf2a* was not induced by TCDD as compared with DMSO. We also collected 48 hpf expression data for *nrf2a* following the protocol used in this study and in Timme-Laragy *et al.* (2012) and obtained results similar to those of Timme-Laragy *et al.* (Supplementary Figure 1), whereas Hahn *et al.* (2015) and Alexeyenko *et al.* (2010) found induction of *nrf2a* at 48hpf. Again, the differences in

experimental design may explain the different results of these studies. Thus, it is imperative to follow the same exposure regime in order to compare gene expression profiles between experiments. Furthermore, these additional data point to temporal differences in gene expression patterns that are worthy of future experiments, which may include the use of qPCR, RNA-sequencing, whole mount *in situ* hybridization, and ChIP.

The AHR-dependent up-regulation of *nrf1b* and *nfe2* in our study is a novel finding that has not previously been observed in mammalian systems. This result suggests that the regulation of genes involved in redox signaling and glutathione synthesis after AHR activation may not be carried out solely by Nrf2a. One possibility, which remains to be tested, is that AHR1b up-regulates *nrf* genes and then physically interacts or otherwise cooperates with their protein products to drive the transcription of shared antioxidant targets that contain XREs and AREs, such as *nqo1* and *gsta1* (Rousseau *et al.*, 2015; Wang *et al.*, 2013).

An alternative idea is that TCDD may not be causing oxidative stress, as suggested by several studies (Alexeyenko *et al.*, 2010; Wang *et al.*, 2013). The AHR1b-dependent induction of *nrf*s may regulate additional downstream targets that are distinct from those involved in antioxidant defense. The identity of genes that are regulated through AHR1b-NRF crosstalk could be ascertained through a RNA-seq experiment following knockdown or knockout of *ahr1b* and *nrf* genes.

Although much of the research on AHR has centered around exogenous ligands, AHR is also constitutively active in the absence of such compounds (Chang and Puga, 1998). We showed that the basal expression of *nrf1a* is AHR1b dependent (Figure 3A) but that *nrf1a* is not inducible by TCDD. To date, there are no known targets of *nrf1a*, although its ortholog in mammals is critical to both regulating genes involved in the oxidative stress response as well as basal functions like proteostasis and metabolism (Kim *et al.*, 2016). Further determination of *nrf1* paralog targets in zebrafish may elucidate the importance of this crosstalk during development.

Gene expression data reported in this study relied on the use of MO technology. This technology has been shown to be effective at temporally knocking down the expression of protein in developing zebrafish (Corey and Abrams, 2001), and in particular AHR2 (Massarsky *et al.*, 2016; Mathew *et al.*, 2006; Prasch *et al.*, 2003). Although morpholinos can induce off-target effects (Bedell *et al.*, 2011), their use avoids genetic compensation, which can occur in null mutants (Rossi *et al.*, 2015). This is especially powerful when dealing with paralogous genes that have arisen through genome duplication, such as the AHR family (Hahn, 2002), which may have similar biological functions including promoter binding. However, future experiments should consider using an *ahr1b* germ line knockout (Karchner *et al.*, 2017; Sugden *et al.*, 2017) as important differences have been reported between mutants and morphants in zebrafish (Kok *et al.*, 2015).

Although it is important to understand the cellular pathways that may be affected by AHR1b-NRF crosstalk, this study is also the first to demonstrate binding specificity of the AHR1b protein to *cis*-promoter elements *in vivo*. In the presence of its binding partner ARNT, AHR is known to bind to the DRE consensus sequences 5'TNGCGTG-3' (Li *et al.*, 2014; Lusska *et al.*, 1993). In cell culture, AHR1b/ARNT2b has been shown to drive the expression of luciferase (pGudLuc6.1) in vehicle control conditions and upon exposure to TCDD (Karchner *et al.*, 2005). In this plasmid, the expression of luciferase is under the control of four 5'GCGTG-3′ DRE core sequences (Han *et al.*, 2004). In this study, those genes whose expression was altered by the knockdown of AHR1b, namely *nrf1a*, *nrf1b*, *nrf2a*, and *nfe2* (Figs. 3A–C and E), directly bound AHR1b to XRE sequences (Figs. 5A–C and E). The expression of *nrf1a* via AHR1b is most dependent upon XRE2 with a core sequence of GCGTG. This core sequence has been shown to bind AHR efficiently in both murine and zebrafish assays (Li *et al.*, 2014; ZeRuth *et al.*, 2007). Interestingly, however, the flanking sequence around XRE2 (GGGG**GCGTG**TCTGC) more closely matches zebrafish *cyp1a* XRE 1, 3, and 6 at position 4 (T vs the consensus nucleotides C/A), and XRE 1, 2, and 6 at position 6 (T vs the consensus nucleotides A/C) (ZeRuth *et al.*, 2007), all of which do not have *in vivo* activity. It does, however, match the consensus sequence at position 8, which was hypothesized to be an important residue in AHR binding (ZeRuth *et al.*, 2007). The difference in binding could be due to differences between AHR/AHR2 and AHR1b.

With respect to *nrf1b*, there was a single XRE (TTA**GCGTG**CCGAGT) that closely matched the XRE consensus sequence across species (Li *et al.*, 2014; Lusska *et al.*, 1993; ZeRuth *et al.*, 2007). This sequence bound AHR1b under both DMSO and TCDD treatment (Figure 5B). In cell culture, a single murine XRE of this same core sequence has been shown to mediate the activation of transcription (Li *et al.*, 2014). Combined with our data, this finding points to AHR orthologs and paralogs being able to regulate transcription with a single XRE. Multiple XREs were shown to bind AHR1b in the *cis*-promoter of *nrf2a*, with the strongest binding in XRE1 (GC**GCGTG**CAGACG) and XRE4 (GC**GCGTG**CTATTA), with the greatest occupancy on XRE4, the more proximal element to the transcriptional start site (Figs. 1 and 5C). In the *cyp1a* promoter region, positions 4, 5, 6, and 8 were important for *in vivo* activity; for *nrf2a* these XREs had conserved residues at position 4 with the consensus sequence were not conserved at position 5, and XRE4 was conserved at position 6 and XRE1 at position 8. A mutation study similar to that of ZeRuth and Pollenz (2007) for the *nrf2a* promoter would elucidate the importance of particular residues and their positions for activation of transcription by AHR1b *in vivo*.

The *cis*-promoter of *nfe2* also bound AHR1b in 24 hpf embryos (Figure 5E). Of those XREs (Figure 1), XRE1 for *nfe2* (CACGCCA) had the strongest binding of AHR1b as compared with XRE2 (CACGCTC). In the murine *cyp1a1* promoter, where the consensus sequence is CACGCNA, T or C residues at the "N" position increase transcriptional efficiency (Li *et al.*, 2014); *nfe2* XREs both have a T or C at this "Nth" position but XRE2 deviates from the consensus sequence where it has a C rather than an A at the next position which may explain the difference in promoter occupancy.

It is unclear why XREs in *nrf2b* and *nrf3* do not bind AHR1b at 24 hpf. One possibility is that there may be temporal control of binding during development. Extending the ChIP assay to other time points would reveal whether AHR1b binds similarly to *nrf* genes during different developmental stages. Since *nrf2b* is transcriptionally activated by TCDD at 48 hpf (Timme-Laragy *et al.*, 2012), it is possible that AHR1b is involved in regulation at this point in development, where it is not at 24 hpf.

This study is the first to show both direct binding of AHR1b to promoters *in vivo* and provide evidence of AHR1b-NRFcrosstalk during development. It is also the first evidence for AHR crosstalk with Nfe2, Nrf1, and Nrf2, extending the previously identified AHR-NRF2 interaction to other NRF family members. Future studies should focus on determining cell- or tissue-specific effects of AHR1b-*nrf* interactions. Since the whole embryo was used to determine changes in gene expression and AHR1b binding, we may have missed cell-to-cell

variation, especially since the majority of AHR1b expression is in the eye (Karchner *et al.*, 2017; Sugden *et al.*, 2017). Methods that tag single-cell types such as the translating ribosome affinity purification approach (TRAP) (Doyle *et al.*, 2008; Heiman *et al.*, 2008; Tryon *et al.*, 2013) or laser capture microdissection (Bonner *et al.*, 1997) may be able to isolate enough embryonic material for gene expression and binding analyses. In order to determine the role of AHR1b as compared with AHR2, the AHR form most responsible in mediating the developmental toxicity of TCDD in zebrafish (Antkiewicz *et al.*, 2006; Dong *et al.*, 2003; Jönsson *et al.*, 2007; Prasch *et al.*, 2003), *nrf* gene expression could be ascertained in AHR2 knockouts as well as in AHR1b/AHR2 double knockouts (Chlebowski *et al.*, 2017; Sugden *et al.*, 2017). In order to complete ChIP assays, though, an effective AHR2 antibody must first be generated. Furthermore, by expanding this work to understand the downstream effects of this transcriptional regulation, the importance of AHR-NRF crosstalk will be better understood.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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