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Nrf2 and Nrf2-Related Proteins in Development and Developmental Toxicity: Insights from studies in Zebrafish (Danio rerio)

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Abstract

Oxidative stress is an important mechanism of chemical toxicity, contributing to developmental toxicity and teratogenesis as well as to cardiovascular and neurodegenerative diseases and diabetic embryopathy. Developing animals are especially sensitive to effects of chemicals that disrupt the balance of processes generating reactive species and oxidative stress, and those anti-oxidant defenses that protect against oxidative stress. The expression and inducibility of anti-oxidant defenses through activation of NFE2-related factor 2 (Nrf2) and related proteins is an essential process affecting the susceptibility to oxidants, but the complex interactions of Nrf2 in determining embryonic response to oxidants and oxidative stress are only beginning to be understood. The zebrafish (*Danio rerio*) is an established model in developmental biology and now also in developmental toxicology and redox signaling. Here we review the regulation of genes involved in protection against oxidative stress in developing vertebrates, with a focus on Nrf2 and related cap'n'collar (CNC)-basic-leucine zipper (bZIP) transcription factors. Vertebrate animals including zebrafish share Nfe2, Nrf1, Nrf2, and Nrf3 as well as a core set of genes that respond to oxidative stress, contributing to the value of zebrafish as a model system with which to investigate the mechanisms involved in regulation of redox signaling and the response to oxidative stress during embryolarval development. Moreover, studies in zebrafish have revealed *nrf* and *keap1* gene duplications that provide an opportunity to dissect multiple functions of vertebrate *NRF* genes, including multiple sensing mechanisms involved in chemical-specific effects.

Keywords

oxidative stress; NRF2; NFE2L2; embryo; zebrafish; development

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Introduction[1]

Oxidative stress occurs when the cellular redox balance is altered, disrupting redox signaling and regulation [1-3]. It can occur through the generation and action of reactive species such as superoxide anion, hydrogen peroxide, peroxynitrite, and other reactive products that can alter thiol redox circuits [4-6]. Oxidative stress is increasingly recognized as a significant mechanism of chemical toxicity and as a contributing factor in a wide range of pathological conditions and diseases in adults, and in developing animals. The roles of reactive species and redox status in normal physiology, and the mechanisms underlying adverse effects of oxidative stress, have been studied extensively in adult animals and cells. However, less is known about the role of these processes in developmental disorders and chemical toxicity during embryonic and fetal development.

Developing animals are uniquely sensitive to oxidative stress because of the rapid changes in cell proliferation and differentiation that occur at this time, and because enzymatic systems that protect against or repair toxic damage may not be fully mature early in development. The expression and inducibility of anti-oxidant defenses are critical factors affecting susceptibility to oxidative stress, but the ontogenic development of antioxidant defenses and their regulation in at early life stages are only beginning to be understood. The zebrafish (*Danio rerio*), an established model in developmental biology, has emerged recently as a valuable system for studying the expression and regulation of antioxidant defenses during development, and in particular the role of Nfe2-related transcription factors. Here we provide a review of the development and regulation of genes involved in protection against oxidative stress, with a focus on insights obtained from studies in zebrafish early life stages (embryos and larvae). For an additional perspective on the use of zebrafish as a model to study oxidative mechanisms, please see a recent review [7].

Oxidant signaling and oxidative stress in development and developmental toxicology

Importance of redox status and oxidant signaling during development

Embryonic development involves precisely orchestrated processes--including proliferation, differentiation, apoptosis, establishment of left-right asymmetry, gene expression, and epigenetic modifications--that increasingly are found to depend on redox signaling and intracellular redox potentials (E_h) [8-16]. Because endogenous oxidants and cellular redox potential play such important and fundamental roles in normal embryonic development, tight regulation of redox balance is critical. Toxicant exposures or other environmental stressors that increase oxidant levels or disrupt redox balance can fundamentally alter cell fate decisions. This can result in functional or structural changes, some of which could have long-term consequences that may only become apparent with subsequent stress or age [17, 18].

[1]Abbreviations used: ARE: anti-oxidant response element; CNC-bZIP: cap'n'collar basic-leucine zipper; CRISPR: clustered regularly interspaced short palindromic repeats; DEM: diethylmaleate; DQ: diquat; GFP: green fluorescent protein; GSH: glutathione; MO: morpholino oligonucleotide; NRF: NFE2-related factor; RT-PCR: reverse transcription-polymerase chain reaction; SFN: sulforaphane; tBHQ: *tert*-butylhydroquinone; tBOOH: *tert*-butylhydroperoxide; TILLING: Targeting Induced Local Lesions IN Genomes

Role of oxidative stress in developmental toxicity

Oxidative stress contributes to a variety of human diseases [19-23] and has been implicated in the mechanism of action of human teratogens including valproic acid, phenytoin, ethanol, and thalidomide [16, 24-29], as well as in congenital malformations associated with PAH exposure [30, 31] and diabetic embryopathy [27, 32]. A variety of chemicals can generate reactive species or disrupt redox balance in other ways, through redox cycling, following biotransformation to reactive intermediates, or through induction or uncoupling of enzymes that can generate oxidants [33-36].

Studies of oxidative stress in adult organisms and cultured cells dominate the literature. However, developing animals (vertebrates including mammals and fish) are often more sensitive than young adults to chemical toxicity [37-40] and embryos appear to be especially sensitive to oxidative damage [24-27, 40, 41]. The risk of embryotoxicity and teratogenicity following exposure to pro-oxidant chemicals depends on the balance between reactions generating oxidative stress and reactions that are protective or repair the damage [24, 25, 41-45]. Consistent with this, supplementation with small molecular anti-oxidants or stimulation of anti-oxidant enzymes protects embryos against the developmental toxicity of a variety of chemicals [46-55] and embryos with reduced antioxidant capacity are at increased risk for developmental toxicity and teratogenicity [24, 30, 44, 56-58].

Constitutive and inducible antioxidant defenses in embryos

Anti-oxidant defenses can be constitutive, protecting against and balancing redox potential under normal physiological conditions, and inducible defenses, which protect against toxicant-stimulated levels of oxidants or redox imbalance. Embryos express relatively low basal (constitutive) levels of most anti-oxidants and anti-oxidant enzymes [59-67], which can put them at increased risk for oxidative toxicity [24, 25, 27]. Less is known about the inducible component of the anti-oxidant defense in embryos. A number of studies have examined inducible anti-oxidant defenses and the oxidative stress response in mammalian development [16, 17, 65, 68-70]. These studies have demonstrated the capacity of vertebrate embryos to respond to oxidative stress with increased expression of anti-oxidant genes, and in some cases have demonstrated a role for NFE2-related factor 2 (Nrf2)[2] [70, 71]. Several groups including ours have begun to examine the oxidative stress response in zebrafish embryos (see below). These reports suggest that the responsiveness of embryos to oxidative stress can vary dramatically with developmental stage, and that this involves Nrf proteins. However, there is much yet to learn concerning the oxidative stress response in embryos and how it is regulated. Before discussing what has been learned from studies in zebrafish embryos, we briefly review what is known about the oxidative stress response and its regulation by NRF proteins in mammals. Additional details can be found in other reviews in this volume.

[2]*Nomenclature: NRF* is a commonly used notation for NFE2-Related Factor genes, which are officially designated as *NFE2L* (NFE2-Like). For example, *NRF2* is officially designated as *NFE2L2*, and *NRF1* as *NFE2L1*. Throughout the paper, we use the more common *NRF* designation. Otherwise, we utilize the approved format for designating genes and proteins (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines). In particular, human genes and proteins are designated using all capitals (*NRF2* and NRF2, respectively), whereas zebrafish genes are designated *nrf2* and Nrf2 for genes and proteins, respectively. When not referring to a specific species, we have used the human notation as a default format.

The oxidative stress response – regulation by Nfe2-related factors

Vertebrate animals possess an inducible enzymatic defense system that acts to detoxify reactive species and replenish the supply of small molecule anti-oxidants. Oxidants, electrophiles, and some so-called anti-oxidant chemicals activate an oxidative stress response via a family of related, cap'n'collar (CNC)-basic-leucine zipper (bZIP) transcription factors that interact with an anti-oxidant response element (ARE; a.k.a. electrophile response element or EpRE: 5'-TGACnnnGC-3') in the promoters of target genes [72-76]. Known target genes in mammals include GSTA1, GSTP, NQO1, GCL, SOD, heme oxygenase (HO-1), and others.

The best-characterized oxidative stress response mechanism is that involving NRF2. NRF2 is normally found in the cytosol as an inactive complex with Kelch-like-ECH-associated protein (Keap1, also called iNRF2). Keap1 both retains NRF2 in the cytoplasm and enhances its proteasomal degradation [77-80]. Oxidative stress disrupts the interaction between NRF2 and Keap1 through a mechanism that may involve phosphorylation and/or disruption of sulfhydryl interactions [72, 81-87] affecting the nucleocytoplasmic shuttling of NRF2 [88, 89]. Once free of the repressor Keap1, NRF2 protein accumulates in the cell, enters the nucleus and forms a heterodimer with one of several small Maf proteins (MafF, MafG, MafK), which also contain bZIP motifs [90-92]. The NRF2-Maf dimer binds to AREs and activates transcription. A few studies suggest that Nrf2 may not dissociate from Keap1, but that the disruption of ubiquitination maintains Keap1 in a Nrf2-saturated state, permitting newly synthesized Nrf2 to travel unimpeded to the nucleus (e.g. reviewed in [93]). However, this remains controversial and has not yet been examined in zebrafish. Irrespective of whether Nrf2 dissociates from Keap1, or whether the *de novo*/Keap1 saturation model proves correct, accumulation of Nrf2 in the nucleus results in ARE-mediated gene regulation. An additional layer of regulation is provided by the related proteins BACH1 (BTB and CNC homology 1) and BACH2, which can also act as repressors of NRF2 [94, 95].

An oxidative stress response involving CNC proteins appears to be evolutionarily conserved in animals, with NRF homologs in *Drosophila melanogaster* [96, 97] and *C. elegans* [98, 99]. However, most of our current understanding of the oxidative stress response has been obtained from studies of the four mammalian CNC-bZIP proteins.

Nuclear factor erythroid-2 (NFE2)—NFE2 (also called p45) is a hematopoietic cell-specific transcription factor controlling globin gene expression as a complex with one of several small Maf proteins (p18) [100, 101]. NFE2 null mice are deficient in platelets and exhibit hemorrhaging and high mortality [102].

NFE2-related factor-1 (NRF1; also called NFE2L1, TCF11 and LCR-F1)—NRF1 is expressed in a variety of tissues [103] and as multiple isoforms [104]. Mice in which the *Nrf1* gene has been disrupted die *in utero* either by day 7.5 or day 17.5 and exhibit a defect in definitive erythropoiesis [105, 106]. Studies in $Nrf1^{-/-}/Nrf1^{+/+}$ chimeric mice show that NRF1 is required for the development of the liver but not other tissues, that the effect is specific to hepatocytes, and that $Nrf1^{-/-}$ hepatocytes exhibit increased oxidative stress and

undergo apoptosis late in pre-natal development [107]. NRF1 interacts with the ARE and regulates the basal expression and inducibility of GCL [108] and NQO1 [109, 110]; it is also important for glutathione homeostasis [111]. NRF1 can also act as a repressor of gene expression [104, 112]. Unlike NRF2 (below), NRF1 is localized to the endoplasmic reticulum and may play a role in the response to ER stress [113]. NRF1 is involved in regulating lipid metabolism [112, 114] and the expression of proteasome subunits in response to oxidant-damaged proteins [115, 116].

NRF2 (NFE2L2)—NRF2 was initially cloned from humans [117] and chickens [118] (called ECH). NRF2 binds to ARE sequences *in vitro* [119] and experiments in NRF2-deficient mice show that it regulates the basal and inducible expression of genes in the oxidative stress response, including NQO1, GSTP, GSTA, GSTM, GCL, ferritin, HO-1 [120-123] and other genes [124, 125]. Unlike targeted disruption of NRF1, which is embryo-lethal, NRF2 knock-out mice develop and reproduce normally [126]. Interestingly, constitutive activation of NRF2 in Keap1-deficient mice leads to postnatal lethality [127]. Moreover, disruption of both NRF2 and NRF1 results in pronounced oxidative stress, apoptosis, and embryolethality at an earlier stage than seen in the $Nrf1^{-/-}$ mice, demonstrating that NRF1 and NRF2 both have developmentally important, partially overlapping roles [128]. In addition, although the $Nrf2^{-/-}$ single knock-out mice are viable, as adults they are much more sensitive to chemicals that cause oxidative stress [129-132], including benzo[a]pyrene (BaP) [133, 134]. Thus, NRF2 has a key role in mediating the oxidative stress response in adult animals.

NRF3 (NFE2L3)—NRF3 is expressed in several tissues, most notably in the placenta and in B-cell lineages [135, 136], and like NRF1 exists as several isoforms and is localized to the endoplasmic reticulum [137, 138]. NRF3-null mice develop normally and compound NRF3/NRF2 and NRF3/NF-E2 mutants exhibit no additional phenotypes [139]. However, NRF3-null mice have increased susceptibility to benzo[a]pyrene-induced T-cell lymphoblastic lymphoma [140]. NRF3 can activate or repress gene expression and may act as a negative regulator of NRF2 [138, 141]. The role of NRF3 in protection against oxidative stress is currently unclear.

Most information about the regulation of the oxidative stress response by NRF proteins has been obtained in adult mammals and mammalian cells. What is the role of NRF-related transcription factors in regulating the response to oxidative stress in developing animals?

Zebrafish as a model for studying development roles of Nrf2 and related proteins

The zebrafish has emerged as a powerful system in which to examine mechanisms involved in the regulation of the oxidative stress response by Nrf2 and related proteins in developing animals. The zebrafish embryo is an important vertebrate model (or “tool” [142]) in embryology and developmental biology [143-153]. Because zebrafish are vertebrates, results obtained in this system have more direct relevance for humans as compared to studies in invertebrates. The fundamental features of developmental signaling pathways are conserved

between fish and mammals, facilitating extrapolation of results from zebrafish to humans [151, 154-156].

A key technical advantage of zebrafish is the external development of nearly transparent embryos, facilitating direct observation of all stages of embryonic development, something not possible in mammals. The small size, rapid development (~48-72 hr from fertilization to hatch), and short generation time (2-3 mo) of zebrafish allow for large numbers of animals to be produced and housed [157]. Transgenic technologies are well developed in zebrafish [158]; transient and stable (germline) expression of transgenes can be used to visualize cell lineages [159], test promoter function [160, 161], and map regulatory elements [162-164], all *in vivo*. Heterologous promoters and proteins have been shown to function faithfully in zebrafish, recapitulating native expression patterns [162] or rescuing mutant phenotypes [165]. The coupling of green fluorescent protein (GFP)-based reporters [166-168] and transparent zebrafish embryos provides a powerful system for visualizing *in vivo* gene expression. Saturation mutagenesis screens have been performed using chemical or insertional mutagenesis [169-172] and many of the mutants reproduce human genetic diseases [173, 174].

Powerful loss-of-function approaches contribute to the utility of zebrafish in studying gene function. An anti-sense approach using morpholino-modified oligonucleotides (MOs) has been widely used to produce targeted gene “knock-downs” in developing zebrafish [175-177]. This approach has been useful for identifying gene function during development [175, 178-183] and to understand the role of specific genes in mechanisms of toxicity [184-191]. More recently, a trio of gene targeting methods have been developed and applied to zebrafish, making it possible to generate null alleles and homozygous null mutants at any locus. These methods include DNA-based targeted endonuclease approaches involving zinc-finger nucleases (ZFN) and transcription activator-like effector nuclease (TALENS) [192-195], as well as the RNA-guided clustered regularly interspaced short palindromic repeats *(*CRISPR)-Cas9 approach [196, 197]. Recent results comparing mutant and morphant phenotypes have highlighted the strengths and limitations of each approach [198-200].

A whole genome sequence supports a variety of genomic tools for studies in zebrafish [201, 202], and affords explicit comparisons with other species. Thus, zebrafish chromosomes exhibit large regions of conserved synteny with human chromosomes, facilitating studies of genome evolution and the identification of orthologous genes [203, 204]. An important advantage of zebrafish as a model is the existence of multiple copies of some human genes.

In general, zebrafish share with humans most features of key biochemical pathways (including signaling pathways). However, because of genome duplication in the teleost fish lineage shortly after its divergence from the tetrapod lineage [205-208], teleost fish have retained extra copies (paralogs) of some transcription factors, not found in humans. The existence of paralogs in fish provides an opportunity for new mechanistic insights, because duplicated fish genes might be exploited to obtain new information about the function of their single human counterpart [205-207, 209, 210]. This might apply especially to human genes with multiple functions or expression domains. The duplication, degeneration,

complementation model of gene evolution [209, 210] predicts that the multiple functions or complex expression patterns of such a gene in humans may be partitioned between its fish "co-orthologs," so that each of the duplicates retains a subset of the original functions. By studying each paralog individually (e.g. by loss-of-function approaches), their distinct roles (subsets of the original roles or expression patterns) can be examined in isolation and thereby elucidated. This approach may be especially informative in studying pleiotropic transcription factors such as Nrf2, because it can help to reveal some of the subtle functions of the mammalian homolog.

Because of these advantages, zebrafish were prominent among a group of alternative models recommended by the National Research Council for research in developmental toxicology [20]. More recently, the zebrafish model has been adopted and embraced by regulatory agencies and scientists in the U.S. and Europe [211-216] and by the pharmaceutical industry [217-224]. The number of studies in which this model system has been used to investigate mechanisms underlying the oxidative stress response was initially small [225-229], but has been rapidly growing. The emerging picture is that the fundamental features of the oxidative stress response are conserved in vertebrates, and thus that zebrafish is a powerful model with which to investigate oxidative phenomena during development.

Although there are many strengths of zebrafish as a model in which to study developmental processes, it is important to keep in mind that early in development (prior to the onset of circulation at ~26 hpf [230]), zebrafish embryos may experience a somewhat different oxygen environment than that experienced by mammalian embryos. Zebrafish embryos are typically cultured and develop at ambient [O_2] [231, 232], whereas at early stages mammalian embryos experience lower oxygen tension, and higher concentrations can be toxic [233]. After the onset of circulation, however, zebrafish embryos are likely to experience oxygen environments similar to those of mammalian embryos. Thus, while the zebrafish embryo may be a valuable system for investigating fundamental mechanisms underlying the developmental roles of Nrf2 and related proteins, as with any model there may also be unique features that must be considered in interpreting the results.

Diversity of CNC-bZip transcription factors and related genes in zebrafish

Studies over the past 15 years have begun to establish the molecular mechanisms underlying the regulation of the oxidative stress response in fish and their similarity to those in mammals. Early studies [225, 226] demonstrated that reporter gene constructs containing a luciferase gene under control of mammalian ARE sequences, when transfected into fish cells, could be induced by exposure to *tert*-butylhydroquinone (tBHQ), suggesting that fish oxidant-responsive transcription factors are able to recognize mammalian ARE sequences. Subsequently, it was shown that exposure of zebrafish embryos or larvae to tBHQ or *tert*-butylhydroperoxide (tBOOH) induces an oxidative stress response [227, 234-237]. The first direct evidence for an oxidative stress response in fish and its mechanistic similarity to that in mammals was from Kobayashi *et al.* [227, 229], who cloned the cDNAs for zebrafish Nrf2 and Keap1 and showed that these proteins mediated the induction of *gstp1*, *nqo1*, and *gclc* by tBHQ in zebrafish embryos. Zebrafish also possess small Maf proteins (including a novel form, MafT) that form heterodimers with Nrf2 [238]. An evolutionarily conserved

ARE 50-bp upstream of the gstp1 transcriptional start site was shown to interact with Nrf2 to regulate *gstp1* expression [228].

Since the initial studies in zebrafish cells and embryos, the number and identity of Nrf genes and their partners has become clear, revealing in some cases enhanced diversity in zebrafish as compared to humans. As noted above, four CNC-bZIP family members exist in humans and other mammals: *NFE2*, *NRF1* (*NFE2L1*), *NRF2* (*NFE2L2*), and *NRF3* (*NFE2L3*). Zebrafish have a single *nfe2* gene that is expressed in erythroid cells and in the developing ear [239]. The function of zebrafish *nfe2* is not well understood, but loss-of-function studies involving knockdown of Nfe2 by morpholino oligonucleotides suggested that *nfe2* may be required for proper cellular organization in the pneumatic duct and subsequent swim bladder function, as well as for proper formation of the otic vesicles [240]. In contrast to the single NRF1 gene in humans, zebrafish have two *nrf1* paralogs (*nrf1a*, *nrf1b*)[3] [234, 241], whose functions are not yet known. In addition to the original *nrf2* gene [227] (now known as *nrf2a*), a second *nrf2* gene (*nrf2b* [234]) has been identified. Overall, Timme-Laragy *et al.* [234] found that zebrafish (*Danio rerio*) possess six *nrf* genes, including *nfe2*, *nrf3*, and duplicated *nrf1* and *nrf2* genes. Comparative genomic analysis demonstrated extensive conserved synteny involving human and zebrafish *nrf2* and *hox* genes **(**Fig. 1**)**, indicating that *nrf2a* and *nrf2b* are co-orthologs of human *NRF2*, and *nrf1a* and *nrf1b* are co-orthologs of *NRF1*. 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 [207, 242]. In general, the comparative genomic analyses demonstrated 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 *NRF* genes. Zebrafish *nrf* genes are summarized in Table 1.

The *HOX* genes have important and well-established roles in embryonic development [245]. The conservation of the syntenic relationship between the *HOX* clusters and all of the *NFE2*-related genes suggest that there has been strong selective pressure to preserve this arrangement over 500 million years of evolution. This has been seen also for other *HOX*-associated genes and in comparisons of human and pufferfish (*Takifugu rubripes*) genomes [246, 247]. An intriguing possibility is that there may be coordinated regulation of these genes during development—a possible example of “genomic regulatory blocks”—large regions of conserved synteny and conserved gene order with overlapping regulatory features that resist recombination [248, 249]. Notably, all six of the NFE2-related genes are expressed in embryos [234, 240] (see below), supporting a plausible regulatory association with the *HOX* developmental patterning genes.

Similar to the findings with *nrf1* and *nrf2*, zebrafish also possess duplicated *keap1* genes (*keap1a* and *keap1b* [227, 229]) and duplicated *bach1* genes (*bach1a*, *bach1b* [250, 251]). Together, these results demonstrate that duplicated zebrafish genes are common in the Nrf

[3]The designation of duplicated genes (and their encoded proteins) as “a” (as in *nrf1a*) or “b” (*nrf1b*) is according to the approved zebrafish nomenclature for duplicates resulting from the fish-specific whole-genome duplication (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines).

pathways, as they are in some other pathways involved in regulation of response to environmental stressors [252, 253].

Ontogeny of glutathione redox dynamics and constitutive antioxidant defenses in developing embryos

In addition to the oxidative stress response and developmental events regulated by the Nrf-family of transcription factors, other endogenous antioxidant defenses play important roles in embryonic development. Glutathione (GSH) is the most abundant cellular antioxidant, present in mM concentrations, and is required for successful embryonic development [254, 255]. Importantly, numerous studies have demonstrated a strong relationship between concentrations and nuclear localization of GSH and cell cycle progression [256-259]. Cell fate decisions have also been shown to be closely related to GSH E_h, with more oxidized E_h associated with differentiation, and more reduced E_h with proliferation [260-262].

Timme-Laragy *et al.* [263] assessed GSH homeostasis during embryonic development by measuring GSH redox dynamics over 0-120 hours of zebrafish development. They used high-performance liquid chromatography (HPLC) to measure reduced and oxidized glutathione (GSH, GSSG) and calculated the whole embryo total glutathione (GSH_T) concentrations and redox potentials (E_h) at each of twelve stages. The results **(**Fig. 2**)** revealed distinct windows of GSH concentration and redox status that correspond to major developmental events. Initially (at fertilization and shortly after), embryos have relatively low concentrations of GSH_T and reducing conditions. Subsequently (3-24 hpf) concentrations of GSH_T remain low (2-3 mM) but the redox potential becomes more oxidized. From 30-48 hpf, concentrations of GSH_T increase while the redox potential remains oxidized. This is a period of cellular differentiation, which would be promoted at a cellular level by an oxidized redox status [260-262]. After hatching, GSH concentrations remain high and the redox potential becomes more reduced, conditions that promote cellular proliferation [261]. These results suggest that embryonic development involves a highly regulated trajectory of glutathione status involving changes in both the amount and oxidation state of this important regulator of cellular redox status. Studies in other fish demonstrate similar cycles of GSH regulation during development [264, 265].

Timme-Laragy *et al.* [263] also identified the set of zebrafish orthologs of mammalian genes involved in glutathione synthesis, recycling, and utilization and measured their expression during development. They identified in the zebrafish genome extensive duplication of genes encoding proteins involved in GSH synthesis and recycling, and these genes exhibited complex patterns of gene expression in developing embryos. A number of the genes involved in GSH synthesis were maternally loaded and/or expressed highly in early embryos. Overall, ontogenic changes in the expression of GSH-related genes supported the hypothesis that GSH redox status is tightly regulated during development. However, the role of Nrf proteins and other transcription factors in regulating the constitutive expression of these genes in embryos is not yet well understood.

Developmental expression of Nfe2-related genes and inducible antioxidant defenses

The constitutive expression of genes involved in antioxidant defense may help to ensure that redox conditions are appropriate for normal embryonic development, and may provide some immediate protection against challenges posed by exposure to chemicals or other stressors. In addition, embryos possess the ability to up-regulate antioxidant defenses in response to challenges, but how these inducible responses vary by cell type and developmental stage and how they are regulated during development are poorly known.

Kobayashi *et al.* [227] found that exposure of zebrafish larvae to tBHQ (30 μM) for 6 hr at 4- or 7-days post fertilization (dpf) caused the induction of *gstp1*, *nqo1*, and *gclc*. However, exposure of 24-hpf embryos to tBHQ for 6 hours did not induce *gstp1*, suggesting that 24-hr old zebrafish embryos are less capable of mounting an oxidative stress response. Timme-Laragy *et al.* [235] found induction of *gstp1*, *gpx1*, and *gclc* in zebrafish embryos exposed to tBOOH or ß-naphthoflavone for 24 hr beginning at 24-hpf, suggesting that at some point during the 24-48-hpf period the embryos are capable of responding to these chemicals. A recent study [237] showed that tBHQ exposure for 6 hr caused strong induction of *gstp1* in 1- and 2-dpf embryos. tBHQ also induced *gclc* (1-, 2-, and 4-dpf), but not *sod1* or *nqo1*. Together, these reports suggest that there are stage- or chemical-specific differences in responsiveness to oxidative stress during development.

Gene expression profiles

A few studies have gene expression profiles in embryos exposed to oxidant chemicals. In a comparison of responses of zebrafish larvae to eleven different chemicals using microarrays, Yang *et al.* [236] found that tBHQ at 5-dpf produced a distinct profile that included a number of genes known from studies in mammals to be involved in the oxidative stress response. Nakajima et al. [266] reported that exposure of 4-dpf larvae to the sulfhydryl-reactive chemical diethylmaleate (DEM) induced 42 genes by more than 2-fold, including *gclc*, *txnrd1* (thioredoxin reductase 1), *peroxiredoxin 1*, and several *gst* genes. In another study [237], microarray analysis of gene expression in 4-dpf larvae acutely exposed to tBHQ showed a robust response, with both up-regulated genes (220 genes induced by more than 2-fold) and down-regulated genes (109 genes). The induced genes included several involved in the synthesis and utilization of GSH and other sulfhydryl-reactive anti-oxidants (thioredoxin) and their regulation, including *gclc*, *gclm*, glutathione synthase (*gss*), GSH reductase (*gr1*), gamma-glutamyl transferase (*ggt1a*), and cystathionine beta-synthase (*cbsb*). There was substantial overlap (10/15) between genes induced in these larvae and a set of marker genes induced by oxidative stress in mammals (Table 2), demonstrating the evolutionary conservation in the oxidative stress response.

Developmental expression of nrf genes

How the expression of *nrf* genes and Nrf proteins varies during development is not fully understood, but current data suggest that there are substantial differences in developmental patterns (ontogeny), levels, and localization. Expression of *nrf2a* mRNA as assessed by RT-PCR is low in early embryos (≤24 hpf), but increases substantially from 2- to 7-dpf [268]. A

similar pattern was seen using quantitative RT-PCR [234] and microarray [240]. Interestingly, microarray data suggest that *nrf3* mRNA is maternally loaded and that levels of *nrf3* expression through the first two days of embryonic development are much greater (by several orders of magnitude) than those of any other *nrf* gene [240].

Localization of *nrf* gene expression also varies by gene and developmental stage. As noted earlier, zebrafish *nfe2* is expressed in erythroid cells and in the developing ear [239]. The expression of *nrf2a* mRNA at 24 hpf is restricted to the olfactory system, but by 5-dpf this gene is more widely expressed, appearing also in gill, liver, and intestine [266]. Expression of *nrf2a* mRNA is also seen in the exocrine pancreas at 84 hpf [250]. The expression of *nrf3* mRNA is localized to fore, mid- and hindbrain and pectoral fin buds between 24- and 36-hpf [240]. The localization of *nrf1* paralogs and *nrf2b* is not known. These patterns of tissue-, cell- and developmental stage-specific expression suggest that there are specific and varying needs for regulating redox status, and also that there are target cells, tissues, or stages that may be more susceptible to non-physiological oxidant challenge; neither of these is well understood.

Functional assessment of Nfe2-related genes in development and developmental toxicity

Loss-of-function approaches to define the roles of Nrf proteins

Measurements of gene expression—whether of nrf genes themselves or their putative target genes—provide intriguing associations but do not reveal regulatory relationships or mechanisms. In contrast, loss-of-function experiments in which the expression or function of a protein is reduced or eliminated can provide valuable mechanistic information about the role of that protein. The primary approach used so far to elucidate the functional roles of Nrf proteins in zebrafish embryos has been knock-down of protein expression using morpholino-modified anti-sense oligonucleotides (MOs) [175]. Although questions have arisen recently about the use of the MO approach to identify functions of novel genes [198-200], MO-based knock-down remains a valuable method for testing specific hypotheses about the role of transcription factors in regulating putative target genes.

Several groups have used MO-mediated knock down of Nrf2a to assess the role of this protein in the regulation of gene expression in zebrafish embryos in response to oxidant exposure. Some of these results are summarized in Table 3. These studies show that a number of oxidant-inducible genes (and at least one repressed gene) are under control of Nrf2a; the genes include some well-known Nrf2 targets such as *gstp1*, *gclc*, *hmox1*, and *prdx1*. However, other oxidant-inducible genes do not appear to be regulated via Nrf2a **(**Table 3**)**, suggesting that other transcription factors may be involved in controlling their induction.

A recent study [244] compared the tBHQ-induced gene expression profiles in 2-dpf zebrafish embryos in which either Nrf2a or Nrf2b had been knocked down. Knockdown of Nrf2a or Nrf2b blocked some but not all of the tBHQ-induced changes in gene expression and the effects of Nrf2a-MO and Nrf2b-MO were distinct. The results indicated that Nrf2 paralogs regulate distinct gene sets, with some overlapping targets, in response to oxidative

stress in embryos. This study also highlighted the importance of gene down-regulation as a component of the oxidative stress response during embryonic development.

Much less is known about the role of other Nrf proteins, including the Nrf1 paralogs and Nrf3, in regulating specific genes in response to oxidative stress. Studies in mammals (e.g. [269, 270]) suggest that much will be learned by defining the sets of genes regulated by each Nrf protein in embryos. In addition, other redox-sensitive transcription factors may be involved in regulating the response to oxidant exposure (see below).

In addition to regulating the *induction* (or repression) of oxidant-responsive genes, Nrf proteins are likely to be involved in the regulation of *constitutive* (basal) expression of some genes. For example, Timme-Laragy *et al.* [234] performed gene expression profiling of Nrf2a-MO-injected embryos and Nrf2b-MO-injected embryos and found that Nrf2a and Nrf2b regulate distinct but partially overlapping gene sets even in the absence of chemical exposure. In Nrf2a-morphants, 198 probes were up-regulated, whereas 310 were down-regulated as compared with embryos injected with the control-MO. 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.

The loss-of-function caused by MO-based knock-down is partial and temporary. Thus, while MO experiments can help to identify functions during early development, they may miss functions occurring later or that can proceed in the presence of reduced Nrf protein expression. A more powerful approach to defining gene function is to generate null mutants in which the expression of a gene or function of its encoded protein is completely eliminated. While such an approach is common in mice and has been applied extensively to understand the role of murine Nrf proteins (see above), application of this approach to understand Nrf function in zebrafish is in its infancy.

Two point mutants of zebrafish *nrf2a* have been generated using the approach known as TILLING (Targeting Induced Local Lesions in Genomes [274, 275]). Each of these mutants (nfe2l2^{fh318} (R485L) and nfe2l2^{fh319} (N494I)) possesses a single amino acid change in a conserved residue in the basic region near the C-terminal end of the Nrf2 protein [243]. Kobayashi and colleagues [243] found that the protein encoded by the nfe2l2^{fh319} mutant retained activity, but that the transcriptional activity of the protein encoded by the nfe2l2^{fh318} allele was greatly reduced. Homozygous nfe2l2^{fh318} mutant fish were viable and fertile, similar to what is seen in Nrf2-knock-out mice [126]. However, mutant fish were more sensitive to the toxicity of H_2O_2 and other peroxides and certain electrophilic compounds, but not to some other oxidants [243]. The mechanism responsible for this increased sensitivity may involve a reduction in the ability to mount an oxidative stress response. Consistent with this, the induction of *gstp1*, *prdx1*, *txn1*, and *gclc* by H_2O_2 was greatly reduced in homozygous mutant larvae as compared to wild-type larvae [243].

The nfe2l2^{fh318} fish are also being used by other groups [273, 276, 277] to explore the role of *nrf2a* in protection against chemicals that cause oxidative stress.

Chemical specificity: patterns and mechanisms

A variety of chemicals can disrupt redox balance by generating oxidants or reacting with sulfhydryl groups, and thereby activate an oxidative stress response. There are questions about the mechanisms by which these chemicals activate this response in embryos. For example, do they act through Nrf2 or other Nrf proteins? Do chemicals that act by different mechanisms produce the same gene expression response?

A number of genes that are induced by oxidant exposure are shared in zebrafish and humans, and in a given species exposed to different oxidants [236, 237, 266, 267]. A recent study [278] examined the set of oxidant responsive genes that is induced by structurally distinct activators of the oxidative stress response in developing zebrafish. Zebrafish larvae (4 dpf) were exposed to tBHQ, tBOOH, diquat (DQ), or sulforaphane (SFN)) and gene expression was measured 6 hr later by microarray and Q-RT-PCR. The compounds caused overlapping but distinct patterns of altered gene expression. A core set of genes responded to all oxidants. However, other genes exhibited oxidant-specific changes in expression. Principal components analysis revealed that the changes in gene expression caused by SFN, a sulfhydryl-reactive agent, were distinct from those produced by the other oxidants. The results demonstrate that the oxidative stress response in developing animals is dependent upon the nature of the oxidative stress.

Kobayashi and colleagues [268, 279, 280] have begun to elucidate some of the mechanisms by which distinct responses might be produced for different chemicals. Their studies of this “multiple sensing mechanism” have identified six classes of inducers based in part upon which cysteines in Keap1 are targeted for oxidation. Additional mechanisms may involve activation of different Nrf paralogs. For example, MO knockdown studies in zebrafish embryos have shown that Nrf2a and Nrf2b regulate distinct gene sets, with only a small number of overlapping target genes [244]. The roles of other Nrf proteins—including Nrf1a, Nrf1b, and Nrf3—in regulating the response of developing animals to oxidative stress are completely unknown. Beyond the Nrf gene family, there are other redox-sensitive transcription factors such as AP-1, NFκB, p53, and HIF [281, 282] that may be involved in generating chemical-specific effects.

Conclusions and Future Directions

Regulation of constitutive and inducible defenses against oxidative stress by Nfe2-related factors is an evolutionarily conserved feature of animals. Conservation is especially evident among vertebrate animals, which share Nfe2, Nrf1, Nrf2, and Nrf3, as well as a core set of genes that respond to oxidative stress [227, 234, 237]. This conservation contributes to the value of zebrafish as a model system with which to investigate the mechanisms involved in regulation of redox signaling and the response to oxidative stress during embryo-larval development. Studies in zebrafish have revealed *nrf* and *keap1* gene duplications that provide an opportunity to dissect multiple functions of vertebrate *NRF* genes, including multiple sensing mechanisms involved in chemical-specific effects.

Important questions about the regulation of the oxidative stress response in developing animals remain to be answered. We highlight three areas where substantial progress can be

expected. One concerns the role of different Nrf homologs and paralogs in such regulation, and how it may vary by cell type and stage of development. Understanding the roles of Nrf proteins will be facilitated by gene-targeting and genome-editing approaches such as CRISPR-Cas9 [196, 197]. This method, first used for gene inactivation in 2013, has now exploded and is being used for a variety of applications, including single-nucleotide genome editing [283, 284]. This is a transformative technology that is dramatically enhancing our ability to manipulate systems to better understand gene function. It is possible, for example, to introduce known human polymorphisms into zebrafish *nrf* or *keap1* genes to better understand how they affect Nrf protein function during development.

Another important goal is to understand the localized generation of oxidants and oxidative stress and the consequent responses. Use of transgenic technologies [158, 285] will facilitate new insight into the cell and tissue specificity of the oxidative stress response in embryos. Initial studies have begun to address such questions, using molecular probes to localize oxidative stress [7] and transient [228, 286] and germ-line transgenics [279, 287] to measure the response.

A third topic of great interest and where progress should be substantial in the near future is cross-talk between Nrf signaling pathways and other stress-response and developmental signaling pathways. One example is the aryl hydrocarbon receptor (AHR) signaling pathway, involved in both developmental processes and the response to endogenous and exogenous chemicals. Interactions between AHR and NRF signaling occur in mammals [288-291] and recent studies suggest that such interactions also occur in zebrafish embryos [234, 277, 292]. In addition to the AHR pathway, there are several other signaling pathways that engage in cross-talk with Nrf signaling in mammalian systems. Some of these pathways include those involving Hsf1, Notch, NFκB, PPARγ/RXR, and the unfolded protein response [93, 293-298]. Whether any of these interact with Nrf signaling during development remains to be determined.

Although substantial progress has been made in using the zebrafish embryo system to understand the role of Nrf proteins in regulating the oxidative stress response in developing animals, much remains to be done. The next few years will be an exciting time as new approaches are applied in novel ways to address longstanding questions about Nrf function during development.

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Highlights

- The zebrafish is a valuable model for studying developmental roles of Nrf proteins.
- Zebrafish and mammals share a core set of oxidative stress response genes.
- Zebrafish and mammals share four types of NRF family transcription factors.
- Zebrafish *nrf* gene duplicates can help elucidate pleotropic roles of Nrf proteins.
- Loss-of-function approaches can reveal Nrf target genes in embryos.

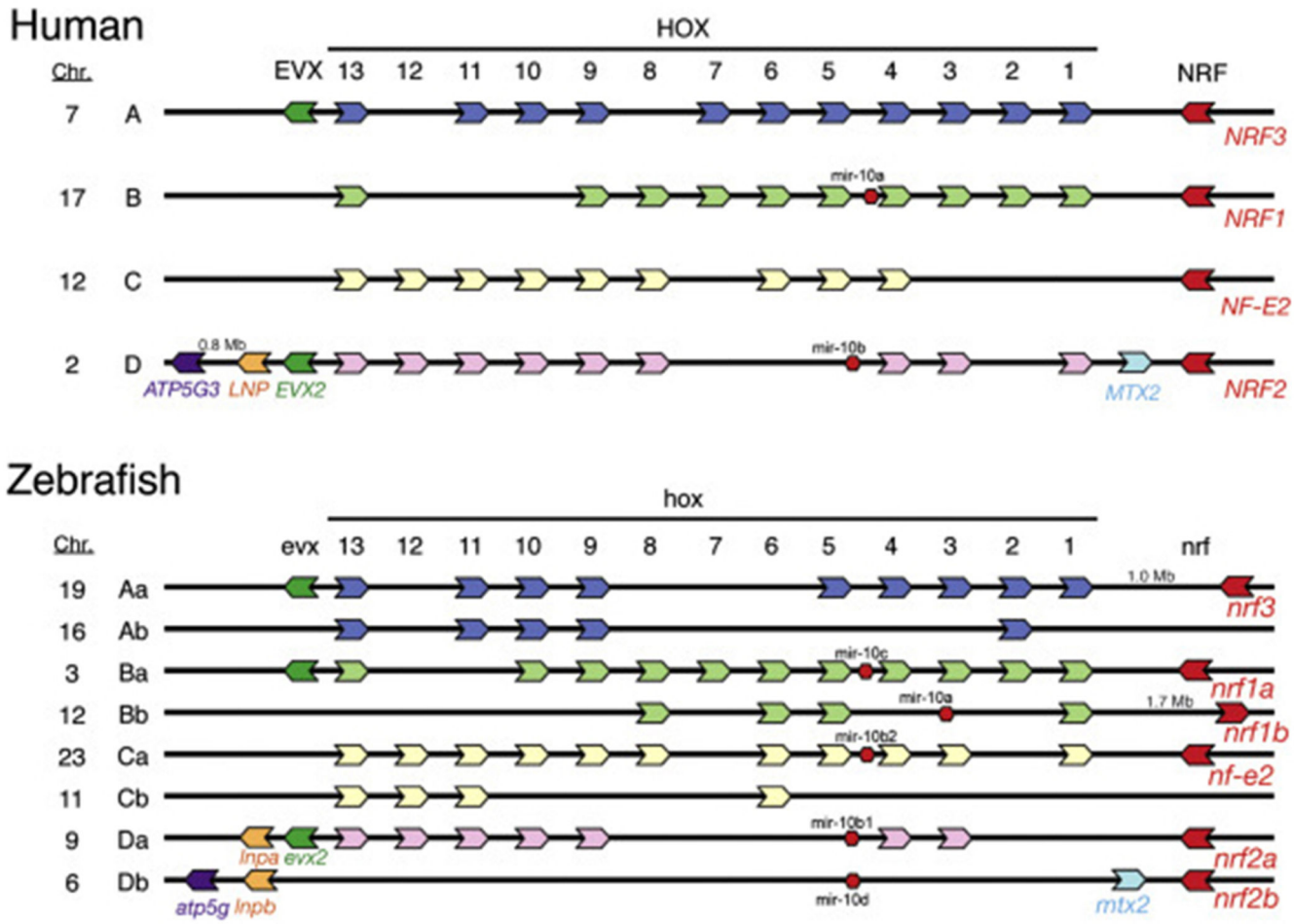

Fig. 1. Comparative genomics of NFE2-related genes in zebrafish

Location and shared synteny involving NFE2-related genes and HOX genes in the human genome (top) and zebrafish genome (bottom). This research was originally published in The Journal of Biological Chemistry. Timme-Laragy *et al.*, Nrf2b, Novel Zebrafish Paralog of Oxidant-responsive Transcription Factor NF-E2-related Factor 2 (NRF2). *The Journal of Biological Chemistry*. 2012; Vol. 287: 4609-4627. [234] © the American Society for Biochemistry and Molecular Biology.

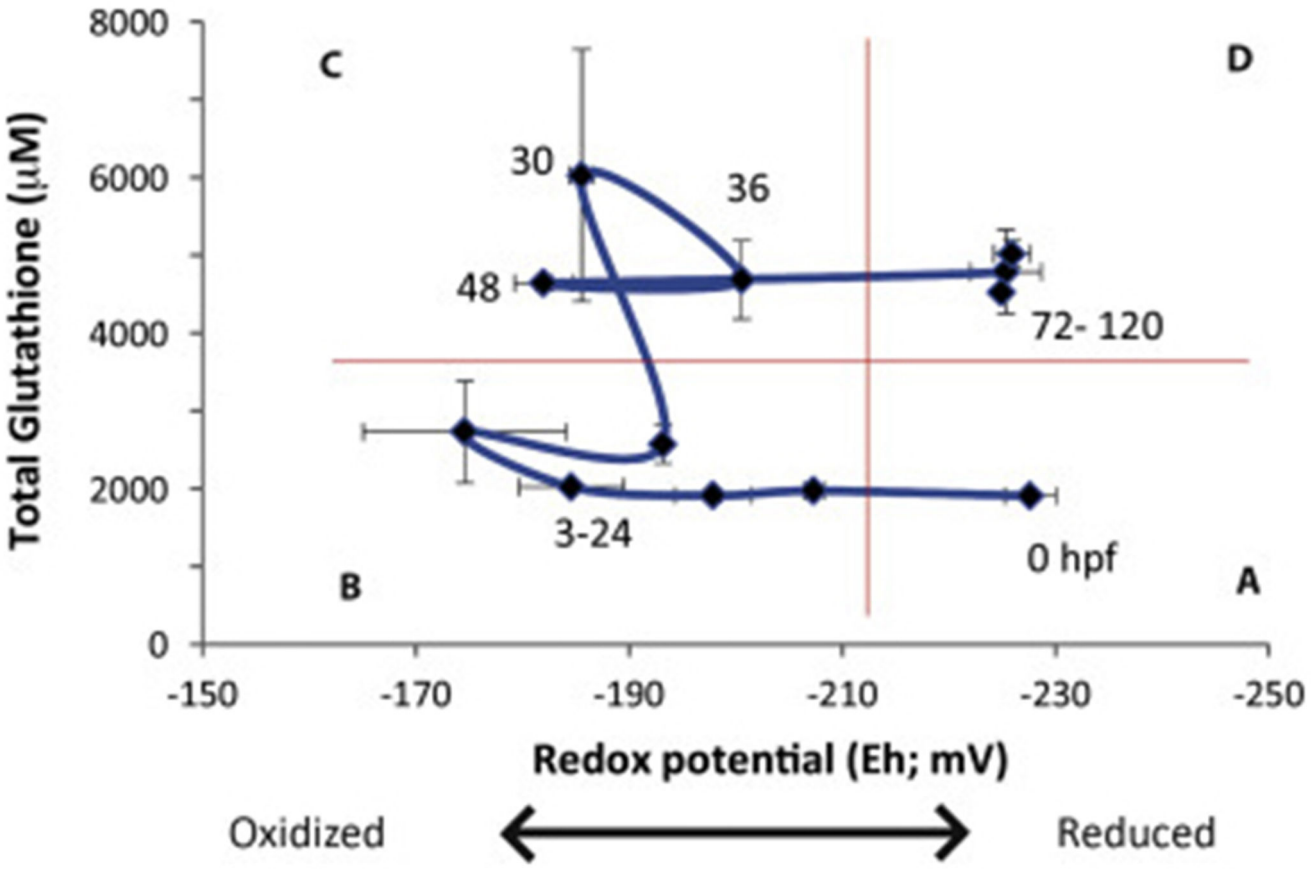

Fig. 2. Concentrations of GSH_T and E_h during the first 120 hours of zebrafish development

Four "windows" of dynamic glutathione conditions during embryonic development are observed. A) reduced E_h and low GSH_T, observed in mature oocytes; B) oxidized E_h and low GSH_T, observed in embryos from the mid-blastula transition through somitogenesis (3-24 hpf); C) oxidized E_h and high GSH_T, observed in embryos undergoing organ differentiation (30-48 hpf), and D) reduced E_h and high GSH_T, observed in post-hatch eleutheroembryos. Reproduced, with permission, from Timme-Laragy *et al.* [263].

Table 1

nfe2-related genes in zebrafish

gene[a]	protein	Expression during development	Morphant phenotype	Mutants available	References
nfe2	Nfe2	yes; localized	swim bladder, otic vesicles	no	[234, 239, 240]
nrf1a (*nfe2l1a*)	Nrf1a	yes	none[b]	no	[234, 240]
nrf1b (*nfe2l1b*)	Nrf1b	yes	none	no	[234, 240]
nrf2a (*nfe2l2a*)	Nf2a	yes, localized; increasing 0-5 dpf	gene expression	nfe2l2^{fh318} (R479L)	[227, 234, 240, 243, 244]
nrf2b (*nfe2l2b*)	Nrf2b	yes	gene expression	no	[234, 240, 244]
nrf3 (*nfe2l3*)	Nrf3	high	none	no	[234, 240]

Notes:

[a] Commonly used gene name is given. When different from the common name, the official gene name is provided in parentheses.

[b] None = no phenotype detected so far, but phenotypes may be revealed upon further analysis.

Table 2
Genes induced by exposure to tBHQ: Comparison of mammals and zebrafish

Marker gene in mammals	Zebrafish (co)-orthologs	tBHQ / DMSO
HSP70 1A, 1B, 6	**hsp70 (Chr.3)**	**52.3**
	hsp70l (Chr.8)	**15.5**
HSP70 9B	hspa9 (Chr.14)	1.2
HSP90 1 alpha	hsp90a (Chr.20)	1.2
	hsp90a2 (Chr.20)	**10.4**
DnaJ(Hsp40) B1	dnajb1a (Chr.3)	1.4
	dnajb1b (Chr.1)	**10.5**
NAD(P)H quinone oxidoreductase-1 (NQO1)	nqo1	0.7
Glutamate-cysteine ligase, modifier subunit	**gclm**	**4.1**
Thioredoxin (TXN)	**txn1 (Chr.7)**	**5.5**
	txn2 (Chr.1)	0.8
Thioredoxin reductase-1	**txnrd1**	**1.6**
Glutathione reductase	**glutathione reductase 1**	**3.8**
Ferritin, heavy polypeptide-1	fth1 (Chr.7)	1.0
	ferritin-like (Chr.3)	**6.8**
	ferritin-like (Chr.25)	0.7
Ferritin light polypeptide	ferritin L	0.9
Carbonyl reductase-1	cbr1	1.1
Phosphogluconate dehydrogenase	**pgd**	**2.5**
Sequestosome-1	**sqstm1**	**7.6**
Ubiquitin thioesterase	usp4	1.0

A set of 20 candidate genes, based on the multiple data sets of genes responding to oxidative stress in mammalian cells, was compiled by Johnson and colleagues, as listed in Table 2 of Li *et al.* [267]. Here, the 20 mammalian candidate genes have been collapsed into 15 sets based on orthologous or co-orthologous relationships with zebrafish genes and including only those genes with orthologs represented on the Agilent zebrafish microarray. Of these 15 genes, 10 have at least one co-ortholog induced by tBHQ in 4-dpf zebrafish (indicated in **bold** type and green shading), whereas 5 are not induced under these conditions. This table is modified from Table 1 of Hahn *et al.* [237]; use is permitted by the Creative Commons Attribution license.

Table 3

Use of MO-mediated knock-down to determine Nrf-dependence[a] of altered gene expression in oxidant-exposed zebrafish embryos

Gene	Inducer	Age	Nrf2a-dependence?	Reference
bcat1	DEM	4 dpf	Yes	[266]
ferric-chelate reductase 1	DEM	4 dpf	Yes	[266]
ferritin heavy chain	DEM	4 dpf	Yes	[266]
gclc	tBOOH, ANF + BNF	2 dpf	Yes	[235]
gclc	$CdCl_2$	4 dpf	Yes	[271]
gpx1	tBOOH, ANF + BNF	2 dpf	Yes	[235]
gsta1	DEM	4 dpf	Yes	[266]
gstp	tBHQ	4 dpf	Yes	[227]
gstp1/gstp2	tBOOH, ANF + BNF	2 dpf	Yes	[235]
gstp	$CdCl_2$	4 dpf	Yes	[271]
gstp1	DEM	4 dpf	Yes	[266]
gstp1	tBHQ	2 dpf	Yes	[234]
hmox1	$CdCl_2$	4 dpf	Yes	[271]
hmox1	PFOS	4 dpf	Yes	[272]
mitfa	tBHQ	2 dpf	Yes[b]	[234]
peroxiredoxin 1	DEM	4 dpf	Yes	[266]
prdx1	$CdCl_2$	4 dpf	Yes	[271]
prdx1	GSNORi	54 hpf	Yes	[273]
sepw2b	DEM	4 dpf	Yes	[266]
sod1	ANF + BNF	2 dpf	Yes	[235]
sod2	ANF + BNF	2 dpf	Yes	[235]
abcc2	DEM	4 dpf	No	[266]
atf3	tBHQ	2 dpf	No	[234]
bc2	DEM	4 dpf	No	[266]
cx32.3	DEM	4 dpf	No	[266]
hsp70	tBHQ	2 dpf	No	[234]
txnrd1	DEM	4 dpf	No	[266]
ugdh	DEM	4 dpf	No	[266]

Abbreviations used: ANF: alpha-naphthoflavone; BNF: beta-naphthoflavone; DEM: diethylmaleate; GSNORi: S-nitrosoglutathione reductase (GSNOR) inhibitor N6547; PFOS: perfluorooctane sulfonate; tBHQ: *tert*-butylhydroquinone; tBOOH: *tert*-butylhydroperoxide.

[a] Nrf2a-dependence is defined as genes showing a reduction of induction (or repression) by chemical in embryos injected with Nrf2a-MO as compared to a control MO.

[b] Gene is repressed, rather than induced, by chemical treatment.