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Genetic hyperactivation of Nrf2 causes larval lethality in Keap1a and Keap1b-double-knockout zebrafish

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ABSTRACT

The Keap1–Nrf2 pathway is an evolutionarily conserved mechanism that protects cells from oxidative stress and electrophiles. Keap1 is a repressor of Nrf2 in normal cellular conditions but also a stress sensor for Nrf2 activation. Interestingly, fish and amphibians have two Keap1s (Keap1a and Keap1b), of which Keap1b is the ortholog of mammalian Keap1. Keap1a, on the other hand, is a gene found only in fish and amphibians, having been lost during the evolution to amniotes. We have previously shown that *keap1b*-knockout zebrafish have increased Nrf2 activity and reduced response to certain Nrf2-activating compounds but that they grow normally to adulthood. This may be because the remaining *keap1a* suppresses the hyperactivation of Nrf2, which is responsible for the post-natal lethality of *Keap1*-knockout mice. In this study, we analyzed *keap1a;keap1b*-double-knockout zebrafish to test this hypothesis. We found that *keap1a;keap1b*-double-knockout zebrafish, like *Keap1*-knockout mice, showed eating defects and were lethal within a week of hatching. Genetic introduction of the Nrf2 mutation rescued both the eating defects and the larval lethality, indicating that Nrf2 hyperactivation is the cause. However, unlike *Keap1*-knockout mice, *keap1a;keap1b*-double-knockout zebrafish showed no physical blockage of the food pathway; moreover, the cause of death was not directly related to eating defects. RNA-sequencing analysis revealed that *keap1a;keap1b*-double-knockout larvae showed extraordinarily high expression of known Nrf2-target genes as well as decreased expression of visual cycle genes. Finally, trigonelline or brusatol partially rescued the lethality of *keap1a;keap1b*-double-knockout larvae, suggesting that they can serve as an *in vivo* evaluation system for Nrf2-inhibiting compounds.

1. Introduction

The Keap1–Nrf2 pathway is a cellular defense system against oxidative and electrophilic stresses and is widely conserved among vertebrates [1,2]. Nrf2 is a transcriptional activator that regulates antioxidant and drug-metabolizing genes and is normally degraded by ubiquitin-proteasome-dependent proteolysis but is stabilized when oxidative stress or electrophiles accumulate in the cell. Keap1 suppresses Nrf2 as a mediator of Nrf2-specific ubiquitination and functions as a sensor molecule to sense Nrf2-activating compounds [3–5]. Interestingly, mammals, birds, and reptiles have only one Keap1 gene, whilst fish and amphibians have two isoforms, Keap1a and Keap1b [6–8]. Phylogenetic analysis revealed that Keap1b corresponds to Keap1 in mammals/birds/reptiles, whilst Keap1a disappeared during the evolution to amniotes [2,8].

Keap1-knockout (KO) mice were lethal within 3 weeks of birth, and this lethality was rescued by disruption of the *Nrf2* gene, indicating that Nrf2 hyperactivation caused the lethality [9]. In contrast, *keap1b*-KO zebrafish were viable to adulthood, although they showed some activation of Nrf2 [8]. We hypothesized that the *keap1b*-KO zebrafish did not accumulate Nrf2 as much as did the *Keap1*-KO mice because of the presence of *keap1a*, which can also suppress Nrf2. In this study, we generated *keap1a;keap1b*-double-KO (DKO) zebrafish and analyzed their phenotypes to test this hypothesis.

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2. Materials and methods

2.1. Zebrafish

keap1a-KO (*keap1a*^{it302}) [8], *keap1b*-KO (*keap1b*^{it308}) [8], Nrf2 mutant (*nfe2l2a*^{fh318}) [10], and wild-type (AB strain) zebrafish were used. Genotyping was performed as previously described [8,11]. *keap1a*^{it302} and *keap1b*^{it308} are available from the National BioResource Project (https://shigen.nig.ac.jp/zebra), and *nfe2l2a*^{fh318}, from the Zebrafish International Resource Center (http://zebrafish.org).

2.2. Other methods

See the Supplemental Materials and Methods for chemicals, details of the survival assay, eating analysis, histological analysis, Alcian blue staining, gene expression analysis, statistical analysis, regulation for animal experiments, and primers used in quantitative reverse transcription PCR (qRT-PCR).

3. Results

3.1. keap1a;keap1b-DKO larvae were lethal and showed eating defects

Heterozygous *keap1a*^{it302};*keap1b*^{it308} DKO (Ka1Kb1) parents were mated to generate homozygous DKO (Ka0Kb0) offspring together with siblings. At 7, 14, and 21 days-post-fertilization (dpf), about 100 of each offspring were collected and genotyped (Fig. 1A). All the genotypes appeared at 7 dpf, whilst there were no Ka0Kb0 larvae but others at 14 or 21 days, suggesting their lethality at the larval stage. We next raised 276 offspring from Ka1Kb1 parents (Fig. 1B, left) and 288 offspring from Ka0Kb1 parents (Fig. 1B, right) to 3 months-post-fertilization (mpf) adult fish and genotyped them and found no Ka0Kb0 adults. All these results indicate that zebrafish without Keap1a and Keap1b are lethal during larval development.

Keap1-KO post-natal mice die probably because of eating defects caused by a narrowed esophagus and forestomach [9]. Zebrafish larvae hatch at 2.5 dpf and start to eat after 5 dpf. To elucidate the reason for the lethality of *keap1a;keap1b*-DKO larvae, the eating defects of the larvae obtained from Ka0Kb1 parents were analyzed (Fig. 1C). The results showed that the majority of the Ka0Kb0 larvae were not eating even at 8 dpf, whilst their siblings started to eat from 5 dpf. To investigate the cause of the eating defect, the thicknesses of the esophagus and gut of Ka0Kb0 and Ka0Kb2 larvae were histologically analyzed (Fig. 1D). Unexpectedly, no significant difference was found between the Ka0Kb0 and the Ka0Kb2 larvae, suggesting that the gastrointestinal tract was normal. The structure of the mouth was also analyzed by means of cartilage staining, and again no obvious difference between the Ka0Kb0 and the Ka0Kb2 larvae was found (Fig. 1E). These results suggest that the eating defect in *keap1a;keap1b*-DKO larvae was not due to physical disturbances in the food pathway. We next performed a survival assay of larvae without feeding (Fig. 1F). The results showed that Ka0Kb2 and Ka0Kb1 larvae could survive without feeding at 11 dpf, whereas all the Ka0Kb0 larvae died by 10 dpf. This result suggests that the eating defect was not the only cause of the lethality of *keap1a;keap1b*-DKO larvae.

3.2. Lethality of keap1a;keap1b-DKO larvae was rescued by Nrf2 mutation

The lethality of *Keap1*-KO mice was rescued by Nrf2 disruption, indicating that the lethality is due to Nrf2 hyperactivation [9]. To determine if the lethality of the *keap1a;keap1b*-DKO larvae was also due to Nrf2 hyperactivation, the Nrf2 mutation (*nfe2l2a*^{fh318}) was introduced into Ka0Kb0 zebrafish. We first generated *keap1a*^{it302} homozygous, *keap1b*^{it308} heterozygous, and *nfe2l2a*^{fh318} heterozygous adults (Ka0Kb1N1) and then performed a survival assay using their offspring (Fig. 2A). Ka0Kb0N2 larvae, like the Ka0Kb0 larvae shown in Fig. 1A, were not found at later than 7 dpf, whilst Ka0Kb0N1 and Ka0Kb0N0 larvae were present at up to 21 dpf and 28 dpf, respectively. We also raised 205 offspring from Ka0Kb1N1 parents until 3- to 6-mpf adults and found Ka0Kb0N0, but not Ka0Kb0N2 and Ka0Kb0N1, adults among them (Fig. 2B). These results suggest that the lethality of the *keap1a;keap1b*-DKO larvae was due to Nrf2 hyperactivation, as in *Keap1*-KO mice, although the Nrf2-independent effects cannot be ruled out.

To investigate the effect of Nrf2 mutation on eating defects in *keap1a;keap1b*-DKO larvae, we next performed eating analysis using 8-dpf larvae obtained from Ka0Kb1N1 parents (Fig. 2C). The eating defects found in Ka0Kb0N2 larvae were partially rescued in Ka0Kb0N1 and fully rescued in Ka0Kb0N0 larvae. Similarly, survival assays without feeding were performed showing that the lethality was also rescued in Ka0Kb0N1 and Ka0Kb0N0 larvae (Fig. 2D). All these results indicate that Nrf2 hyperactivation is the cause of both eating defects and lethality in non-feeding conditions of *keap1a;keap1b*-DKO larvae.

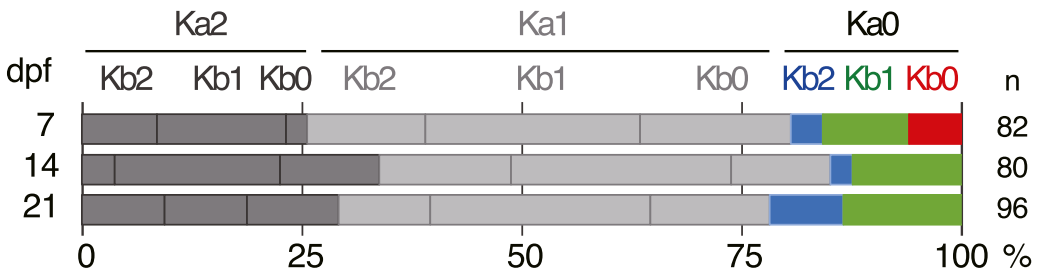
3.3. Nrf2 hyperactivation in keap1a;keap1b-DKO larvae

We next examined the expression of Nrf2 target genes in *keap1a;keap1b*-DKO larvae. First, *gstp1.2* expression in 5-dpf larvae was analyzed by whole-mount *in situ* hybridization (WISH) (Fig. 3A). The results showed that *gstp1.2* was expressed drastically higher in Ka0Kb0N2 larvae than in their siblings or wild-type controls. This expression was reduced in Ka0Kb0N0 larvae and partially so in Ka0Kb0N1 larvae, indicating that it is Nrf2-dependent. Next, we quantified the expression of *gstp1*.2 by means of qRT-PCR (Fig. 3B). The results showed that *gstp1.2* expression in Ka0Kb0N2 larvae was 14.35-fold higher than in wild-type larvae, whereas in Ka0Kb0N0 larvae, it was only 5.13-fold higher. Because the upregulated expression of *gstp1.2* was not statistically significant, we examined the expression of other Nrf2 target genes (*prdx1*, *txn*, and *gss*) and found a similar Nrf2-dependent upregulation that was statistically significant in Ka0Kb0N2 larvae (Fig. 3B). These results suggest that Nrf2 is abnormally activated in *keap1a;keap1b*-DKO larvae.

3.4. Genes abnormally expressed in keap1a;keap1b-DKO larvae

We next performed RNA-sequencing analysis (RNA-seq) using Ka0Kb0N2, Ka0Kb0N0, Ka2Kb2N0, and wild-type larvae to comprehensively identify genes that are aberrantly expressed in *keap1a;keap1b*-DKO larvae (Fig. 4A, Tables S1 and S2). As a result, we identified 599 genes as those expressed more than 2-fold higher and 595 genes as those expressed less than half in Ka0Kb0N2 larvae than in wild-type larvae. Using these genes, gene ontology (GO) analysis was performed to identify biological processes modulated by *keap1a;keap1b*-DKO (Fig. 4B, Table S3). We did not exclude 99 and 314 genes that were expected to be Nrf2-independent (see Fig. 4A) because known target genes of zebrafish Nrf2, such as *prdx1*, *txn*, *fthl31*, *sqor*, *gsr*, *histh1l1*, *gpx1a*, *tcnbb*, and *fthl27* [12–14], were included in these categories (see also Table S1). Most of the biological processes that emerged in the GO analysis were known processes that are regulated by the Keap1–Nrf2 pathway (Fig. 4B). On the other hand, “vitamin A metabolic process” and “visual perception” identified as biological processes downregulated in *keap1a;keap1b*-DKO larvae (Fig. 4B, lower panel) have not been previously reported. We therefore examined the expression of visual cycle genes by qRT-PCR. Although Nrf2 dependence was unclear, there was an overall trend indicating that these visual cycle genes were downregulated in *keap1a;keap1b*-DKO larvae (Fig. 4C). Finally, the effects of known Nrf2 inhibitors on the lethality of *keap1a;keap1b*-DKO larvae were examined, as a result of which the lethality was shown to be partially rescued by treatment with trigonelline or brusatol [15,16].

A

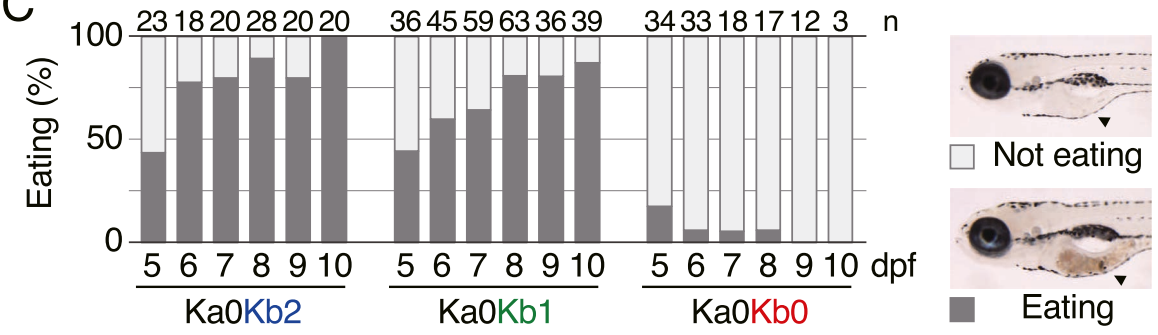


B

	WT	*keap1a*$^{-/+}$	*keap1a*$^{-/-}$
WT	26 (9.2%)	34 (12.3)	7 (2.5)
keap1b$^{-/+}$	35 (12.7)	78 (28.3)	39 (14.1)
keap1b$^{-/-}$	23 (8.3)	34 (12.3)	0 (0.0)

	keap1a$^{-/-}$
WT	104 (36.1%)
keap1b$^{-/+}$	184 (63.9)
keap1b$^{-/-}$	0 (0.0)

C



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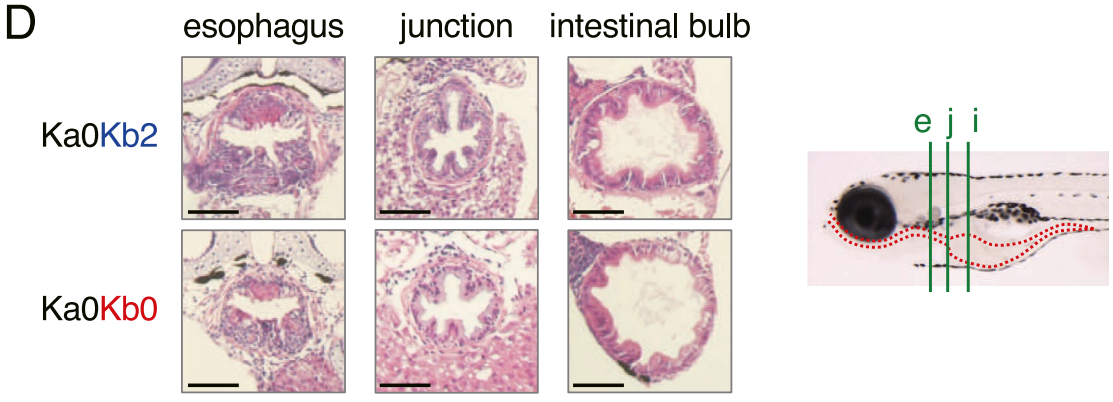

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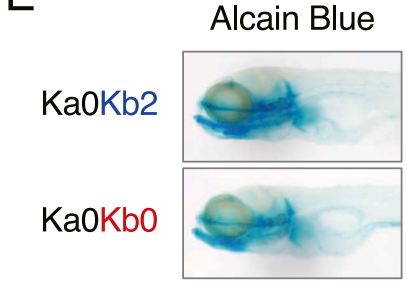

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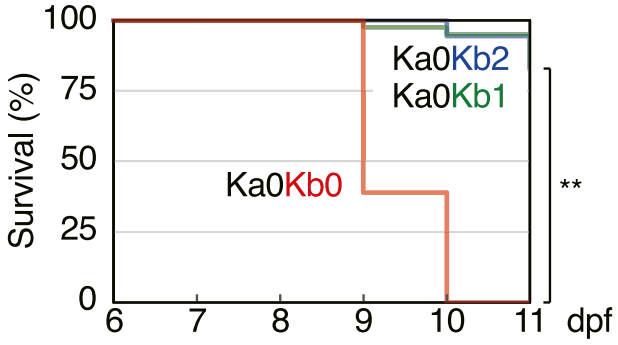

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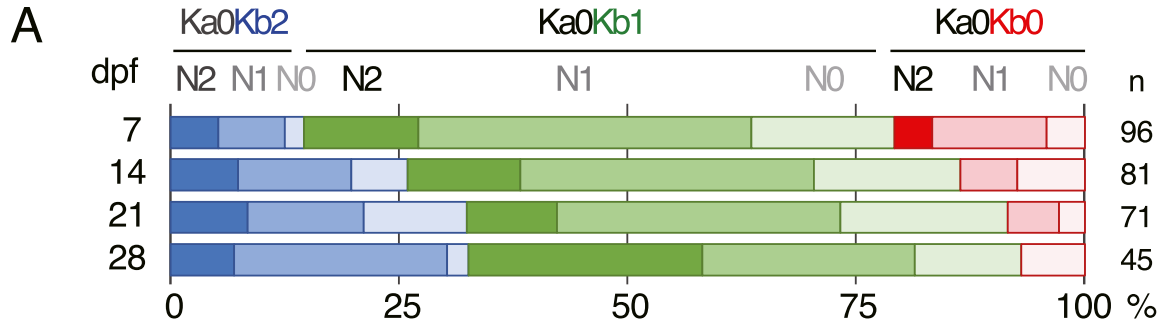
Fig. 1. Phenotypes of *keap1a;keap1b*-DKO zebrafish. (A) Survival rates of offspring from heterozygous *keap1a;keap1b*-DKO (Ka1Kb1) parents at 7, 14, and 21 dpf. The red bar represents homozygous *keap1a;keap1b*-DKO (Ka0Kb0) larvae. (B) Survival rates of 3-mpf offspring from Ka1Kb1 parents (left) and Ka0Kb1 parents (right). WT, −/+, and −/− indicate wild-type, heterozygous, and homozygous fish, respectively. Data from 3 independent experiments were combined. (C) Eating analysis of 5- to 10-dpf offspring from Ka0Kb1 parents. The numbers at the top are the number of larvae tested. It was noted that eating in Ka0Kb0 larvae was reduced. The black arrowheads in the right panels indicate the food inside the gut of the larvae. (D) Hematoxylin and eosin staining of paraffin sections of 7-dpf larvae. In the right panel, the larval digestive tract is indicated with red dotted lines, and vertical sections at the locations of the esophagus (e, green line), the esophagus-gut junction (j), and the intestinal bulb (i) are shown on the left panel. Scale bars, 50 μm. (E) Alcian blue staining of head cartilages of 7-dpf larvae. (F) Survival rates of offspring from Ka0Kb1 parents without feeding. Survival was observed every 24 h from 6 to 11 dpf. The tested numbers were as follows: Ka0Kb2 n = 18, Ka0Kb1 n = 40, Ka0Kb0 n = 18. The asterisks indicate significant differences (**$p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



B

		Ka0Kb2	Ka0Kb1	Ka0Kb0
nfe2l2a$^{+/+}$	(N2)	16 (7.8%)	24 (11.7)	0 (0.0)
nfe2l2a$^{fh318/+}$	(N1)	33 (16.1)	66 (32.2)	0 (0.0)
nfe2l2a$^{fh318/fh318}$	(N0)	19 (9.3)	36 (17.6)	11 (5.4)

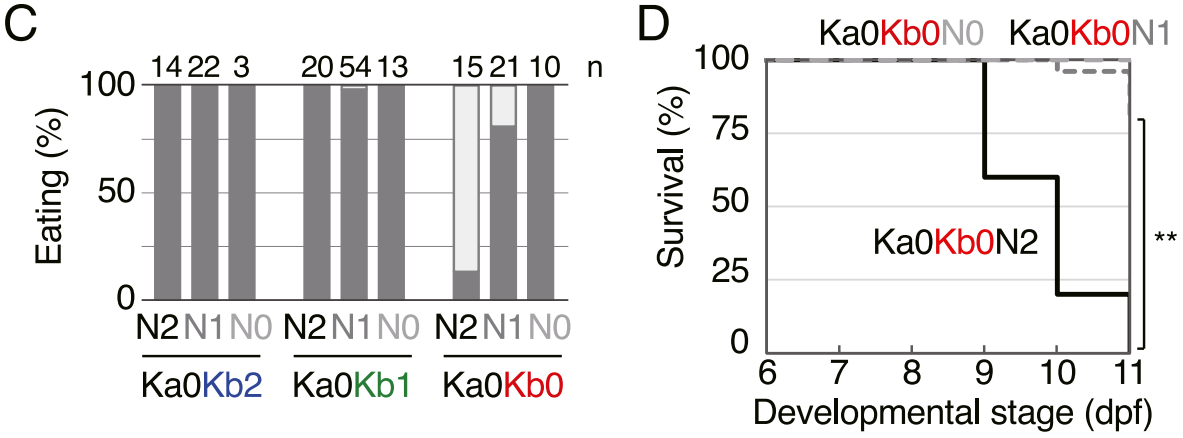


Fig. 2. Effects of the Nrf2 mutation on the phenotypes of *keap1a;keap1b*-DKO zebrafish. (A) Survival rates of offspring from Ka0Kb1N1 parents at 7, 14, 21, and 28 dpf. The red, pink, and lavenderblush bars represent Ka0Kb0N2, Ka0Kb0N1, and Ka0Kb0N0 larvae, respectively. N0, N1, and N2 designate homozygous, heterozygous, and wild-type of the Nrf2 mutant (*nfe2l2a*^{fh318}), respectively. (B) Survival rates of 3- to 6-mpf offspring from Ka0Kb1N1 parents. Data from 3 independent experiments were combined. (C) Eating analysis of offspring from Ka0Kb1N1 parents at 8 dpf. Data from 3 independent experiments were combined. (D) Survival rates of offspring from Ka1Kb0N1 parents without feeding. The survival was observed every 24 h from 6 to 11 dpf. The tested numbers were as follows: Ka0Kb0N2 n = 5, Ka0Kb0N1 n = 25, Ka0Kb0N0 n = 11. The asterisks indicate significant differences (**$p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

In the present study, we found that *keap1a;keap1b*-DKO zebrafish are lethal at the larval stage and have eating and other defects and that their larvae showed extraordinarily high expression of Nrf2 target genes. Both the larval lethality and the high expression of Nrf2 targets were rescued by the Nrf2 mutation, resulting in viable and fertile adults. The lethality of *keap1a;keap1b*-DKO zebrafish, which die around a week after hatching (10 dpf), is similar to that of *Keap1*-KO mice, which die within 3 weeks of birth [9]. Furthermore, the lethality of *keap1a;keap1b*-DKO zebrafish was rescued by the Nrf2 mutation, which is also similar to that of *Keap1*-KO mice [9]. These facts indicate that Nrf2 hyperactivation is fatal to post-natal mammals and post-hatched fish larvae. In both zebrafish and mice, one of the critical targets for hyperactivated Nrf2

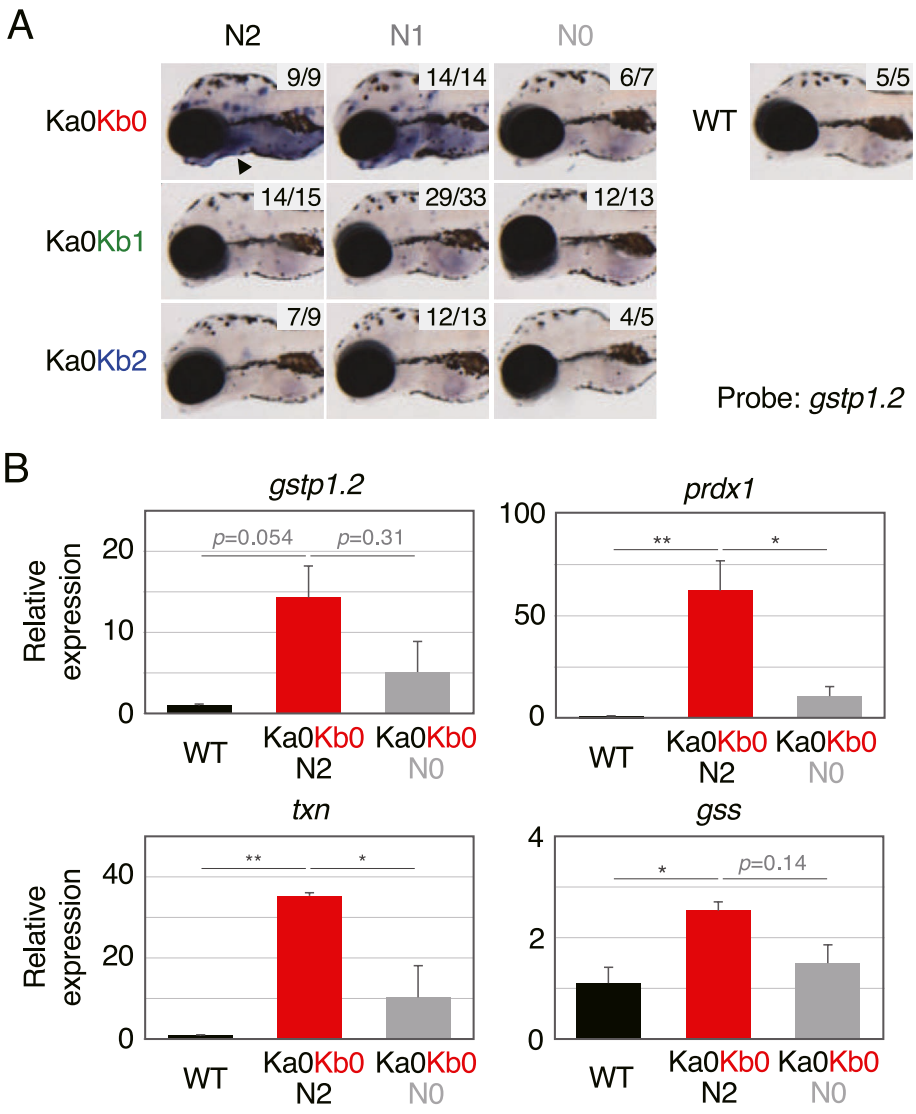


Fig. 3. Upregulation of Nrf2-target genes in *keap1a;keap1b*-DKO larvae. (A) WISH analysis of *gstp1.2* expression in 5-dpf offspring from Ka0Kb1N1 parents. The arrowhead indicates the gill. The numbers in each picture indicate the numbers of larvae with similar staining profiles and tested larvae. (B) qRT-PCR analysis of the relative expressions of *gstp1.2*, *prdx1*, *txn*, and *gss* in wild-type, Ka0Kb0N2, and Ka0Kb0N0 larvae at 5 dpf. The expression of the wild-type specimens was normalized to 1. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$).

was eating. In *Keap1*-KO mice, hyperkeratosis of the esophagus and forestomach inhibited food intake into the gastrointestinal tract [9], and its lethality was rescued by *Keratin 5*-Cre-specific KO of Nrf2 [17], suggesting that gastric obstruction of food may be the cause of the eating defects. In zebrafish, however, no such physical disturbance was found in the gastrointestinal tract, therefore suggesting another reason. Moreover, the lethality of *keap1a;keap1b*-DKO larvae has a cause different from eating defects. Whether these phenotypes are also present in mice remains to be clarified.

All these defects in *keap1a;keap1b*-DKO zebrafish and *Keap1*-KO mice were rescued by reducing Nrf2 activity, so Nrf2 hyperactivation is definitely the cause of the effects. Intriguingly, lethality did not appear at the embryonic stage, but after hatching/birth. Similar regulation may exist in invertebrates, because the disruption of Keap1 in *Drosophila* was also lethal in the larval stage [18,19]. A recently study of Keap1 in a group of birds, Neoaves, is also interesting [20]. The Neoaves Keap1 protein has a deletion in the Nrf2-binding domain and is unable to inhibit Nrf2, but Neoave chicks are viable and have no eating problems. It will be interesting to elucidate how Neoaves overcome the detrimental effects of Nrf2 hyperactivation. In any case, the lethality of *keap1a;keap1b*-DKO larvae fully rescued by Nrf2 mutation and partially so by chemical Nrf2 inhibitors could be a useful animal assay system to evaluate the efficacy/toxicity of Nrf2-inhibiting compounds currently under development to inhibit cancer growth.

We previously performed microarray analysis using Nrf2-overexpressing embryos and found that proteasome subunit genes were targets of zebrafish Nrf2 [14]. In contrast, none of the proteasome subunit genes were upregulated in *keap1a;keap1b*-DKO larvae. Because it has recently been shown that the expression of proteasome subunit genes is regulated by Nrf1 in mammalian cells [21], it is likely that these are not the original Nrf2 targets, but only artificially induced in embryos overexpressing Nrf2 by mRNA injection. On the other hand, a group of cancer-promoting genes observed in Nrf2-hyperactivated cancer cells [22,23], including growth-related factors, serine/glycine metabolic enzymes, and oncogenic proteins, were not upregulated in *keap1a;keap1b*-DKO larvae. Hyperactivation of Nrf2 alone may not be sufficient to upregulate these cancer-promoting genes.

The only downregulated pathways in *keap1a;keap1b*-DKO larvae that emerged in the current GO analysis were “vitamin A metabolic process” and “visual perception” pathways, to which the same genes belong, with no reduction in proinflammatory cytokine genes observed in *Keap1*-knockdown mice [24]. Some genes in these pathways are known to be induced by retinoic acid [25], and it is possible that abnormally increased Nrf2 interacted with retinoic acid receptor α [26] and/or retinoid X receptor α [27] to inhibit transcriptional activation of their target genes. Interestingly, the expression of visual cycle genes such as *lrata* and *rdh5*, which are downregulated in *keap1a;keap1b*-DKO larvae, is known to increase with age in mice [28]. Considering that Nrf2

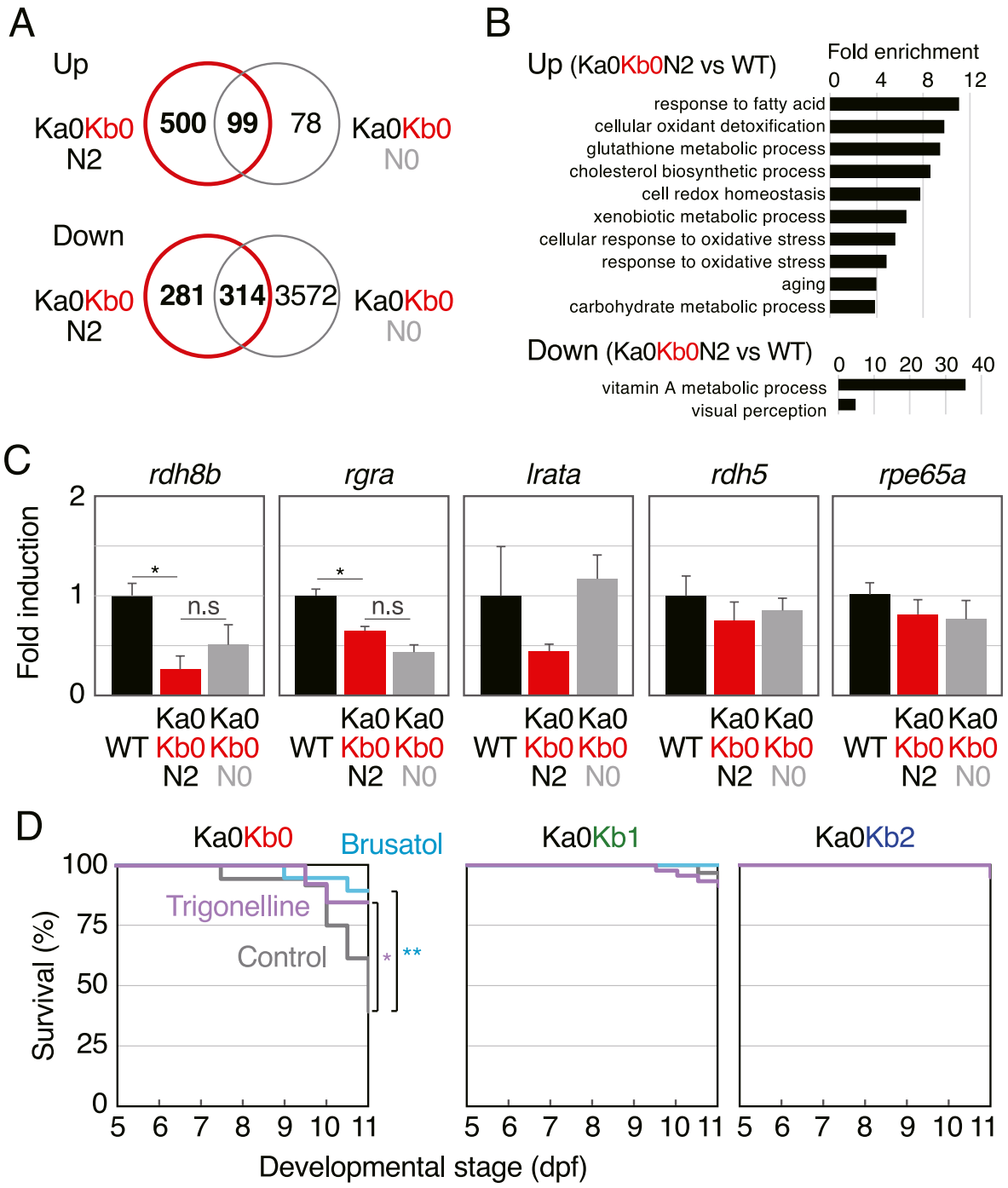


Fig. 4. RNA-seq and the effects of Nrf2 inhibitors. (A) Venn diagrams showing upregulated and downregulated genes in 5-dpf Ka0Kb0N2 and Ka0Kb0N0 larvae identified by means of RNA-seq in comparison with wild-type larvae. (B) GO enrichment analysis of the biological processes in *keap1a;keap1b*-DKO larvae. (C) qRT-PCR analysis of the relative expression of visual cycle genes (*rdh8b*, *rgra*, *lrata*, *rdh5*, and *rpe65a*) in wild-type, Ka0Kb0N2, and Ka0Kb0N0 larvae at 5 dpf. Asterisk and n.s. denote significant difference ($*p < 0.05$) and not significant, respectively. For *rpe65a, lrata*, and *rdh5*, one-way analysis of variance did not show significant differences. (D) Survival rates of offspring from Ka0Kb1 parents without feeding. Larvae were treated without (control, gray) or with 0.3 μM trigonelline (purple) or 0.3 μM brusatol (turquoise blue) from 5 to 11 dpf (replaced every 2 days). The tested numbers were as follows: brusatol n = 19, trigonelline n = 13, control n = 36 (Ka0Kb0); brusatol n = 39, trigonelline n = 45, control n = 63 (Ka0Kb1); brusatol n = 19, trigonelline n = 20, control n = 26 (Ka0Kb2). Asterisks indicate significant differences ($*p < 0.05$, $**p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

activity attenuates with age [29], it is possible that Nrf2 suppression is released with age, resulting in increased expression of visual cycle genes.

Authors contributions

Investigation, L.B., V.T.N.; materials, Y.E., G.D., A.S.; data analysis, J.T.; writing, L.B., M.K.

Declaration of competing interest

The authors have no conflict of interests to declare.

Data availability

No data was used for the research described in the article.

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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.2023.102673.

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