

Nitro-fatty acids and cyclopentenone prostaglandins share strategies to activate the Keap1-Nrf2 system: a study using GFP transgenic zebrafish

Tadayuki Tsujita^{1,2†}, Li Li^{1†}, Hitomi Nakajima¹, Noriko Iwamoto³, Yaeko Nakajima-Takagi¹, Ken Ohashi¹, Koichi Kawakami⁴, Yoshito Kumagai³, Bruce A. Freeman⁵, Masayuki Yamamoto^{2,6} and Makoto Kobayashi^{1,2*}

¹*Institute of Basic Medical Sciences, Graduate School of Comprehensive Human Sciences, Center for TARA, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8577, Japan*

²*ERATO Environmental Response Project, Japan Science and Technology Agency, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8577, Japan*

³*Institute of Community Medicine, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan*

⁴*Division of Molecular and Developmental Biology, National Institute of Genetics, and Department of Genetics, Graduate University for Advanced Studies (SOKENDAI), 1111 Yata, Mishima 411-8570, Japan*

⁵*Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15213, USA*

⁶*Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, 2-1 Seiryō-cho, Aoba-ku, Sendai 980-8575, Japan*

Running title: Nrf2 activation by nitro-fatty acids

**Correspondence:*

FAX number, +81-29-853-5977

e-mail, makobayash@md.tsukuba.ac.jp

[†]These authors equally contributed to this work.

Total character count: 33,789

Abstract

Nitro-fatty acids are electrophilic fatty acids produced *in vivo* from nitrogen peroxide that have many physiological activities. We recently demonstrated that nitro-fatty acids activate the Keap1-Nrf2 system, which protects cells from damage due to electrophilic or oxidative stresses via transactivating an array of cytoprotective genes, although the molecular mechanism how they activate Nrf2 is unclear. A number of chemical compounds with different structures have been reported to activate the Keap1-Nrf2 system, which can be categorized into at least six classes based on their sensing pathways. In this study, we showed that nitro-oleic acid (OA-NO₂), one of major nitro-fatty acids, activates Nrf2 in the same manner that of a cyclopentenone prostaglandin 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) using transgenic zebrafish that expresses green fluorescent protein (GFP) in response to Nrf2 activators. In transgenic embryos, GFP was induced in the whole body by treatment with OA-NO₂, 15d-PGJ₂ or diethylmaleate (DEM), but not with hydrogen peroxide (H₂O₂), when exogenous Nrf2 and Keap1 were co-overexpressed. Induction by OA-NO₂ or 15d-PGJ₂ but not DEM was observed, even when a C151S mutation was introduced in Keap1. Our results support the contention that OA-NO₂ and 15d-PGJ₂ share an analogous cysteine code as electrophiles and also have similar anti-inflammatory roles.

Introduction

The Keap1-Nrf2 system plays a central role in the cellular defense against electrophilic and oxidative stresses by orchestrating gene expression of detoxifying and antioxidant enzymes (Kobayashi & Yamamoto 2006; Kensler & Wakabayashi 2010). Nrf2 is a transcription factor that heterodimerizes with small Maf proteins and binds to the antioxidant responsive element (ARE)/electrophile responsive element (EpRE) within the regulatory region of its target genes. Keap1 is a substrate-specific adaptor protein for a Cul3-dependent E3 ubiquitin ligase complex that homodimerizes and interacts with the ETGE and DLG motifs of Nrf2. Under basal conditions, Nrf2 is maintained at low levels due to Keap1-dependent ubiquitination and proteasomal degradation. Upon exposure to electrophiles or oxidative stress, Nrf2 is stabilized and accumulates in the nucleus, where it transactivates ARE/EpRE-regulated genes.

A variety of Nrf2 activators have been reported (Kobayashi & Yamamoto 2005). Some of these activators have protective activities against carcinogenesis, neuronal damage and inflammation that can be ingested as dietary agents for the prevention and therapy of age-related diseases such as cancer, cardiovascular diseases, chronic inflammation, and neurodegenerative diseases (Calabrese *et al.* 2008). On the other hand, positive roles of Nrf2 in cancer tumorigenesis and chemoresistance have recently been uncovered (Hayes & McMahon 2009; Lau *et al.* 2008). It will be important to understand the differences between the Nrf2 activators, in particular the mechanisms of Keap1-Nrf2 activation, the activation of other systems, and which Nrf2-target genes they induce.

The new field has focused on the identification of sensor molecules that activate Nrf2. These activators have a common ability to modify sulfhydryl groups by alkylation, oxidation, or reduction. This observation suggests that cells possess a primary sensor for Nrf2 activators that is equipped with highly reactive cysteine residues. Cys-151 of mouse Keap1 is a target site for many Nrf2 activators such as DEM (Kobayashi *et al.* 2009; Sekhar *et al.* 2010). We recently found that

cyclopentenone prostaglandins, 15d-PGJ₂ and prostaglandin A₂ (PGA₂), target residues other than Cys-151 in Keap1, perhaps Cys-273 (Kobayashi *et al.* 2009). Furthermore, sensor proteins for H₂O₂, cadmium chloride, and gold-compound auranofin were not Keap1 or Nrf2 (Kobayashi *et al.* 2009). The target selections of a variety of Nrf2 activators should be investigated comprehensively.

The Keap1-Nrf2 system is conserved among vertebrates, including zebrafish (Kobayashi *et al.* 2002; Takagi *et al.* 2004; Li *et al.* 2008). Among the known endogenous targets of zebrafish Nrf2, the pi-class glutathione *S*-transferase 1 gene (*gstp1*) showed the strongest induction in both electrophile-treated larvae and Nrf2-overexpressing embryos. The gene regulatory region of *gstp1* was examined by a GFP reporter gene analysis using microinjection into zebrafish embryos, and an ARE/EpRE-like sequence located 30 bp upstream of the transcription initiation site was shown to be necessary and sufficient for the induction by Nrf2 (Suzuki *et al.* 2005).

In this study, we tried to generate stable transgenic zebrafish lines that express GFP in response to Nrf2 activators using a 3.5 kb gene regulatory region of *gstp1* to develop a rapid and easy method for screening and classifying Nrf2 activators. Two stable transgenic lines that express GFP in the larval olfactory regions in response to Nrf2 activators were isolated. No GFP induction was detected in transgenic embryos, but strong induction in response to DEM and 15d-PGJ₂, but not H₂O₂, was observed when both Nrf2 and Keap1 were overexpressed. Using this system, we classified a newly identified Nrf2 activator, OA-NO₂, into the same category as 15d-PGJ₂.

Results

Generation of stable transgenic lines that express GFP in response to Nrf2 activators

In transient assays, GFP expression from the p3.5gstp1GFP construct which contains a 3.5 kb promoter region of the zebrafish *gstp1* gene is strongly transactivated by Nrf2 in zebrafish embryos (Fig. 1A; Suzuki *et al.* 2005). Stable transgenic lines carrying this construct were isolated by genotyping F1 embryos from p3.5gstp1GFP-injected founders. Although three stable lines were isolated, none of the three lines exhibited GFP expression upon treatment with DEM, thus suggesting a position effect of the transgene (data not shown; Table S1 in Supporting Information). To circumvent this problem, a highly efficient *Tol2* transposon system (Kawakami *et al.* 2004) was used to generate additional stable lines. A pT3.5gstp1GFP construct was made by introducing *Tol2* sequences into p3.5gstp1GFP and were co-injected into zebrafish embryos with mRNA encoding *Tol2*-specific transposase. Injected founders (n=104) were raised and 12 transgenic lines, which showed GFP expression in F1 larvae, were isolated (Table S1 in Supporting Information). Out of 12 *Tg(-3.5gstp1:GFP)* lines, two lines exhibited GFP induction in response to DEM, and the rest displayed only basal GFP expression. In the larvae of these two lines (*it416a* and *it416b*), strong GFP induction was observed in the olfactory regions as for endogenous *gstp1*, while constitutive GFP expression was also detected in the lens, ears, fins, pericardium, and lateral lines (Figs. S1 and S2 in Supporting Information). We used the *it416b* line for further experiments because it exhibited stronger GFP induction than the *it416a* line.

In day 4 larvae, induction of endogenous *gstp1* expression began approximately 3 hours after DEM treatment and reached maximum expression levels around 6 to 9 hours (data not shown). GFP induction in the olfactory regions of *Tg(-3.5gstp1:GFP)it416b* larvae was first detected at 6 hours after DEM treatment and later became stronger (Fig. 1B; Movie S1 in Supporting Information). This induction was greatly reduced when *nrf2* was knocked down with the *nrf2*-specific

antisense-morpholino oligonucleotide (*nrf2*MO) (Kobayashi *et al.* 2002), suggesting that GFP induction was mediated by Nrf2 (Fig. 1C).

Quantification of GFP induction

To quantify the level of the GFP expression, a fluorescent intensity in the olfactory regions was measured using an imaging analyzer equipped with the BZ-8000 microscope (Keyence) (Fig. 2A). The average fluorescent intensities of DEM-induced GFP expression in the olfactory regions were measured using 3, 10, or 30 individual larvae, and all of these showed similar values (Fig. 2B). We therefore used three individuals for quantification of the GFP induction in further analyses. According to this quantification, the reduction of DEM-induced GFP expression by the *nrf2*-knockdown was calculated as one-third (Fig. 2C).

We first quantified the time- and dose-dependencies of DEM-induced GFP expression. GFP expression in the olfactory regions of *Tg(-3.5gstp1:GFP)it416b* larvae treated with various DEM concentrations was measured at 3, 6, 10 and 14 hours following treatment (Fig. 2D). GFP induction is detectable with treatment over 30 μ M DEM, which is similar for detecting endogenous *gstp1* induction by a real-time reverse transcription (RT)-PCR examination (Fig. 2E) or by a whole-mount *in situ* hybridization (data not shown). The onset of GFP induction (6 h) was later than that of endogenous *gstp1* induction (3 h), most likely due to the time required for the accumulation of GFP protein. Altogether, these results suggest that the measurement sensitivity of DEM-induced GFP expression using the fluorescence intensities in the olfactory regions is comparable to that of endogenous *gstp1* induction in the whole body using a real time RT-PCR analysis.

Quantification of reduced GFP induction in the *it567* mutant larvae

We have screened and isolated zebrafish mutants that have defects in *gstp1* induction by Nrf2

activators (Kobayashi *et al.* 2009). One of these mutants, *it567*, has reduced induction of *gstp1* in response to DEM. To visualize this reduction in living larvae, we crossed *it567* fish with *Tg(-3.5gstp1:GFP)it416b* fish. As expected, DEM-induced GFP expression in the olfactory regions of *it567* homozygous larvae was significantly reduced compared to that of sibling wild-type larvae (Fig. 3; Movie S2 in Supporting Information). The result indicates that homozygous mutant larvae are easily distinguishable from wild-type siblings by monitoring GFP induction upon treatment with DEM, although we have not identified the gene responsible for the *it567* mutation.

GFP induction by a variety of Nrf2 activators

To elucidate whether *Tg(-3.5gstp1:GFP)it416b* can monitor the induction by a variety of Nrf2 activators, five Nrf2-activating compounds in different classes were examined (Kobayashi *et al.* 2009). Day 4 larvae of *Tg(-3.5gstp1:GFP)it416b* fish were treated with sulforaphane, 15d-PGJ₂, H₂O₂, auranofin or CdCl₂, and the GFP expression in the olfactory regions was monitored in three individuals (Fig. 4A). GFP induction was observed in larvae treated with all tested Nrf2 activators, although it was a bit weaker upon induction with H₂O₂ and CdCl₂. The results suggest that *Tg(-3.5gstp1:GFP)it416b* can be used for the analysis of Nrf2 activator sensing mechanisms and also to screen for novel and unidentified activators.

We recently classified eleven Nrf2 activators based on the requirement of these activators for an Nrf2 response (Kobayashi *et al.* 2009). The classification was carried out using zebrafish embryos overexpressing Nrf2 and Keap1. The embryos were treated with each Nrf2 activator and the induction of the endogenous *gstp1* gene was analyzed by a whole-mount *in situ* hybridization or by an RT-PCR analysis. It will be time- and labor-saving if *Tg(-3.5gstp1:GFP)it416b* can be utilized for this analysis. To test this possibility, Nrf2 was overexpressed by an mRNA injection in embryos obtained from crossing heterozygous transgenic males and non-transgenic females and GFP expression was analyzed during gastrulation stages (Fig. S3 in Supporting Information). Robust

GFP expression was observed in the embryos injected with Nrf2 mRNA, while no GFP expression was observed in uninjected embryos.

We then generated homozygous transgenic lines to make all the injected embryos positive for GFP. Embryos obtained from homozygous transgenic males and non-transgenic females were used for the further analyses, because maternal GFP expression was observed in embryos derived from transgenic females (data not shown). When wild-type or C151S mutant Keap1 was overexpressed with Nrf2, no GFP expression was observed, indicating that Keap1 suppressed GFP inducing activity of Nrf2 (Fig. 4B). Treatment of DEM cancelled the Nrf2-suppression by wild-type Keap1 but not by C151S Keap1, while 15d-PGJ₂ released Nrf2 activity from both. H₂O₂ treatment had no effect at all. These results suggest that Keap1 is a sensor for DEM and 15d-PGJ₂, but not for H₂O₂, and Cys-151 is required for DEM but not 15d-PGJ₂ sensing. All these results were identical as in the case of the previous analyses conducted using endogenous *gstp1* induction (Kobayashi *et al.* 2009).

OA-NO₂ activates Nrf2 as 15d-PGJ₂

OA-NO₂ was next analyzed as an uncharacterized Nrf2 activator using *Tg(-3.5gstp1:GFP)it416b*. OA-NO₂ is a nitro-fatty acid that has been recently shown to activate Nrf2 (Freeman *et al.* 2008). Nitro-fatty acids are electrophilic fatty acids produced *in vivo* from nitrogen peroxide that have many physiological activities, such as cGMP-dependent vessel relaxation, the inhibition of the inflammatory cell function, the induction of heme oxygenase 1 expression, the inhibition of NFκB, peroxisome proliferator-activated receptor (PPAR) activation, and Nrf2 activation. GFP expression in the olfactory regions of *Tg(-3.5gstp1:GFP)it416b* larvae was analyzed after treatment with OA-NO₂ at 4 days post-fertilization (dpf) (Fig. 5A,B). A significant GFP induction was observed in the olfactory regions of OA-NO₂-treated larvae and was decreased upon Nrf2 knockdown after injecting *nrf2*MO. GFP induction was also observed with OA-NO₂ treatment of embryos

overexpressing Nrf2 and Keap1 (Fig. 5C). Significantly, the Keap1 C151S mutation could not eliminate this induction, suggesting that Cys-151 is not a sensing site for OA-NO₂ in the Keap1-Nrf2 system. Identical results were obtained when the induction of endogenous *gstp1* was analyzed by RT-PCR (Fig. 5D). We also showed that a Cys-to-Ser mutation in Cys-288, another key reactive cysteine in Keap1, had no effect on the response to OA-NO₂ (Fig. S4 in Supporting Information). It must be noted that these are same results as in the case of 15d-PGJ₂ (Kobayashi *et al.* 2009).

To determine the sites in Keap1 modified by OA-NO₂, mouse Keap1 protein treated with or without OA-NO₂ was digested with trypsin and analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Peptide mass mapping by an MALDI-TOF MS analysis of the trypsin fragments of native mouse Keap1 made it possible to identify the peptides, thus accounting for ~80% of the protein sequence (see Table S2 in the Supporting Information). Compared with the calculated masses of the unmodified peptides, modified peptides P-1 to P-6 had an increased mass of +327.4 Da, thus corresponding to the addition of a single equivalent of OA-NO₂ (Fig. 6). The sequences and masses of the peptides were: P-1 (CHALTPR, m/z = 1124.8), P-2 (SGVGVAVTMEPCR, m/z = 1632.7), P-3 (LNSAECYYPER, m/z = 1671.7), P-4 (LSQQLCDVTLQVK, m/z = 1802.1), P-5 (SGLAGCVVGGLLYAVGGR, m/z = 1976.2), and P-6 (QEEFFNLSHCQLATLISR, m/z = 2463.3), indicating that mouse Keap1 is modified by OA-NO₂ at Cys-77, Cys-226, Cys-273, Cys-368, Cys-489 and Cys-613. It should be noted that Cys-273 was included in the amino acids to which 15d-PGJ₂ and PGA₂ were shown to bind in our previous analyses (Fig. 6B; Kobayashi *et al.* 2009).

Another criterion of the classification is dependency to *it275* gene. *it275* is a zebrafish mutant which has defects in the response to some, but not all, Nrf2 activators. For example, induction of endogenous *gstp1* was decreased in *it275* larvae in response to 15d-PGJ₂, auranofin and CdCl₂, and was similar with wild-type larvae in response to DEM, sulforaphane and H₂O₂. *gstp1* expression

was analyzed in *it275* larvae or sibling wild-type larvae with or without OA-NO₂ treatment at 4 dpf by an *in situ* hybridization (Fig. 7). OA-NO₂-dependent *gstp1* induction in gills or olfactory regions was dramatically reduced in *it275* larvae compared to wild-type larvae. The reduction was not observed in larvae treated with DEM. This result suggests that Nrf2 activation mediated by OA-NO₂ requires the *it275* gene, which is also similar to 15d-PGJ₂.

Taken together, OA-NO₂ was classified as a class 4 Nrf2 activator, as are 15d-PGJ₂ and PGA₂ (Kobayashi *et al.* 2009). We thus hypothesize that nitro-fatty acids and cyclopentenone prostaglandins share strategies to activate the Keap1-Nrf2 system.

Discussion

We previously demonstrated that nitrolinoleic acid (LNO₂), a nitro-fatty acid similar to OA-NO₂, also activates Nrf2 in cultured cells (Villacorta *et al.* 2007). The present study verifies that nitro-fatty acids are indeed effective Nrf2 activators in living animals. The covalent binding of OA-NO₂ with reactive cysteines and histidines has been demonstrated previously in a variety of proteins (Batthyany *et al.* 2006). It is quite possible that OA-NO₂ directly binds Keap1, most likely at Cys-273. Importantly, OA-NO₂ appears to belong to the same Nrf2 activator class as 15d-PGJ₂ as we have described herein. Strikingly the chemical structures of nitro-fatty acids (OA-NO₂ and LNO₂) and cyclopentenone prostaglandins (15d-PGJ₂ and PGA₂) are similar (Fig. 7A). We speculate that unique structures common between these compounds are essential for targeting identical sites in the Keap1 protein. Besides activating Nrf2, nitro-fatty acids can activate PPAR γ as an endogenous ligand with covalent binding to Cys-285 (Schopfer *et al.* 2005; 2010), and can inhibit NF κ B-DNA binding through direct interaction with p65 (Cui *et al.* 2006). Interestingly, 15d-PGJ₂ also exhibits these properties via direct binding to PPAR γ at Cys-285 (Shiraki *et al.* 2006) and p65 of NF κ B at Cys-38 (Straus *et al.* 2000). It is plausible that OA-NO₂ can also bind to Cys-38 of NF κ B p65. We hypothesize that electrophilic signaling compounds, such as nitro-fatty acids and cyclopentenone prostaglandins, can bind to a series of key target proteins (*e.g.*, Keap1, PPAR γ and NF κ B) and thereby exert their physiological functions (for example, anti-inflammatory effects) by the combined actions of their target proteins (Fig. 7B) (the cysteine code hypothesis, Kobayashi *et al.* 2009).

The *Tg(-3.5gstp1:GFP)it416b* line established in the current study can monitor electrophile-induced gene expression *in vivo* and will be useful for many situations, for example in gene analyses, small molecule screens, and mutant fish screens. Generation of real-time monitoring

systems of electrophile-induced Nrf2 activation has been studied for many years. Stable cell lines that can monitor Nrf2 activation were previously produced using multiple ARE/EpRE sequences as transcriptional drivers and GFP or luciferase as reporter genes (Zhu & Fahl 2001; Wang *et al.* 2006). The results obtained by use of these cell lines are reproducible and are more reliable than experiments relying on transient transfection. Transgenic mice which have luciferase reporter genes fused to multiple ARE/EpRE sequences were also generated (Yates *et al.* 2007; Fisher *et al.* 2007). These transgenic mice are useful since they can allow the monitoring of tissue- and developmental stage-specific profiles of Nrf2 activation. Real-time activation of Nrf2 in disease models can also be analyzed by crossing these mice with a variety of gene-disrupted mice. However, this model has shortcomings, in that administration of the substrate luciferin into mice is required, whose disposition and side-effects may affect the proper analysis of results. The use of GFP instead of luciferase will be helpful to overcome this problem: however, it is difficult to analyze GFP expression in the internal regions of a mouse. Trials to generate stable transgenic zebrafish that harbor ARE/EpRE-driven GFP reporter gene have been carried out (Carvan *et al.* 2000; Kusik *et al.* 2008) but were unsuccessful. *Tg(-3.5gstp1:GFP)it416b* is the first ARE/EpRE-GFP reporter line that can be used to monitor Nrf2 activation at a practical and physiologically relevant level. Our success was due to a screen of effective lines from substantial numbers of stable transgenic lines. In that sense, the use of the *Tol2* transposon system was an important strategy which greatly improved the transgene integration efficiency (Kawakami 2007).

At the onset of this study, we hoped to establish transgenic lines that can analyze tissue-specific differences in GFP induction upon induction by a variety of Nrf2 activators. Unfortunately, this method did not succeed. GFP induction in gills and livers, in which endogenous *gstp1* was induced (in addition to the olfactory regions), was not observed in the present lines. We supposed that *cis*- and *trans*-activating factors required for *gstp1* induction differ between tissues. Indeed, cell-specific differences in the induction levels of the ARE/EpRE-reporter gene have been demonstrated using

cultured cell lines (Wang *et al.* 2006). Gene regulatory elements required for the expression in gills and liver other than ARE/EpRE sequences may not be included in the 3.5-kb *gstp1* region. The negative and positive roles of Nrf2 in human health have both been recently demonstrated (Hayes & McMahon 2009; Lau *et al.* 2008). It will be important to elucidate the molecular mechanisms of the cell-specific induction of Nrf2 target genes, particularly in cancer cells. Modified bacterial artificial chromosomes for transgenesis will be useful to identify *cis*-activating factors for cell specificity (Yang *et al.* 2009).

The zebrafish is an attractive model for analyzing gene expression profiles since it has transparent embryos and larvae. Moreover, a transparent adult line, termed *casper*, has been recently generated (White *et al.* 2008). Thus, utilizing a GFP reporter will facilitate the visualization of a variety of stress responses in living animals. Indeed, cellular responses to metals, such as cadmium, copper, and arsenate, were monitored in GFP transgenic zebrafish using heat shock promoters (Blechinger *et al.* 2007; Wu *et al.* 2008; Seok *et al.* 2007). GFP induction by environmental estrogens and dioxin was also analyzed by GFP transgenic zebrafish using estrogen response elements + *vitellogenin* promoter (Chen *et al.* 2010) and *cytochrome P4501A1* promoter (Mattingly *et al.* 2001), respectively, as transcriptional drivers. The zebrafish provides opportunities to accelerate the process of drug discovery (Zon & Peterson 2005). These GFP transgenic lines, including *Tg(-3.5gstp1:GFP)it416b*, will contribute to several aspects of the drug development process, including target identification by genetic and morpholino-oligonucleotide screens, lead discovery by small-molecule screens, and the testing of drug efficacy and toxicity. The application of *Tg(-3.5gstp1:GFP)it416b* to screen novel Nrf2 activators and inhibitors will be therefore be pursued in future studies.

Experimental procedures

Zebrafish

Embryos and larvae were obtained by natural mating. The wild-type strain AB and the mutant strain *it567* (Kobayashi *et al.* 2009) were used. The stable transgenic lines *Tg(-3.5gstp1:GFP)it416a* and *Tg(-3.5gstp1:GFP)it416b* were maintained as homozygous lines. Homozygous transgenic lines were crossed with the AB strain to obtain embryos and larvae for experiments. The genotyping of transgenic lines was performed by PCR using genomic DNA as templates and the primers 5'-CCTCGTGACCACCCTGAC-3' and 5'-TGGCGGATCTTGAAGTTCAC-3'.

The transgenic line, *Tg(-3.5gstp1:GFP)it416b*, can be obtained upon request under the support of Zebrafish National Bioresource Project of Japan (http://www.shigen.nig.ac.jp/zebra/index_en.html).

Chemical Treatments

For the induction studies, zebrafish were placed in culture dishes containing 100 μ M DEM (Wako, Osaka, Japan), 40 μ M sulforaphane (LKT laboratories, St. Paul, MN), 2.5 μ M 15d-PGJ₂ (Cayman Chemical, Ann Arbor, MI), 1 μ M auranofin (Enzo Life Sciences, Farmingdale, NY), 1 mM H₂O₂ (Wako), 20 μ M CdCl₂ (Wako) and 1.5 or 2.5 μ M OA-NO₂. OA-NO₂ was prepared as previously described (Baker *et al.* 2005).

Plasmids

pT3.5gstp1GFP was generated by replacing the *Xenopus* EF1 α enhancer/promoter region in pT2KXIG (Kawakami *et al.* 2004) with a *Xho*I-*Not*I fragment of p3.5gstp1GFP (Suzuki *et al.* 2005). The construct was verified by DNA sequencing. Plasmids pCS2nrf2, pCS2FLmKeap1, pCS2FLmKeap1C151S, pCS2FLmKeap1C288S, pKSgstp1N, and pCS-TP were described

previously (Kobayashi *et al.* 2002; 2009; Kawakami *et al.* 2004).

Microinjection

For the overexpression of Nrf2 and Keap1 proteins, synthetic capped mRNA was generated with an SP6 mMESSAGE mMACHINE *in vitro* transcription kit (Ambion, Austin, TX) using linearized DNA of the pCS2 derivatives described above and injected into yolk at the one-cell stage using an IM300 microinjector (Narishige, Tokyo, Japan). For knocking-down zebrafish Nrf2 (*nrf2*), antisense morpholino-oligonucleotides (Gene Tools, Philomath, OR) specific for *nrf2* (*nrf2*MO) (Kobayashi *et al.* 2002) were injected into yolk at the one-cell stage. For generating stable transgenic lines, 25 pg of pT3.5gstp1GFP or p3.5gstpGFP was co-injected into a blastomere of early one-cell stage embryos with 25 pg of *Tol2*-transposase mRNA synthesized from *NotI*-linearized pCS-TP as previously described (Takeuchi *et al.* 2010).

Expression Analysis

GFP expression during various embryonic stages was examined under a GFP2 (480 nm excitation, 510 nm barrier) filter of a MZFLIII microscope (Leica, Wetzlar, Germany) equipped with a 600CL-CU digital camera (Pixera Los Galos, CA). GFP expression during larval stages was analyzed under a GFP-BP BZ filter (470 nm excitation, 535 nm emission) of a Biozero BZ-8000 microscope (Keyence, Osaka, Japan). For taking time-lapse photographs, larvae were anesthetized with 0.016% 3-aminobenzoic acid ethyl ester (Sigma-Aldrich, St. Louis, MO) and placed on a hole slide glass containing 3% methylcellulose (Sigma Aldrich) for immobilization. To quantify GFP expression, fluorescence intensity in the olfactory regions was measured by an image processing system equipped with the BZ-8000 microscope. The gene expressions of endogenous *gstp1* were examined by an *in situ* hybridization analysis, a RT-PCR analysis, or a real-time RT-PCR analysis, as previously described (Li *et al.* 2008; Kobayashi *et al.* 2001).

MALDI-TOF MS analysis. The recombinant mouse Keap1 protein expressed by *E. coli* was incubated with or without 100 μ M OA-NO₂ for 60 min at 25°C in buffer containing 20 mM Tris-HCl, pH 8.5. The trypsin-digested mouse Keap1 was mixed with dithiothreitol and trifluoroacetic acid. To improve the ionization efficiency of MS, samples were purified with Zip-tip μ C18 (Millipore, Bedford, MA) before MS analysis. Peptides were mixed with α -cyano-4-hydroxycinnamic acid (2.5 mg/ml) containing 50% acetonitrile and 0.1% trifluoroacetic acid and dried on stainless steel targets at room temperature. The analyses were performed using an AXIMA-TOF2 (Shimadzu, Tokyo, Japan) with a nitrogen laser. All analyses took place in the positive ion mode and the instrument was calibrated immediately prior to each series of studies.

Acknowledgements

We thank Sakura Motion Picture Co. Ltd for their assistance with the movies. We also thank T. Kinoshita, H. Niu, T. Shimokoube and Y. Terashita for help in fish maintenance, and T. Suzuki, M. Takeuchi and K. Mukaigasa for help and discussion. We also thank T. Kudoh for critical reading of the manuscript. This work was supported by Grants-in-Aid from the Japan Science and Technology Corporation (ERATO) (to M.Y.), and the Ministry of Education, Science, Sports and Culture of Japan (to M.K. and M.Y.).

References

- Baker, P.R.S., Lin, Y., Schopfer, F.J., Woodcock, S.R., Groeger, A.L., Batthyany, C., Sweeney, S., Long, M.H., Iles, K.E., Baker, L.M.S., Branchaud, B.P., Chen, Y.E. & Freeman, B.A. (2005) Fatty acid transduction of nitric oxide signaling: multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands. *J. Biol. Chem.* **280**, 42464-42475.
- Batthyany, C., Schopfer, F.J., Baker, P.R.S., Durán, R., Baker, L.M.S., Huang, Y., Cerveñansky, C., Branchaud, B.P. & Freeman, B.A. (2006) Reversible post-translational modification of proteins by nitrated fatty acids *in vivo*. *J. Biol. Chem.* **281**, 20450-20463.
- Blechinger, S.R., Kusch, R.C., Haugo, K., Matz, C., Chivers, D.P. & Krone, P.H. (2007) Brief embryonic cadmium exposure induces a stress response and cell death in the developing olfactory system followed by long-term olfactory deficits in juvenile zebrafish. *Toxicol. Appl. Pharmacol.* **224**, 72-80.
- Calabrese, V., Cornelius, C., Mancuso, C., Pennisi, G., Calafato, S., Bellia, F., Bates, T.E., Giuffrida-Stella, A.M., Schapira, T., Dinkova-Kostova, A.T. & Rizzarelli, E. (2008) Cellular stress response: a novel target for chemoprevention and nutritional neuroprotection in aging, neurodegenerative disorders and longevity. *Neurochem. Res.* **33**, 2444-2471.
- Carvan, M.J., 3rd, Dalton, T.P., Stuart, G.W. & Nebert, D.W. (2000) Transgenic zebrafish as sentinels for aquatic pollution. *Ann. N. Y. Acad. Sci.* **919**, 133-147.
- Chen, H., Hu, J., Yang, J., Wang, Y., Xu, H., Jiang, Q., Gong, Y., Gu, Y. & Song, H. (2010) Generation of a fluorescent transgenic zebrafish for detection of environmental estrogens. *Aquat. Toxicol.* **96**, 53-61.
- Cui, T., Schopfer, F.J., Zhang, J., Chen, K., Ichikawa, T., Baker, P.R.S., Batthyany, C., Chacko, B.K., Feng, X., Patel, R.P., Agarwal, A., Freeman, B.A. & Chen, Y.E. (2006) Nitrated fatty acids: Endogenous anti-inflammatory signaling mediators. *J. Biol. Chem.* **281**, 35686-35698.

- Fisher, C.D., Augustine, L.M., Maher, J.M., Nelson, D.M., Slitt, A.L., Klaassen, C.D., Lehman-McKeeman, L.D. & Cherrington, N.J. (2007) Induction of drug-metabolizing enzymes by garlic and allyl sulfide compounds via activation of constitutive androstane receptor and nuclear factor E2-related factor 2. *Drug Metab. Dispos.* **35**, 995-1000.
- Freeman, B.A., Baker, P.R.S., Schopfer, F.J., Woodcock, S.R., Napolitano, A. & d'Ischia, M. (2008) Nitro-fatty acid formation and signaling. *J. Biol. Chem.* **283**, 15515-15519.
- Hayes, J.D. & McMahon, M. (2009) *NRF2* and *KEAP1* mutations: permanent activation of an adaptive response in cancer. *Trends Biochem. Sci.* **34**, 176-188.
- Kawakami, K. (2007) *Tol2*: a versatile gene transfer vector in vertebrates. *Genome Biol.* **8 Suppl 1**, S7.
- Kawakami, K., Takeda, H., Kawakami, N., Kobayashi, M., Matsuda, N. & Mishina, M. (2004) A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. *Dev. Cell* **7**, 133-144.
- Kensler, T.W. & Wakabayashi, N. (2010) Nrf2: friend or foe for chemoprevention? *Carcinogenesis* **31**, 90-99.
- Kobayashi, M., Itoh, K., Suzuki, T., Osanai, H., Nishikawa, K., Katoh, Y., Takagi, Y. & Yamamoto, M. (2002) Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. *Genes Cells* **7**, 807-820.
- Kobayashi, M., Li, L., Iwamoto, N., Nakajima-Takagi, Y., Kaneko, H., Nakayama, Y., Eguchi, M., Wada, Y., Kumagai, Y. & Yamamoto, M. (2009) The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. *Mol. Cell. Biol.* **29**, 493-502.
- Kobayashi, M., Nishikawa, K., Suzuki, T. & Yamamoto, M. (2001) The homeobox protein Six3 interacts with the Groucho corepressor and acts as a transcriptional repressor in eye and forebrain formation. *Dev. Biol.* **232**, 315-326.

- Kobayashi, M. & Yamamoto, M. (2005) Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid. Redox Signal.* **7**, 385-394.
- Kobayashi, M. & Yamamoto, M. (2006) Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv. Enzyme Regul.* **46**, 113-140.
- Kusik, B.W., Carvan, M.J., 3rd & Udvardia, A.J. (2008) Detection of mercury in aquatic environments using EPRE reporter zebrafish. *Mar. Biotechnol.* **10**, 750-757.
- Lau, A., Villeneuve, N.F., Sun, Z., Wong, P.K. & Zhang, D.D. (2008) Dual roles of Nrf2 in cancer. *Pharmacol. Res.* **58**, 262-270.
- Li, L., Kobayashi, M., Kaneko, H., Nakajima-Takagi, Y., Nakayama, Y. & Yamamoto, M. (2008) Molecular Evolution of Keap1: Two Keap1 molecules with distinctive intervening region structures are conserved among fish. *J. Biol. Chem.* **283**, 3248-3255.
- Mattingly, C.J., McLachlan, J.A. & Toscano, W.A., Jr. (2001) Green fluorescent protein (GFP) as a marker of aryl hydrocarbon receptor (AhR) function in developing zebrafish (*Danio rerio*). *Environ. Health Perspect.* **109**, 845-849.
- Schopfer, F.J., Cole, M.P., Groeger, A.L. *et al.* (2010) Covalent peroxisome proliferator-activated receptor γ binding by nitro-fatty acids: Endogenous ligands act as selective modulators. *J. Biol. Chem.* **285**, 12321-12333.
- Schopfer, F.J., Lin, Y., Baker, P.R.S., Cui, T., Garcia-Barrio, M., Zhang, J., Chen, K., Chen, Y.E. & Freeman, B.A. (2005) Nitrolinoleic acid: an endogenous peroxisome proliferator-activated receptor γ ligand. *Proc. Natl. Acad. Sci. USA* **102**, 2340-2345.
- Sekhar, K.R., Rachakonda, G. & Freeman, M.L. (2010) Cysteine-based regulation of the CUL3 adaptor protein Keap1. *Toxicol. Appl. Pharmacol.* **224**, 21-26.
- Seok, S.H., Baek, M.W., Lee, H.Y., Kim, D.J., Na, Y.R., Noh, K.J., Park, S.H., Lee, H.K., Lee, B.H., Ryu, D.Y. & Park, J.H. (2007) Quantitative GFP fluorescence as an indicator of arsenite

developmental toxicity in mosaic heat shock protein 70 transgenic zebrafish. *Toxicol. Appl. Pharmacol.* **225**, 154-161.

Shiraki, T., Kodama, T.S., Shiki, S., Nakagawa, T. & Jingami, H. (2006) Spectroscopic analyses of the binding kinetics of 15d-PGJ₂ to the PPAR γ ligand-binding domain by multi-wavelength global fitting. *Biochem. J.* **393**, 749-755.

Straus, D.S., Pascual, G., Li, M., Welch, J.S., Ricote, M., Hsiang, C.H., Sengchanthalangsy, L.L., Ghosh, G. & Glass, C.K. (2000) 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ inhibits multiple steps in the NF- κ B signaling pathway. *Proc. Natl. Acad. Sci. USA* **97**, 4844-4849.

Suzuki, T., Takagi, Y., Osanai, H., Li, L., Takeuchi, M., Katoh, Y., Kobayashi, M. & Yamamoto, M. (2005) Pi-class glutathione S-transferase genes are regulated by Nrf2 through an evolutionarily conserved regulatory element in zebrafish. *Biochem. J.* **388**, 65-73.

Takagi, Y., Kobayashi, M., Li, L., Suzuki, T., Nishikawa, K. & Yamamoto, M. (2004) MafT, a new member of the small Maf protein family in zebrafish. *Biochem. Biophys. Res. Commun.* **320**, 62-69.

Takeuchi, M., Kaneko, H., Nishikawa, K., Kawakami, K., Yamamoto, M. & Kobayashi, M. (2010) Efficient transient rescue of hematopoietic mutant phenotypes in zebrafish using Tol2-mediated transgenesis. *Dev. Growth Differ.* **52**, 245-250.

Villacorta, L., Zhang, J., Garcia-Barrio, M.T., Chen, X.L., Freeman, B.A., Chen, Y.E. & Cui, T. (2007) Nitro-linoleic acid inhibits vascular smooth muscle cell proliferation via the Keap1/Nrf2 signaling pathway. *Am J. Physiol. Heart Circ. Physiol.* **293**, H770-H776.

Wang, X.J., Hayes, J.D. & Wolf, C.R. (2006) Generation of a stable antioxidant response element-driven reporter gene cell line and its use to show redox-dependent activation of Nrf2 by cancer chemotherapeutic agents. *Cancer Res.* **66**, 10983-10994.

White, R.M., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., Bourque, C., Dovey, M., Goessling, W., Burns, C.E. & Zon, L.I. (2008) Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell* **2**, 183-189.

- Wu, Y.L., Pan, X., Mudumana, S.P., Wang, H., Kee, P.W. & Gong, Z. (2008) Development of a heat shock inducible *gfp* transgenic zebrafish line by using the zebrafish *hsp27* promoter. *Gene* **408**, 85-94.
- Yang, Z., Jiang, H. & Lin, S. (2009) Bacterial artificial chromosome transgenesis for zebrafish. *Methods Mol. Biol.* **546**, 103-116.
- Yates, M.S., Tauchi, M., Katsuoka, F., Flanders, K.C., Liby, K.T., Honda, T., Gribble, G.W., Johnson, D.A., Johnson, J.A., Burton, N.C., Guilarte, T.R., Yamamoto, M., Sporn, M.B. & Kensler, T.W. (2007) Pharmacodynamic characterization of chemopreventive triterpenoids as exceptionally potent inducers of Nrf2-regulated genes. *Mol. Cancer Ther.* **6**, 154-162.
- Zhu, M. & Fahl, W.E. (2001) Functional characterization of transcription regulators that interact with the electrophile response element. *Biochem. Biophys. Res. Commun.* **289**, 212-219.
- Zon, L.I. & Peterson, R.T. (2005) *In vivo* drug discovery in the zebrafish. *Nat. Rev. Drug. Discov.* **4**, 35-44.

Figure Legends

Figure 1 DEM-induced GFP expression in *Tg(-3.5gstp1:GFP)it416b* larvae. (A) A schematic diagram of the p3.5gstp1GFP construct. ExI and ExII denote exon 1 and exon 2, respectively, of the *gstp1* gene. (B) Time-course of GFP expression in the olfactory regions (arrowheads) of *Tg(-3.5gstp1:GFP)it416b* larvae at 4 dpf after treatment with 100 μ M DEM. The asterisks and arrows denote the constitutive GFP expression in the lens and lateral lines, respectively. Dorsal views. (C) Effects of *nrf2* knockdown on DEM-induced GFP expression. Day 4 larvae of the *Tg(-3.5gstp1:GFP)it416b* line injected with *nrf2*MO at the one-cell stage were treated with 100 μ M DEM treatment for 12 hours.

Figure 2 Quantification of DEM-induced GFP expression. (A) Fluorescence intensities in the areas enclosed by the triangles were analyzed. (B) Determination of sample numbers. The indicated numbers of *Tg(-3.5gstp1:GFP)it416b* larvae were treated with 100 μ M DEM for 12 hours at 4 dpf, and the average fluorescence intensities in the olfactory regions were calculated. Error bars indicate standard deviation values. (C) The effects of *nrf2* knockdown on 100 μ M DEM-induced GFP expression at 4 dpf. The average fluorescence intensities in the olfactory regions of three individual *Tg(-3.5gstp1:GFP)it416b* larvae were analyzed: larvae were injected with or without *nrf2*MO at the one-cell embryo stage. (D) Time- and dose-effects on DEM-induced GFP expression at 4 dpf. The average fluorescence intensities in the olfactory regions of three individual *Tg(-3.5gstp1:GFP)it416b* larvae were analyzed. (E) Real time RT-PCR analysis. Time- and dose-dependent effects on DEM-induced expression of endogenous *gstp1* at 4 dpf.

Figure 3 Effect of the *it567* mutation on DEM-induced GFP expression. (A) GFP expression in the *it567* mutant larvae at 4 dpf. *Tg(-3.5gstp1:GFP)it416b;it567/it567* larvae or wild-type siblings were treated with 100 μ M DEM for 6 hours. Arrowheads indicate the olfactory regions. (B) Average fluorescence intensities of three individual larvae were analyzed. Error bars indicate the standard deviation values.

Figure 4 GFP induction by a variety of Nrf2 activators. (A) GFP induction in the olfactory regions of *Tg(-3.5gstp1:GFP)it416b* larvae at 4 dpf by sulforaphane (SF), 15d-PGJ₂, H₂O₂, auranofin (AF), and CdCl₂. Ctr denotes non-treated control larvae. Error bars indicate the standard deviation values of three individual larvae. (B) GFP induction in *Tg(-3.5gstp1:GFP)it416b* embryos by DEM,

15d-PGJ₂ and H₂O₂. *Tg(-3.5gstp1:GFP)it416b* embryos co-injected with wild-type (WT) or C151S Keap1 mRNA and Nrf2 mRNA were treated with Nrf2 activators for 6 hours.

Figure 5 OA-NO₂ induced GFP expression in *Tg(-3.5gstp1:GFP)it416b* larvae and embryos. (A, B) GFP induction by 1.5 μM OA-NO₂ in the olfactory regions (arrowheads) of *Tg(-3.5gstp1:GFP)it416b* larvae at 4 dpf. The effects of Nrf2 knockdown were analyzed using *nrf2MO*. Error bars indicate the standard deviation values of three individual larvae. (C) OA-NO₂ induced GFP expression in *Tg(-3.5gstp1:GFP)it416b* embryos. *Tg(-3.5gstp1:GFP)it416b* embryos were co-injected with wild-type (WT) or C151S Keap1 mRNA and Nrf2 mRNA were treated with 2.5 μM OA-NO₂ for 6 hours. (D) An RT-PCR analysis of OA-NO₂-induced endogenous *gstp1* expression. Embryos co-injected with wild-type (WT) or C151S Keap1 mRNA and Nrf2 mRNA were treated with 2.5 μM OA-NO₂ for 6 hours.

Figure 6 Identification of the sensor cysteines for OA-NO₂. (A) MS analysis of peptides from mouse Keap1 digested with trypsin following incubation with or without OA-NO₂. Mouse Keap1 (3 μg) was incubated at 25°C for 60 minutes in the absence (top) or presence (bottom) of 100 μM OA-NO₂ in a total volume of 10 μl containing 20 mM Tris-HCl (pH8.5). (B) Modification sites in mouse Keap1 for Nrf2-activating compounds was determined by MS analysis. The modification sites for 15d-PGJ₂ and PGA₂ have been analyzed previously (Kobayashi *et al.* 2009).

Figure 7 Effect of the *it275* mutation on OA-NO₂-induced endogenous *gstp1* expression. An *in situ* hybridization analysis of endogenous *gstp1* expression (arrowheads) of homozygous *it275* larvae and wild-type siblings treated with 1.5 μM OA-NO₂ or 100 μM DEM for 6 hours at 4 dpf.

Figure 8 Nitro-fatty acids and cyclopentenone prostaglandins. (A) Chemical structures of nitro-fatty acids (OA-NO₂ and LNO₂) and cyclopentenone prostaglandins (15d-PGJ₂ and PGA₂). (B) Cysteine code hypothesis. OA-NO₂ and 15d-PGJ₂ share an analogous cysteine code as nucleophilic targets and play similar anti-inflammation roles.

Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Table S1 Generation of stable transgenic lines harboring p3.5gstp1GFP or pT3.5gstp1GFP constructs.

Table S2 Peptides identified by MALDI-TOF MS from mouse Keap1 following treatment with OA-NO₂.

Figure S1 DEM-induced GFP expression in stable transgenic lines.

Figure S2 Constitutive GFP expression in *Tg(-3.5gstp1:GFP)it416b* larvae at 6 dpf.

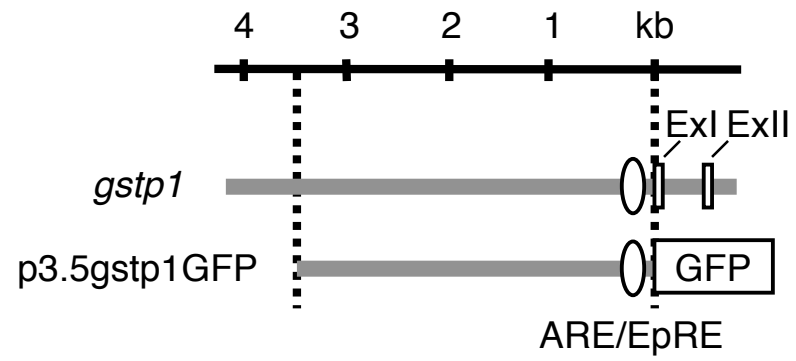
Figure S3 Nrf2-induced GFP expression in *Tg(-3.5gstp1:GFP)it416b* embryos.

Figure S4 The effects of the C288S mutation in Keap1 on the OA-NO₂ response.

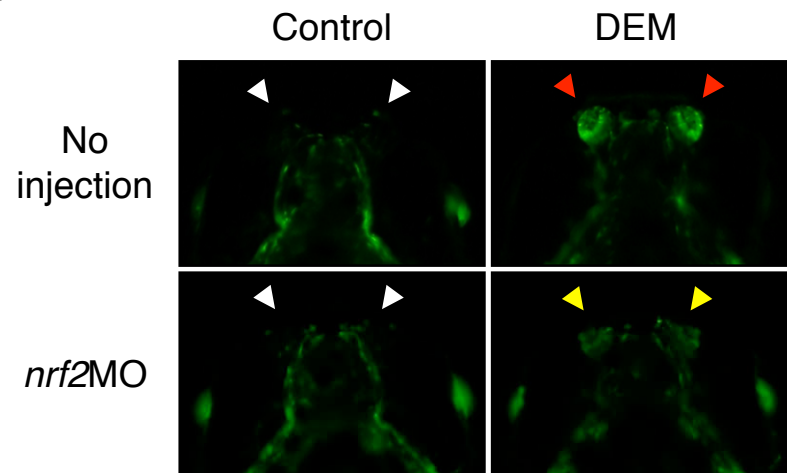
Movie S1 Real-time monitoring of DEM-induced GFP expression in *Tg(-3.5gstp1:GFP)it416b* larvae.

Movie S2 DEM-induced GFP expression in *it567* and its sibling wild-type larvae.

A



C



B

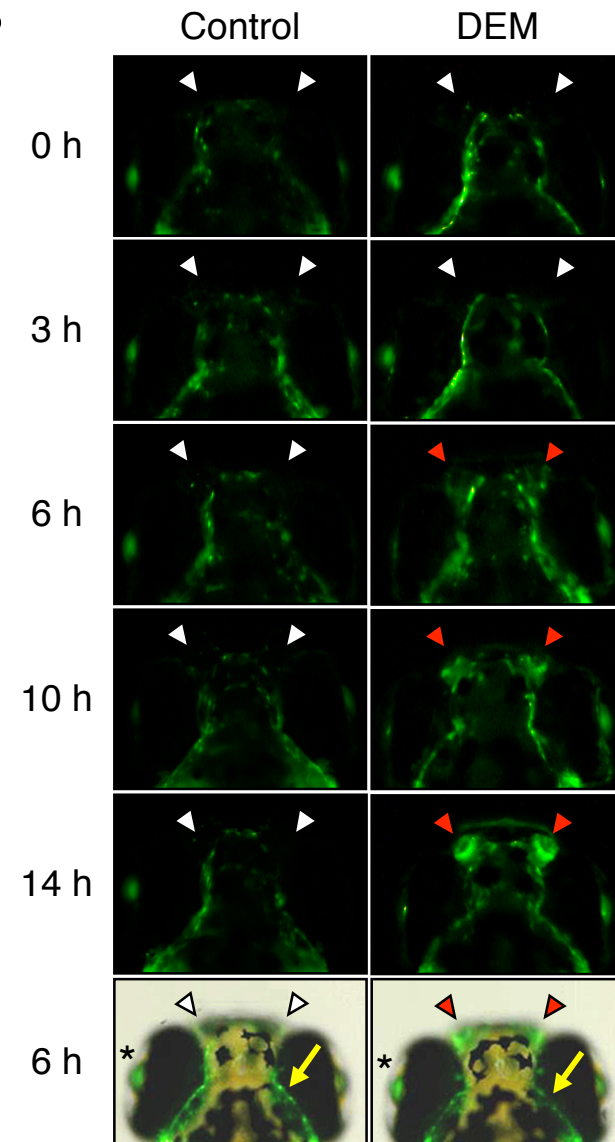


Fig. 1 Tsujita et al.

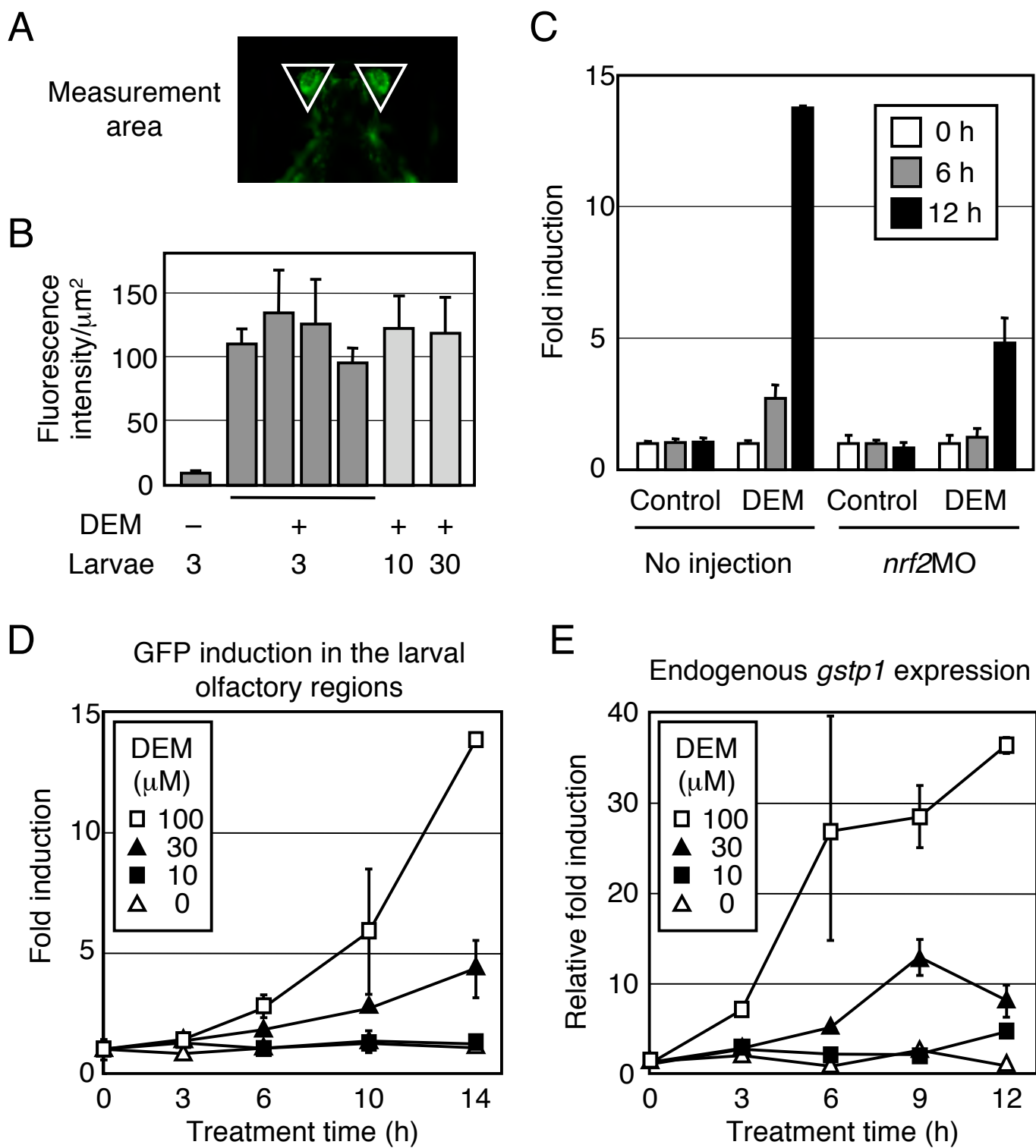


Fig. 2 Tsujita et al.

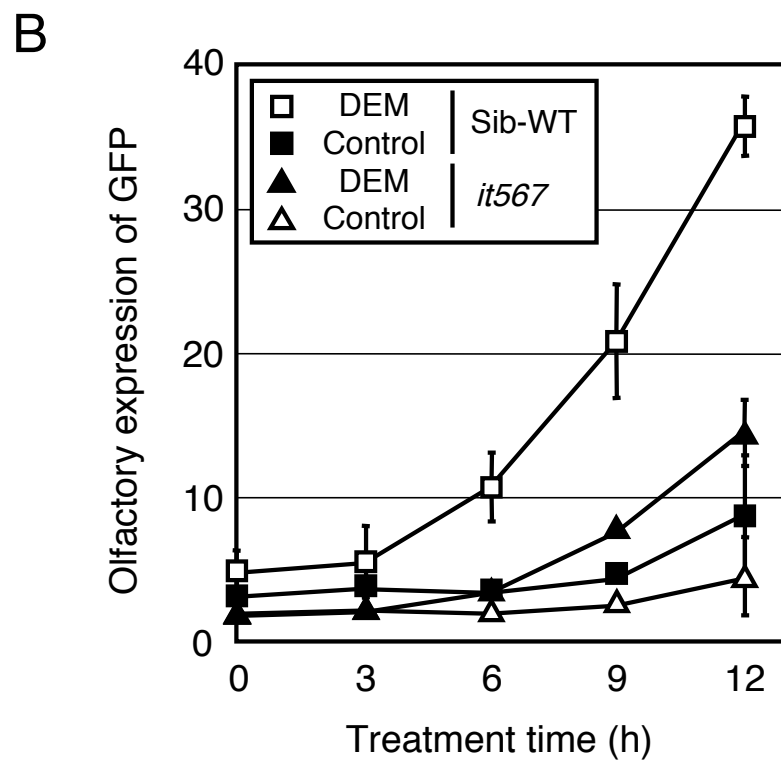
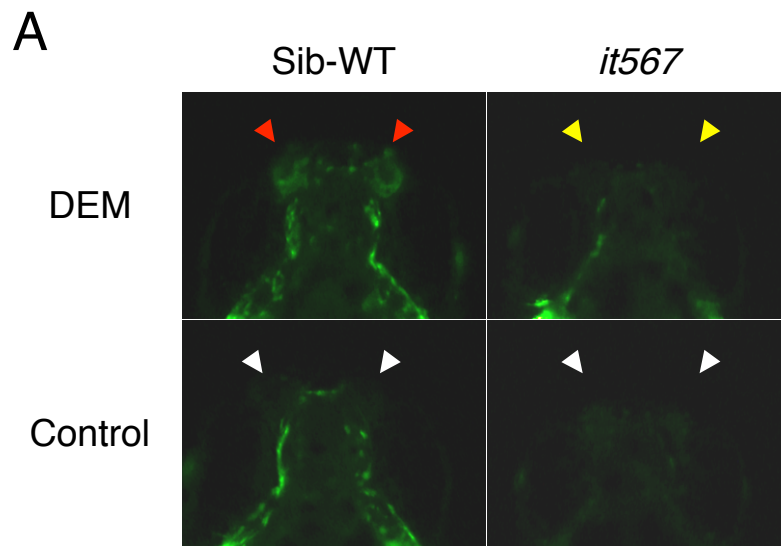
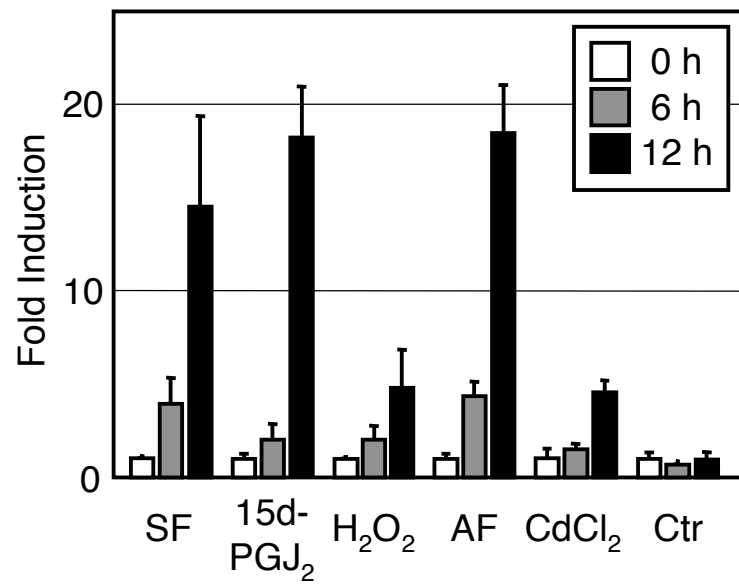


Fig. 3 Tsujita et al.

A GFP induction in the larval olfactory regions



B

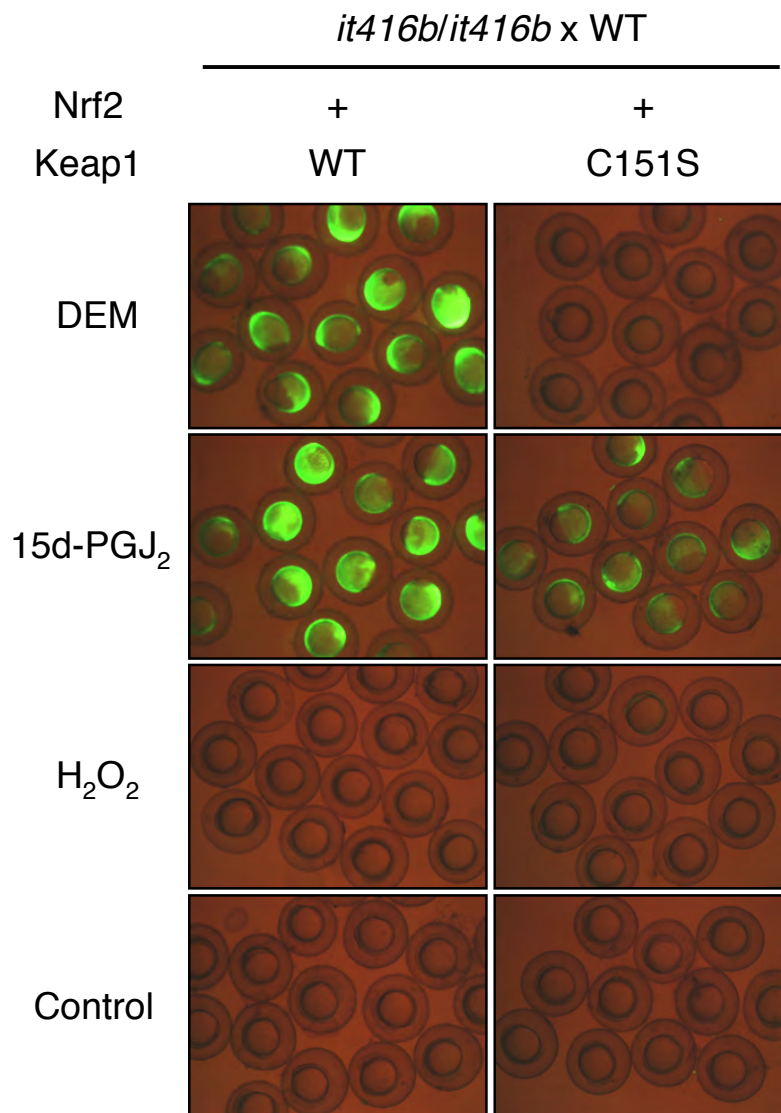


Fig. 4 Tsujita et al.

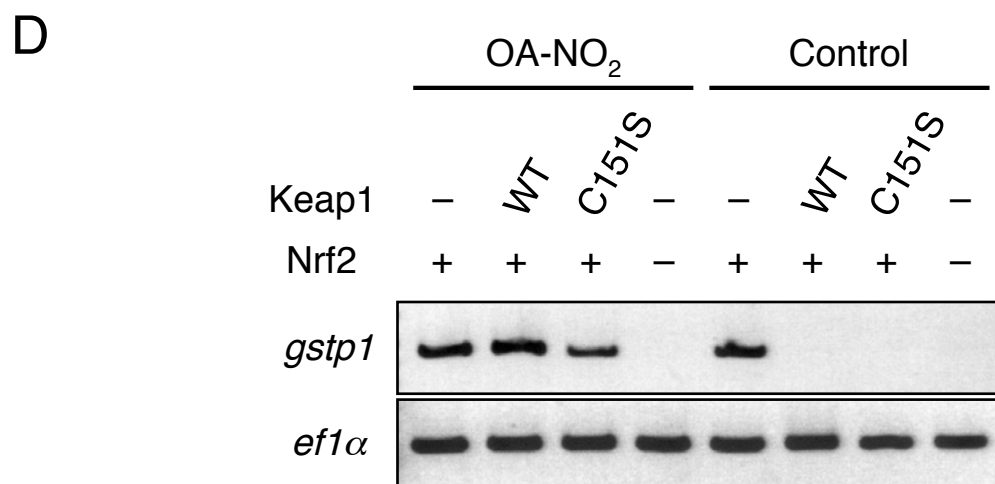
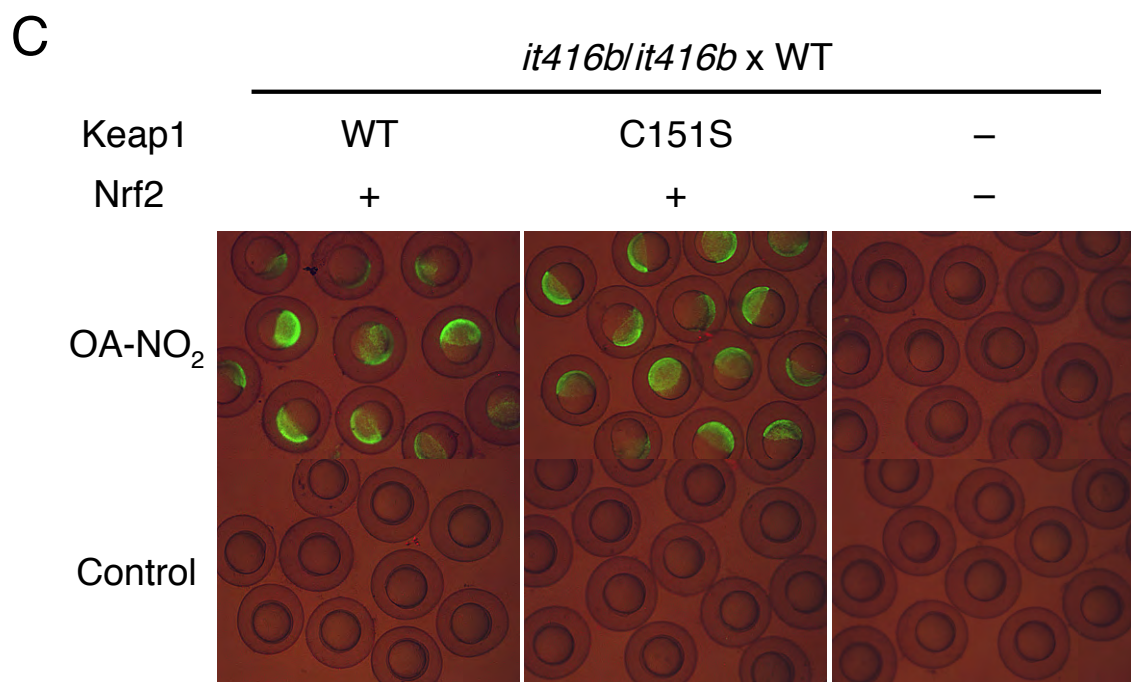
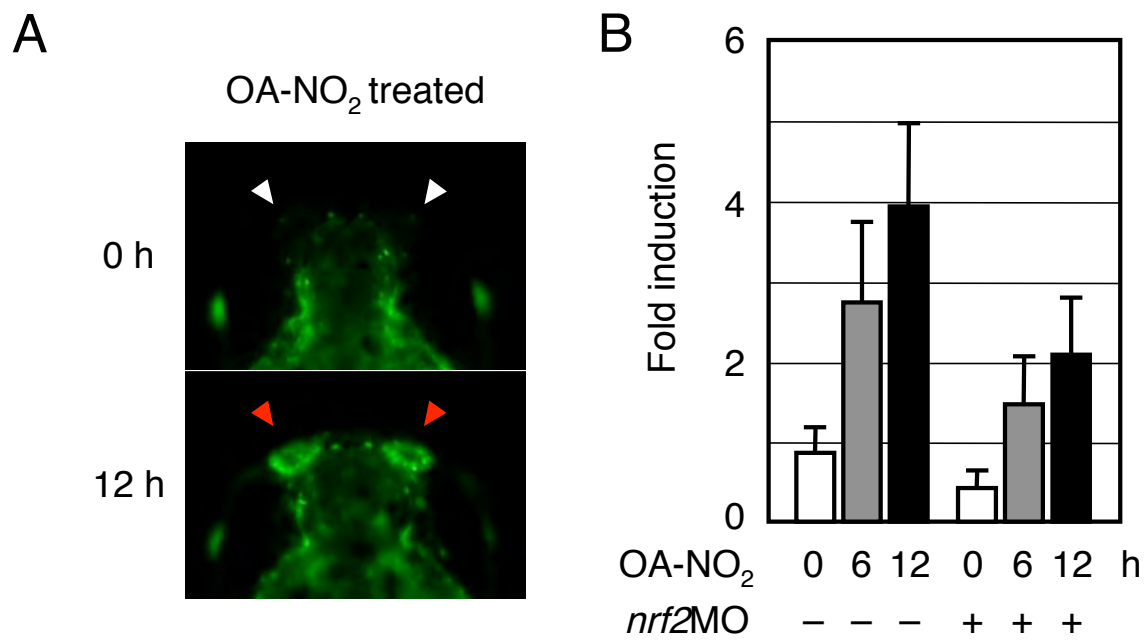
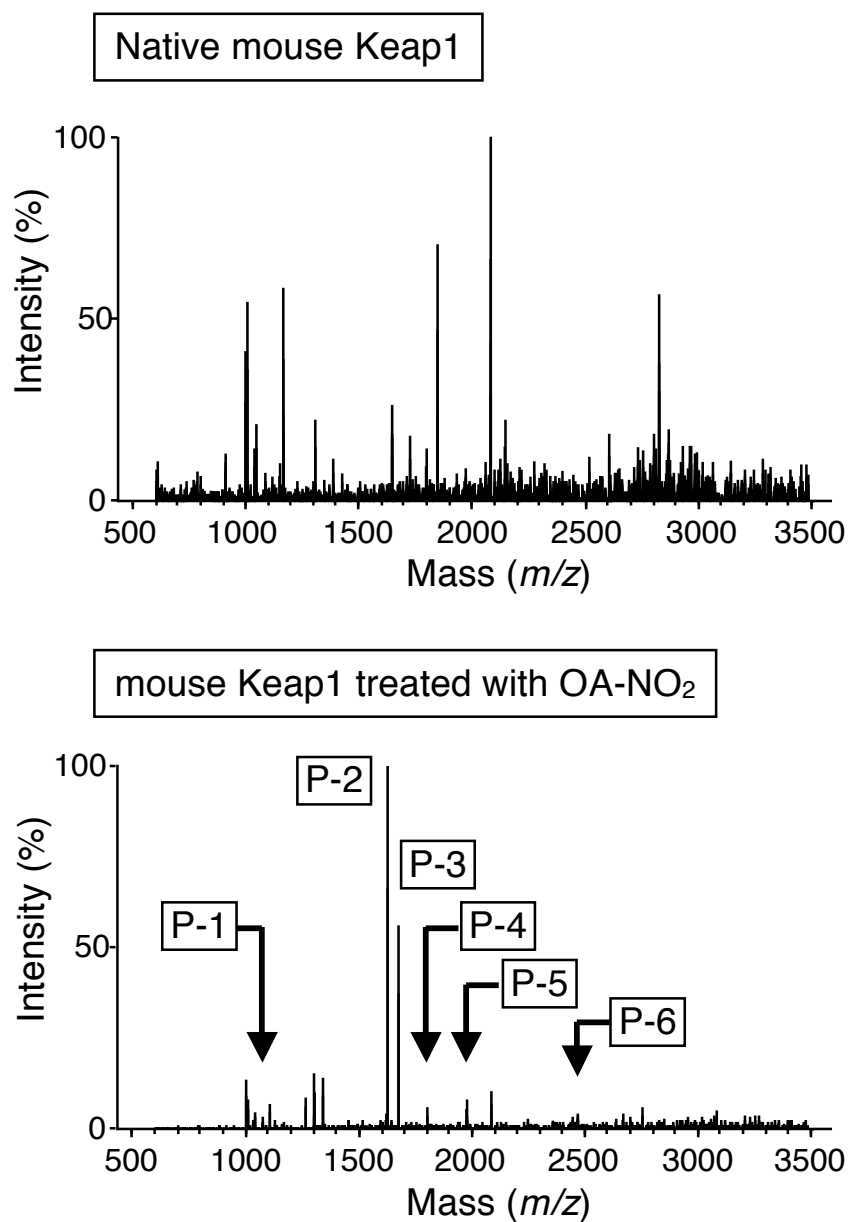


Fig. 5 Tsujita et al.

A



B

Activators	Cysteines					
OA-NO ₂	77	226	273	368	489	613
15d-PGJ ₂	273	288				
PGA ₂	273	297	489			

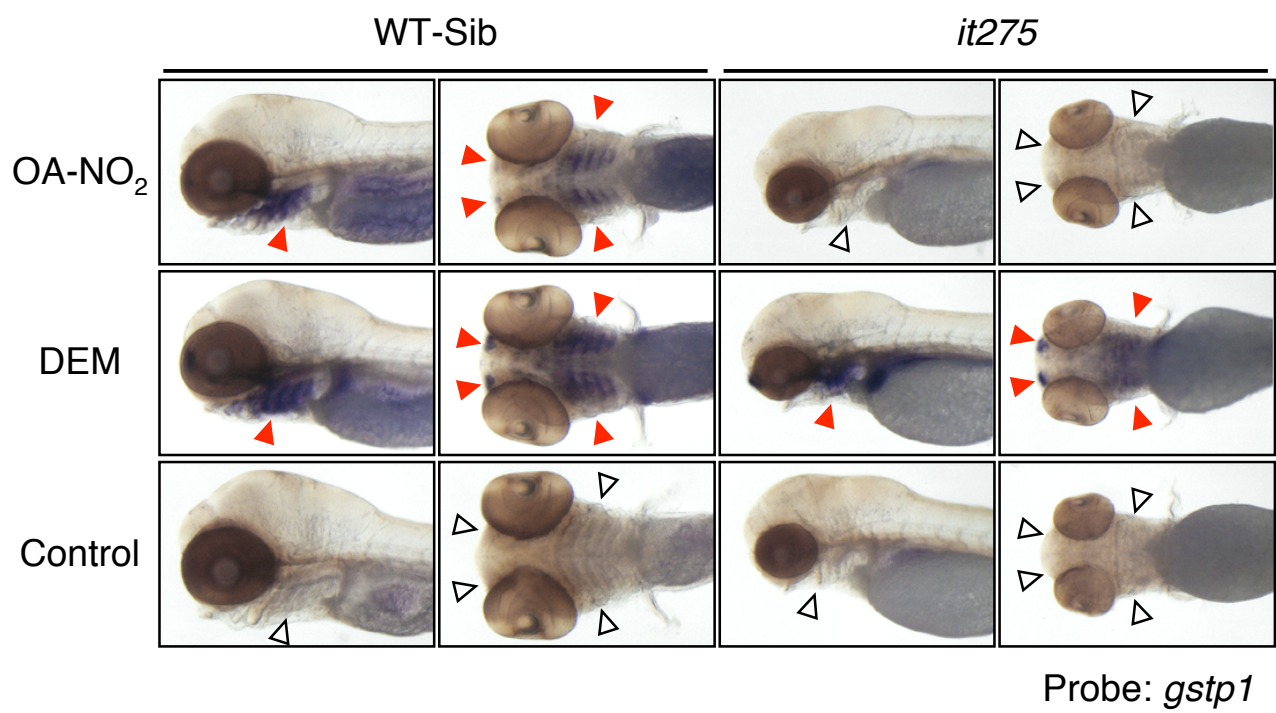
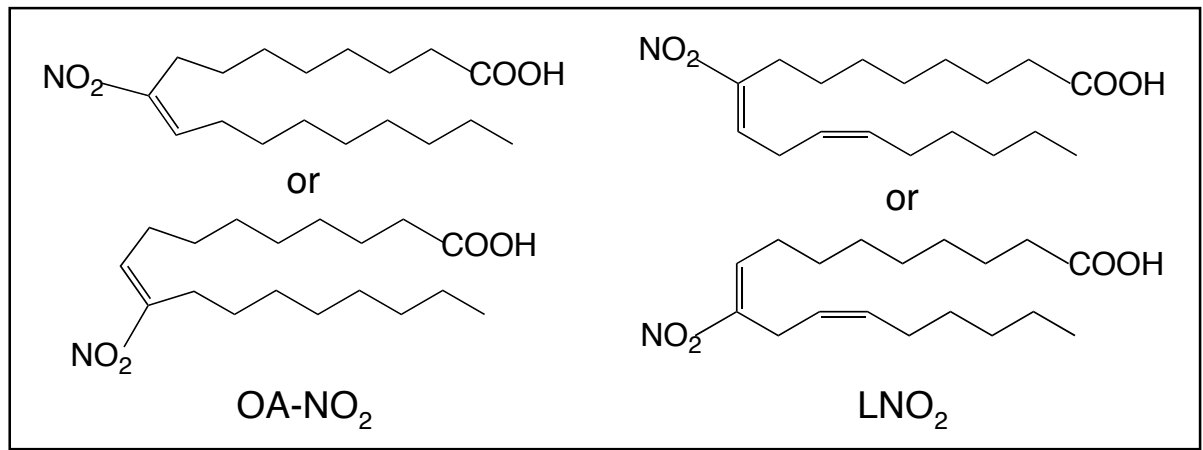
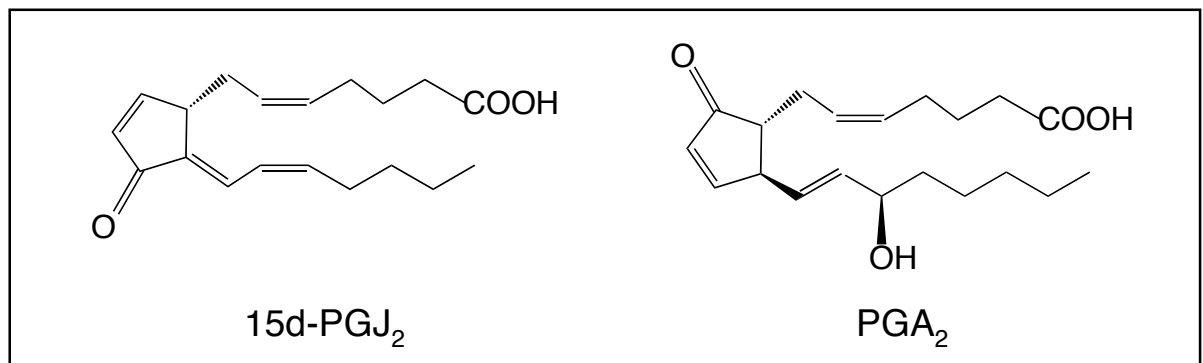


Fig. 7 Tsujita et al.

A



Nitro-fatty acids



Cyclopentenone prostaglandins

B

