Nrf2b, Novel Zebrafish Paralog of Oxidant-responsive Transcription Factor NF-E2-related Factor 2 (NRF2)**

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**This work was supported, in whole or in part, by National Institutes of Health grants F32ES017585 (to A. R. T.-L.), R01ES017044 (to M. J. J.), R01ES015912 (to J. V. G.), and R01ES016366 and R01ES006272 (to M. E. H.). This work was also supported by a Woods Hole Oceanographic Institution postdoctoral scholar award and by Walter A. and Hope Noyes Smith.

Background: NRF2 is a transcription factor that regulates the oxidative stress response.

Results: Zebrafish have duplicate nrf2 genes, nrf2a and nrf2b, with distinct functions during embryonic development.

Conclusion: nrf2a and nrf2b have undergone subfunction partitioning; Nrf2b is a negative regulator of embryonic gene expression.

Significance: Duplicate zebrafish nrf2 genes provide opportunities for new insights into developmental roles of NRF2.

NF-E2-related factor 2 (NRF2; also called NFE2L2) and related NRF family members regulate antioxidant defenses by activating gene expression via antioxidant response elements (AREs), but their roles in embryonic development are not well understood. We report here that zebrafish (Danio rerio), an important developmental model species, possesses six nrf genes, including duplicated nrf1 and nrf2 genes. We cloned a novel zebrafish nrf2 paralog, nrf2b. The predicted Nrf2b protein sequence shares several domains with the original Nrf2 (now Nrf2a) but lacks the Neh4 transactivation domain. Zebrafish-human comparisons demonstrate conserved synteny involving nrf2 and hox genes, indicating that nrf2a and nrf2b are co-orthologs of human NRF2. nrf2a and nrf2b displayed distinct patterns of expression during embryonic development; nrf2b was more highly expressed at all stages. Embryos in which Nrf2a expression had been knocked down with morpholino oligonucleotides were more sensitive to tert-butythylhydroperoxide but not tert-butythylhydroquinone, whereas knockdown of Nrf2b did not affect sensitivity of embryos to either chemical. Gene expression profiling by microarray identified a specific role for Nrf2b as a negative regulator of several genes, including p53, cyclin G1, and heme oxygenase 1, in embryos. Nrf2a and Nrf2b exhibited different mechanisms of cross-talk with the Ahr2 signaling pathway. Together, these results demonstrate distinct roles for nrf2a and nrf2b, consistent with subfunction partitioning, and identify a novel negative regulatory role for Nrf2b during development. The identification of zebrafish nrf2 co-orthologs will facilitate new understanding of the multiple roles of NRF2 in protecting vertebrate embryos from oxidative damage.

Nuclear factor erythroid 2 (NF-E2)/p45-related factor 2 (NRF2; also called NFE2L2), a member of the cap’n’collar (CNC)-basic-leucine zipper (bZIP) protein family, plays an important role in the regulation of antioxidant genes and Phase II metabolism in vertebrates. This transcription factor, which activates gene transcription through its interactions with antioxidant/electrophile response elements (ARE/EpRe), is a key regulator of the oxidative stress response, influencing numerous biological processes, such as aerobic respiration, embryonic development, inflammation, and carcinogenesis (1, 2).

Under normal conditions, NRF2 is maintained in the cytoplasm by a repressor protein, Kelch-like ECH-associated protein (KEAP1), which targets it for ubiquitination by the 26 S proteasome. KEAP1 contains redox-sensitive cysteines that release NRF2 in the presence of redox imbalances or oxidative stress (3). NRF2 then translocates to the nucleus, where it dimerizes with small MAF proteins to activate ARE-regulated genes.

††1 This article contains supplemental Tables S1–S3 and Figs. S1 and S2.
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Nrf2b, Novel NRF2 Paralog in Zebrafish

The NRF2 protein consists of six Neh domains, originally assigned based upon regions identified as homologous between cross-species orthologues (4). Within each domain, particular features have been identified that contribute to either the transactivation or stability of the protein. Neh1 contains the DNA binding domain and serves to heterodimerize with small MAF proteins. Transactivation activities are promoted by Neh3, Neh4, and Neh5. Degrons are located in Neh6 and the KEAP1 binding domain in Neh2.

NRF2 can also participate in cross-talk with the aryl hydrocarbon receptor (AHR) pathway. The AHR is a bHLH/PAS (basic helix-loop-helix/Per-Arnt-Sim) family transcription factor that, upon ligand binding in the cytoplasm, translocates to the nucleus, where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). This dimer then recognizes xenobiotic response elements (XREs) in the promoter regions of numerous genes, such as the CYP1 family of xenobiotic-metabolizing enzymes. In the mouse, Nrf2 expression can be regulated by the AHR via three functional XREs in the promoter and first intron of Nrf2 (5). Yeager et al. (6) described a “TCDD-inducible Nrf2 gene battery,” demonstrating that, in adult mice, NRF2 is required for up-regulation of some Phase II genes that are classically thought of as part of the AHR battery of genes. NRF2 also plays a role in sustaining basal levels of AHR in mouse liver, and knock-out of Nrf2 resulted in lower expression and activity of numerous Phase I, II, and III drug-metabolizing enzymes and multidrug transporters (7).

The zebrafish is an important vertebrate model for studying developmental toxicity, with implications for understanding human embryonic development and teratogenesis (8). An advantage of the zebrafish model is that it often contains duplicate copies of genes that are present as only single copies in mammals, thus allowing for additional insight into the multiple functions of the human counterpart (9). With this in mind, we sought to characterize the oxidative stress response in zebrafish embryos and the role of zebrafish homologs of genes in the Nrf2 gene family. Previous studies carried out in zebrafish or zebrafish cells have established the evolutionary conservation of the response to oxidative stress, including the roles of Nrf2 (10–12), Keap1 (10, 11, 13), and AREs (14–17). Because of a lack of the response to oxidative stress, including the roles of Nrf2 and Keap1 (10, 11, 13), and AREs (14–17). Because of a whole-genome duplication that occurred after the divergence of the fish and mammalian lineages, teleost fish often possess whole-genome duplication that occurred after the divergence (10–12), Keap1 (10, 11, 13), and AREs (14–17). Because of a whole-genome duplication that occurred after the divergence of the fish and mammalian lineages, teleost fish often possess.

We demonstrate that nrf2a and nrf2b5 have undergone subfunction partitioning and provide evidence that the Nrf2b protein functions as a repressor to regulate constitutive gene expression during embryonic development.

EXPERIMENTAL PROCEDURES

Fish Husbandry—Zebrafish (Danio rerio) of the Tupfel/Long fin mutation (TL) wild-type strain were used in all experiments. Fish were maintained, and embryos were collected under standard light and temperature conditions as described previously (19). All procedures were approved by the Woods Hole Oceanographic Institution Animal Care and Use Committee.

Chemicals—t-Butylhydroquinone (tBHQ), tert-butylhydroperoxide (tBOOH), and dimethyl sulfoxide (DMSO) were obtained from Acros Organics (Geel, Belgium). 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were from Ultra Scientific (Hope, RI).

Gene Searches and Phylogenetic Analysis—Zebrafish NRF homologs were identified by using mammalian NRF2 protein sequences to search draft assemblies of the zebrafish genome (see the Zebrafish Sequencing Project Web site) using BLAST. Sequences of NF-E2-related transcripts were obtained from GenBank™ (predicted Nrf1a, Nrf1b, and Nrf3) or determined by cDNA cloning and sequencing (Nrf2b; see below). Multiple sequence alignments of the deduced proteins were performed using ClustalX (20) and Muscle (version 3.8.31 (21)). After masking regions of uncertain alignment, the aligned amino acid sequences were used to construct phylogenetic trees using maximum parsimony and minimum evolution (distance) criteria in PAUP4.0b8 (22) or the maximum likelihood criterion with RAxML (version 7.2.6 (23)) using the PROTCATWAG model of amino acid substitution followed by likelihood calculations using the GAMMA model. Bootstrap analysis with 100 or 1000 resamplings was used to assess confidence in individual nodes. Trees were rooted using the Drosophila CNC_C protein. Additional details can be found in the figure legends.

Expression of nrf Genes in Embryos—To determine whether all of these nrf genes are expressed in embryos, PCR primers were designed based on the predicted sequences (see supplemental Table S1). Samples at 24 and 48 h postfertilization (hpf) were pools of 10 embryos each, and four embryos were pooled for the 96 hpf time point. Total RNA was isolated using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX). Poly(A)+ RNA was purified using the MicroPoly(A)Purist kit (Ambion). cDNA was synthesized from 2 μg of total RNA using Omniscript reverse transcriptase (Qiagen, Valencia, CA). PCR was performed using AmpliTaq Gold polymerase (Applied Biosystems, Carlsbad, CA), with a PCR cycle of 94 °C for 10 min followed by 35 cycles of 94 °C for 15 s, 60 or 65 °C for 30 s, and 72 °C for 30 s, followed by 7 min at 72 °C. Products were visualized with gel electrophoresis.

cDNA Cloning—The full-length cDNA for nrf2b was obtained using 5′ and 3′ RACE PCR. The Marathon cDNA amplification kit (Clontech) was used to generate double-stranded cDNA from 1 μg of poly(A)+ RNA from pooled zebrafish liver. Adaptors were ligated to both ends of the cDNAs as per the manufacturer’s instructions. Nested genespecific primers were designed and used with adaptor primers.

5The designation of duplicated genes (and their encoded proteins) as “a” (as in nrf2a) or “b” (nrf2b) is according to the approved zebrafish nomenclature for duplicates resulting from the fish-specific whole-genome duplication (see the ZFIN Zebrafish Nomenclature Web site).
(AP1 and AP2) provided with the Marathon kit. The 5’ RACE used 5’/GCGAAGTGTAGCTAGACTCTCC-3’ in combination with AP1 and 5’/AAGCAGGAGGCAAGACAG-AGG-3’ in combination with AP2; the 3’ RACE used 5’/CCTC-ACGTGTTACCCAGATCCCTC-3’ in combination with AP1 and then 5’/TCACCTGTACCAGAATCCCTTG-3’ in combination with AP2, and the PCR programs were as instructed by the manufacturer. The products were cloned into the pGEM T-easy vector (Promega, Madison, WI), plasmids were isolated (PureYield Plasmid Miniprep System, Promega), restriction digest was performed, and products were sequenced (MWG Operon, Huntsville, AL).

After obtaining the full-length sequence with RACE, we then amplified the full-length cDNA with forward primer 5’/AGCTGGAGACATGACG-3’ and reverse primer 5’/ACAGCAACATTTAATCCTCG-3’, using the proofreading Pfu Ultra II Fusion HA DNA polymerase (Agilent Technologies, Santa Clara, CA). The PCR cycle was 95 °C for 1 min and then 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 50 s for 38 cycles, followed by 3 min at 72 °C. The PCR product was cloned into the pENTR/D-TOPO vector, and then the insert was transferred into pCS2, or 1 μg of plasmid DNA using X-tremeGENE HP DNA transfection reagent (Roche Applied Science) as per the manufacturer’s instructions. Cells were treated 24 h after transfection with water or 100 μM tBOOH for 1 h and fixed with 4% paraformaldehyde (28). To stain nuclei, coverslips were washed with PBS, and cell membranes were permeabilized with 0.25% Triton for 10 min. Cells were washed in PBS again, incubated with RNase and propidium iodide (1 μg/ml) for 30 min, and then washed with PBS. Slides were imaged immediately using a Zeiss Axio Imager.Z2 with Axiovision software (Carl Zeiss). Ten representative fields were imaged at ×200 for each condition. The number and intensity of GFP-positive pixels that either overlapped with propidium iodide-positive pixels (nucleus) or did not overlap (cytoplasmic localization) were quantified using Axiovision co-localization software (Carl Zeiss) with access provided by the Marine Biological Laboratory (Woods Hole, MA). Each field of cells was corrected for background fluorescence and exposure time. To provide an independent measure of subcellular localization, we also conducted a blind cell count that categorized up to 10 cells/field (61–93 cells/group) according to the localization of GFP expression within each cell: predominantly in the nucleus, in both nucleus and cytoplasm, or predominantly in the cytoplasm (no cells were found in the latter category).

**Bioinformatics**—Promoter analysis for putative XREs and AREs was conducted using the JASPAR Core vertebrate data set (24). Using the position weight matrix algorithm for NFE2L2 (MA0150.1) for AREs and the ARNT:AHR (MA0006.1) algorithm for XREs, we searched 3000 bp upstream of the Nrf2a translation start site and the entire first intron and 1712 bp upstream of the Nrf2b translation start site and its first intron. A relative score of 0.80 was accepted for AREs, and 0.90 was accepted for the XREs. Potential sites were then manually evaluated, and sequences that did not contain bases known to be essential for binding activity were excluded. A manual search for AREs was also conducted to include a previously described functional zebrafish ARE variant not identified by the position weight matrix (TGA(G/C)nnnTC) (16), here designated ARE_{Tn}. CpG islands were identified using CpG Island Searcher (25). Sequence logos were generated using WebLogo 3 (26).

To compare the protein structure and important motif features of Nrf2b, a ClustalW multiple alignment of mouse Nrf2, the three isoforms of the human NRF2 (which differ by use of an alternate promoter and also an alternate splice site), chicken ECH, and the two zebrafish Nrf2s was conducted using the BioEdit program (27). Percentage identity between proteins and within the Neh regions was calculated using BioEdit’s Sequence Identity Matrix.

**GFP-Nrf2 Fusion Constructs**—Nrf2a and Nrf2b cDNAs were amplified with the primer pairs Nrf2a-GFP/Nrf2a-GFPR and Nrf2b-GFP/Nrf2b-GFPR (supplemental Table S1), respectively, using Pfu Ultra II DNA Polymerase (Agilent Technologies). The pCS2-nrf2a (a generous gift from Dr. Makoto Kobayashi (10)) and pENTR-Nrf2b plasmids were used as templates for the PCR. The cycling condition was 95 °C for 1 min and then 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 90 s for 20 cycles, followed by 10 min at 72 °C. The PCR products were purified and A-tailed prior to ligation into the pcDNA3.1/NT-GFP-TOPO (Invitrogen). Constructs were sequenced, and expression was confirmed by the TNT Quick Coupled Reticulocyte Lysate System (Promega) with [35S]methionine.

**Transient Transfections and Co-localization Studies**—COS-7 monkey kidney cells (ATCC, Manassas, VA) were plated on coverslips in 6-well plates as described previously (28) and transfected with 3 μg of plasmid DNA using X-tremeGENE HP DNA transfection reagent (Roche Applied Science) as per the manufacturer’s instructions. Cells were treated 24 h after transfection with water or 100 μM tBOOH for 1 h and fixed with 4% paraformaldehyde (28). To stain nuclei, coverslips were washed with PBS, and cell membranes were permeabilized with 0.25% Triton for 10 min. Cells were washed in PBS again, incubated with RNase and propidium iodide (1 μg/ml) for 30 min, and then washed with PBS. Slides were imaged immediately using a Zeiss Axio Imager.Z2 with Axiovision software (Carl Zeiss). Ten representative fields were imaged at ×200 for each condition. The number and intensity of GFP-positive pixels that either overlapped with propidium iodide-positive pixels (nucleus) or did not overlap (cytoplasmic localization) were quantified using Axiovision co-localization software (Carl Zeiss) with access provided by the Marine Biological Laboratory (Woods Hole, MA). Each field of cells was corrected for background fluorescence and exposure time. To provide an independent measure of subcellular localization, we also conducted a blind cell count that categorized up to 10 cells/field (61–93 cells/group) according to the localization of GFP expression within each cell: predominantly in the nucleus, in both nucleus and cytoplasm, or predominantly in the cytoplasm (no cells were found in the latter category).

**Morpholino Antisense Oligonucleotides (MOs)**—MOs (Gene Tools, LLC, Philomath, OR) were targeted to knockdown both maternally loaded and embryonic mRNAs by inhibiting translation at the ATG start site. The Nrf2a-MO was previously described (5’-CATTTCACATCTCCATGTCTCAG-3’) (10). The sequence for the Nrf2b-MO was 5’-AGCTGAAAGGTCGTTAGCTGTCGATCGTGTC-3’. The zebrafish Ahr2-MO was also previously described (5’-TGACGGACAGACCAATTTA-3’) (29). The standard control-MO from Gene Tools was also used (5’-CTCTTACCTCATGTTACATATATATA-3’). For the Nrf2a+MO combination injection, the MOs were mixed in an injection solution containing 0.1 mM Nrf2-MO and 0.1 mM Nrf2b-MO and matched with a control-MO concentration of 0.2 mM. All MOs were tagged with a 3’-end carboxyfluorescein modification in order to monitor injection success.

To confirm that the MOs were able to prevent protein synthesis in a specific manner, we used the TNT Quick Coupled Reticulocyte Lysate System (Promega) for in vitro target protein transcription and translation. [35S]Methionine-labeled zebrafish Nrf2a and Nrf2b proteins were synthesized as per the manufacturer’s protocols. TNT reagents were combined with 1 μl of [35S]methionine (>1000 Ci/mmol at 10 μCi/ml), 1 μg of nrf2a in pCS2, or 1 μg of nrf2b in pcDNA 3.2/V5-DEST. To test the efficacy of the target MOs, the standard control-MO, gene-specific MO, or an MO specific for the paralogous gene was...
Nrf2b, Novel NRF2 Paralog in Zebrafish

added to the reaction for a final concentration of 500 nM. Mixtures were incubated at 30 °C for 90 min. The labeled proteins were resolved by gel electrophoresis, dried onto Whatman filter paper, and visualized on film. Densitometric analysis was performed with AlphaView (AlphaInnotech/Cell Biosciences, Santa Clara, CA). The relative densitometric units were determined by normalizing the target MO treatments to the control-MO treatments after all densitometric values were adjusted for local background and band size.

Microinjection of MOs and Embryo Chemical Exposures—Embryos at the 1–4-cell stage were injected with 3–5 nl of Nrf2a-MO, Nrf2b-MO, control-MO, or Nrf2a-MO+Nrf2b-MO using a Narishige IM-300 Microinjector (Tokyo, Japan). Only healthy embryos exhibiting strong, uniform distribution of the fluorescent MO at 24 hpf were used in experiments.

MO-injected or non-injected embryos were exposed to 2 μM tBHQ or DMSO (0.02%) for 4 h starting at 48 or 72 hpf in triplicate glass scintillation vials containing five embryos in 5 ml of 0.3× Danieau’s solution. Following exposure, embryos were immediately placed in RNA later and stored at −80 °C until total RNA isolation and gene expression analysis. Exposures to tBOOH were conducted in 96-well plastic dishes (Corning Glass) in 200 μl of 0.3× Danieau’s solution with one fish per well. At 48 hpf, embryos were exposed to tBOOH (0, 0.5, 0.75, 1, 1.5, and 2 mM); exposure solutions were renewed at 72 hpf. Cumulative mortality and survival were assessed at 96 hpf. Non-injected controls were included in at least one row on every plate to control for positional and plate effects.

TCDD exposure of embryos microinjected with control-MO or Ahr2-MO as well as non-injected embryos was conducted as described previously (30). Briefly, embryos were exposed to either 0.1% DMSO or 2 nm TCDD for 1 h at 6 hpf. After exposure, the embryos were washed and placed in Petri dishes with 25 ml of fresh 0.3× Danieau’s solution and maintained at 28 °C with a 14-h light/10-h dark cycle. Three biological replicates of 20 pooled embryos were collected for each treatment at 48 hpf. Embryos were flash frozen in liquid nitrogen and stored at −80 °C until total RNA isolation and analysis of gene expression.

Adult TL zebrafish (~12 months in age) were separated by sex and maintained in large glass beakers at 4 fish/liter of zebrafish system water with constant aeration. The zebrafish were euthanized by decapitation, and the organs (liver, gill, gut, kidney, ovary, testes, heart, brain, and eye) were removed by dissection. Three replicates per exposure were collected, resulting in four males or four females pooled per replicate for each organ. The dissected organs were placed in RNA later and stored at −80 °C until RNA isolation and analysis of gene expression.

Sampling, RNA Extraction, and cDNA Synthesis—For the developmental series, four pools of 30 carefully staged embryos from a single clutch kept at low density at 28.5 °C were flash frozen in liquid nitrogen at 6, 12, 24, 48, 60, 72, 96, and 120 hpf. At the 48 and 60 hpf time points, hatched and unhatched embryos were collected and analyzed separately. Eggs for the 0 hpf time point were manually stripped from three females and combined.

Isolation of RNA from embryonic and adult tissues was conducted using RNA STAT-60 according to the manufacturer’s instructions. Two female liver DMSO samples did not meet RNA quality standards, and these were excluded. Samples from the MO-injected embryo experiments contained much lower starting tissue amounts, and RNA was isolated according to a modified protocol using the RNeasy microkit (Qiagen) (31). cDNAs were synthesized from 2 μg of total RNA (embryo development series and adult tissues) or 500 ng of total RNA (morphant embryo samples), using random hexamers and the Omniscript cDNA synthesis kit (Qiagen).

Measurement of Gene Expression by Quantitative Real-time RT-PCR (QPCR)—QPCR was performed using iQ SYBR Green Supermix (Bio-Rad) in a MyiQ single-color real-time PCR detection system (Bio-Rad). Each reaction was run in duplicate wells containing cDNA from 5 ng of RNA for embryos, and 40 ng of RNA for adult tissue samples. Primers and extension temperatures are provided in supplemental Table S1. The PCR conditions used were 95 °C for 3.5 min followed by 35–40 cycles of 95 °C for 15 s and 25 s at the gene-specific temperature (see supplemental Table S1). Each run included melt curve analysis to ensure that only a single product was amplified, as well as a no-template control. All primers were tested for amplification efficacy (100 ± 10%). In addition, standard curves of serially diluted plasmids containing a full-length copy of each gene were used for β-actin, nrf2a, and nrf2b. Housekeeping genes were selected to be most appropriate for both embryonic development with chemical exposure (β-actin) and tissue differences with chemical exposure (ef1α) (32). Total molecule numbers were calculated and normalized by the housekeeping gene correction factor. Other genes were analyzed using the comparative ΔΔCt method (33).

Statistical Analyses of QPCR Data—Data were analyzed with Statview for Windows (version 5.0.1; SAS Institute, Cary, NC) and BioStat 2009 (AnalystSoft, Inc.). Data were log-normalized for statistical analysis, and six statistical outliers were removed from the nrf2b development series (one data point from each of six time points: 0, 6, 24, 48 (unhatched), 48 (hatched), and 96 hpf). When ANOVA yielded significance (p < 0.05), Fisher’s protected least significant differences test was used as a post hoc test with Bonferroni correction as noted in the figure legends. Data are presented as mean ± S.E., and n is defined as the number of pools of embryos or pools of tissues from four individuals as specified in the figure legends. Survival data were analyzed using Probit analysis. Following statistical analysis, non-injected and control-MO-injected embryos were combined for graphical simplicity.

Gene Expression Profiling—RNA from the embryos injected with Nrf2a-MO, Nrf2b-MO, control-MO, or Nrf2a-MO+Nrf2b-MO and treated with tBHQ or DMSO for 4 h at 48 hpf (described above) was used for gene expression profiling. The RNA samples (n = 3 biological replicates per treatment/MO combination) were labeled with Cy3 and hybridized to the Agilent V3 4 × 44K zebrafish microarray (catalog no. G2519F-026437) at the Genome Technology Core of the Whitehead
Institute (Cambridge, MA) using methods described in detail previously (34).

Raw array data obtained from the Whitehead Institute were analyzed essentially as described by Goldstone et al. (34). Briefly, data were extracted using Agilent’s feature extraction software using background detrending (spatial and multiplicative). Prior to normalization, Cy3 values below 5 were set to 5. The data were then normalized using the non-linear scaling method based on rank invariant probes, as described (34). After normalization but before statistical analyses, probes not significantly above background in all microarrays were removed (3147 probes in all; based on Agilent’s 2.6 S.D. method). None of these probes were saturated for Cy3 signal on any microarray, so (3147 probes in all; based on Agilent’s 2.6 S.D. method). None of these probes were saturated for Cy3 signal on any microarray, so no further filtering was applied. There were a total of 40,456 probes for statistical analyses.

Statistical tests were performed using MeV version 4.3 (35). Data were log-transformed and median-centered for each probe. A two-factor ANOVA was run for morpholino, compound, and their interaction with p value based on 1000 permutations of the data and a of 0.01. The probes found significant in the two-factor ANOVA were subsequently examined using rank product analysis (36) to identify probes up- and down-regulated by the MO injection (i.e. DMSO-treated only), probes affected by BHQ treatment, and the effect of MO injection on transcriptional response to BHQ. For each rank product test, a two-class unpaired rank product analysis was performed using 100 permutations of the data with a false discovery rate of ≤10%. Only the data from DMSO-treated embryos are presented here; a more complete analysis will be published separately. Microarray data have been deposited in the Gene Expression Omnibus (GEO) database (GEO GSE32594).

**In Vivo Reporter Gene Expression—**Plasmid DNAs were linearized by digesting the plasmids with XhoI (nrf2a) or Apal (nrf2b). Capped mRNA was synthesized using the mMessage mMACHINE Ultra kit (Ambion), as per the manufacturer’s instructions. mRNA (100 pg) was injected into the blastomere of early one-cell stage embryos along with 50 ng of the pT3.5gstp1GFP reporter construct (a generous gift from Dr. M. Kobayashi), which contains the ARE-rich promoter of the pT3.5gstp1GFP reporter construct (a generous gift from Dr. M. Kobayashi), which contains the ARE-rich promoter of the zebrafish gstp1 gene fused to a GFP reporter (16, 37). Embryos were imaged at shield stage (6–7 hpf) using a Zeiss Axioscope and GFP filter set. All images were collected using a 700-ms exposure.

**RESULTS**

**Identification of NF-E2-related Factors in Zebrafish—**We sought initially to assess the diversity of the NF-E2-related CNC-bZIP family in zebrafish as compared with the four members found in humans: NF-E2, NRF1 (NFE2L1), NRF2 (NFE2L2), and NRF3 (NFE2L3). Single zebrafish homologs of NF-E2 and NRF2 have been described previously (10, 38). In examining the zebrafish genome and predicted protein set, we identified two predicted zebrafish homologs of mammalian NRF1, a predicted NRF3 ortholog, and a second predicted NRF2 form. The NRF1 homologs were noted earlier from expressed sequence tag data (39). The new predicted Nrf2 (XM_001344745.1) was supported by two expressed sequence tag sequences (BQ133267.1 and BI326455.1). Based on phylogenetic and comparative genomic analyses (see below), the new nrf2 gene has been named nrf2b, and the originally identified zebrafish nrf2 has been designated nrf2a. Thus, there are at least six zebrafish CNC-bZIP genes in zebrafish: nfe2, nrf1a, nrf1b, nrf2a, nrf2b, and nrf3 (nomenclature issues are addressed further below and under “Discussion”). All of these genes are expressed in zebrafish embryos and early larvae (Fig. 1A).

Searches of the pufferfish fugu (Takifugu rubripes) genome (40) also revealed several NRF isoforms, suggesting that the presence of NRF duplicates is not unique to zebrafish. However, a second Nrf2 was not found in fugu.

**Phylogenetic Analysis and Comparative Genomics of Vertebrate NF-E2-related Proteins—**To better understand the relationships of zebrafish Nrf proteins to their mammalian homologs, we performed multiple phylogenetic analyses on the amino acid sequences of NF-E2-related proteins from humans, mice, zebrafish, and fugu. The zebrafish Nfe2 and Nrf3 sequences each grouped within a strongly supported clade containing their mouse, human, and fugu orthologs (Fig. 1B and supplemental Fig. S1). Zebrafish Nrf1a and Nrf1b were part of a clade containing mouse and human NRF1 proteins along with two Nrf1 paralogs from fugu. Zebrafish Nrf2a and Nrf2b both were part of a strongly supported clade containing fugu NRF2 and avian and mammalian NRF2 proteins, supporting the designation of Nrf2b as an Nrf2 paralog. However, Nrf2b was more divergent than Nrf2a, appearing basally in the NRF2 clade, a position inconsistent with an origin as part of the fish-specific genome duplication (Fig. 1B and supplemental Fig. S1). Although it is clear that zebrafish possess at least one ortholog of each of the four mammalian NRF-related CNC-bZIP transcription factors (see also supplemental Table S2), the precise relationships between zebrafish Nrf duplicates and their mammalian homologs were not fully resolved in these trees.

Shared synteny is known to be a powerful tool for assigning orthology (41–43). Therefore, we used information from comparative genomic mapping to gain additional insight into the relationship among fish and mammalian NRF genes. Each of the four human NRF genes is present as a single copy near one of the two hoxa11 clusters, which contains only a single copy of the human HOXD cluster (45). Zebrafish and other teleosts have additional clusters, including duplicated zebrafish Nrf duplicates and their mammalian homologs were not fully resolved in these trees.

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**CONCLUSIONS**

The zebrafish genome contains at least six CNC-bZIP genes, of which the two Nrf2 paralogs are likely derived from a fish-specific whole genome duplication. The situation in fugu is consistent with this model, but Zebrafish nrf2a and nrf2b both were part of a strongly supported clade containing fugu NRF2 and avian and mammalian NRF2 proteins, supporting the designation of Nrf2b as an Nrf2 paralog. However, Nrf2b was more divergent than Nrf2a, appearing basally in the NRF2 clade, a position inconsistent with an origin as part of the fish-specific genome duplication (Fig. 1B and supplemental Fig. S1). Although it is clear that zebrafish possess at least one ortholog of each of the four mammalian NRF-related CNC-bZIP transcription factors (see also supplemental Table S2), the precise relationships between zebrafish Nrf duplicates and their mammalian homologs were not fully resolved in these trees.
**nrf2a**, is found on chromosome 9 containing the single *hoxda* cluster, near the anterior *hox* genes; this is similar to the position of the human *NRF2* gene adjacent to the HOXD cluster. The second, novel zebrafish *nrf2* gene (*nrf2b*) occurs on chromosome 6, near *miR-10d2* and other *Hox*-associated genes (*atp5g, lnp*, and *mtx2*) in the region corresponding to the location of the degenerate *hoxdb* gene cluster (Fig. 1C). Overall, the genomic mapping data demonstrate extensive conserved synteny involving *nrf* and *hox* genes, providing strong support for the hypothesis that the zebrafish *nrf* genes are orthologs (*nfe2; nrf3*) or co-orthologs (*nrf1a and nrf1b; nrf2a and nrf2b*) of the corresponding human *NRF2* genes.

**nrf2b** cDNA Cloning, Gene Structure, and Putative Regulatory Motifs—Because the role of NRF2 is particularly widespread in mammals (46 – 49), we hypothesized that the novel *nrf2b* gene in zebrafish may hold important insights for understanding *NRF2* function in humans, particularly if the two zebrafish *nrf2* genes have undergone subfunction partitioning. We thus focused subsequent efforts on the characterization of the novel gene, *nrf2b*.

We used RACE PCR to obtain the sequence and RT-PCR to clone the full-length cDNA corresponding to the predicted *nrf2b* transcript. The cDNA sequence for exon 1 differed from that of the predicted transcript, possibly due to an apparent error in the genome assembly (Ensembl Zv8); no evidence for the predicted exon 1 was found in any of the multiple 5’-RACE products sequenced. The *nrf2b* cDNA and predicted protein sequences have been deposited in the GenBank™ database (accession numbers HQ661166 and ADX30690, respectively). *nrf2a* and *nrf2b* cDNA sequences share 46.5% sequence identity. Mapping of the cDNA sequences to the zebrafish genome showed that, although the *nrf2* paralogs have similar gene structures, *nrf2b* is lacking an exon corresponding to exon 3 of *nrf2a*, thus having only four exons rather than five (Fig. 2A and Table 1).

To examine whether subfunctionalization has occurred, we first assessed the conservation of regulatory elements. Based on the presence of functional AREs and XREs in the promoters and first introns of mammalian *NRF2* genes and target genes (5, 50, 51), we searched promoters and first introns of *nrf2a* and *nrf2b*...
genes for these motifs. For nrf2a, we identified 14 predicted AREs: one ARETC in the promoter (−91 from the ATG start site) and 13 (including two ARETC) in the first intron (Fig. 2B). For nrf2b, we found nine predicted ARE sites: two ARETC variants at −1399 and −1222 and seven additional ARE-like sequences (three ARETC) in the first intron (Fig. 2B). The sequence logos of the ARE-like sequences found in nrf2a and nrf2b were similar (Fig. 2C). The presence of AREs in the promoter and first introns suggests that the two nrf2 paralogs may exhibit auto- or cross-regulation.

We found 10 predicted XREs in nrf2a: one in the promoter (−198) and nine in intron 1 (Fig. 2B). In nrf2b, we found 11 predicted XREs: two in its promoter (−78 and −80) and nine in the first intron (Fig. 2B). The presence of XREs suggests that both zebrafish nrf2 genes could be regulated by one or more of the zebrafish AhR proteins.

Identification of CpG islands, important regulatory elements that play a role in epigenetic regulation of gene activity, has also been used to predict genomic areas proximal to functional transcription start sites (52). Both nrf2 promoters contain a single CpG island. The CpG island for nrf2a is located −302 to −97 bp upstream of the translation start site and contains 13 CpG sites in the 206-bp island. The CpG island in the nrf2b promoter is located −891 to −566 bp upstream of the translation start site and contains 18 CpG sites in the 326-bp island (Fig. 2B). Taken together, conservation of the regulatory elements AREs, XREs, and CpG islands between nrf2a and nrf2b provides evidence of conserved regulation of these two genes.

Conservation of Nrf2 Protein Features and Neh Domains—Amino acid sequence alignments show that Nrf2b and Nrf2a share 25.1% sequence identity overall (supplemental Table S3), with greater identity found in the conserved Neh (Nrf2 ECH homology) domains (Fig. 3A). The Neh domains were originally assigned based upon regions identified as highly conserved between orthologues (4). Within each domain, particular features have been identified that contribute to either the function or stability of the protein. The zebrafish Nrf2b retains all but one of the Neh domains, lacking the Neh4 transactivation domain (Fig. 3).

In comparing Neh domains of both of the zebrafish Nrf2s with those of the human NRF2, Neh1 is the most conserved, with Nrf2a and Nrf2b sharing 73 and 41% identity, respectively, in this region (Fig. 3A). Neh1 contains the CNC homology region and the basic-leucine zipper domains that allow NRF2 to heterodimerize with small MAF proteins and bind to DNA. Within the DNA binding domain, Nrf2b differs from the other Nrf2s by only two residues, one of which is not well conserved among other NRFS. This basic region contains a redox-sensitive cysteine (53) that is conserved. Neh1 contains several lysine residues that contribute to promoter-binding activity by serving as sites for acetylation by the transcription coactivator p300/CBP and may confer some gene promoter selectivity among AREs (54). The Neh1 domain of human NRF2 contains 18 lysines; Nrf2a has 16, and Nrf2b has 12. Neh1 also contains a nuclear localization signal that overlaps with the DNA binding domains and is well conserved, differing by the same two residues. A nuclear export signal found in the Neh1 domain of human NRF2 is not well conserved in either of the zebrafish Nrf2 proteins (Table 2).

The Neh2 domain at the N terminus contains the KEAP1 binding domain and plays an important role in regulating the activity and degradation of NRF2. KEAP1 binds NRF2 as a homodimer; the “hinge-latch” model identifies two KEAP1 recognition sites in Neh2, with differing affinities (55–57). The strongest KEAP1 recognition element, the ETGE motif (residues 79–82), is conserved in Nrf2b (Fig. 3B and Table 2). The second recognition site is a hydrophobic region toward the N terminus, which binds a redox-sensitive degron, the DIIDLID element (58). This element contains a weaker KEAP1 binding site, the DLG motif (59), that is subject to disruption by redox-related cysteine alterations on KEAP1, which release the “latch” of the homodimer. This binding site is also conserved in both Nrf2b and Nrf2a (Fig. 3B and Table 2). Between these two KEAP1 recognition sites are lysine residues that serve as ubiquitination sites. The mammalian NRF2 proteins have seven lysines in this region; the presence of any of these is sufficient for KEAP-1-mediated ubiquitination (60). None of these are conserved in Nrf2b, and only two are conserved in Nrf2a. However, Nrf2b does have two other lysines that could conceivably serve this function.

The Neh3 domain plays a dual role, influencing both protein stability and transactivation. The motif VFLVPK was found to be critical for transactivation activity through binding of CDH6, a chromodomain and DNA-helicase protein that is not well understood (61). Whereas Nrf2a is missing the last residue of this sequence, Nrf2b has only three of the six (Fig. 3B and Table 2). The role of Neh3 in NRF2 stability involves a nuclear export signal that targets the protein for FYN-mediated ubiquitination and degradation (62), a redox-responsive process (63). This nuclear export sequence is conserved in Nrf2b but not Nrf2a.

There are two other transactivation domains, Neh4 and Neh5, both of which have acidic characteristics. Neh4 is completely missing from Nrf2b, largely due to the loss of exon 3. The Neh5 domains of Nrf2a and Nrf2b both have five acidic residues, compared with eight in human NRF2. Both Neh4 and Neh5 can bind CBP and promote gene transcription. Neh5 binds BRG1 (Brahma-related gene 1), which facilitates activation of the heme oxygenase promoter (64). Zhang et al. (64) identified amino acids critical for Neh5-mediated activation of luciferase expression, termed the actin-related motif (D/E)(M/I/L)XXW. Although Nrf2a has 2 of 4 critical residues (SLDQAW), none of these residues is conserved in Nrf2b (Fig. 3B and Table 2).

Neh6 contains another degron that retains its ability to promote NRF2 degradation even under conditions of oxidative stress (when binding of KEAP1 to the Neh2 degron is weakened and NRF2 is released). Within Neh6, there are two regions that are highly conserved, corresponding to residues 329–339 and 363–379 of mouse NRF2 (58). This degron contains a group of serines used as a phosphorylation site(s) by GSK3β for SCF/β-TrCP-dependent degradation via CULLIN1 (65). Unlike Nrf2a, Nrf2b does not share many amino acids with the human NRF2 and mouse NRF2 sequences in this region (Fig. 3B and Table 2). Overall, comparative analysis of the predicted protein sequence
of Nrf2b suggests that some but not all NRF2 functions are likely to be conserved in this protein.

Localization of Nrf2a and Nrf2b under Control and Oxidative Stress Conditions—Mammalian forms of NRF2 have been shown to undergo nuclear translocation following oxidative stress (66, 67). Subcellular localization of the original zebrafish Nrf2 (Nrf2a) has not been investigated previously. To determine whether both zebrafish Nrf2 proteins maintain the ability to undergo nuclear translocation, we prepared fusion constructs GFP-Nrf2a and GFP-Nrf2b and conducted transient transfection experiments in COS-7 cells to measure the subcellular localization of the proteins under normal and oxidative stress conditions. All cells expressing GFP-Nrf2 exhibited green fluorescence in the nucleus. In a subset of cells transfected with each construct, GFP-Nrf2a fluorescence was shown to undergo nuclear translocation following oxidative stress (66, 67). Quantification of the digital images using an intensity-weighted co-localization coefficient showed that under both control and oxidative stress conditions (100 μM BOOH for 1 h), 60–80% of the GFP was located in the nucleus (Fig. 4A). This pattern of localization is similar to that seen for human NRF2 when expressed by transient transfection in COS-7 cells in the absence of KEAP1 or oxidant treatment (68). Quantification of the digital images using an intensity-weighted co-localization coefficient showed that under both control and oxidative stress conditions (100 μM BOOH for 1 h), 60–80% of the GFP was located in the nucleus (Fig. 4B). We also conducted a blind count of GFP-positive cells and categorized each cell according to the location of GFP fluorescence (predominantly in the nucleus or in both nucleus and cytoplasm; Fig. 4C). For both GFP-Nrf2a and GFP-Nrf2b, exposure to rBOOH caused a slight enhancement in the proportion of cells expressing GFP in the nucleus. Overall, the results demonstrate that, like mammalian NRF2, both Nrf2a and Nrf2b are capable of undergoing nuclear localization. A complete understanding of the regulation of this cellular localization and the role of Keap1 and chemical oxidants will require further study.

Differential Expression of nrf2a and nrf2b in Embryos and Adult Tissues—Patterns of gene expression may provide insight into distinct roles for nrf2a and nrf2b. We used QPCR to measure expression of nrf2a and nrf2b in unfertilized eggs (time 0) and developing embryos (6–120 hpf). Transcripts of both nrf2a and nrf2b were found in unfertilized eggs and decreased from these levels by 6 hpf (Fig. 5A). Expression of nrf2a was initially low but steadily increased through 120 hpf. nrf2b levels were higher and somewhat more variable and exhibited a significant difference between embryos in the hatched and unhatched state at 60 hpf (Fig. 5A). Most notably, expression of nrf2b was 10–100-fold greater than that of nrf2a at nearly every developmental stage (Fig. 5A).

We measured expression of nrf2a and nrf2b in adult tissues, including brain, eye, gill, gut, heart, kidney, liver, ovary, and testes from male and female adult zebrafish. For all tissues, expression of nrf2a was greater than that of nrf2b (Fig. 5B). The expression of each nrf2 gene varied among tissues. The tissue with the highest expression of nrf2a was the gill, followed by the eye, brain, kidney, testis, gut, heart, liver, and ovary (lowest expression) (see Table 3 for p values). nrf2b was most highly expressed in the ovary, followed by the gut, gill, testis, and brain and the heart, kidney, and eye (lowest expression). The only sex-related difference in basal adult expression levels was in the female gonads, where the ratio of nrf2a/nrf2b was lowest, reflecting the contribution of higher amounts of nrf2b transcripts from the mature eggs (supplemental Fig. S2). Efforts to obtain antibodies to confirm these findings at the protein level are under way.

Distinct Functions of Nrf2a and Nrf2b during Development—To identify whether Nrf2b plays an important role in embryonic development, we used start site MOs to transiently knock down translation of nrf2a and nrf2b transcripts in embryos. To first establish the specificity of the MOs, we determined their ability to inhibit protein synthesis, assessed by measuring incorporation of 35S-labeled methionine in vitro. Both MOs were successful in reducing synthesis of their specific targets without any cross-reactivity between paralogs. Densitometry measurements showed a 60% reduction in Nrf2a by Nrf2a-MO and an 80% reduction in Nrf2b by Nrf2b-MO (Fig. 6A).

We compared the phenotypes of embryos injected with Nrf2a-MO alone, Nrf2b-MO alone, or Nrf2a-MO + Nrf2b-MO; controls included embryos injected with a control-MO and
Nrf2b, Novel NRF2 Paralog in Zebrafish

Localization of zebrafish Nrf2a and Nrf2b under control and oxidative stress conditions. COS-7 cells were transfected with plasmids encoding GFP-Nrf2a or GFP-Nrf2b fusion proteins and exposed to PBS or 100 μM tBOOH for 1 h. Nuclei were labeled with propidium iodide (PI; red fluorescence) as described under “Experimental Procedures.” Images were captured using an Axio Imager.Z2 fluorescence microscope at ×200 magnification. Green and red channels were overlaid, and the GFP-positive and propidium iodide-positive pixels in the cytoplasm and nuclei of 10 fields, including the cells shown, were quantified using Axiovision co-localization software (Zeiss). Images were also subjected to a blinded assessment of the number of cells expressing GFP in the nucleus, cytoplasm, or both. Data are representative of two independent experiments. A, localization of GFP-Nrf2a and GFP-Nrf2b was nuclear, with some cells also expressing GFP in the cytoplasm. B, proportion of the digitally quantified GFP pixels that co-localize with propidium iodide pixels under each condition. C, percentage of cells expressing GFP predominantly in the nucleus or in the nucleus and cytoplasm. Error bars, S.E.

To determine the potential role of Nrf2b in regulating gene expression in response to oxidative stress, we measured expression of four known oxidant-responsive genes in morphant embryos exposed to tBOOH for 4 h at 48 hpf. In an earlier study (69), we showed that these genes were either induced (gstp1, atf3, hsp70) or repressed (mitfa) by embryo exposure to tBOOH. MO knockdown of Nrf2a reduced the basal expression of gstp1 slightly and prevented the induction of mitfa by tBHQ, whereas the induction of atf3 and hsp70 were not affected (Fig. 6C). Knockdown of Nrf2b did not affect the response of any of the genes to tBHQ.

We then asked whether Nrf2b has a role in regulating expression of nrf2a. To do this, we measured gene expression of nrf2a in Nrf2b-morphant embryos exposed to 2 μM tBHQ for 4 h at 48 or 72 hpf. No changes in expression of nrf2a were found at 48 hpf, but at 72 hpf, we observed a slight up-regulation of nrf2a following tBHQ exposure that was significant in the Nrf2b-morphant embryos (Fig. 7A). We also asked the reverse question, whether Nrf2a plays a role in regulating expression of nrf2b. Again, there were no significant changes in nrf2b expression at 48 hpf with tBHQ treatment in control or morphant embryos, but at 72 hpf, we found a slight induction of nrf2b following tBHQ exposure that was significant in the Nrf2a-morphant embryos (Fig. 7B). Basal transcription levels of nrf2b appear to be at least partly regulated by Nrf2a; expression levels were restored by exposure to tBHQ (Fig. 7B).

Nrf2a and Nrf2b Regulate Distinct Gene Sets in Embryos—To determine whether Nrf2a and Nrf2b regulate the same set of genes, we performed loss-of-function experiments with microarray-based gene expression profiling on 52-hpf embryos in which expression of Nrf2a, Nrf2b, or both paralogs had been knocked down by specific MOs, as described above. We focus here on genes regulated constitutively by the two Nrf2 paralogs, as indicated by changes in gene expression following knockdown of Nrf2a or Nrf2b in the absence of oxidant treatment.

Comparison of gene expression patterns in Nrf2a-morphants and Nrf2b-morphants as compared with embryos injected with the control-MO revealed that Nrf2a and Nrf2b regulate distinct but partially overlapping gene sets. Overall, of the 398 probes up-regulated after knockdown of Nrf2a or Nrf2b, only 80 (20%) were regulated in common by both paralogs. Of the 426 down-regulated probes, only 138 (32%) were regulated in common (Fig. 8A).

In Nrf2a-morphants, 198 probes were up-regulated, whereas 310 were down-regulated as compared with embryos injected with the control-MO. Of the 198 probes up-regulated, 80 (41%) were also up-regulated in Nrf2b-morphants. Conversely, only 138 (68%) of the 200 down-regulated probes in Nrf2a-morphants were also down-regulated in Nrf2b-morphants. Overall, 178 of the 398 (45%) of the probes up-regulated after knockdown of Nrf2a were not up-regulated following knockdown of Nrf2b, and 240 of the 426 (56%) of the probes down-regulated in Nrf2a were not down-regulated in Nrf2b. Thus, Nrf2a and Nrf2b regulate distinct but partially overlapping gene sets.
with the control-MO (Fig. 8A). The greater number of down-regulated probes in Nrf2a-morphants is consistent with a primary role of Nrf2a as a constitutive activator of transcription, a result similar to that observed in tissues or cells from Nrf2-null mice (70, 71). In contrast, Nrf2b-morphants had more up-regulated probes (280) than down-regulated probes (254), suggesting that Nrf2b can act as a repressor of constitutive gene transcription during development.

To verify the apparent repressive function of Nrf2b, we performed QPCR on three genes suggested by array data to be up-regulated by Nrf2b knockdown. For each of these genes (p53, hmox1, and ccng1), QPCR confirmed the enhancement of expression that was seen in Nrf2b-morphants (Fig. 8B). Overall, the microarray data suggest that although both Nrf2a and Nrf2b may act as activators and repressors of transcription, Nrf2a is predominantly a transcriptional activator, whereas Nrf2b appears to be more active in repressing gene expression.

**Nrf2b Repression of Reporter Gene Expression in Vivo**—To further investigate the function of Nrf2b in vivo, we synthesized capped mRNA for both nrf2a and nrf2b and co-injected zebrafish embryos with mRNA along with a GFP reporter construct under control of an ARE-rich promoter (16, 37). In embryos injected with ARE-GFP reporter alone (but not in uninjected embryos), there was low level GFP fluorescence, reflecting some basal transcription of the reporter gene in 61% of embryos injected (84 of 137 over two experiments) (Fig. 9, A (top panels) and B). Overexpression of nrf2a mRNA dramatically increased the occurrence of GFP expression in embryos to 91% (145 of 159) (Fig. 9B) and caused an increase in the intensity of fluorescence (Fig. 9A, middle panels). In contrast, overexpression of nrf2b mRNA reduced the background activity of this reporter construct, resulting in minimal GFP expression in only 11% of injected embryos (24 of 209) (Fig. 9A, bottom panels). This finding supports a role for Nrf2b as a repressor of ARE-regulated gene expression in embryos.

**Nrf2 Cross-talk with Ahr**—The presence of multiple potential XREs in both the promoters and first introns of nrf2a and nrf2b suggested that there may be cross-talk with the AHR pathway. NRF2-AHR cross-talk has been demonstrated in adult mam-

![FIGURE 5. Expression of nrf2a and nrf2b during embryonic development and in adult tissues. A, embryos or eggs (time 0) over the course of development up to 120 hpf, with early or late hatchers treated as separate samples for the time points at 48 and 60 hpf. β-Actin was used as a housekeeping gene. Data are presented as the mean ± S.E. (error bars), and n = 4 pools of 30 embryos. Following significance with ANOVA, differences between hatching state were assessed with Fisher’s PLSD (*, p ≤ 0.05; n = 3–4 pools of 30 embryos). B, adult tissues, analyzed by QPCR with ef1α as a housekeeping gene. Males and females were analyzed separately (see supplemental Fig. S2) and combined for graphical representation (n = 6 individuals/tissue except for liver, where n = 4).](image-url)

**TABLE 3**

Comparison of p values of nrf2a and nrf2b adult basal tissue expression

| p-value | Brain | Eye | Gill | Gut | Heart | Kidney | Liver | Ovary | Testis |
|---------|-------|-----|------|-----|-------|--------|-------|-------|--------|
| Brain   |       | -   | 0.0001 | 0.0334 | 0.0014 | -      | 0.0008 | <0.0001 | -      |
| Eye     | 0.0050 |      | 0.0042 | 0.0015 | <0.0001 | -      | <0.0001 | <0.0001 | 0.0136 |
| Gill    | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| Gut     | 0.0012 | <0.0001 | 0.0097 | -    | -     | <0.0001 | -      | -      | -      |
| Heart   | -     | -   | <0.0001 | <0.0001 | -      | 0.0030 | -      | <0.0001 | -      |
| Kidney  | 0.0086 | <0.0001 | <0.0001 | <0.0001 | -      | -      | 0.0016 | <0.0001 | -      |
| Liver   | 0.0009 | <0.0001 | 0.0248 | -    | <0.0001 | <0.0001 | <0.0001 | <0.0001 | -      |
| Ovary   | <0.0001 | <0.0001 | -    | -    | <0.0001 | <0.0001 | <0.0001 | -      | <0.0001 |
| Testis  | -     | 0.0001 | 0.0018 | -    | 0.0070 | 0.0002 | -      | <0.0001 | -      |

**Nrf2a**

**Nrf2b**
Effects of Nrf2 knockdown in embryos.

A. TNT protein synthesis reaction with [35S]methionine showing that both Nrf2 MOs work in vitro and that they do not have any cross-reactivity between paralogs. Densitometry measurements showed a 60% reduction in Nrf2a and an 80% reduction in Nrf2b in vitro protein translation by their respective MOs. Nrf2a (lanes 1–4) and Nrf2b (lanes 5–8) panels represent different autoradiography exposure periods for the signal to be in the linear range.

B. MO knockdown of Nrf2a and Nrf2a/H11001 Nrf2b, but not Nrf2b alone, results in increased sensitivity to tBOOH compared with control-MO and non-injected controls.

C. Expression of four oxidative stress-responsive genes in Nrf2-morphant embryos. Embryos injected with Nrf2a-MO, Nrf2b-MO, Nrf2a+b-MO, or a control-MO were exposed alongside non-injected controls to 2 μM tBHQ for 4 h starting at 48 hpf and were then sampled for QPCR analysis. Data were analyzed using ANOVA and Fisher’s PLSD (*, treatment differences; #, MO differences) and Bonferroni correction (** or #). Non-injected controls and control-MO controls were analyzed separately and combined after statistical analysis for graphical simplicity. Data presented are the mean ± S.E. (error bars), where n = 5–6 pools of five embryos from two independent experiments.

FIGURE 6. Effects of Nrf2 knockdown in embryos. A. TNT protein synthesis reaction with [35S]methionine showing that both Nrf2 MOs work in vitro and that they do not have any cross-reactivity between paralogs. Densitometry measurements showed a 60% reduction in Nrf2a and an 80% reduction in Nrf2b in vitro protein translation by their respective MOs. Nrf2a (lanes 1–4) and Nrf2b (lanes 5–8) panels represent different autoradiography exposure periods for the signal to be in the linear range. B. MO knockdown of Nrf2a and Nrf2a/H11001 Nrf2b, but not Nrf2b alone, results in increased sensitivity to tBOOH compared with control-MO and non-injected controls. n = 10–26 individual embryos/group/dose. C. Expression of four oxidative stress-responsive genes in Nrf2-morphant embryos. Embryos injected with Nrf2a-MO, Nrf2b-MO, Nrf2a+b-MO, or a control-MO were exposed alongside non-injected controls to 2 μM tBHQ for 4 h starting at 48 hpf and were then sampled for QPCR analysis. Data were analyzed using ANOVA and Fisher’s PLSD (*, treatment differences; #, MO differences) and Bonferroni correction (** or #). Non-injected controls and control-MO controls were analyzed separately and combined after statistical analysis for graphical simplicity. Data presented are the mean ± S.E. (error bars), where n = 5–6 pools of five embryos from two independent experiments.
Nrf2b, Novel NRF2 Paralog in Zebrafish

![Graph](image)

**FIGURE 7. Expression of nrf2a (A) and nrf2b (B) in non-injected and MO-injected embryos at 48 and 72 hpf.** Each time point was normalized to its own DMSO in order to compare induction by 2 μM tBHQ for 4 h from two independent experiments. Non-injected controls and control-MO samples were analyzed separately and combined after statistical analysis for graphical simplicity. *, significant ANOVA and Fisher's PLSD p < 0.05 for difference between treatment within a time point; #, significant p < 0.05 for difference between control and MO embryos. Error bars, S.E.

mals or mammalian cells but has not been investigated in zebrafish or in any vertebrate embryos. To determine whether expression of the nrf2 paralogs is inducible by AHR agonists, we exposed adult zebrafish to a potent AHR ligand, PCB-126, and measured changes in expression of both nrf2a and nrf2b in several tissues. There were significant differences in responses between males and females in the eye, gill, and gut (Table 4). In males, both nrf2a and nrf2b were induced in these tissues. In the gill, nrf2b was also significantly induced in female fish but to a lesser extent than in the males (2.26-fold for females and 6.55-fold for males; Table 4).

To determine the inducibility of these genes during development, we exposed embryos to another potent AHR ligand, TCDD. At 48 hpf, TCDD-exposed embryos showed significant up-regulation of nrf2b but not nrf2a (Fig. 10). To determine whether this was dependent on Ahr2, embryos at the 1–4-cell stage were injected with either Ahr2-MO or a control-MO, exposed to TCDD, and sampled for QPCR analysis at 48 hpf. Knockdown of Ahr2 completely inhibited the induction of nrf2b by TCDD (Fig. 10), demonstrating a key role for Ahr2 in this response. In addition, the basal expression of nrf2a was significantly reduced in Ahr2-morphant embryos. Thus, both zebrafish Nrf2s are capable of participating in cross-talk with the Ahr during embryonic development, but they do so in different ways.

**DISCUSSION**

*NRF Diversity in Zebrafish*—To understand the oxidative stress response and its regulation in zebrafish embryos, it is important to identify the set of oxidant-responsive transcription factors and their relationships to their human homologs. Through genome searches, targeted cloning, and phylogenetic analysis, we showed that zebrafish have orthologs of each of the four human NF-E2-related genes (NF-E2, NRF1, NRF2, and NRF3) and that for two of these (NRF1 and NRF2), zebrafish possess duplicates (paralogs). The zebrafish and human NF-E2-related genes exhibited extensive conserved synteny with the HOX clusters in each species, providing strong evidence that the two sets of zebrafish paralogs (nrf1a and nrf1b; nrf2a and nrf2b) are co-orthologs of the human NRF1 and NRF2 genes, respectively. The conserved synteny suggests that the nrf1 and nrf2 duplicates, like the hox clusters to which they are linked, arose as part of the fish-specific whole-genome duplication that occurred after divergence of fish and mammalian lineages (9, 18).

**Subfunction Partitioning between nrf2 Paralogs**—The nrf2 duplicates are of particular interest because of the demonstrated importance of their mammalian ortholog, NRF2, in the response to oxidative stress. We present here the discovery and initial characterization of nrf2b in the zebrafish. Although many fish have duplicate copies of genes found as only single copies in mammals, this is the first identification of a duplicate nrf2 gene. Duplicate genes generally are subject to one of three fates: nonfunctionalization (i.e. becoming a pseudo-gene), neofunctionalization, or subfunctionalization (subfunction partitioning) (9). Based on the data presented here, we propose that the two nrf2 paralogs have undergone subfunction partitioning. In mammals, NRF2 is highly pleiotropic, serving a wide array of functions in processes as diverse as inflammation, DNA repair, lipid metabolism, Phase II and Phase III metabolism, autophagy, and glutathione homeostasis (7, 48, 49, 72–74). Because NRF2 plays such diverse roles, its characterization can be challenging. The zebrafish nrf2 gene paralogs offer a valuable opportunity for new insight into the evolution and functions of the orthologous human NRF2 gene.

Subfunction partitioning can involve regulatory (spatial, temporal, quantitative) or structural features (9). Comparing the two nrf2 paralogs, we find evidence for several kinds of subfunction partitioning. Conserved synteny with the hox gene clusters and hox-associated genes demonstrate that nrf2a and nrf2b are co-orthologous to the human NRF2 (Fig. 1C). However, direct sequence comparisons and phylogenetic analyses indicate that the zebrafish nrf2b has evolved at a faster rate than nrf2a, suggesting that these paralogs are likely to exhibit distinct functions.

Consistent with regulatory partitioning of nrf2a and nrf2b, we found striking quantitative, spatial, and temporal differences in the expression patterns of these two paralogs, particularly between the adult and embryo stages. Expression of each
paralog varied among adult tissues, but the expression of nrf2a was consistently higher than that of nrf2b. During embryonic development, at all time points sampled through the first 5 days of development, the expression of nrf2b was much greater than that of nrf2a, suggesting that Nrf2b may have important functions during development. However, the functions of Nrf2a and Nrf2b during embryonic development are not yet clear. Just as Nrf2−/− mice develop normally (75), so do zebrafish embryos with one or both of the zebrafish Nrf2s knocked down, in the absence of oxidant exposure. Nrf2a-morphant embryos were more sensitive to tBOOH, whereas Nrf2b-morphants were similar to controls in their sensitivity to this compound. The abundant nrf2b expression in embryos could explain why the Nrf2b knockdown (which, like all MO-mediated knockdowns, is incomplete) did not show more dramatic effects. It is possible that the nrf2b gene would need to be knocked out completely in order to detect a role in response to oxidative stress; such studies are under way.

We also found that Nrf2a and Nrf2b differ with respect to their roles in regulating gene expression in embryos exposed to oxidative stress. It is well known that exposure to tBHQ or tBOOH induces gstp1, an effect inhibited by knockdown of Nrf2a (10, 12, 16); similar results were obtained in our experiments (Fig. 6). However, knockdown of Nrf2b did not impact induction of gstp1 by tBHQ exposure. Similarly, knockdown of Nrf2a but not Nrf2b blocked the tBHQ-mediated inhibition of mitfa expression. This suggests that Nrf2a and Nrf2b differ in their ability to regulate embryonic gene transcription in response to oxidants, consistent with the hypothesis of subfunction partitioning. Conceivably, Nrf2a and Nrf2b may exhibit different chemical sensitivities, possibly linked to regulation by one or both of the zebrafish Keap1 paralogs (Keap1a and Keap1b) (13).

Nrf2b Is Negative Regulator of Gene Expression during Development—Although the targeted analysis of known oxidant-responsive genes provided evidence that Nrf2a and Nrf2b differ in their ability to regulate gene expression in response to oxidant exposure, it did not reveal a specific role for Nrf2b. We therefore conducted gene expression profiling in embryos injected with Nrf2a-MO or Nrf2b-MO to determine the sets of genes regulated by each NRF2 paralog. We focus here on genes regulated in the absence of oxidant exposure (i.e., those genes whose constitutive expression during development is regulated by one or both paralogs).

The microarray studies yielded two important results. First, they revealed that Nrf2a and Nrf2b regulate distinct gene sets, with only partial overlap (20–32%), providing compelling evidence for distinct functions of Nrf2a and Nrf2b. Second, these experiments revealed a fundamental difference between the two proteins in the nature of their gene regulatory roles. Knockdown of Nrf2a caused mostly decreases in gene expression, consistent with the well known role of vertebrate NRF2 proteins as activators of transcription (1). In contrast, knockdown of Nrf2b caused mostly increases in gene expression, suggesting that this protein acts primarily as a negative regulator of gene expression in embryos. This proposed role of Nrf2b is consistent with predictions arising from sequence analysis of the two zebrafish NRF2 paralogs. Whereas sequences important for KEAP1 interactions, protein stability, and DNA binding are largely conserved in both zebrafish Nrf2 proteins, Nrf2b lacks the Neh4 transactivation domain found in Nrf2a and mammalian NRF proteins.
We confirmed the apparent repressive function of Nrf2b for three genes, p53, ccng1, and hmox1, that suggest possible roles of Nrf2b in cell cycle regulation and in the regulation of heme degradation. As shown in Fig. 8B, knockdown of Nrf2b resulted in increased expression of p53, a well known regulator of cell cycle progression and apoptosis (76), and cyclin G1 (ccng1), which is important in regulating the G1/S transition of the cell cycle (77). Activation of p53 has been noted as an off-target effect of some MOs (78). However, this effect involves increased p53 protein without increased levels of full-length p53 mRNA (78) and thus is distinct from the results shown here. In addition, neuronal cell death and craniofacial malformations, the hallmark signs of off-target effects involving p53 activation (76, 78), were not observed in our study. Thus, the increased p53 mRNA expression that we measured in Nrf2b-morphants (and which was not observed in the control-MO or Nrf2a-MO groups) most likely reflects a specific effect on p53 gene expression rather than an off-target effect.

Nrf2b also repressed basal expression of hmox1, the inducible isozyme of heme oxygenase, the rate-limiting enzyme in heme degradation. Interestingly, in mammalian cells, constitutive expression of HMOX1 is positively regulated by NRF2 (71, 79) but is repressed by BACH1 (80, 81). Our results suggest a novel role for the Nrf2b paralog in maintaining low hmox1 expression during development.

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Nrf2b, Novel NRF2 Paralog in Zebrafish

Cross-talk with Aryl Hydrocarbon Receptor Pathway—We found direct evidence that both Nrf2a and Nrf2b are capable of participating in cross-talk with Ahr2, but they do so in different ways, consistent with the hypothesis of sub-function partitioning. Although basal expression of nrf2a was higher than that of nrf2b in all adult tissues examined, nrf2b was more inducible by exposure to the potent AHR agonist PCB-126, specifically in the eye, gill, and gut.

Although AHR-NRF2 cross-talk has been demonstrated previously in mammalian cells and adult tissues, whether such cross-talk occurs also in developing embryos is not known. In the present study, we found that nrf2b, but not nrf2a, was inducible in embryos exposed to the potent AHR agonist TCDD. The induction of nrf2b was dependent on expression of Ahr2, thus demonstrating direct regulatory cross-talk between the AHR and NRF2 pathways in embryos. We also found that nrf2a also participates in cross-talk with Ahr2 but in a different way. Although nrf2a was not up-regulated by TCDD exposure, the basal expression of this gene was significantly reduced when Ahr2 expression was knocked down. These results provide evidence that Ahr2 plays a direct role in maintaining basal levels of nrf2a and inducible levels of nrf2b during embryonic development.

Cross-talk between NRF2 and AHR has emerged recently as an important area of research. There are three XREs in the murine Nrf2 promoter and first intron, and Nrf2 has been shown to be up-regulated by dioxin in mice (5). In mice, induction of certain genes by TCDD was found to require NRF2 (6); AHR- and NRF2-dependent induction was seen for Nqo1, Ugt1a6, and Gsta1 (6), which are classically considered part of the AHR battery of genes (84, 85), as well as for other Ugt and Gst isoforms (6). Other studies have also found cross-talk in either mouse models or cell lines (7, 86–88). Our results in zebrafish provide the first direct evidence of AHR-NRF2 cross-talk during embryonic development in any system. This presents an opportunity to use the zebrafish system to provide new insights into the multiple mechanisms of NRF2-AHR cross-talk in vertebrate animals.

In summary, we have identified a novel NRF2 protein, Nrf2b, that is prominently expressed in developing embryos and is distinct from its paralog Nrf2a in multiple respects, including expression patterns, regulation, target genes, mode of action, and ability to interact with the AHR signaling pathway. We provide evidence that Nrf2a and Nrf2b have undergone sub-function partitioning and that a primary role of Nrf2b is as a negative regulator of gene expression in embryos. Further investigation of Nrf2b in comparison with Nrf2a is likely to yield additional new insights regarding the function and regulation of the NRF2-signaling pathway and its roles in development and in protecting vertebrate embryos from oxidative damage.

Acknowledgments—We greatly appreciate assistance provided by Bruce Woodin, Akira Kubota, and Neelakanteswar Aluru; imaging assistance provided by Louis Kerr (Marine Biological Laboratory); and the excellent fish care provided by Gale Clark and Brandy Joyce. We also are grateful to Dr. Makoto Kobayashi (University of Tsukuba) for generously sharing plasmids pCS2nrf2a and pT3.5gstp1GFP.

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