Generation and characterization of *keap1a* and *keap1b*-knockout zebrafish

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**ABSTRACT**

The Keap1–Nrf2 pathway is an evolutionarily conserved mechanism that protects cells from oxidative stress and electrophiles. Under homeostatic conditions, Keap1 interacts with Nrf2 and leads to its rapid proteasomal degradation, but when cells are exposed to oxidative stress/electrophiles, Keap1 senses them, resulting in an improper Keap1–Nrf2 interaction and Nrf2 stabilization. Keap1 is therefore considered both an “inhibitor” of and “stress sensor” for Nrf2 activation. Interestingly, fish and amphibians have two Keap1s (Keap1a and Keap1b), while there is only one in mammals, birds and reptiles. A phylogenetic analysis suggested that mammalian Keap1 is an ortholog of fish Keap1b, not Keap1a. In this study, we investigated the differences and similarities between Keap1a and Keap1b using zebrafish genetics. We generated zebrafish knockout lines of *keap1a* and *keap1b*. Homozygous mutants of both knockout lines were viable and fertile. In both mutant larvae, the basal expression of Nrf2 target genes and antioxidant activity were up-regulated in an Nrf2-dependent manner, suggesting that both Keap1a and Keap1b can function as Nrf2 inhibitors. We also analyzed the effects of the Nrf2 activator sulforaphane in these mutants and found that *keap1a*-, but not *keap1b*-, knockout larvae responded to sulforaphane, suggesting that the stress/chemical-sensing abilities of the two Keap1s are different.

1. Introduction

The Kelch-like ECH-associated protein 1 (Keap1)–Nrf2 pathway is an evolutionarily conserved mechanism that protects cells against oxidative stress and electrophilic xenobiotics [1–4]. Nrf2 is a transcription factor that transactivates a variety of cytoprotective genes in response to many types of insults/stresses. The physiological importance of the Nrf2-dependent cytoprotection has been demonstrated in Nrf2-deficient mice [5], rats [6] and zebrafish [7]. Keap1 is an Nrf2-specific adaptor protein for the Cullin 3-E3 ubiquitin ligase that binds to the Nrf2-ECH homology 2 (Neh2) domain of Nrf2 and facilitates its ubiquitination and proteasomal degradation, leading to the down-regulation of Nrf2 target genes under homeostatic conditions [8–11]. In addition to this negative function in the Nrf2-dependent gene regulation, Keap1 also plays a role as a sensor for various Nrf2-activating electrophiles and oxidative stress using its reactive cysteine residues [12–15].

We have been studying the Keap1–Nrf2 pathway in zebrafish and found that its physiological roles and molecular mechanisms are quite similar to those in mice, suggesting that the Keap1–Nrf2 pathway is highly conserved among vertebrates [7,16–18]. Keap1 is a single gene in mammals, but there are two co-orthologs (*keap1a* and *keap1b*) in zebrafish [16,19].

In the present study, we generated and characterized *keap1a*– and *keap1b*-knockout zebrafish and compared their phenotypes to elucidate the differences and similarities between Keap1a and Keap1b as “inhibitors” of and “stress sensors” for Nrf2 activation.

2. Materials and methods

2.1. Zebrafish and chemicals

AB (wild-type), *nfe2l2a*\(^{\text{fh318}}\) [7], *keap1a*\(^{\text{it302}}\) and *keap1b*\(^{\text{it308}}\) strains were used. For genotyping *keap1a*\(^{\text{it302}}\) and *keap1b*\(^{\text{it308}}\), polymerase chain reaction (PCR) was carried out using the following primer sets: 5′-CTGCTTGAGCTGATCAGTCAGG and 5′-CGGCTCTGAGATCAGTCAGG...
was performed as previously described [20]. Before using for experiments in this paper, we backcrossed keap1a<sup>it302</sup> and keap1b<sup>it308</sup> lines with wild-type AB strain more than 4 times after their generation. Both lines are available from the National BioResource Project of Japan (https://shigen.nig.ac.jp/zebra/index-en.html). Surhofane and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were purchased from LKT Laboratories (St. Paul, MN) and Wako (Osaka, Japan), respectively.

All animal experiments were performed in accordance with the animal protocol approved by the Animal Experiment Committee of the University of Tsukuba. All methods were carried out in accordance with the Regulation for Animal Experiments in our university and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

2.2. Phylogenetic analyses

The phylogenetic tree was constructed using the CLUSTAL W program (http://clustalw.ddbj.nig.ac.jp) and plotted with NJplot (http://dou.prabi.fr/software/njplot).

2.3. Gene knockout

Knockout lines of zebrafish keap1a and keap1b were generated using the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated sequences 9 (Cas9) technology as previously described [21]. In brief, guide RNAs for keap1a- or keap1b-specific (25 pg each) and Cas9 mRNA (250 pg) were co-injected into the yolk of single-cell-stage wild-type AB embryos. Plasmids for the guide RNAs were constructed using the plasmid vector pDR274 (Addgene, Waterford, MA, USA) and oligonucleotides: 5′-taggGGAGG-ACCGAGGAGTCTACA and 5′-aaacTGTAGAGCTCGCTCTCGG (keap1a); 5′-tagggCGCTGCTAGCCCGGGTGGG and 5′-aaacCCACCCGGGTA-CAGCAGG (keap1b). RNAs were transcribed using the T7 MAXiscript Kit (Ambion, Austin, TX, USA), and Cas9 mRNA was synthesized by the mMESSAGE mMACHINE SP6 kit (Ambion) using pCS2+hsCas9 (Addgene) as a template.

2.4. RNA sequence (RNA-seq) analyses

Total RNA was extracted from 5-days-post-fertilization (dpf) larvae of keap1a- and keap1b-knockout lines and wild-type AB treated with or without surhofane using ISOGEN II (Nippon Gene, Tokyo, Japan). Larvae were treated with surhofane from 4.5 dpf to 5 dpf (12 h). RNA-seq library was constructed with 500 ng of total RNA using NEB-Next Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Beverly, MA, USA) and NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). Libraries were validated using Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to determine the size distribution and concentration. Sequencing was performed by Tsukuba i-Laboratory LLP using NextSeq500 (Illumina, San Diego, CA, USA) with a paired-end 36-base read option. Sequencing reads in FASTQ format were imported to CLC Genomics Workbench (CLC-GW, ver. 10.1.1, Qiagen, Hilden, Germany) and mapped on the zebrafish reference genome assembly GRCz10. Reads were quantified for 31,701 genes using CLC GW, and quantities were used for isoform discovery. The expression genome assembly GRCz10. Reads were quantified for 31,701 genes in ENSEMBLE annotation provided in CLC-GW, and quantities were per kilobase per million mapped reads (RPKM) value were obtained. RPKM values from 4 samples (untreated and surhofane-treated wild-type AB, untreated keap1a-knockout, untreated keap1b-knockout) were normalized using Normalization Tool in CLC-GW with mean-scaling, with median of means as reference value, 5% trimming option settings. Fold-changes (FC) of normalized RPKM values were calculated between two samples, and differentially expressed genes were identified by threshold of |FC| > 1.5. A gene ontology (GO) analysis was carried out using DAVID 6.8 with GOTERM_BP_DIRECT (BP: biological processes) [22] after converting identified zebrafish genes into their human homologs using bioDBnet [23] and ZFIN (https://zfin.org/).

2.5. Gene expression analyses

 Quantitative real-time PCR (qPCR) was carried out as previously described [17]. For qPCR analysis, larvae were treated with surhofane for 6 h at 5 dpf. Primer sets for keap1a and keap1b are as follows: 5′-GGCTGAAAGCTGTTCTGGGC and 5′-GAGTGGGACAACTGGTCT (keap1a); 5′-AGTAAGCGCATGCTCG and 5′-TGAA-GAATCTGCTCTGGTGC (keap1b). Primers for prdx1 and gsp1 were designed previously [17].

2.6. Survival assays

Survival assays were performed as previously described [24]. At 4 dpf, larvae were exposed to 2 mM of H<sub>2</sub>O<sub>2</sub> for 120 h. Surhoffane was administered at 3.5 dpf, 12 h prior to H<sub>2</sub>O<sub>2</sub> exposure. Dead larvae were collected and stored at -20 °C until genotyping together with surviving larvae.

2.7. Statistical analyses

The survival data were calculated using the Kaplan–Meier method and analyzed by the log-rank test. The statistical significance of gene induction was determined by two-tailed t-test. Comparison of gene induction levels between different genotypes was performed using a one-way analysis of variance followed by Bonferroni’s multiple comparisons test. All statistical analyses were performed using EZR [25], which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). P values of <0.05 were considered to be statistically significant and indicated with asterisks (* < p < 0.05, ** < p < 0.01, *** p < 0.001).

3. Results

3.1. Keap1b is an authentic ortholog of mammalian Keap1, and Keap1a is a fish/amphibian-specific gene

We initially believed that the Keap1a and Keap1b genes are restricted to fish that had been generated by the teleost-specific whole genome duplication (WGDD) [26,27] (Fig. 1A, green rectangle), but our assumption was wrong. In addition to teleosts, Keap1a/Keap1b are also present in non-teleost fish, such as polypteri, sharks and coelacanths, and even in anuran and urodele amphibians (Fig. 1B). Intriguingly, phylogenetic analyses showed that mammalian Keap1 belongs to the Keap1b subfamily, and no reptiles, birds and mammals have Keap1a genes, suggesting that both Keap1a and Keap1b were conserved during the evolution process from fish to tetrapods, with Keap1a subsequently lost during further evolution to amniotes (Fig. 1A). These findings led us to hypothesize that Keap1b is an authentic ortholog of mammalian Keap1, while Keap1a is a fish/amphibian-specific gene that may have unique roles that Keap1/Keap1b do not have.

3.2. keap1a- and keap1b-knockout zebrafish were viable and fertile

To investigate the differences and similarities between Keap1a and Keap1b, we generated keap1a- and keap1b-knockout zebrafish lines using CRISPR-Cas9 technology [28,29]. Target sites for CRISPR–Cas9 were designed in exon 4, which led to the deletion of the Nrf2-interacting diglycine repeat (DGR) domains in both Keap1a and Keap1b (Fig. 2A). Two mutant lines, keap1a<sup>it302</sup> and keap1b<sup>it308</sup>, were generated: the keap1a<sup>it302</sup> line has an 18-base pair (bp) deletion and 28-bp insertion, and the keap1b<sup>it308</sup> line has a 7-bp deletion in the
CRISPR target sites, resulting in the C-terminal deletion of Keap1a after Val215 and that of Keap1b after Ala347 due to frameshift mutations (Fig. 2A).

As shown in Fig. 2B, no obvious abnormality was found in either the keap1a- or keap1b-homozygous knockout larvae at 5 dpf derived from heterozygous parents. For genotyping, we designed specific PCR primers to amplify 10-bp-longer and 7-bp-shorter PCR products for knockout alleles compared with the wild-type allele (Fig. 2B, right). We raised these larvae to adulthood and then genotyped them at four months-post fertilization (mpf). The results showed that the genotypes of both knockout adults were roughly according to the expected Mendelian ratio, suggesting that the homozygous knockout adults were both viable (Fig. 2C, right). No obvious difference was found between homozygous knockout mutant and wild-type in both larval (Fig. 2B, left) and adult stages (Fig. 2C, left). To eliminate the possibility of genetic compensation of deleterious gene mutations by the up-regulation of their paralog genes [30], we next examined the gene expression of keap1a and keap1b in keap1b- and keap1a-homozygous knockout larvae (Fig. 2D). Homozygous knockout larvae used in this experiment were derived from homozygous parents, suggesting that both keap1a- and keap1b-knockout lines were fertile. The expression of keap1a and keap1b in keap1b- and keap1a-knockout larvae, respectively, was not significantly different from that in wild-type animals, implying that the phenotypes (viable and fertile) of keap1a- and keap1b-knockout zebrafish were not due to genetic compensation for each other.

3.3. The effects of keap1a- and keap1b-disruption on gene expression profiles were similar

To detect specific molecular alterations in keap1a-knockout

Fig. 1. The comparison of Keap1 proteins.

(A) Simplified phylogeny of vertebrate and invertebrate animals. Black and green rectangles represent two WGD events that occurred early in vertebrate evolution and an additional WGD in the teleost ancestor, respectively.

(B) Phylogenetic tree of Keap1 family proteins. Amino acid sequences in the broad complex, tramtrack and bric-a-brac domains-intervening region (BTB-IVR) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/) and NewtBase (http://newtbase.eko.uj.edu.pl/). Accession numbers: lmK1a, c152694_g1_i1 (NewtBase contig); xtK1a, XM_012953965; lcK1a, XM_014486756; dkK1a, NM_182864; trK1a, XM_011618924; okK1a, XM_004082216; ecK1a, XM_028816380; rtK1a, XM_020523471; hsK1, NM_203500; mmK1, NM_016679; ggK1, KU321503; arK1, XM_026068028; ptK1, XM_026711369; acK1, XM_003216399; drK1b, NM_001113477; trK1b, XM_003972594; olK1b, XM_023955710; eckK1b, XM_028823679; lcK1b, XM_005994311; lmkK1b, c118729_g1_i1 (NewtBase contig); xtK1b, NM_001008023; rtK1b, XM_020519602; cikK1, XM_002128019; dmK1, NM_142337. Abbreviations: ac, Anolis carolinensis (lizard); ar, Apteryx rowi (kiwi); ci, Ciona intestinalis (ascidian); dm, Drosophila melanogaster (fly); dr, Danio rerio (zebrafish); ec, Epetoichthys calabaricus (snakefish); gg, Gallus gallus (chicken); hs, Homo sapiens (human); lc, Latimeria chalumnae (coelacanth); lm, Lissotriton montandoni (newt); mm, Mus musculus (mouse); ol, Oryzias latipes (medaka); pt, Pseudanajja textilis (snake); rt, Rhinodon typus (shark); tr, Takifugu rubripes (pufferfish); xt, Xenopus tropicalis (frog). Of note, all 74 teleosts in the Ensembl Genome Browser have both Keap1a and Keap1b genes, while all 104 mammals, 13 birds and 11 reptiles have only Keap1a/Keap1b. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
zebrafish, we performed a whole-transcriptome analysis by RNA-seq. When 5-dpf keap1a- and keap1b-knockout larvae were compared against wild-type AB larvae in standard condition (Table S1), 162 and 129 genes were up-regulated in keap1a- and keap1b-knockout larvae, while 413 and 949 genes were down-regulated, respectively (Fig. 3A, red and blue circles, 1.5-fold). Out of 162 up- and 413 down-regulated genes in keap1a-knockout larvae, 94 (58.0%) and 388 (93.9%) overlapped with those in keap1b-knockout larvae, respectively, suggesting that most of the affected genes were common between the two types of knockout larvae, especially in the case of down-regulated genes.
To compare the effects of Keap1 disruption and Nrf2 activation, we further performed an RNA-seq analysis of wild-type AB larvae in the presence of sulforaphane, an isothiocyanate found abundantly in broccoli sprouts and known to be a potent Nrf2 activator [31]. The analysis identified 251 up- and 97 down-regulated genes (Fig. 3A, green circles, 1.5-fold) by sulforaphane-treatment. 43.2% (70 genes) and 12.1% (50 genes) of those genes were also altered by keap1a-knockout larvae, respectively.

To identify and compare the biological processes that were altered by keap1a disruption, keap1b disruption or sulforaphane treatment, a GO analysis was performed using DAVID bioinformatics resources [22] (Tables S2–S7). Fig. 3B shows the major biological processes that were significantly enriched by keap1a-disruption (p < 0.01). Of the 162 up- and 413 down-regulated genes in keap1a-knockout larvae, 119 (73.4%) and 370 (89.6%) genes were mapped to human homologs, respectively, and used for the GO analysis. The analysis of the up-regulated genes demonstrated that affected biological processes were mainly downstream targets of Nrf2 [4,17,32], such as oxidation-reduction process and xenobiotic metabolic process, which were common in all keap1a-, keap1b-knockout and sulforaphane-treated larvae (black bars), and the glutathione metabolic process and cellular oxidant detoxification, which were common in keap1a-knockout and sulforaphane-treated larvae (green bars)(see also Tables S2–S4). The enriched biological processes in the GO analysis of the down-regulated genes were markedly similar between keap1a- and keap1b-knockout larvae (blue bars)(see also Tables S5–S7), and the processes that have been shown to be related to Nrf2, such as desmosome organization and circadian rhythm [33–35], were high ranked. Some biological processes were found to be specific to keap1a-knockout larvae (red bars). To identify the processes only related to keap1a, we analyzed the genes that were up-regulated in keap1a- but not in keap1b-knockout larvae (Fig. 3B, keap1a–/– vs keap1b–/–), but target processes of Nrf2 still seemed to account for the majority, except DNA replication initiation (Tables S8 and S9).

Taken together, these results suggested that a major role of keap1a in
zebrafish larvae is to inhibit Nrf2 functions, which is quite similar to that of keap1b. To compare the contribution of keap1a and keap1b in suppressing Nrf2 activities, the expression of peroxiredoxin 1 (prdx1) and glutathione S-transferase P1 (gstp1) was examined by qPCR (Fig. 3C). These are two typical target genes of zebrafish Nrf2 that were identified by an RNA-seq analysis under all three conditions here, namely the results of RNA-seq analyses using keap1a knockout larvae, keap1b-knockout larvae, and sulforaphane-treated wild-type larvae, as well as by a microarray analysis using diethyl maleate-treated 4-dpf larvae [36] and Nrf2-overexpressing embryos at 8 h post-fertilization [17]. Compared with wild-type AB, the expression of prdx1 and gstp1 was significantly higher in keap1a- (prdx1: 3.2-fold; gstp1: 1.8-fold) and keap1b- (prdx1: 2.4-fold; gstp1: 1.5-fold) knockout larvae. The results indicated that both zebrafish Keap1a and Keap1b contribute to Nrf2 inhibition in a similar manner at the larval stage.

3.4. The antioxidant activity was up-regulated in both keap1a- and keap1b-knockout larvae in an Nrf2-dependent manner

The up-regulation of Nrf2 target gene expression under basal conditions suggests that the antioxidant activity may also be up-regulated in keap1a- and keap1b-knockout larvae. To test this possibility, the effects of keap1a- and keap1b-knockout on sensitivities against oxidative stress were analyzed by a survival assay. We previously showed that the activation of Nrf2 up-regulated the survival rates of zebrafish larvae against H2O2 [7]. Larvae at 4 dpf were treated with 2 mM H2O2, and their survival was analyzed using the Kaplan-Meier method (Fig. 4A). For keap1a-knockout mutants, 90% of heterozygous mutants and wild-type larvae died within 24 h after H2O2 treatment, while 25%–30% of homozygous mutants were survived. Similarly, more than 80% of heterozygous mutants and wild-type larvae died among keap1b-knockout mutants, while 30%–50% of homozygous mutants survived. These results suggest that the antioxidant activity is up-regulated in keap1a- and keap1b-knockout larvae.

We assumed that the up-regulated antioxidant activities in keap1a- and keap1b-knockout larvae to be due to the up-regulation of the Nrf2 activity. To confirm this assumption, we examined the effects of Nrf2 mutation on antioxidant activities in keap1a- and keap1b-knockout larvae by generating and using compound mutant lines with nfe2l2a/h2o2 (nfe2l2a: zebrafish ortholog of mammalian Nrf2 gene) [7]. As shown in Fig. 4B, the survival rates against H2O2 toxicity of both keap1a- and keap1b-knockout larvae were markedly reduced by introducing an Nrf2 mutation, indicating that the up-regulated antioxidant activities in these knockout mutants were Nrf2-dependent. All of these results suggest that Keap1a and Keap1b have similar Nrf2-inhibitory activities in vivo.

3.5. Keap1b showed significantly higher sensing ability to sulforaphane than Keap1a

In addition to Nrf2-inhibitory activity, Keap1 also plays a role as a...
sensor for a variety of Nrf2 activators. We previously showed that the pretreatment of sulforaphane 12 h prior to H₂O₂ treatment enhanced the survival rates of H₂O₂-treated zebrafish larvae in an Nrf2-dependent manner [7,37]. To clarify whether Keap1a and/or Keap1b are required for the antioxidant activity of sulforaphane, we performed a survival analysis using keap1a- and keap1b-knockout larvae generated from heterozygous matings (Fig. 5A). Pretreatment of sulforaphane increased the survival against H₂O₂ toxicity in keap1a-homozygous knockout larvae as well as wild-type larvae, but no such effects were noted in keap1b-homozygous knockout larvae (Fig. 5B and C). We previously showed that only Keap1b, not Keap1a, responded to sulforaphane in Keap1–Nrf2 co-overexpressed 8-h-post-fermentation embryos [13]. Our current and previous results suggested that Keap1b but not Keap1a is sensitive to sulforaphane.

We also analyzed the sulforaphane-induced expression of the Nrf2 target genes prdx1 and gap1 by qPCR in keap1a- and keap1b-knockout larvae (Fig. 5D, Tables S11 and 13). In wild-type larvae, a 17.8- and 4.4-fold increase in prdx1 and gap1 mRNA, respectively, was observed 6 h after treatment with 40 μM sulforaphane. This sulforaphane-induced expression of both genes was significantly reduced in keap1b-knockout larvae (prdx1, 4.8-fold; gap1, 2.3-fold) but not in keap1a-knockout larvae (prdx1, 11.0-fold; gap1, 6.2-fold), suggesting that both Keap1a and Keap1b proteins have the ability to sense sulforaphane, although the ability of Keap1a is weaker than that of Keap1b. To confirm this possibility, the dose-dependent effects of sulforaphane (10–40 μM) on the induction of these genes was analyzed. As shown in Fig. 5D and also Fig. S2, Tables S11 and 12), 10–30 μM sulforaphane showed almost no effects on the up-regulation of gap1 in keap1b-knockout larvae (10 μM, 1.4-fold [p = 0.18]; 20 μM, 1.5-fold [p = 0.063]; 30 μM, 1.5-fold [p = 0.23]) but had significant gene-inducing activities in keap1a-knockout larvae (10 μM, 2.7-fold [p = 0.025]; 20 μM, 3.8-fold [p = 0.0041]; 30 μM, 5.0-fold [p = 0.0024]). Taken together, these results indicated that Keap1b is better at sensing sulforaphane than Keap1a.

4. Discussion

In this paper, we showed that neither keap1a nor keap1b are essential for zebrafish growth and fertility using knockout lines of these genes. With knockout of either gene in larvae, Nrf2 was activated, and the antioxidant activity was enhanced. Furthermore, we found that the antioxidant activity of sulforaphane was significantly lower in keap1b-knockout larvae than in wild-type larvae and keap1a-knockout larvae, demonstrating that Keap1b, but not Keap1a, is a physiological sensor protein for the group of electrophiles including sulforaphane.

Since all 77 fish and amphibians in the Ensembl Genome Browser (http://www.ensembl.org) have both Keap1a and Keap1b, we assumed that Keap1a must have an important role during developmental period, especially at the larval stage, but the keap1a-homozygous knockout larvae showed no obvious phenotypes and became normal adults in a Mendelian ratio. We therefore next examined the effects of keap1a-disruption on the larval gene expression by an RNA-seq analysis and detected 162 up- and 413-down regulated genes. However, the gene lineups in keap1a-knockout larvae were similar to those in keap1b-knockout larvae, and many of them, especially up-regulated genes, are included in the gene lineups in sulforaphane-treated wild-type larvae, which we consider to be Nrf2 target genes. The only category found as included in the gene lineups in sulforaphane-treated wild-type larvae, knockout larvae, and many of them, especially up-regulated genes, are detected 162 up- and 413-down regulated genes. However, the gene disruption on the larval gene expression by an RNA-seq analysis and larvae showed no obvious phenotypes and became normal adults in a Mendelian ratio. We therefore next examined the effects of sulforaphane (40 μM) on the expression of both genes was significantly reduced in keap1b-knockout larvae (keap1a and keap1b-homozygous knockout larvae under uninduced conditions and was strongly activated after sulforaphane treatment, suggesting that both Keap1a and Keap1b were able to inhibit Nrf2 without the help of their counterpart. Since fish Keap1a and Keap1b do not have cysteine residues corresponding to mouse Cys273 and Cys288, respectively [2], which are thought to be involved in Nrf2 inhibition [41], we hypothesized that Keap1a–Keap1b heterodimers formed between over-expressed and endogenous proteins, which were able to inhibit the Nrf2 activity in contrast to their unfunctional homodimers in Keap1a- and Keap1b-overexpressing embryos [19]. Indeed, we showed that Keap1a and Keap1b form heterodimers in vitro [19], and Wakabayashi et al. demonstrated that mouse C273A and C288A mutant Keap1s, which do not function alone, inhibited the Nrf2 activity when co-expressed in cultured cells [41]. However, our hypothesis turned out to be not correct. The results here clearly demonstrated that both Keap1a- and Keap1b-homodimers were able to inhibit the Nrf2 activity.

Cys151 has been identified as a specific sensor for sulforaphane in mammalian Keap1 [13,42,43]. Although both zebrafish Keap1a and Keap1b have cysteine residues that correspond to mammalian Cys151, the residue corresponding to Lys150 that may enhance the reactivity of Keap1, which seems to be important for the Keap1–Sqstm1/p62 interaction [40], was not conserved in Keap1a [2]. The binding specificity to Sqstm1/p62 protein may differ between Keap1a and Keap1b, which may lead to different responses to lysosomal stress. It will be interesting to examine the differences between keap1a- and keap1b-knockout zebrafish in the phenotypic response to a variety of environment stresses in the future. For down-regulated genes in keap1a-knockout larvae, 94% were overlapped with those in keap1b-knockout larvae but only 12% with those in sulforaphane-treated wild-type larvae. It may be due to secondary effects of the persistent up-regulation of Nrf2 in both keap1a- and keap1b-knockout larvae. To confirm this possibility, gene expression analyses of keap1a,keap1b-compound knockout larvae should be performed in the future.

Regarding the Nrf2-inhibitory activity, we expected to find no significant difference between Keap1a and Keap1b, since similar degradation activity of Nrf2 was previously shown in Keap1a- and Keap1b-overexpressing embryos [19]. The current findings support this notion, since the up-regulation of the Nrf2 target genes and antioxidant activities was observed in both keap1a- and keap1b-knockout larvae. In addition, we were interested to find that Nrf2 was inhibited to some extent in keap1a- and keap1b-knockout larvae under uninduced conditions and was strongly activated after sulforaphane treatment, suggesting that both Keap1a and Keap1b were able to inhibit Nrf2 without the help of their counterpart. Since fish Keap1a and Keap1b do not have cysteine residues corresponding to mouse Cys273 and Cys288, respectively [2], which are thought to be involved in Nrf2 inhibition [41], we hypothesized that Keap1a–Keap1b heterodimers formed between over-expressed and endogenous proteins, which were able to inhibit the Nrf2 activity in contrast to their unfunctional homodimers in Keap1a- and Keap1b-overexpressing embryos [19]. Indeed, we showed that Keap1a and Keap1b form heterodimers in vitro [19], and Wakabayashi et al. demonstrated that mouse C273A and C288A mutant Keap1s, which do not function alone, inhibited the Nrf2 activity when co-expressed in cultured cells [41]. However, our hypothesis turned out to be not correct. The results here clearly demonstrated that both Keap1a- and Keap1b-homodimers were able to inhibit the Nrf2 activity.

Cys151 has been identified as a specific sensor for sulforaphane in mammalian Keap1 [13,42,43]. Although both zebrafish Keap1a and Keap1b have cysteine residues that correspond to mammalian Cys151, the residue corresponding to Lys150 that may enhance the reactivity of Cys151 is threonine in Keap1a [13]. Indeed, we previously showed using Keap1-overexpressing embryos that zebrafish Keap1a has a lower affinity for Cys151-targeting type electrophiles, such as sulforaphane, than Keap1b [13]. The results here not only support our notion that zebrafish mainly use Keap1b to sense sulforaphane-type antioxidants but also prompts our hypothesis that the expression ratio between Keap1a and Keap1b affects the animal’s reactivity to such molecules. Water environments vary in their oxygen concentration, vegetation, flow, salt concentration, water pressure, temperature and other aspects. It may be possible that animals living in water, such as fish and amphibians, have advantage over amniotes in that they can more easily adapt to changes in their environment. The identification of Nrf2 activators mediated by Keap1a that may be specific to water environments will help clarify this possibility in the future.

Authors contributions

V.T.N., L.B. and S.O. performed the experiments; S.O. and A.K designed constructs for genome editing; J.T. and M.M. conducted data analysis; V.T.N. and M.K. designed the study and wrote the paper.

Declaration of competing interest

The authors have no conflict of interests to declare.
Fig. 5. The effect of sulforaphane pretreatment on the survival of zebrafish larvae after exposure to H₂O₂.

(A) A schematic illustration of the experiment. Larvae were treated with 2 mM of H₂O₂ at 4 dpf after 12-h pretreatment with 40 μM sulforaphane (SF).

(B) The survival rate of keap1a<sup>it302</sup> larvae.

(C) The survival rate of keap1b<sup>it308</sup> larvae.

(D) The fold increase in the expression of Nrf2 target genes in keap1a- and keap1b-homozygous knockout and wild-type larvae with sulforaphane treatment. Larvae at 5 dpf were treated with or without sulforaphane at the indicated concentration for 6 h, and the expression of prdx1 and gstp1 was analyzed by qPCR. The expression of untreated larvae was normalized to 1 (arrowheads, white dotted lines). Each experiment was conducted at least three times with duplicate samples. Asterisks indicate significant differences (*p < 0.05, **p < 0.01, ***p < 0.001). ns, not significant.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101667.

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