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# Genetic Evidence of an Evolutionarily Conserved Role for Nrf2 in the Protection against Oxidative Stress

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**Transcription factor Nrf2 is considered a master regulator of antioxidant defense in mammals. However, it is unclear whether this concept is applicable to nonmammalian vertebrates, because no animal model other than Nrf2 knockout mice has been generated to examine the effects of Nrf2 deficiency. Here, we characterized a recessive loss-of-function mutant of Nrf2 (*nrf2*$^{fh318}$) in a lower vertebrate, the zebrafish (*Danio rerio*). In keeping with the findings in the mouse model, *nrf2*$^{fh318}$ mutants exhibited reduced induction of the Nrf2 target genes in response to oxidative stress and electrophiles but were viable and fertile, and their embryos developed normally. The *nrf2*$^{fh318}$ larvae displayed enhanced sensitivity to oxidative stress and electrophiles, especially peroxides, and pretreatment with an Nrf2-activating compound, sulforaphane, decreased peroxide-induced lethality in the wild type but not *nrf2*$^{fh318}$ mutants, indicating that resistance to oxidative stress is highly dependent on Nrf2 functions. These results reveal an evolutionarily conserved role of vertebrate Nrf2 in protection against oxidative stress. Interestingly, there were no significant differences between wild-type and *nrf2*$^{fh318}$ larvae with regard to their sensitivity to superoxide and singlet oxygen generators, suggesting that the importance of Nrf2 in oxidative stress protection varies based on the type of reactive oxygen species (ROS).**

Oxidative stress causes damage to multiple cellular components, such as DNA, proteins, and lipids, and is implicated in various human pathological conditions, including cancer, neurodegeneration, and inflammatory diseases (37). Several proteins such as superoxide dismutase (SOD), catalase, glutathione peroxidase (Gpx), peroxiredoxin (Prdx), and the small thiol molecules glutathione (GSH) and thioredoxin (Txn), are directly involved in the removal of oxidative stress. Recent discoveries in the cellular antioxidant system gave rise to the novel concept of "indirect antioxidants," which act through the augmentation of cellular antioxidant capacity by enhancing the gene expression driven by the transcription factor Nrf2 (21, 22). Nrf2 is a basic-region leucine zipper (bZIP) transcription factor that heterodimerizes with small Maf proteins and binds to the antioxidant response element (ARE) within the regulatory region of its target genes (20, 27). A variety of cytoprotective genes that encode phase 2 detoxifying enzymes and antioxidant proteins, such as glutathione *S*-transferases (GST), NAD(P)H:quinone oxidoreductase, and glutamate-cysteine ligase, are induced by Nrf2 via ARE sequences (14). Under basal conditions, Nrf2 is constantly degraded through the ubiquitin-proteasome pathway in a Keap1-dependent manner (28, 50). Upon exposure to electrophiles or oxidative stress, Nrf2 escapes from proteasomal degradation, accumulates in the nucleus, and transcriptionally activates its target genes.

We previously isolated zebrafish homologs of Nrf2 and its regulator Keap1 genes (*nrf2*, *keap1a*, and *keap1b*) and demonstrated that the induction of cytoprotective genes by the Keap1-Nrf2 system is conserved among vertebrates (26, 30, 34). We also isolated zebrafish small Maf (*mafg1*, *mafg2*, *mafk*, and *maft*) and Pi-class GST (*gstp1* and *gstp2*) genes and determined that the Nrf2–small-Maf heterodimer binds and transactivates *gstp1* expression through a critical ARE sequence (41, 44). Using this zebrafish system, we have provided new insights into the functions of Nrf2 (26, 29, 34, 47), e.g., the identification of an evolutionarily conserved ETGE motif in Nrf2 that is necessary for its interaction with Keap1, the finding that there is more than one sensor cysteine residue in Keap1 that can sense different sorts of Nrf2-activating compounds, the isolation of several lines of mutant zebrafish that exhibit impaired responses to specific Nrf2-activating compounds, and the demonstration of tissue-specific expression of Nrf2 and its target genes. However, the physiological roles of Nrf2 in zebrafish remain unclear and need to be elucidated to understand Nrf2 functions from an evolutionary standpoint. In the present study, we isolated and characterized *nrf2* mutant zebrafish by the TILLING (targeting induced local lesions in genomes) method (32, 53). Our results revealed that *nrf2* mutant zebrafish are viable and fertile but show an increased susceptibility to oxidative stress and electrophiles.

## MATERIALS AND METHODS

**Zebrafish.** The wild-type strain (AB) and the mutant strain (*nrf2*$^{fh318}$) of zebrafish were used for the present studies. To genotype the *nrf2*$^{fh318}$ fish, genomic DNA was extracted from single embryos or larvae or amputated tail fins of adult fish by incubating the sample in extraction buffer [10 mM Tris-HCl (pH 8.2), 10 mM EDTA (pH 8.0), 200 mM NaCl, 0.5% sodium dodecyl sulfate (SDS), 800 μg/ml proteinase K (Nacalai Tesque, Kyoto, Japan)] at 55°C for more than 4 h, followed by PCR amplification using the primers: 5′-ATATTCCCATTGGTGTACCTC and 5′-AGCTTGCGC TTGCGGCAGTTCTGCGCCGCCACTTTGTTCTTGACT. The PCR products were treated with SpeI, which digests products derived from the *nrf2*$^{fh318}$ mutant allele but not the wild-type allele, and then these products

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were analyzed by polyacrylamide gel electrophoresis. Body weight and length were measured at 55 days postfertilization (dpf), after the fish were anesthetized with 100 μg/ml Tricaine (Sigma-Aldrich, St. Louis, MO).

**Identification of *nrf2* mutant zebrafish.** Genomic DNA from a library of *N*-ethyl-*N*-nitrosourea (ENU)-mutagenized zebrafish was screened for mutations in exon 5 of *nrf2*, which contains the basic region required for DNA binding (18). Among the 10 missense mutations identified in the screening, R485L, N494I, and N494K were found within the basic region (see Fig. S1 in the supplemental material). Since Arg-485 and Asn-494 are the amino acid residues that are conserved among vertebrate Nrf2 and related bZIP proteins (see Fig. S2 in the supplemental material), it was thought to be possible that these mutations would reduce the DNA binding activity of Nrf2.

To determine whether mutations in Arg-485 and/or Asn-494 affect the transactivation activity of Nrf2, the abilities of Nrf2 mutant proteins (Nrf2R485L and Nrf2N494I) to induce endogenous *gstp1* expression were examined in zebrafish embryos. Mutant or wild-type Nrf2 proteins were tagged with the FLAG epitope and overexpressed in zebrafish embryos by mRNA injection, and the expression of endogenous *gstp1* in the embryos was analyzed by reverse transcription-PCR (RT-PCR) (see Fig. S3A in the supplemental material). A significant induction of *gstp1* expression was observed in embryos overexpressing both wild-type Nrf2 and Nrf2N494I, while almost no induction was detected in Nrf2R485L-overexpressing embryos, suggesting that the mutation in Arg-485, but not Asn-494, reduced the transactivational activity of Nrf2. To exclude the possibility that the R485L mutation decreases the protein stability of Nrf2, the expression of the Nrf2R485L protein was confirmed by an immunoblot analysis using an anti-FLAG antibody (see Fig. S3B in the supplemental material). Based on these results, we chose to use the *nrf2*$^{fh318}$ mutant for further studies.

**Microinjection.** The constructs pCS2FLnrf2R485L and pCS2FLnrf2N494I were made by introducing Arg-to-Leu and Asn-to-Ile point mutations into pCS2FLnrf2 (30) by PCR. Synthetic capped RNA was made with an SP6 mMESSAGEmMACHINE *in vitro* transcription kit (Ambion, Austin, TX) using linearized DNA of the pCS2 derivatives described above. For expression in whole bodies, the RNA was injected into the yolk at the one-cell stage using an IM300 microinjector (Narishige, Tokyo, Japan).

**Chemical treatment.** For the induction experiments and survival assays, larvae were placed in culture dishes with E3 medium (13) containing diethyl maleate (DEM) (Wako, Osaka, Japan), hydrogen peroxide ($H_2O_2$) (Wako), *tert*-butyl hydroperoxide (tBHP) (Tokyo Chemical Industry, Tokyo, Japan), paraquat (Tokyo Chemical Industry), menadione (Nacalai Tesque), rose bengal (Nacalai Tesque), sulforaphane (LKT Laboratories, St. Paul, MN), acetaminophen (Tokyo Chemical Industry), methylmercuric chloride (MeHg) (Tokyo Chemical Industry), and/or buthionine sulfoximine (BSO) (Wako). In the case of survival assays, the E3 medium containing each compound was exchanged every 2 days. Both dead and surviving larvae at 5 days postexposure were collected for genotyping in the genome extraction buffer as described above.

**Gene expression analysis.** A semiquantitative RT-PCR analysis was performed using the PCR primers listed in Table S1 in the supplemental material as described previously (30). Real-time RT-PCR was performed on the ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA) using TaqMan technology as described previously (30) except that FastStart Universal Probe Master (ROX) (Roche Diagnostics, Mannheim, Germany) was used for the reagent mixture. Each PCR amplification was performed in triplicate, and the quantitative values of target mRNA were normalized to that of *ef1α* mRNA expression. The sequences of the TaqMan probes and primers used in real-time RT-PCR are listed in Table S2 in the supplemental material. Whole-mount *in situ* hybridization for *gstp1* was performed using an RNA probe transcribed from pKSgstp1N, as described previously (26). For immunoblot analysis, proteins extracted from whole embryos at 6 h after injection of FLAG-tagged Nrf2 mRNA were separated by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting using an anti-FLAG antibody (M2, peroxidase conjugate; Sigma-Aldrich) as described previously (25).

**Senescence-associated β-galactosidase (SA-β-Gal) staining.** SA-β-Gal staining was performed as described previously (23). Briefly, zebrafish larvae were fixed overnight in 4% paraformaldehyde at 4°C, washed 3 times for 1 h in phosphate-buffered saline (PBS) pH 7.4 and for a further hour in PBS pH 6.0 at 4°C, and then incubated overnight at 37°C in β-Gal staining solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ and 1 mg/ml X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside] in PBS [pH 6.0]).

## RESULTS

**Nrf2 is not essential for zebrafish development or fertility.** To elucidate the physiological roles of Nrf2 in zebrafish, *nrf2* mutant zebrafish were isolated using the TILLING method (32, 53). Among the 10 identified mutations in the screening, we chose to use the *nrf2*$^{fh318}$ mutant, which has the R485L mutation in the Nrf2 protein, since the transcriptional activity of Nrf2 was found to be greatly reduced by this mutation (see Materials and Methods; also, see Fig. S3 in the supplemental material). To examine the effects of the R485L mutation on the Nrf2 activity *in vivo*, the *gstp1* induction by Nrf2-activating compounds was analyzed in *nrf2*$^{fh318}$ larvae by whole-mount *in situ* hybridization (Fig. 1). An electrophile, DEM, and $H_2O_2$ were used as Nrf2-activating compounds. At 5 dpf, exposure to 100 μM DEM or 1 mM $H_2O_2$ for 6 h induced *gstp1* expression in the gills of wild-type (DEM, 100%, $n = 18$; $H_2O_2$, 100%, $n = 20$) and *nrf2*$^{fh318}$ heterozygous mutant (*nrf2*$^{fh318/+}$) (DEM, 95%, $n = 40$; $H_2O_2$, 90%, $n = 49$) larvae. In contrast, the induction was significantly decreased in *nrf2*$^{fh318}$ homozygous mutant larvae (*nrf2*$^{fh318/fh318}$) (DEM, 13%, $n = 23$; $H_2O_2$, 0%, $n = 17$). The results suggest that the Nrf2 activity was greatly reduced in *nrf2*$^{fh318}$ homozygous mutants.

Nrf2 knockout mice have been demonstrated to be viable and fertile (6, 19). To test whether Nrf2 is essential for zebrafish development, 96 embryos obtained from intercrosses of heterozygous *nrf2*$^{fh318}$ fish were raised to the adult stage (50 dpf), and their genotypes were determined. The distribution of genotypes was consistent with Mendelian inheritance (28.1% wild type [$n = 27$], 50.0% *nrf2*$^{fh318/+}$ [$n = 48$] and 21.9% *nrf2*$^{fh318/fh318}$ [$n = 21$]), suggesting that Nrf2 is not necessary for zebrafish development or viability, at least under normal conditions. Indeed, no differences in body weight and length were observed among the three genotypes in adult fish (Table 1). Furthermore, the fertility of *nrf2* mutant zebrafish was evaluated by natural mating using homozygous *nrf2*$^{fh318/fh318}$ males and females (data not shown). Fertilized eggs obtained from *nrf2*$^{fh318/fh318}$ homozygous parents developed normally, suggesting that Nrf2 is not required for zebrafish reproduction.

***nrf2* mutant zebrafish are hypersensitive to peroxides but not to all reactive oxygen species (ROS).** Since no differences were observed in the development and viability between *nrf2*$^{fh318/fh318}$ and wild-type zebrafish under normal conditions, we next tested their sensitivity to oxidative stress. Larvae at 4 dpf were treated with $H_2O_2$, one of the major ROS, and their survival was analyzed using the Kaplan-Meier method (Fig. 2A). After treatment with 1 mM $H_2O_2$, more than 70% of wild-type larvae and about 60% of *nrf2*$^{fh318/+}$ larvae, which were slightly weaker than wild-type larvae, had survived for 5 days. In contrast, more than 80% of *nrf2*$^{fh318/fh318}$ larvae were dead within 24 h after $H_2O_2$ treatment. This result indicates that the resistance of the larvae to oxidative stress was reduced by the Nrf2 mutation.

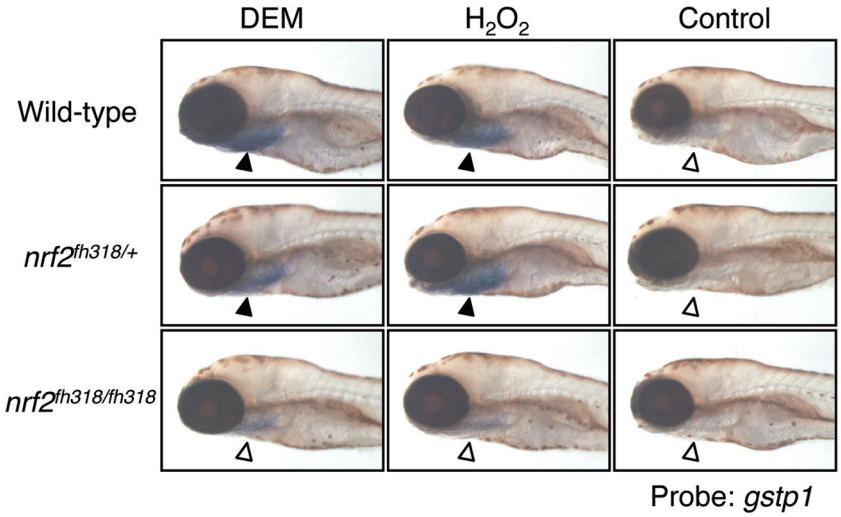


**FIG 1** *gstp1* expression in *nrf2*$^{fh318}$ mutant larvae. At 5 dpf, larvae were treated with 100 μM DEM or 1 mM $H_2O_2$ for 6 h, and their *gstp1* expression was analyzed by whole-mount *in situ* hybridization. Lateral views, with the anterior side on the left, are shown. Black and white arrowheads indicate positive and negative expression in gills, respectively.

To examine the effects of the Nrf2 mutation on the sensitivity of the larvae to different types of ROS, survival assays were carried out using organic hydroperoxide tBHP, the superoxide generators paraquat and menadione, and a singlet oxygen generator, rose bengal (Fig. 2B to E). As with $H_2O_2$, more than 80% of *nrf2*$^{fh318/fh318}$ larvae were dead within 36 h after 0.8 mM tBHP treatment, while about 70% of both wild-type and *nrf2*$^{fh318/+}$ larvae survived for 96 h (Fig. 2B). Approximately 80% of *nrf2*$^{fh318/fh318}$ larvae treated with 0.6 mM paraquat were dead after 72 h, while 80% of wild-type and 70% of *nrf2*$^{fh318/+}$ larvae survived (Fig. 2C). The survival rate of larvae treated with 2.4 μM menadione or 0.1 μM rose bengal was not significantly different among the different genotypes (Fig. 2D and E). These results indicate that *nrf2* mutant zebrafish were hypersensitive to the peroxides, $H_2O_2$ and tBHP, moderately sensitive to paraquat, and not sensitive to menadione and rose bengal, thus suggesting that the effects of the Nrf2 mutation in zebrafish vary for the different types of ROS.

Since Nrf2 is also important in protection against electrophiles in mammals (21, 22, 28), sensitivity of Nrf2 mutant zebrafish to electrophiles or their metabolic precursors was examined (Fig. 2F to H). The survival rate of *nrf2*$^{fh318/fh318}$ larvae exposed to the analgesic acetaminophen (7.5 mM) was 32% at 48 h postexposure, while that of wild-type larvae was 84%. Similarly, more than 90% of *nrf2*$^{fh318/fh318}$ larvae were dead within 48 h after the treatment of the isothiocyanate sulforaphane (80 μM), while about 80% of wild-type larvae survived. In the case of the heavy metal MeHg (0.1 μM), 23% of wild-type larvae, but only 4% of *nrf2*$^{fh318/fh318}$ larvae, survived for 96 h after the treatment. The survival rate of *nrf2*$^{fh318/+}$ larvae was intermediate between those of wild-type and *nrf2*$^{fh318/fh318}$ larvae in all three cases. These results suggest that the resistance to toxic doses of electrophiles was also reduced by the Nrf2 mutation in zebrafish, consistent with the findings from mammalian studies (7, 10, 15, 46).

**TABLE 1** Body weights and lengths of 55-day-old *nrf2* mutant zebrafish[a]

| Genotype | Wt (g) | Length (cm) |
|---|---|---|
| Wild type | 0.24 ± 0.07 | 2.79 ± 0.26 |
| *nrf2*$^{fh318/+}$ | 0.22 ± 0.10 | 2.63 ± 0.48 |
| *nrf2*$^{fh318/fh318}$ | 0.23 ± 0.12 | 2.56 ± 0.72 |

[a] Data are means ± standard deviations.

To understand how Nrf2 is associated with peroxide sensitivity, we examined the $H_2O_2$-induced expression of 25 genes by semiquantitative RT-PCR in 5-dpf larvae which encoded ROS-scavenging enzymes [SOD (*sod1* to *sod3*), catalase (*cat*), Gpx (*gpx1* to *gpx8*), Prdx (*prdx1* to *prdx6*)], GSH-generating enzymes [glutamate-cysteine ligase (*gclc* and *gclm*), and GSH synthetase (*gss*)], Txn (*txn1* and *txn2*), and Txn reductase (*txnrd1* to *txnrd3*) (Fig. 3A; also, see Fig. S4 in the supplemental material). $H_2O_2$-induced expression of *prdx1*, *txn1*, and *gclc* was downregulated in *nrf2*$^{fh318/fh318}$ larvae, which may be responsible for the increased sensitivity to peroxides. No differences were observed between the wild-type and *nrf2*$^{fh318/fh318}$ larvae regarding their expression of SOD, which scavenges superoxide, and the other tested genes. Unexpectedly, the basal expression of *gpx1b* was upregulated in the *nrf2*$^{fh318/fh318}$ larvae. The expression profiles of *prdx1*, *txn1*, *gclc*, *sod1*, *sod2*, *sod3*, and *gpx1b* were confirmed by quantitative real-time RT-PCR (Fig. 3B). Gpx1 is a selenoprotein whose loss has been demonstrated to be counterbalanced by activation of the Nrf2 pathway (42). Likewise, it is possible that inactivation of Nrf2 may be compensated for, at least in part, by the accumulation of selenoproteins.

**Administration of an Nrf2 activator increases Nrf2-dependent protection against $H_2O_2$.** Sulforaphane, an isothiocyanate originally isolated from broccoli sprouts, is a potent Nrf2-activating compound, and administration of nontoxic doses of sulforaphane has been demonstrated to inhibit chemically induced cancer in mice (11, 54, 55). Since sulforaphane has also been demonstrated to activate Nrf2 in zebrafish (29), we hypothesized that the mortality of peroxide-treated larvae would be reduced by sulforaphane administration through activation of Nrf2. To test this possibility, the effects of sulforaphane pretreatment on the survival of $H_2O_2$-treated larvae were examined (Fig. 4A to C). A sul-

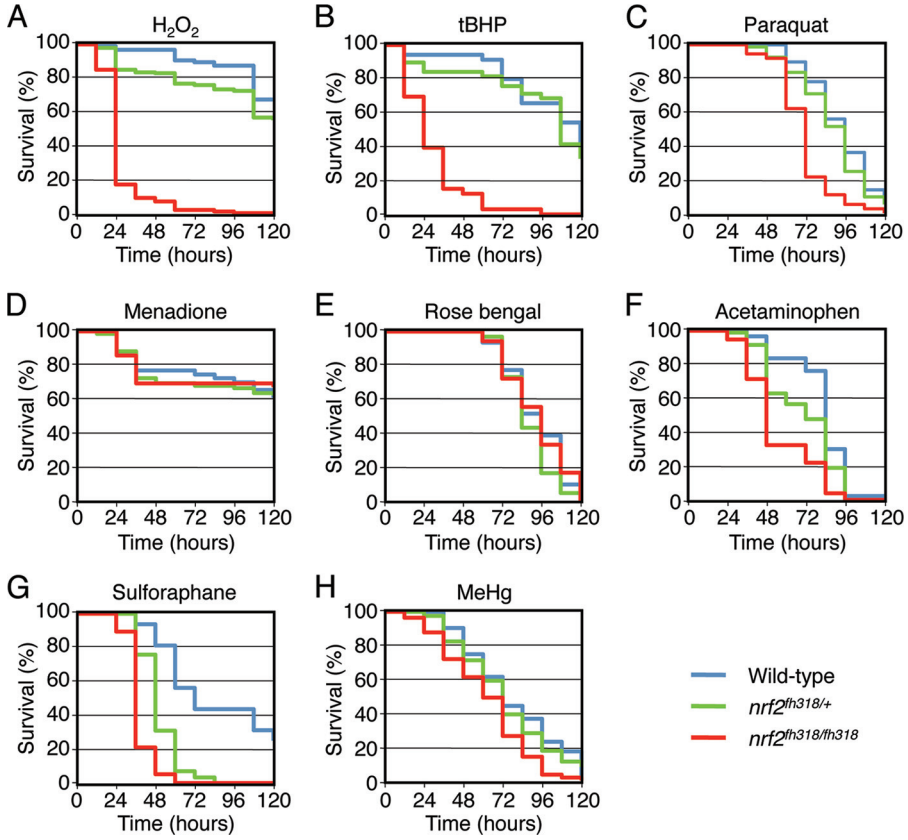


**FIG 2** Survival rate of *nrf2*$^{fh318}$ mutant larvae after exposure to oxidative stress-inducing agents, electrophiles, or their metabolic precursors. At 4 dpf, larvae were exposed to 1 mM $H_2O_2$ (A), 0.8 mM tBHP (B), 0.6 mM paraquat (C), 2.4 μM menadione (D), 0.1 μM rose bengal (E), 7.5 mM acetaminophen (F), or 80 μM sulforaphane (G) for 120 h or to 0.1 μM MeHg for 24 h, followed by a change to fresh medium without MeHg for an additional 96 h (H), and the survival rates were calculated using the Kaplan-Meier method. The data from at least three experiments were combined.

foraphane concentration of 40 μM was used for the analysis because no toxicity was observed in wild-type or *nrf2*$^{fh318/fh318}$ larvae at this concentration (data not shown). After 24 h of 1.5 mM $H_2O_2$ treatment, the survival rates of wild-type and *nrf2*$^{fh318/+}$ larvae were 13.9% and 7.4%, respectively, while 70.5% of the wild-type and 29.5% of *nrf2*$^{fh318/+}$ larvae survived when sulforaphane was used for the pretreatment. In contrast, all *nrf2*$^{fh318/fh318}$ larvae were dead within 24 h after the 1.5 mM $H_2O_2$ treatment, and no survivors were observed even when sulforaphane was administered. These results indicate that the sulforaphane pretreatment reduced the lethality of $H_2O_2$ in wild-type larvae and partially reduced it in *nrf2*$^{fh318/+}$ larvae but did not reduce it in *nrf2*$^{fh318/fh318}$ larvae. The same result was obtained when the $H_2O_2$ concentration was increased to 2.0 mM (see Fig. S5 in the supplemental material). RT-PCR analysis indicated that *prdx1*, *txn1*, and *gclc* were upregulated by sulforaphane in an Nrf2-dependent manner as in the case of $H_2O_2$, suggesting that the induction of these genes may mediate antioxidant effects of sulforaphane (see Fig. S6 in the supplemental material).

To assess the contribution of GSH to the protective action of sulforaphane, the effect of BSO, a specific inhibitor of GSH biosynthesis (3), on the survival of 1.5 mM $H_2O_2$-treated wild-type AB larvae was examined (Fig. 4D and E). Without BSO pretreatment, the survival rate after 120 h of $H_2O_2$ treatment increased from 33% ($n$ = 58) to 84% ($n$ = 64) as a result of sulforaphane administration, while this sulforaphane-dependent increase was reduced to 39% ($n$ = 54) when 1 mM BSO was administered as pretreatment for 24 h, suggesting that the accumulation of GSH may be one of the main factors of the antioxidant activity of sulforaphane, consistent with the findings in mammalian cells (31).

## DISCUSSION

In the present study, we generated and characterized *nrf2* mutant zebrafish by a reverse genetics approach. This was the second animal model used to examine the effects of Nrf2 deficiency since Nrf2 knockout mice were generated more than 15 years ago (6, 19). The present study demonstrated that (i) Nrf2 has a physiological role in protection against oxidative stress and electrophiles in zebrafish, and (ii) Nrf2 is not essential for the normal development and reproduction of zebrafish. The protective role of Nrf2 against oxidative stress and electrophiles in mammalian cells has been reported (15), suggesting that the function of Nrf2 is conserved in vertebrates. The latter conclusion is also consistent with that of the study using Nrf2 knockout mice, which showed no discernible difference in their development compared with wild-type mice (6, 19). These characteristics of the vertebrate Nrf2-deficient mutants are different from those of the invertebrate mutants of the Nrf2-related factors Cnc and Skn-1, in fruit flies and nematodes, respectively, which both showed abnormal development (5, 33). Nrf2, Cnc, and Skn-1 seem to have evolved from a

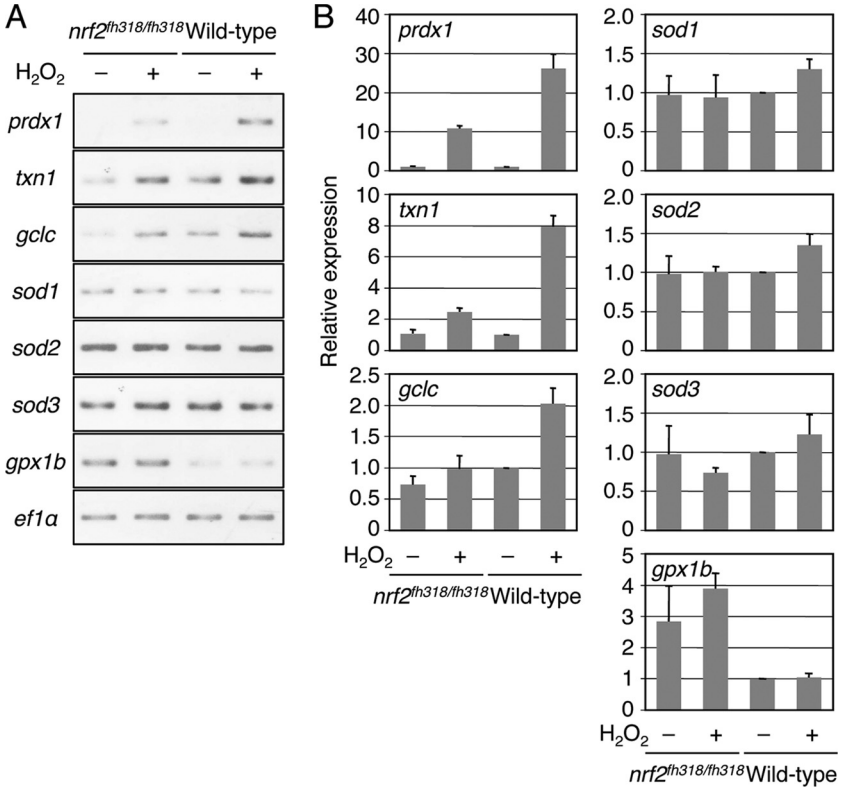


**FIG 3** $H_2O_2$-dependent induction of ROS-scavenging enzyme genes. A semiquantitative RT-PCR (A) and quantitative real-time RT-PCR (B) analyses of indicated genes were performed using total RNA mixtures from whole bodies of 20 *nrf2*$^{fh318/fh318}$ or wild-type larvae treated with 1 mM $H_2O_2$ for 6 h at 5 dpf or not treated. The expression of *ef1*α was used to standardize the amount of cDNA. Error bars indicate standard deviations from three independent experiments.

common ancestor, although Nrf2 appears to have lost its function as a morphogenic factor, while Cnc and Skn-1 retained this function. It has been speculated that the defense against oxidative stress may have become more important for vertebrates and that Nrf2 evolved as a specialized factor for this function.

The *nrf2* mutant zebrafish showed an increased susceptibility to the peroxides $H_2O_2$ and tBHP and lower expression levels of *prdx1*, *gclc*, and *txn1*. It has been reported that in mammalian cells, defense against $H_2O_2$ toxicity is achieved by Prdx1 (4, 35), glutamate-cysteine ligase (9, 39), and Txn (48). Therefore, the downregulation of these genes may be responsible for the increased sensitivity of *nrf2* mutant zebrafish to peroxides. Unlike peroxides, there were moderate or no significant differences in the sensitivity of *nrf2* mutant zebrafish to the superoxide generators paraquat and menadione. It has been reported that SOD plays a role in the defense against paraquat and menadione in mice and cultured cells (8, 16, 17). Since the expression levels of SOD in *nrf2* mutant zebrafish were equivalent to those of wild-type fish, and the protective capacity of *nrf2* mutant zebrafish against superoxide generators is comparable to that of the wild-type, so SOD may not be direct targets of Nrf2. It has been demonstrated that embryonic fibroblasts derived from Nrf2-deficient mice are hypersensitive to $H_2O_2$ and tBHP but not to paraquat and menadione (15), thus suggesting that resistance to peroxides in vertebrates is more highly dependent on Nrf2 function than resistance to superoxide generators. On the other hand, it has been demonstrated that Cnc and Skn-1 contribute to the survival of *Drosophila* and *Caenorhabditis elegans*, respectively, after exposure to paraquat (2, 43). These data imply that minor differences in the protective role against oxidative stress developed among Nrf2-related factors during evolution.

The *nrf2* mutant zebrafish can be used as a convenient animal model to study environmental science as well as the clinical, pharmaceutical, and agricultural applications of the Keap1-Nrf2 system. Zebrafish represent an attractive model for risk assessment of environmental chemicals (1, 38), and therefore, the effects of Nrf2 knockdown have been analyzed to evaluate environmental pollutions using zebrafish (40, 45), although the morpholino-based gene knockdown technique is applicable only to embryos and early-stage larvae. In this regard, the *nrf2*$^{fh318}$ line is extremely useful, since homozygous *nrf2*$^{fh318}$ mutants can develop into adults normally, which allows the long-term effects of environmental chemicals to be examined. Aging is an another important topic in the Nrf2 field. Cnc and Skn-1 have been reported to play a critical role in the regulation of life span in flies and worms, respectively (2, 43), but the roles of Nrf2 in vertebrate longevity have not been sufficiently investigated (36). Recent studies have shown that the zebrafish is a potentially valuable model for aging research (12, 24). The relationship between longevity and Nrf2 functions can be analyzed using the *nrf2*$^{fh318}$ mutants examined in this study. Since there were no differences in SA-β-Gal activity, a biomarker of cellular senescence and aging (23), between *nrf2*$^{fh318}$ mutant and wild-type larvae (see Fig. S7 in the supplemental material), further analyses using aged fish are expected. Furthermore, knowledge from zebrafish studies can be utilized for fish farming. To protect cultured fish from oxidative stress-related diseases, dietary sup-

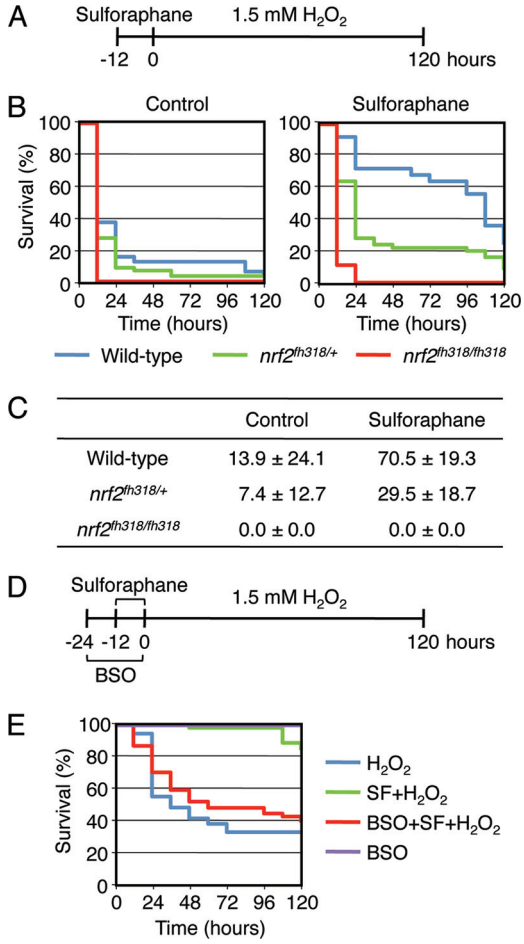


**FIG 4** Effects of sulforaphane pretreatment on the survival rate of $H_2O_2$-treated larvae. (A) Experimental scheme. After pretreatment with 40 μM sulforaphane for 12 h, 4-dpf larvae were treated with 1.5 mM $H_2O_2$ for 120 h. (B) Survival curve of $H_2O_2$-treated larvae with or without sulforaphane pretreatment. The data were combined from four experiments. (C) Survival rates of wild-type, *nrf2*$^{fh318/+}$, and *nrf2*$^{fh318/fh318}$ larvae at 24 h postexposure. Data are means ± standard deviations from four independent experiments. (D) Experimental scheme of BSO pretreatment. Wild-type AB larvae were pretreated with 1 mM BSO for 24 h before 1.5 mM $H_2O_2$ treatment at 4 dpf. (E) Survival curve of $H_2O_2$-treated larvae with or without BSO and sulforaphane (SF) pretreatment.

plementation with antioxidants, such as ascorbic acid and α-tocopherol, has been suggested (49, 51, 52). In the present study, we demonstrated that sulforaphane pretreatment increases the resistance of fish to $H_2O_2$, thus suggesting that the upregulation of Nrf2 by dietary supplementation of indirect antioxidants appears to be a highly effective strategy that could be used to ameliorate oxidative stress in farmed fish.

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