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*Research Article*

# Conservation of the Nrf2-Mediated Gene Regulation of Proteasome Subunits and Glucose Metabolism in Zebrafish

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The Keap1-Nrf2 system is an evolutionarily conserved defense mechanism against oxidative and xenobiotic stress. Besides the exogenous stress response, Nrf2 has been found to regulate numerous cellular functions, including protein turnover and glucose metabolism; however, the evolutionary origins of these functions remain unknown. In the present study, we searched for novel target genes associated with the zebrafish Nrf2 to answer this question. A microarray analysis of zebrafish embryos that overexpressed Nrf2 revealed that 115 candidate genes were targets of Nrf2, including genes encoding proteasome subunits and enzymes involved in glucose metabolism. A real-time quantitative PCR suggested that the expression of 3 proteasome subunits (*psma3*, *psma5*, and *psmb7*) and 2 enzymes involved in glucose metabolism (*pgd* and *fbp1a*) were regulated by zebrafish Nrf2. We thus next examined the upregulation of these genes by an Nrf2 activator, diethyl maleate, using Nrf2 mutant zebrafish larvae. The results of real-time quantitative PCR and whole-mount in situ hybridization showed that all of these 5 genes were upregulated by diethyl maleate treatment in an Nrf2-dependent manner, especially in the liver. These findings implied that the Nrf2-mediated regulation of the proteasome subunits and glucose metabolism is evolutionarily conserved among vertebrates.

## 1. Introduction

The Keap1-Nrf2 system is a mechanism that protects cells against xenobiotic and oxidative stress. Under conditions of stress, transcription factor Nrf2 transactivates a wide array of genes, which perform a range of functions, including (but not limited to) the encoding of antioxidant proteins, glutathione-conjugating enzymes, and xenobiotic transporters. This confers an inducible defense against stressors [1, 2]. Although these genes have been extensively studied as Nrf2 targets, there is increasing evidences to show that Nrf2 activates a wider gene set than was initially considered [3]. Nrf2 regulates the protein turnover by inducing proteasome subunits to confer protective effects against chronic diseases [4] and modifies cellular metabolic processes, for example, the pentose phosphate pathway, which provides NADPH and purine nucleotides that are essential for the redox homeostasis and cellular proliferation [5, 6]. Because of this multifunctionality, Nrf2 research has become an emerging topic in the medical field [7, 8].

The zebrafish has become a popular model vertebrate in basic medical science [9, 10]. Although it is convenient to use lower vertebrate models for medical research, there is always some concern as to whether the findings are applicable to human medicine. We have investigated the Keap1-Nrf2 system using the zebrafish as a model and revealed that the

regulation of the system is based on a similar molecular mechanism to mammals [11–15]. Moreover, an analysis of the Nrf2 mutant zebrafish strain, *nrf2a*$^{fh318}$, revealed that the protective role against oxidative and xenobiotic stress was conserved in the zebrafish [16, 17]. The lineup of the target genes in the zebrafish, which provides the defense function, is also being clarified from recent studies [18]. Our previous study [19] and the study of Hahn et al. [20] reported the performance of microarray analyses using zebrafish larvae treated with potent Nrf2 inducers, diethyl maleate (DEM) and *tert*-butylhydroquinone (tBHQ), respectively, and found that canonical Nrf2 targets, such as detoxification and antioxidative enzymes were conserved in zebrafish. However, it is still unclear whether the Nrf2-mediated regulation of cellular pathways other than antioxidation and detoxification, for example, protein turnover and glucose metabolism, is conserved among vertebrates or whether it is only present in higher vertebrates.

In the present study, we searched for novel target genes for zebrafish Nrf2 to answer this question. We performed a microarray analysis of zebrafish embryos that overexpressed Nrf2, which was a different approach from previous reports [19, 20]. The analysis revealed genes encoding proteasome subunits and enzymes involved in glucose metabolism, suggesting that the Nrf2-mediated regulation of both protein turnover and glucose metabolism is evolutionarily conserved among vertebrates.

## 2. Materials and Methods

*2.1. Zebrafish and Chemical Treatments.* The wild-type (AB) and *nrf2a*$^{fh318}$ mutant [16] zebrafish strains were used in the present study. The *nrf2a*$^{fh318}$ strain was maintained by PCR-based genotyping, as described in Fuse et al. [17]. Embryos were obtained by natural mating. For DEM treatment, the larvae were placed in 3 cm culture dishes at 4 days post-fertilization (dpf) and treated with 100 $\mu$M DEM for 6 h (Wako, Osaka, Japan). All of the experiments were performed according to methods that were approved by the Animal Experiment Committee of the University of Tsukuba.

*2.2. Microarray Analysis.* A DNA microarray analysis was performed using custom-made 16 K MZH chips (GPL14379), as described previously [19]. Biological experiments were carried out in triplicate and microarray analyses were conducted in duplicate or triplicate for each biological experiment. For the overexpression of zebrafish *nrf2a*, capped *nrf2a* RNA was synthesized from pCS2nrf2 [11] using an SP6 mMESSAGE mMACHINE in vitro transcription kit (Ambion, Austin, TX). One hundred pg of mRNA was injected into a 1-cell stage embryo by an IM300 microinjector (Narishige, Tokyo, Japan). At 8 h after injection, the embryos were collected and homogenized with QIAzol reagent (Qiagen, Hilden, Germany) and stored at −80°C. A dual-color ratio methodology was applied to compare *nrf2a* RNA-injected embryos with uninjected embryos (Control), according to the manufacturer's protocol for the AceGene DNA microarray (Hitachi Solutions, Tokyo, Japan). Total RNA was extracted according to the manufacturer's instructions for QIAzol reagent (Qiagen), in combination with the clean-up protocol of the RNeasy Mini Kit (Qiagen). Amino-allyl-modified RNA was synthesized using the amino-allyl RNA amplification kit (Sigma-Aldrich, St. Louis, MO) and labeled with monoreactive Cy3 and Cy5 dyes (GE Healthcare, Little Chalfont, UK). The hybridized MZH chips were scanned using the Affymetrix 428 array scanner (Affymetrix, Santa Clara, CA). The microarray data were processed from raw data image files with Affymetrix Jaguar (Affymetrix) and were analyzed using per-chip normalization. The processed data were subsequently imported into Excel (Microsoft, Redmond, WA) to compare expression profiles of two samples (injected versus uninjected with *nrf2a* mRNA). Genes whose expression was affected by the Nrf2a overexpression were selected based on cut-off values of >1.5-fold up or >1.5-fold down, without considering their significance. A biological pathway analysis was performed using the Reactome database (http://www.reactome.org/). We have deposited the raw data at Gene Expression Omnibus (GEO) under accession number GSE86174, and we can confirm all details are Minimum Information About a Microarray Experiment compliant.

*2.3. Real-Time PCR.* Total RNA from *nrf2a*-overexpressing embryos and DEM-treated larvae was prepared according to the procedure that was performed in the microarray analysis. cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. A quantitative PCR (qPCR) was performed using a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) with THUNDERBIRD SYBR qPCR Mix (Toyobo). The specific primers are listed in Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/5720574. The expression level of each gene was normalized to the level of *ef1α*.

*2.4. In Situ Hybridization Analysis.* A whole-mount in situ hybridization analysis was performed as described previously [21]. To construct pKSpsma3, pKSpsma5, pKSpsmb7, pKSpgd, and pKSfbp1a, PCR fragments were amplified with primers shown in Table S2 using cDNA synthesized from zebrafish larvae (4 dpf), and ligated with pBluescript II KS+ vector using a DNA Ligation Kit Ver.1 (Takara, Otsu, Japan). Plasmids were linearized by restriction enzymes (pKSpsma3, pKSpsmb7, and pKSpgd: *Bam*HI; pKSpsma5: *Eco*RI; pKSfbp1a: *Spe*I) and transcribed with T3 RNA polymerase (Roche Diagnostics, Indianapolis, IN) in the presence of DIG RNA labeling mix (Roche Diagnostics) to make RNA probes. The larvae were photographed using an MZ16 microscope (Leica, Wetzlar, Germany) equipped with a DP73 digital camera (Olympus, Tokyo, Japan), followed by PCR-based genotyping.

*2.5. Statistical Analysis.* The gene expression levels derived from the real-time PCRs were compared using the two-tailed Student's *t*-test. *P* values of <0.05 were considered to indicate statistical significance.

Table 1: The pathways activated in *nrf2a*-overexpressing embryos.

| $p$ value | Pathway name | Genes |
|---|---|---|
| $5.27E-7$ | Detoxification of reactive oxygen species | *txn*, *gstp1*, *prdx1*, *gsr*, *gpx1b*, *prdx5*, *gpx1a* |
| $5.22E-5$ | Glutathione conjugation | *gclc*, *gsto2*, *gstp1*, *gclm*, *gsta.1* |
| $6.47E-4$ | Reduction of cytosolic $Ca^{++}$ levels | *atp2b2*, *calm3a* |
| $7.86E-4$ | Apoptosis | *lmna*, *tradd*, ***psma3***, ***psmb7***, ***psma5***, *tp53bp2a*, *kpna1*, *prkcdb* |
| $1.04E-3$ | Programmed cell death | *lmna*, *tradd*, ***psma3***, ***psmb7***, ***psma5***, *tp53bp2a*, *kpna1*, *prkcdb* |
| $5.09E-3$ | Degradation of GLI1 by the proteasome | *tpk1*, ***psma3***, ***psmb7***, ***psma5*** |
| $5.09E-3$ | GLI3 is processed to GLI3R by the proteasome | *tpk1*, ***psma3***, ***psmb7***, ***psma5*** |
| $5.09E-3$ | Degradation of GLI2 by the proteasome | *tpk1*, ***psma3***, ***psmb7***, ***psma5*** |
| $5.99E-3$ | Gluconeogenesis | *pcxb*, *fbp1a*, *pck1*, *tpk1* |
| $7.01E-3$ | CaM pathway | *tpk1*, *calm3a*, *prkcdb* |

Bold genes encode proteasome subunits.

## 3. Results

*3.1. The Identification of Novel Target Genes for Zebrafish Nrf2 by a Microarray Analysis.* To search for the novel target genes of zebrafish Nrf2, we performed a microarray analysis of zebrafish embryos overexpressing Nrf2. In vitro synthesized mRNA of *nrf2a*, the functional ortholog of mammalian Nrf2 in zebrafish, was injected into 1-cell stage of zebrafish embryos, and the gene expression in the injected embryos at 8 h after the injection was examined using 16 K MZH chips, which contain 16,399 probes [19]. In total, 115 genes were found to be upregulated more than 1.5-fold by the overexpression of *nrf2a* (Table S3).

The lineup of upregulated genes in the microarray of *nrf2a*-overexpressing embryos was further analyzed using the Reactome database to determine the biological functions that could be influenced by these upregulated genes (Table 1). Besides antioxidation and glutathione conjugation, proteasome-related pathways were listed, due to the upregulation of the 3 genes encoding the proteasome subunits, *psma3*, *psma5,* and *psmb7* (proteasome subunits $\alpha 3$, $\alpha 5$, and $\beta 7$, resp.). It should be noted that none of the genes encoding the proteasome subunits, including these 3 genes, have been identified in previous microarray analyses [19, 20].

*3.2. Proteasome Subunits Were Regulated by Zebrafish Nrf2.* To validate the microarray results, we performed a real-time qPCR. *nrf2a*-overexpressing embryos were prepared in the same way as in the microarray analysis. Overexpression of *nrf2a* in mRNA-injected embryos was confirmed by a real-time qPCR (Figure S1, 75.5-fold higher compared to uninjected embryos). As shown in Figure 1(a), *psma3* was significantly induced by the overexpression of *nrf2a* (1.51-fold). *psma5* and *psmb7* also tended to be weakly induced by the overexpression of *nrf2a* (1.28- and 1.18-fold, resp.). These results suggest that, similarly to mammals [4], some of the proteasome subunit genes are targets of Nrf2 in the zebrafish.

We then tested whether the expression of 3 proteasome subunit genes is induced by DEM in an Nrf2-dependent manner by a real-time qPCR. Although DEM did not significantly induce any of proteasome subunit genes at 6 h (Figure 1(b)), *psmb7* was induced after 12 h exposure (Figure 1(c)) in wild-type larvae (1.59-fold) with statistical significance, while the induction in $nrf2a^{fh318/fh318}$ mutant (1.33-fold) was weaker than that of wild-type. *psma3* and *psma5* were also tended to be induced after 12 h exposure to DEM both in wild-type larvae (1.49- and 1.47-fold, resp.) and in $nrf2a^{fh318/fh318}$ mutant larvae (1.27- and 1.63-fold, resp.).

We speculated that the reason of this unclear Nrf2-dependency was due to ubiquitous basal expression of proteasome subunit genes. Thus, we next performed whole-mount in situ hybridization to evaluate tissue-restricted induction of the proteasome subunit genes, since many Nrf2 target genes showed gill-, liver- or nose-specific induction in zebrafish larvae [19]. As we expected, the expression of all three subunit genes was induced in the liver of wild-type and heterozygous mutant ($nrf2a^{fh318/+}$) larvae after 12 h exposure to DEM (Figure 2). This liver-specific induction was not observed in homozygous mutant siblings ($nrf2a^{fh318/fh318}$). These results suggest that Nrf2 regulates the gene expression levels of proteasome subunits in zebrafish, especially in the liver.

*3.3. Zebrafish Nrf2 Regulates Enzymes That Are Involved in Glucose Metabolism.* It is noteworthy that many high-ranked genes in the present microarray analysis were also identified in the previous microarray analyses using DEM- or tBHQ-treated larvae (see Table S3, gray highlighted genes) [19, 20]. This observation indicates that these 27 overlapping genes may be strong candidates for zebrafish Nrf2 targets (Figure 3(a), indicated genes; Table S3, gray highlighted). We therefore analyzed these genes using the Reactome database to search for novel biological pathways that are related to zebrafish Nrf2 (Table 2). As a result, the "Metabolism" pathway was listed within the top 10 categories, in addition to the pathways related to glutathione conjugation and antioxidation. Three catabolic enzymes that are involved in glucose metabolism were included in the "Metabolism" pathway: *pck1* (phosphoenolpyruvate carboxykinase 1), *pcxb* (pyruvate carboxylase b), and *pgd* (phosphogluconate dehydrogenase). Furthermore, looking back on the lineup of genes that were upregulated by the overexpression of *nrf2a* (Table S3), two more related genes were found: *taldo1* (transaldolase 1) and *fbp1a* (fructose-1,6-bisphosphatase 1a).

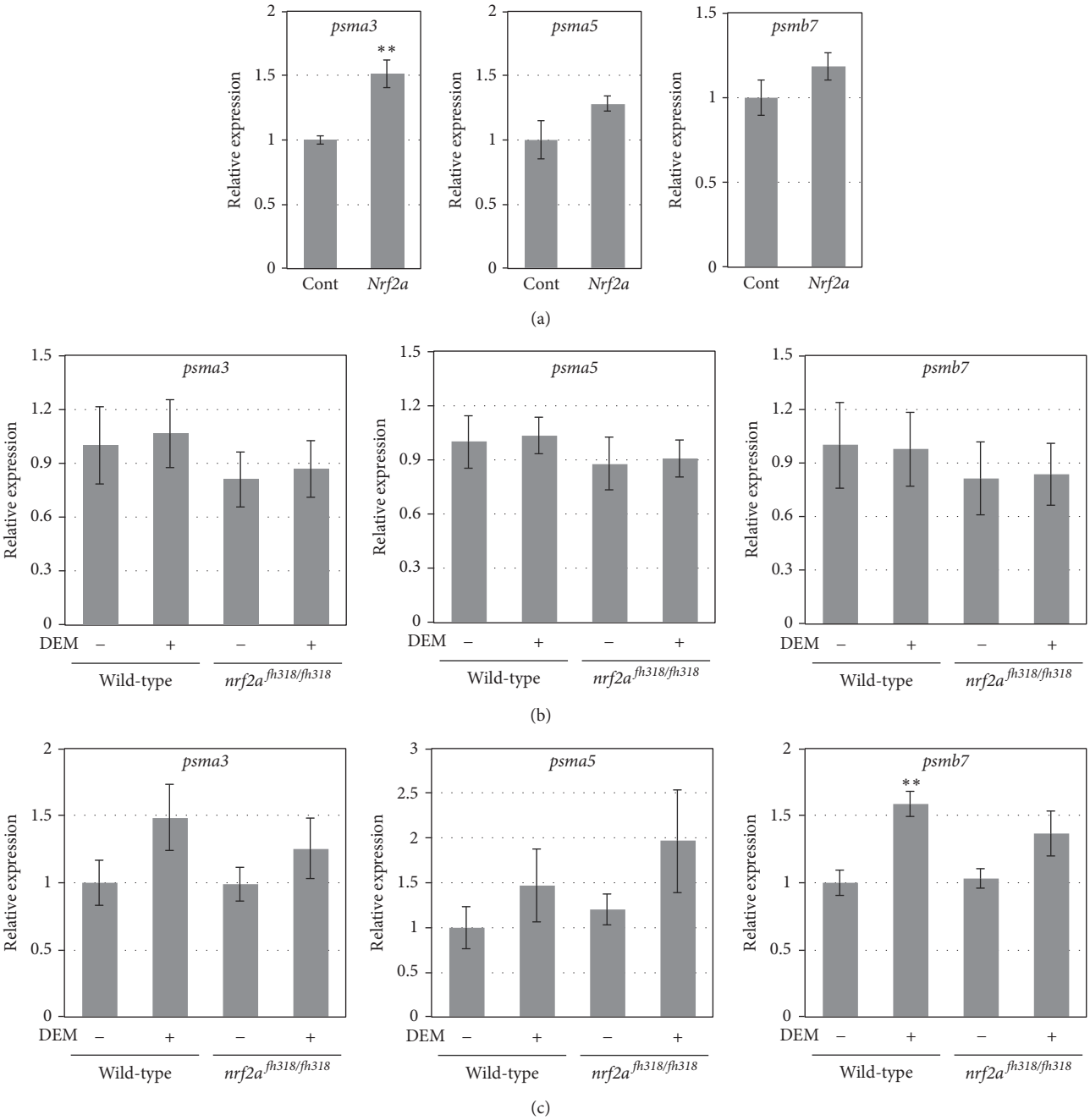


Figure 1: The expression of the proteasome subunit genes. (a) The gene expression of the indicated proteasome subunits in 8 h postfertilization (hpf) wild-type embryos injected with or without 100 pg of *nrf2a* mRNA at the 1-cell stage was analyzed by a real-time qPCR. Total RNA was extracted from 30 embryos for each sample. The expression of each gene was normalized to that of *ef1α* (means ± SEM), and the value in uninjected control was set to 1. Asterisks denote statistical significance (Control versus *nrf2a* overexpression, $^{**}P < 0.01$; Student's *t*-test, $n = 6$ for each group). (b) and (c) The gene expression of the indicated proteasome subunits in 4 dpf wild-type or $nrf2a^{fh318/fh318}$ mutant larvae that were treated (or not treated) with 100 $\mu$M DEM for 6 h (b) and 12 h (c) was analyzed by a real-time qPCR. The expression of each gene was normalized to that of *ef1α* (means ± SEM), and the value in untreated wild-type control was set to 1.

To confirm the Nrf2-dependent upregulation of these genes, we next carried out a real-time qPCR using *nrf2a*-overexpressing embryos. The result indicated that the expression levels of *pgd* and *fbp1a* (1.40- and 2.76-fold, resp.), but not *pck1*, *pcxb,* and *taldo1* (1.21-, 0.75-, and 0.70-fold, resp.), were significantly upregulated by the overexpression of *nrf2a* (Figure 3(b)). For *pgd* and *fbp1a*, we further analyzed the gene expression in DEM-treated larvae. As shown in Figure 3(c), the DEM treatment induced the expression of both *pgd* and *fbp1a* in wild-type larvae (5.85- and 2.18-fold, resp.), and

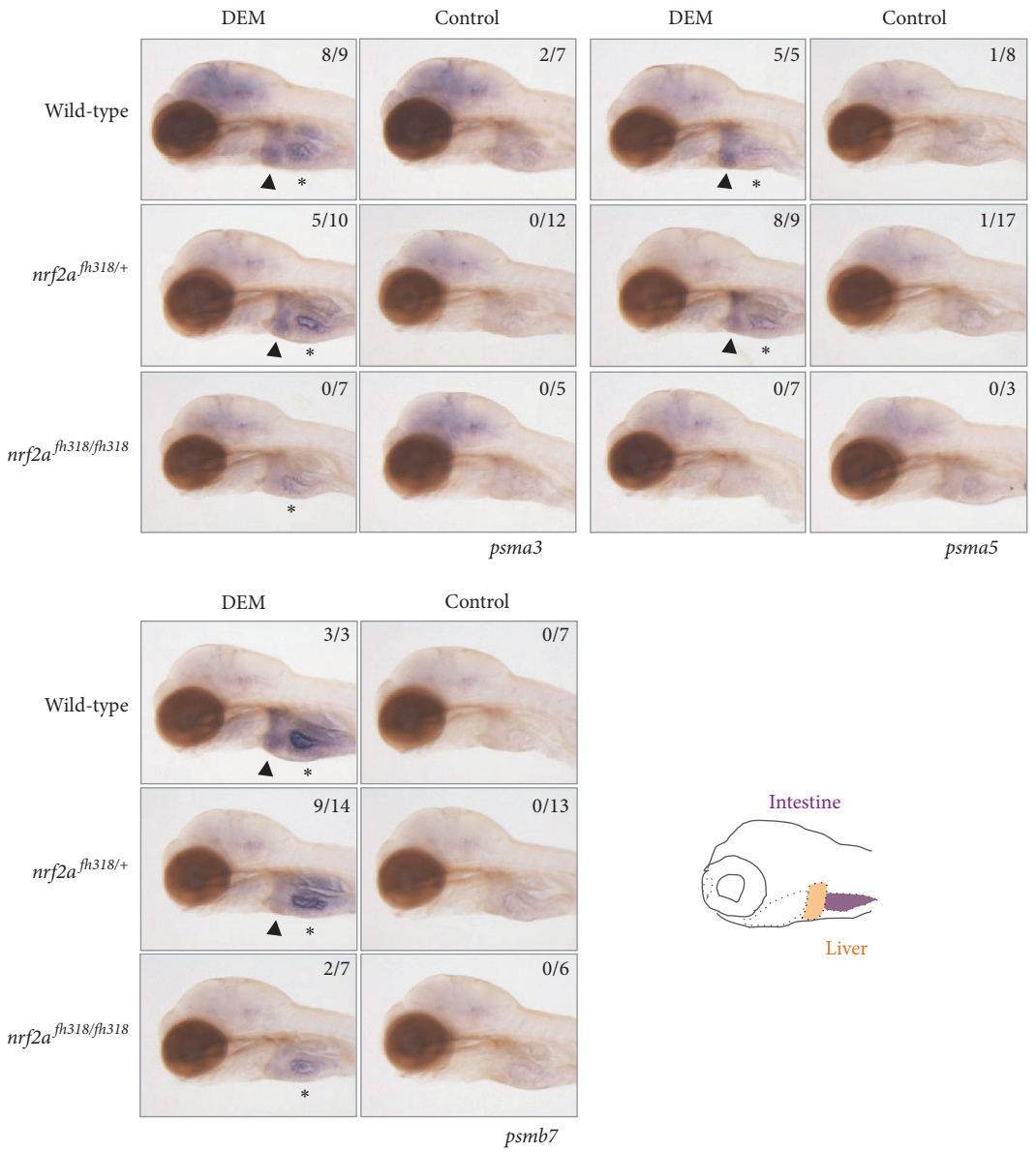


Figure 2: The induction profiles of proteasome subunit genes. Whole-mount in situ hybridization was performed to analyze the induction profiles of *psma3*, *psma5*, and *psmb7* using 4 dpf *nrf2a*$^{fh318}$ mutant larvae treated with or without 100 $\mu$M DEM for 12 h. The arrowheads indicate positive expression in the liver, and asterisks denote the basal expression in the intestine. The numbers in each picture indicate the positive/tested larvae.

the induction was weaker in homozygous *nrf2a*$^{fh318}$ mutant larvae (2.15- and 1.20-fold, resp.), suggesting clear genetic evidence of Nrf2-dependent regulation.

Induction profiles of these two genes were further analyzed by in situ hybridization (Figure 4). In wild-type and *nrf2a*$^{fh318}$ heterozygous mutant larvae, *pgd* was induced specifically in the liver after treatment with DEM for 6 h, while no induction was observed in *nrf2a*$^{fh318}$ homozygous mutant. *fbp1a* was also induced by DEM in the liver, and this induction was stronger in wild-type and heterozygous mutant compared with homozygous mutant. Weak DEM-induced expression of *fbp1a* was also observed in the gills, but this induction was independent of *nrf2a* genotypes. These results indicate that the Keap1-Nrf2 system, at least in part, regulates the transcription of these enzymes involved in glucose metabolism.

*3.4. The Identification of Other Target Genes for Zebrafish Nrf2.* The microarray data offered several more candidates for the conserved Nrf2 target gene in zebrafish. We selected 6 genes from the 27 overlapping genes (see Figure 3(a) and Table S3) identified in the current and previous microarray analyses and analyzed their expression using a real-time qPCR. The genes that were selected included *gclm* (glutamate-cysteine

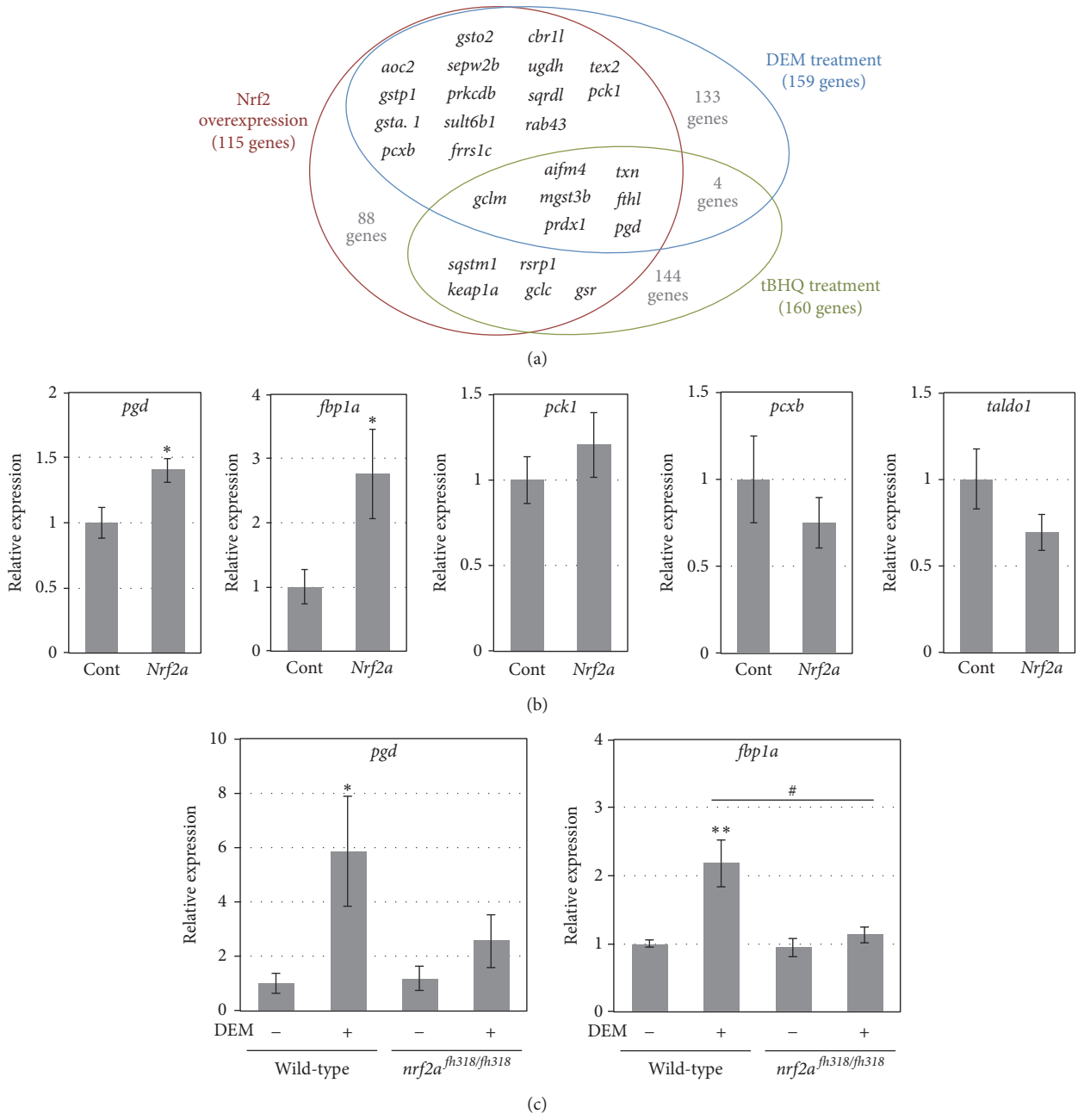


Figure 3: The expression of glucose metabolism-related genes. (a) The upregulated gene lineups from the three microarray experiments were compared. The data of DEM- or tBHQ-treated zebrafish larvae are from Nakajima et al. [19] and Hahn et al. [20], respectively. Numbers in parentheses and in the Venn diagrams denote the numbers of genes which belong to each category. The names of 27 overlapping genes were displayed. (b) The gene expression of the indicated enzymes related to glucose metabolism in 8 hpf wild-type embryos injected with or without 100 pg of *nrf2a* mRNA at the 1-cell stage was analyzed by a real-time qPCR. Asterisks denote statistical significance (Control versus *nrf2a* overexpression, $^{*}P < 0.05$; Student's *t*-test, $n = 6$ for each group). (c) The gene expression of the indicated enzymes related to glucose metabolism in 4 dpf wild-type or *nrf2a*$^{fh318/fh318}$ mutant larvae treated with or without 100 $\mu$M DEM for 6 h was analyzed by a real-time qPCR. Asterisks and hash marks denote statistical significance (DEM+ versus DEM−, $^{*}P < 0.05$ and $^{**}P < 0.01$; wild-type versus *nrf2a*$^{fh318/fh318}$, $^{\#}P < 0.05$; Student's *t*-test, $n = 6$ for each group).

ligase, modifier subunit), *gsto2* (glutathione *S*-transferase omega 2), *gsr* (glutathione reductase), *sqstm1* (sequestosome 1), and *keap1a* (kelch-like ECH-associated protein 1a), together with a well-studied *nrf2a* target, *gstp1* (glutathione *S*-transferase pi 1). As shown in Figure 5(a), *gclm*, *gsto2 gsr,* and *gstp1* were upregulated by the overexpression of *nrf2a* (1.98-, 9.10-, 3.12-, and 6.39-fold, resp.), while *sqstm1* and *keap1a* were only slightly induced (1.28- and 1.21-fold, resp.). The expression of these genes was further investigated in the DEM-treated embryos (Figure 5(b)). All of the genes were

Table 2: The pathways activated in both *nrf2a*-overexpressing embryos and DEM/tBHQ-treated larvae.

| *p* value | Pathway name | Genes |
|---|---|---|
| 3.33*E* − 9 | Glutathione conjugation | *gclc*, *gsto2*, *gstp1*, *gclm*, *gsta.1* |
| 4.13*E* − 7 | Phase II conjugation | *gclc*, *gsto2*, *gstp1*, *ugdh*, *gclm*, *gsta.1* |
| 7.44*E* − 7 | Detoxification of reactive oxygen species | *txn*, *gstp1*, *prdx1*, *gsr* |
| 8.26*E* − 7 | Biological oxidations | *gclc*, *gstp1*, *gsto2*, *aoc2*, *ugdh*, *gclm*, *gsta.1* |
| 1.39*E* − 3 | Vitamin C (ascorbate) metabolism | *gsto2* |
| 1.78*E* − 3 | Sulfur amino acid metabolism | *gclc*, *sqrdl*, *gclm* |
| 4.77*E* − 3 | TP53 regulates metabolic genes | *txn*, *prdx1*, *gsr* |
| 6.76*E* − 3 | Glutathione synthesis and recycling | *gclc*, *gclm* |
| 8.46*E* − 3 | Cellular responses to stress | *txn*, *gstp1*, *prdx1*, *gsr* |
| 8.83*E* − 3 | Metabolism | *gclc*, ***pck1***, *gstp1*, *sqrdl*, *txn*, *gsta.1*, *gsr*, ***pcxb***, *gsto2*, *aoc2*, ***pgd***, *ugdh*, *gclm* |

Bold genes encode enzymes related to the glucose metabolism process.

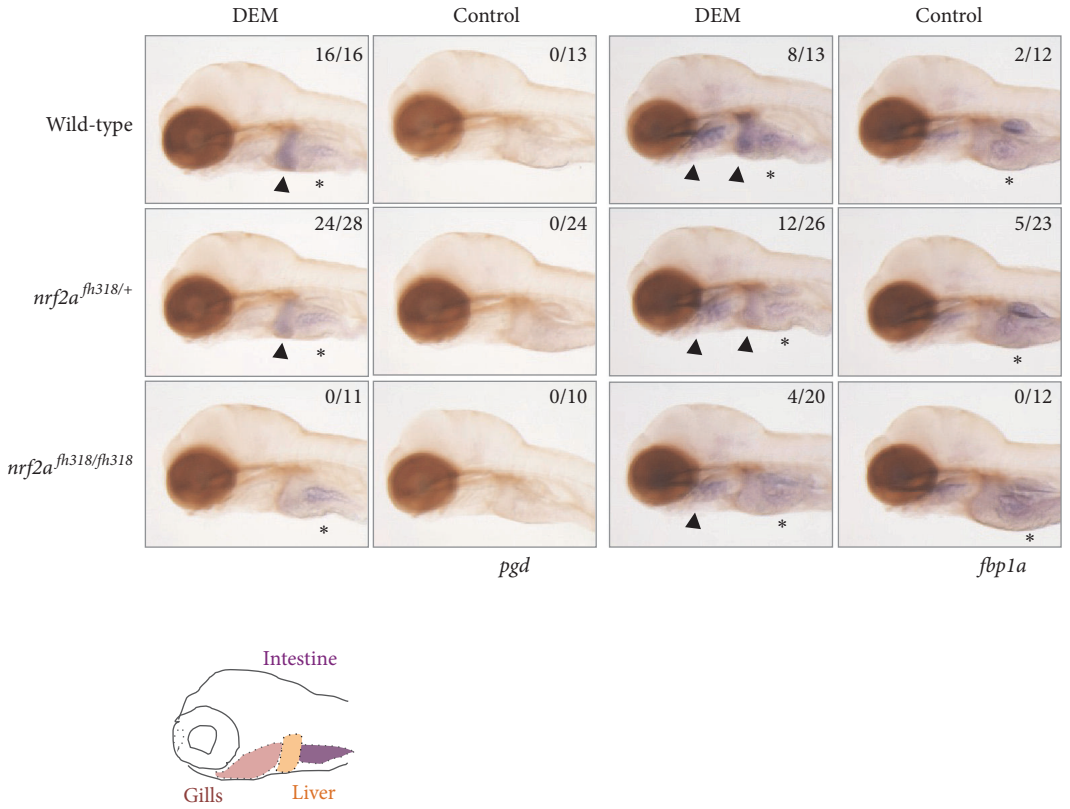


Figure 4: The induction profiles of glucose metabolism-related genes. Whole-mount in situ hybridization was performed to analyze the induction profile of *pgd* and *fbp1a* using 4 pdf *nrf2a*$^{fh318}$ mutant larvae treated with or without 100 $\mu$M DEM for 6 h. The arrowheads indicate positive expression in the liver and gills, and asterisks denote the basal expression in the intestine. The numbers in each picture indicate the positive expression in the liver/tested larvae.

strongly induced by DEM in wild-type larvae (*gclm*, 6.37-fold; *gsto2*, 3.99-fold; *gsr*, 9.40-fold; *gstp1*, 5.88-fold; *sqstm1*, 5.44-fold; *keap1a*, 1.72-fold); the induction was weaker, except for *keap1a*, in *nrf2a*$^{fh318/fh318}$ mutant larvae (*gclm*, 2.80-fold; *gsto2*, 2.08-fold; *gsr*, 6.69-fold; *gstp1*, 1.94-fold; *sqstm1*, 2.35-fold; *keap1a*, 2.10-fold). The results suggest that these genes are target genes for zebrafish Nrf2.

In our microarray analysis, we also found 55 genes that were downregulated by the overexpression of *nrf2a* (Table S4). None of these genes, except *hesx1*, was identified in the previous DEM- or tBHQ-based microarray analyses (25 and 82 genes, resp.) [19, 20]. More importantly, we could not find genes associated with fatty acid metabolism or inflammation—recent studies have implied that these functions are

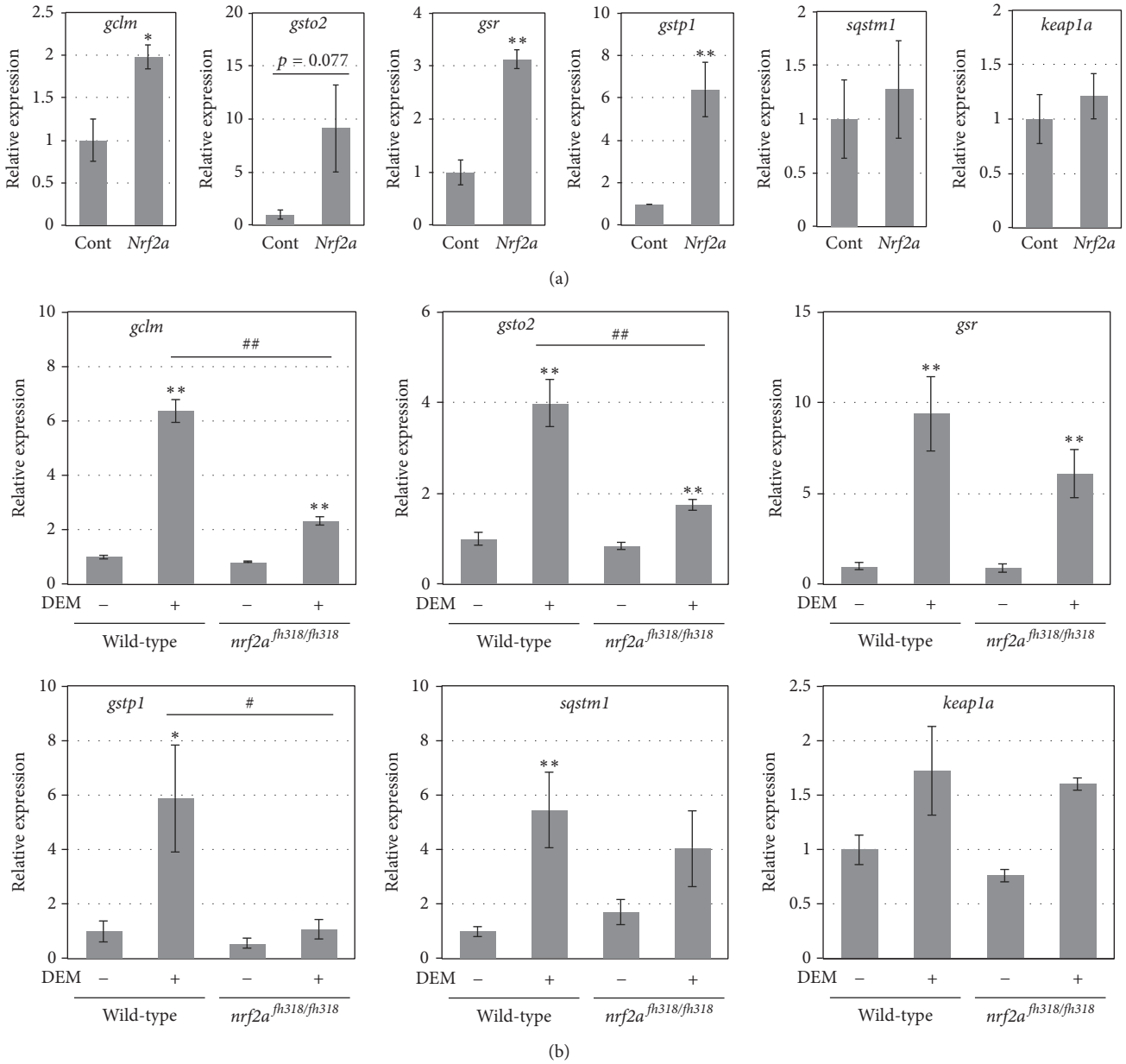


Figure 5: The expression of other candidate genes for zebrafish Nrf2 targets. (a) The expression of the indicated genes in 8 hpf wild-type embryos injected with or without 100 pg of *nrf2a* mRNA at the 1-cell stage was analyzed by a real-time qPCR. Asterisks denote statistical significance (Control versus *nrf2a* overexpression, $^{*}P < 0.05$ and $^{**}P < 0.01$; Student's *t*-test, $n = 6$ for each group). (b) The expression of the indicated genes in 4 dpf wild-type or $nrf2a^{fh318/fh318}$ mutant larvae treated with or without 100 $\mu$M DEM for 6 h was analyzed by a real-time qPCR. Asterisks and hash marks denote statistical significance (DEM+ versus DEM−, $^{*}P < 0.05$ and $^{**}P < 0.01$; wild-type versus $nrf2a^{fh318/fh318}$, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$; Student's *t*-test, $n = 6$ for each group).

negatively regulated by Nrf2 [5, 22, 23]. The evolutional conservation related to the Nrf2-dependent negative regulation of some types of genes remains unclear.

## 4. Discussion

In the present study, we found that the three proteasome subunits in the 20S core particle were regulated by zebrafish Nrf2, one of which (*psmb7*) has a trypsin-like protease activity; the others (*psma3* and *psma5*) comprise the $\alpha$ ring structure [24]. In mammals, multiple subunits of proteasome have also been shown to be regulated by Nrf2 at the transcriptional level [4], suggesting the presence of a conserved regulatory mechanism among vertebrates. Consistent with the report by Kwak et al. [4], which showed the transcriptional induction of proteasome subunits in mice liver after treatment with D3T, an Nrf2-activating antioxidant, induction was observed dominantly in the liver of zebrafish larvae. Although most Nrf2

target genes tested in zebrafish larvae showed the induction in the nose and gills in addition to the liver [19], proteasome subunit genes were induced only in the liver. The molecular basis of this liver specificity together with their slow induction should be characterized in the future. As the Nrf2-dependent upregulation of the proteasome function is suggested to be important in the defense against oxidative stress, endoplasmic reticulum stress, and senescence [25–27], it is anticipated that the regulatory context should be clarified for medical applications. The zebrafish model can also provide a good model for further study.

Enzymes related to glucose metabolism were also targeted by Nrf2 in zebrafish. Phosphogluconate dehydrogenase (Pgd) is an enzyme that converts 6-phosphogluconate to ribulose 5-phosphate in the oxidative branch of the pentose phosphate pathway, which produces NADPH as well as purine nucleotides [28]. The Nrf2-dependent upregulation of *pgd* has also been demonstrated in mice [6]. The upregulation of *pgd* may confer protection against stressors by augmenting the supply of NADPH, an essential cofactor for redox homeostasis [5], and the activation of nucleotide biosynthesis through the pentose phosphate pathway, which may lead to the metabolic remodeling in cancer cells [6]. The zebrafish has recently emerged as an important model in cancer biology [29, 30]. It will be worthwhile to study the Nrf2-dependent metabolic remodeling that takes place in carcinogenesis using zebrafish. In addition to *pgd*, the Nrf2-depedent transcriptional regulation of fructose-1,6-bisphosphatase (Fbp), an enzyme that converts fructose 1,6-bisphosphate to fructose 6-phosphate in gluconeogenesis, was observed specifically in the liver. Further study on the mechanism underlying this liver-specific induction will provide an important clue to understand the biological significance of Nrf2-dependent regulation of glucose metabolism.

In the present microarray analysis, the cut-off value for significant upregulation by the overexpression of *nrf2a* was set at a 1.5-fold change instead of a 2-fold change in the previous studies [19, 20]. It was effective, since 4 out of 6 genes, as we validated the microarray data by a real-time qPCR with statistical significance, showed less than 2-fold change in the microarray analysis (see Figure S2 and Table S3). A correlation in the fold changes between microarray and real-time qPCR data was relatively poor; thus, it is worthwhile to pick up not only high-ranked genes in the microarray analysis but also low-ranked genes for further analyses.

The unique point of the present study is that genes were screened using *nrf2a*-overexpressing embryos. A major approach for the microarray-based screening of Nrf2-target genes is the use of cells/tissues/animals treated with Nrf2-activating compounds [31–33] as is the case for the previous screen studies that used zebrafish [19, 20]. Another approach is the use of Keap1 knockout cells/tissues [5, 34]. Since Keap1 is an E3 ubiquitin ligase that targets Nrf2, the disruption of its gene increases the stability of Nrf2 and leads to the constitutive activation of Nrf2. A study by Yates et al. [22] which compared sets of genes upregulated by treatment with a potent Nrf2-activating compound (CDDO-Im) with those upregulated by liver-specific Keap1 knockout is of particular interest. Keap1-knockout activated Nrf2-dependent transcription more strongly than the pharmacological activation; as a result greater numbers of Nrf2-regulated genes were detected [22]. It is possible that similar effects were observed in the overexpression method of the present study.

## 5. Conclusions

In conclusion, we found novel Nrf2-target genes in zebrafish by a microarray analysis using *nrf2a*-overexpressing zebrafish embryos. The basal expression levels of proteasome subunits were revealed to be regulated by zebrafish Nrf2. In addition, enzymes involved in the pentose phosphate pathway and gluconeogenesis were found to be under Nrf2-dependent transcriptional control. These results suggest that the Nrf2-mediated regulation of the genes related to protein turnover and glucose metabolism, at least a part of them, was evolutionarily conserved in vertebrates.

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

Vu Thanh Nguyen and Yuji Fuse contributed equally to this work.

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